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REVIEW ARTICLE

# Glioblastoma-associated microglia and macrophages: targets for therapies to improve prognosis

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Glioblastoma is the most common and most malignant primary adult human brain tumour. Diagnosis of glioblastoma carries a dismal prognosis. Treatment resistance and tumour recurrence are the result of both cancer cell proliferation and their interaction with the tumour microenvironment. A large proportion of the tumour microenvironment consists of an inflammatory infiltrate predominated by microglia and macrophages, which are thought to be subverted by glioblastoma cells for tumour growth. Thus, glioblastoma-associated microglia and macrophages are logical therapeutic targets. Their emerging roles in glioblastoma progression are reflected in the burgeoning research into therapeutics directed at their modification or elimination. Here, we review the biology of glioblastoma-associated microglia and macrophages, and model systems used to study these cells *in vitro* and *in vivo*. We discuss translation of results using these model systems and review recent advances in immunotherapies targeting microglia and macrophages in glioblastoma. Significant challenges remain but medications that affect glioblastoma-associated microglia and macrophages hold considerable promise to improve the prognosis for patients with this disease.

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**Abbreviation:** GAMM = glioblastoma-associated microglia and macrophage

## Introduction

Glioblastoma comprises the majority of malignant primary adult brain tumours (Ostrom *et al.*, 2014) and has one of the worst survival rates of all cancers (Scott *et al.*, 1999; Krex *et al.*, 2007). The poor prognosis is a product of the transformed cells acting in collusion with a tumour microenvironment (Charles *et al.*, 2012; Zhou and Bao, 2014; Hambardzumyan and Bergers, 2015; Quail *et al.*, 2016) comprised in large part of vascular and stromal cells together with inflammatory infiltrates (Rossi *et al.*, 1987; Hewedi *et al.*, 2013). Glioblastoma-associated microglia and macrophages (GAMMs) predominate this immune infiltrