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Microglia/brain macrophages as central drivers of brain tumor pathobiology

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Summary

One of the most common brain tumors in children and adults is the glioma or astrocytoma. There are few effective therapies for these cancers, and patients with malignant glioma fare poorly, even after aggressive surgery, chemotherapy and radiation. Over the past decade, it is now appreciated that these tumors are comprised of numerous distinct neoplastic and non-neoplastic cell populations, which could each influence overall tumor biology and response to therapy. Among these non-cancerous cell types, monocytes (microglia and macrophages) predominate. In this review, we discuss the complex interactions involving microglia and macrophages relevant to glioma formation, progression, and response to therapy.

Introduction

The most common brain tumor arising in children and adults is the glioma (or astrocytoma). These tumors are classified by the World Health Organization according to histologic features, running the spectrum from low-grade (I and II) to high-grade (III and IV) gliomas. With the advent of molecular diagnostics, the nosology of adult glioma has changed [Louis *et al.*, 2016], now incorporating the presence or absence of specific genetic (e.g., *IDH1* and *IDH2* mutations) and genomic (e.g., chromosome 1p/19q co-deletions) alterations. Similarly, pediatric diffuse gliomas are molecularly defined by mutations in histone H3 genes (H3-K27M-mutant diffuse midline gliomas).

Glioma tissue is not only composed of cancer cells, but is also infiltrated by non-transformed cells, predominantly resident microglia from the brain and circulating blood monocytes (macrophages), which comprise 30–50% of the cellular content of these tumors [Simmons *et al.*, 2011]. Microglia are the brain resident immune cells, first described by Pio del Rio Hortega in 1919 [Sierra *et al.*, 2016], and later reported by Wilder Penfield to infiltrate glioma tissue as cells with an amoeboid morphology, similar to microglia found in multiple sclerosis or Alzheimer disease tissue [Penfield, 1925].

Microglia develop from primitive yolk sac myeloid precursors, and enter the brain during embryogenesis [Ginhoux *et al.*, 2010] to mature into distinct populations of central nervous system (CNS) monocytes. Additionally, there may be other ontologies for brain microglia that reflect different waves of yolk sac hematopoiesis [De *et al.*, 2018]. In contrast to resident microglia, macrophages most typically enter the brain in the context of pathology, either from the blood (bone marrow-derived macrophages) or through direct vascular channels that connect the skull bone marrow to the brain [Herisson *et al.*, 2018].

Over the last decade, it has become clear that glioma associated microglia and infiltrated macrophages from the peripheral system (GAM) are not merely bystander or “reactive” immune system-like cells, but interact with numerous other cell types to actively influence brain tumor biology. However, there is still no consensus on the clinical significance of GAM infiltration relevant to patient outcome. In one study, there was a positive association between the numbers of CD68-, CD163- and CD206-positive GAM in the vital tumor core and prolonged overall survival of patients with IDH1^{R132H}-non-mutant GBM [Zeiner *et al.*, 2018]. Similarly, others found that microglia density, as determined by Iba1 labelling, correlated with improved outcomes for patients with gliomas, whereas CD204 expression in GAM was associated with poorer survival [Sorensen *et al.*, 2018]. In contrast, the expression of microglia/macrophage-related genes in adult and pediatric malignant gliomas of the mesenchymal subtype revealed a negative correlation between GAM accumulation and patient survival in adults, but not children [Engler *et al.*, 2012]. While GAM enrichment is higher in the mesenchymal subtype of high-grade glioma relative to proneural or classical gliomas [Kaffes *et al.*, 2019], the mechanisms underlying GAM-mediated patient survival remain to be determined.

GAM regulate glioma formation and progression

Numerous studies have revealed that GAM are critical for both low-grade and high-grade gliomagenesis and continued tumor growth. In experimental malignant (high-grade) glioma models, microglia depletion reduces glioma growth [Hambardzumyan *et al.*, 2016] (Figure 1). While the mechanisms underlying this pro-tumoral effect likely vary from tumor to tumor, numerous potential etiologies have been identified. In these studies, TGF- β , stress-inducible Protein (STI)-1, IL-6, IL-1 β and EGF are factors released from GAM, which each can promote tumor growth [Hambardzumyan *et al.*, 2016]. Moreover, microglia, but not bone marrow-derived macrophages, induce platelet-derived growth factor receptor expression in a subset of tumor cells of mouse and human low and high-grade glioma. This receptor expression stimulates the migratory capacity of glioma cells, which accelerates tumor progression [Wallmann *et al.*, 2018]. Similarly, GAM are the predominant source of the secreted form of osteopontin/SPP1, which suppresses glioma growth in some contexts [Szulzewsky *et al.*, 2018] and reduces glioma cell apoptosis in other experimental settings [P Chen *et al.*, 2019].

In contrast to their malignant counterparts, low-grade gliomas predominate in children and harbor a paucity of genetic mutations. These tumors arise either sporadically, caused frequently by genomic alterations involving the *BRAF* kinase gene (*KIAA1549:BRAF* fusion) [Pfister *et al.*, 2008] [J Yu *et al.*, 2009], or in the setting of the Neurofibromatosis

type 1 (NF1) cancer predisposition syndrome [Listernick *et al.*, 1989]. Importantly, *Nf1* loss or BRAF fusion is usually not sufficient for glioma formation in rodents, unless coupled with supportive growth factors from the tumor microenvironment. In this regard, *Nf1* loss [Larribere *et al.*, 2015] or *KIAA1549:BRAF* expression [Jacob *et al.*, 2011] in numerous cell types induces cellular senescence, which requires stromal signals to overcome and lead to neoplasia.

To explore the role of these permissive stromal signals, studies using *Nf1* genetically engineered mouse strains that develop optic glioma, the signature brain tumor seen in children with NF1, have uncovered essential roles for microglia in glioma formation and maintenance. First, optic glioma formation is delayed in mice with reduced expression of the key receptor involved in directed microglia migration (CX3CR1) [Pong *et al.*, 2013a]. Second, optic glioma growth *in vivo* is attenuated following treatment with either minocycline (crude microglia inhibitor) [Daginakatte and Gutmann, 2007] [Pan *et al.*, 2017b] or JNK inhibitors (targeting the signalling pathway hyperactivated in *Nf1*-mutant microglia) [Daginakatte *et al.*, 2008]. Third, RNA sequencing of murine *Nf1* optic glioma-associated microglia demonstrated that these GAM secrete CCL5, which is a potent growth factor for glioma cells [Solga *et al.*, 2015]. Treatment of *Nf1* optic glioma-bearing mice with a neutralizing CCL5 antibody dramatically attenuated tumor growth *in vivo*. Consistent with the idea that high-grade gliomas gain some measure of stromal independence, malignant gliomas harboring *NF1* mutations express CCL5, establishing an autocrine loop for mesenchymal glioblastoma survival [Pan *et al.*, 2017a]. The requirement for microglia and microglia-produced Ccl5 is further underscored by studies in which murine *Nf1* optic glioma stem cells are implanted into mice. While wild-type mice support the formation of glioma-like lesions following transplantation [YH Chen *et al.*, 2015], no tumors form in mice lacking the two chemokine receptors that direct monocyte migration (Cx3cr1 and Ccr2) or in mice deficient in Ccl5 expression [Pan *et al.*, 2018] [Guo *et al.*, 2019]. While less well studied, *KIAA1549:BRAF*-expressing low-grade gliomas are also dependent on monocytes in the tumor microenvironment, such that gliomas fail to form following the transplantation of murine *KIAA1549:BRAF*-expressing neural stem cells in *Ccr2*-deficient mice [R Chen *et al.*, 2019].

Glioma cells attract/recruit GAM

There are a variety of glioma-derived factors that can function as chemoattractants for GAM, including CCL2, CX3CL1, SDF-1, CSF-1, GM-CSF, LOX and potentially EGF [Roesch *et al.*, 2018]. This directed monocyte recruitment occurs through the establishment of chemokine gradients that attract monocytes to the evolving tumor bed (Figure 2). Studies employing *Ccr2* and *Cx3cr1* reporter mice have revealed that two of the most important chemoattractants are Ccl2 and Cx3cl1, which traditionally have been thought to drive directional migration of macrophages and microglia, respectively. In this regard, Ccl2 is produced by low-grade glioma stem cells expressing the *KIAA1549:BRAF* genomic alteration, leading to monocyte attraction [R Chen *et al.*, 2019], whereas *NF1* mutation in both low-grade and high-grade gliomas attracts microglia through Cx3cl1 [Z Chen *et al.*, 2017] [Guo *et al.*, 2019]. Similarly, in experimental glioblastoma models, Ccl2 is produced by the tumor cells, which attract macrophages [Platten *et al.*, 2003], such that Ccl2/Ccr2

inhibition prolongs mouse survival [Z Chen et al., 2017] [Hutter et al., 2019]. While there are few studies that specifically focused on chemokine networks responsible for monocyte attraction, other molecules are also likely involved in recruiting monocytes to the tumor [Dijksterhuis et al., 2015] [Brandenburg et al., 2016].

Moreover, emerging evidence supports the view that microglia and macrophages colonize different regions of malignant gliomas, such that macrophages appear to be recruited early during tumorigenesis and occupy perivascular regions [Z Chen et al., 2017]. However, conflicting data exist regarding the major monocyte population in these tumors, with some studies reporting a microglia predominance [Hutter et al., 2019] and others demonstrating that infiltrating macrophages represent the majority of the GAM population [Z Chen et al., 2017] [K Yu et al., 2019]. These differences could reflect specific experimental mouse model systems (RCAS model versus GL261 or T387 cell lines) used in each of these studies, suggesting that variations in GAM populations may be differentially dictated by the molecular properties of the glioma.

Support for potential mutation-specific effects on GAM recruitment comes from two studies: First, murine high-grade gliomas harboring a mutant *IDH1* allele exhibited reduced macrophage and microglia infiltration, which correlated with lower levels of chemokine expression (e.g., CCL2, CXCL2) [Amankulor et al., 2017]. Moreover, the IDH1/2-mediated accumulation of the oncometabolite 2-hydroxyglutarate reduced the expression of pro-inflammatory chemokines [Han et al., 2019]. Second, differences in microglia recruitment were observed in mice genetically engineered to develop low-grade gliomas with distinct patient-derived germline *NF1* gene mutations and cooperating genetic alterations (e.g., heterozygous *Pten* loss) through the elaboration of chemokines [Guo et al., 2019]. These findings suggest that variations in the GAM composition of gliomas could be dictated by types of immune chemoattractant molecules produced by cancer cells with different mutations.

GAM acquire unique phenotypes in the setting of glioma

Once GAM are recruited to the glioma milieu, they adopt new cellular and molecular identities that reflect the enactment of epigenetic or transcriptional programs that create monocyte populations critical for glioma homeostasis and progression. To identify these GAM transcriptional programs, two mouse glioma models, inoculated GL261 cells and the RCAS transgenic system, have been used to compare the expression profiles of glioma-associated microglia/macrophages and naive control cells. Not surprisingly, these profiles do not correspond to the M1/M2 classification scheme often used to describe macrophages/microglia in homeostatic or pro-inflammatory settings. Two genes were increased in GAM, *Gpnmb* and *Spp1*, which have also been described in other specialized microglia populations (e.g., disease-associated microglia [DAM] and proliferative region-associated microglia [PAM]), and were associated with poor prognosis in human GBM [Szulzewsky et al., 2015]. Work in many laboratories, using different microglia isolation methods and RNA analysis platforms have identified other GAM-specific markers; however, no marker has been discovered to date that uniquely distinguishes GAM from other microglia populations.

While microglia are the professional phagocytes of the CNS, in the setting of glioma, they do not attack or phagocytose glioma cells. This may reflect increased expression of the anti-phagocytic (“don’t eat me”) surface protein CD47 that binds to its cognate receptor SIRP α on phagocytic cells to inhibit its phagocytic activity. In this regard, anti-CD47 antibody treatment induces microglial tumor phagocytosis in a glioblastoma xenograft model and reduced tumor expansion [Hutter *et al.*, 2019]. A similar effect of a humanized anti-CD47 antibody was found on five aggressive and etiologically distinct pediatric brain tumors: group 3 medulloblastoma (primary and metastatic), atypical teratoid rhabdoid tumor, primitive neuroectodermal tumor, pediatric glioblastoma, and diffuse intrinsic pontine glioma [Gholamin *et al.*, 2017]. Moreover, in glioma patients, CD47 expression inversely correlated with histopathologic grading (low-grade versus high-grade glioma), and high levels of CD47 were associated with lower overall survival rates [FLi *et al.*, 2018].

How microglia become reprogrammed in the setting of glioma remains to be fully elucidated. One family of receptors expressed on microglia may play an instructive role in the establishment of these new functional states. Toll-like receptors (TLR) were initially recognized as pathogen sensors, but it is now appreciated that they can also be activated by endogenous ligands. TLR2 expression is increased in GAM [Hu *et al.*, 2015], such that mouse GL261 glioma cells implanted into the brains of *Tr2* knockout mice have smaller tumors and enhanced survival. This TLR2-dependency is partially mediated by increased production of a membrane-residing protease (MMP14) essential for activating MMP2 and promoting malignant glioma invasion [Vinnakota *et al.*, 2013]. In addition to MMP14, TLR2 signaling also triggers the release of MMP9 from microglia, which serves as an additional factor for degrading the extracellular matrix to promote glioma invasion and growth [Hu *et al.*, 2014]. TLR2 signaling is also involved in the interaction of GAM with T cells. As such, TLR2-mediated growth glioma is reduced using TLR2 blocking antibodies [Hu *et al.*, 2015]. Beyond its role in glioma growth, TLR2 activation may also function to facilitate glioma immune evasion through reduced MHC class II molecule expression and reduced CD4⁺ T cells activation [J Qian *et al.*, 2018].

In addition to TLR2, TLR4 is also important for regulating microglial IL-6 secretion, a mitogen for glioma stem cells. Analysis of human glioma tissue confirmed that GAM are the major source of IL-6 in gliomas [a Dzaye *et al.*, 2016]. One of the endogenous ligands for TLR4 is the cell adhesion molecule, tenascin C. In tumors with genetically reduced tenascin C expression, microglia acquire a more amoeboid morphology and increased expression of MHCII molecules [Xia *et al.*, 2016], similar to that observed with TLR2 silencing.

Finally, monocytes likely receive instructive cues from other cell types. One of these cell types is the T lymphocyte. Using a low-grade glioma stem cell transplantation system, glioma-like lesions did not form in mice with impaired T cell function [Pan *et al.*, 2018]. These T cells, found in small abundance in both human and mouse *NFI*-mutant tumors, secrete paracrine factors important for inducing microglia to express CCL5 and drive glioma growth. Understanding how these T cells are recruited to the tumor, how they are activated, and the mechanisms that underlie their induction of microglia represent opportunities to dissect other immunologic determinants that underlie glioma formation and maintenance.

Experimental model systems to study glioma-microglia interactions

Current available platforms to examine the interactions between monocytes and glioma cells involve co-culture systems and organotypic systems, as well as small and large animal models. In addition to several genetically engineered mouse models (for review see [Gargiulo, 2018], large animal platforms involving pigs and dogs have been utilized. Spontaneously occurring low- and high-grade gliomas have been observed in dogs, but a systematic study of GAM in these tumors has not been performed [Bentley *et al.*, 2017]. Similarly, swine engineered with a patient germline *NFI* gene mutation develop low-grade optic gliomas, as observed in their human counterparts; however, little is known about the role of GAM populations in these tumors [Isakson *et al.*, 2018]. Moreover, human glioma cell lines can be injected into the brains of immunosuppressed pigs as xenograft models [Selek *et al.*, 2014] [Khoshnevis *et al.*, 2017], but the use of immunocompromised animals limits a full analysis of GAM contributions due to secondary defects in microglia function [Pan *et al.*, 2018].

In vitro culture assays have been employed for many years, typically involving established glioma cell lines (*e.g.*, mouse GL261 or human U87 high-grade glioma cells) and freshly isolated microglia or the BV2 microglia-like cell line [S Muller *et al.*, 2017] [Gu *et al.*, 2017]. These platforms *in vitro* are well suited to determine defined functions of defined cell types, such as quantifying chemoattraction by tumor cells or microglia-induced glioma growth and migratory properties, but have only limited value in determining the interactions of these cell types in the natural context of a glioma tissue.

An intermediate model between *in vivo* and cell culture is represented by the organotypic brain slice model, in which rodent glioma cells are introduced into rodent brain slices. This allows for direct visualization of microglia/tumor interactions using fluorescent protein-expressing tumor cells and transgenic mice with microglia-restricted expression of different fluorescent proteins [Bayerl *et al.*, 2016] [Ghoochani *et al.*, 2016] [Resende *et al.*, 2016]. Further refinements of these platforms enable the analysis of microglia and tumor motility using induced models of glioma, rather than using established cell lines [Juliano *et al.*, 2018].

A newer experimental platform is the zebrafish larval brain. The optically transparent zebrafish larva allows for real-time visualizations of the interactions between implanted mammalian (even human) glioma cells and genetically labelled GAM [Astell and Sieger, 2017] [Yan *et al.*, 2019]. Using the *Irf8*^{-/-} zebrafish mutant, which lacks microglia, the contribution of microglia to the tumor growth has been explored [Hamilton *et al.*, 2016]. While the current xenotransplantation models have employed established human glioblastoma cell lines, future modifications may incorporate reporter fish in which gliomas are induced by specific cancer-causing genetic alterations [Chia *et al.*, 2018] [Jung *et al.*, 2013], thus providing a more physiologic context in which to examine the glioma ecosystem. This model may have good potential as an important tool for drug screening and the development of future immunotherapeutic approaches that target microglia.

While these vertebrate models have been very instructive, they do not fully recapitulate their human counterparts. In addition to structural and lifespan differences between rodents and

people, the human brain contains more white matter relative to the mouse brain. For this reason, the generation of human cerebral organoids from induced pluripotent stem cells (iPSCs) has recently been developed as a proxy for the human brain [X Qian *et al.*, 2019], allowing for the implantation of patient-derived glioma stem cells [Linkous *et al.*, 2019] [Hubert *et al.*, 2016]. Alternatively, brain tumors can be generated by genomic editing. For example, CRISPR/CAS9 insertion of an oncogenic *RAS* allele into the *TP53* locus in human iPSCs was used to generate high-grade gliomas following transplantation into immunodeficient animals [Ogawa *et al.*, 2018]. These humanized systems facilitate a close examination of the interactions between glioma cells and the non-neoplastic cells in the human brain.

Outlook

Unfortunately to date, the clinical application of these basic science advances has been modest. In this regard, numerous strategies aimed at silencing GAM function has not translated well to human clinical trials. For example, the tetracycline analog, minocycline, which blocks microglia activation and reduces glioma expansion in experimental glioma mouse models of both high-grade [Markovic *et al.*, 2011] and low-grade [Daginakatte and Gutmann, 2007; Toonen *et al.*, 2017] glioma, has led to three clinical trials with no clear clinical benefit (NCT01580969, NCT02272270, NCT02770378). Similarly, PLX3397-induced depletion of microglia attenuates malignant glioma growth in mice [Pyonteck *et al.*, 2013]; however, no efficacy was observed in human clinical trials [NCT01349036], despite good patient tolerability and adequate blood-brain-barrier penetration [Butowski *et al.*, 2016]. Finally, antibody targeting of microglial TLRs reduces high-grade glioma growth in a mouse brain tumor slice model [Hu *et al.*, 2015] and exhibits good safety and tolerability profiles in healthy subjects [Reilly *et al.*, 2013], but has not progressed further in clinical trials for glioma.

While targeting microglia and macrophages represents a novel and potentially efficacious therapeutic approach, it is possible that adaptive changes in the tumor ecosystem will occur, creating a treatment-induced whack-a-mole scenario. In this regard, microglia can be reprogrammed by radiation [Monje *et al.*, 2002] [Allen *et al.*, 2014] [Monje *et al.*, 2003] and/or chemotherapy [Gibson *et al.*, 2019] to create new functional states, with different abilities to promote tumor growth. Similarly, hypoxia in necrotic areas of high-grade gliomas harbor high levels of extracellular ATP, which could act on microglia expressing purinergic receptors to increase cell motility, phagocytic activity and cytokine release [Kettenmann *et al.*, 2011]. Moreover, it is possible that immunotherapies, like chimeric antigen receptor T cell therapy, will similarly alter microglia dynamics to establish new functional states. For this reason, it becomes exceeding important to understand the complex relationships established between GAM and the other cell types in the tumor in order to develop treatments that disrupt these interactions that support glioma maintenance (Figure 3).

Additionally, it is also vital to consider other factors that could influence microglia function, including brain location and patient sex. To this end, the incidence of malignant glioma is higher in males [Ostrom *et al.*, 2018], but females generally have better overall survival

[Gittleman *et al.*, 2018]. While these sexually dimorphic differences may reflect direct effects on cancer cells, it is equally possible that they act at the level of GAM. Emerging evidence from numerous laboratories have revealed differences in brain microglia gene expression and function in normal male and female mice [Guneykaya *et al.*, 2018; Villa *et al.*, 2018] [Thion *et al.*, 2018], as well as between male and female mice in the setting of a diverse number of different brain diseases, including murine *Nfl* optic glioma microglia-induced neuronal damage and vision loss [Toonen *et al.*, 2017]

Finally, the lack of robust GAM-specific markers has limited our ability to define the distinct contributions of microglia and macrophages to glioma biology. In most studies, CD45 expression by FACS is used to distinguish microglia (CD45^{low}) from peripheral macrophages (CD45^{high}) [Badie and Schartner, 2000], leading to conflicting conclusions regarding the distribution of microglia and macrophages within gliomas. Moreover, microglia increase CD45 expression in the context of glioma [A Muller *et al.*, 2015], whereas macrophages often express markers traditionally associated with microglia following integration into the cancer ecosystem, including downregulation of CD45 and upregulation of CX3CR1 expression [Pong *et al.*, 2013b] [Z Chen *et al.*, 2017]. These adaptations make it difficult to confidently ascribe unique functions to distinct monocyte populations [S Muller *et al.*, 2017]. However, with the availability of more stably expressed markers and the use of spatial proteomics and single cell RNA sequencing strategies [Q Li *et al.*, 2019] [Keren-Shaul *et al.*, 2017] [Masuda *et al.*, 2019], it should become possible to define the individual contributions of these monocyte populations to overall glioma biology [Haage *et al.*, 2019] [Z Chen *et al.*, 2017] [Bowman *et al.*, 2016] [S Muller *et al.*, 2017]. Understanding how distinct subpopulations of microglia/macrophages contribute to glioma pathobiology may also help to resolve conflicting reports in the literature regarding monocyte content and overall patient survival with glioma. Importantly, defining these different GAM species is necessary in order to design effective therapeutic approaches that selectively impair the monocyte populations most critical for tumor maintenance and progression.

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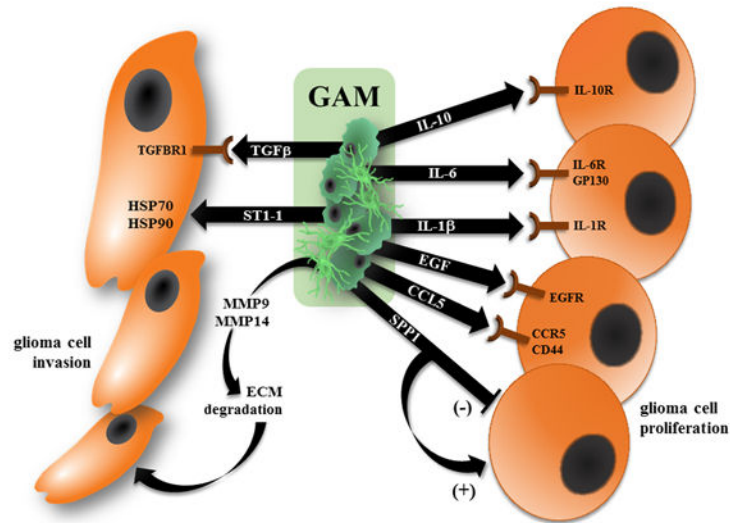


Figure 1. GAM increase glioma growth through the release of mitogens and invasion promoting factors.

Brain microglia and infiltrating peripheral macrophages (GAM) become reprogrammed to produce growth factors that increase glioma cell proliferation, attenuate glioma cell apoptosis, and promote tumor cell migration. In this fashion, GAM produce factors that enhance (IL-10, IL-6, IL-1, EGF, and CCL5) through binding to their cognate receptors (**right**). In addition, SPP1 has been reported to both increase (“+”) and inhibit (“-”) glioma cell growth. Moreover, GAM elaborate other factors that increase extracellular matrix (ECM) degradation and directly promote glioma cell invasion and motility (**left**).

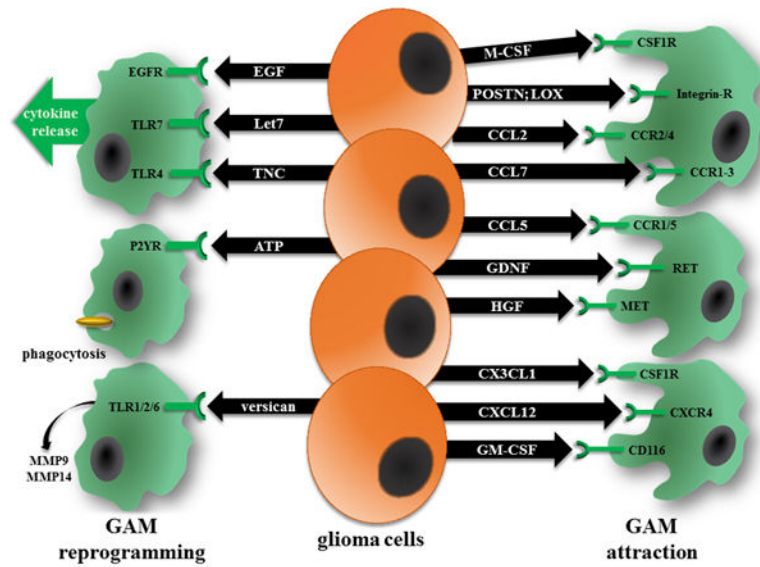


Figure 2. Glioma cells recruit and reprogram GAM through the elaboration of chemokines and other soluble factors.

Chemokines produced by glioma cells actively recruit resident microglia from the brain, as well as macrophages from the blood, through binding to their cognate receptors on GAM (**right**). In addition, glioma cells produce proteins that increase cytokine release (EGF, Let7, and Tenascin-C; TNC), phagocytosis (ATP), and matrix metalloprotease (MMP9, MMP14) expression (versican) (**left**).

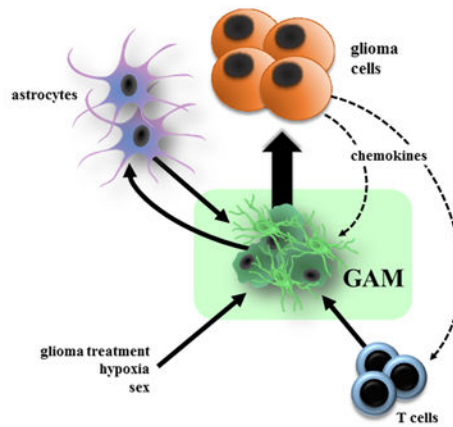


Figure 3. Microglia are reprogrammed by numerous cell types and conditions in the context of glioma.

As highly adaptive cells, GAM gene expression and function can be changed by glioma treatment (chemotherapy, radiation therapy), environmental stresses (hypoxia), interactions with other non-neoplastic cell types (*e.g.*, astrocytes), and signals from the glioma cells themselves. Each of these modifications could alter the properties of GAM in ways that either promote or inhibit continued tumor growth or invasion.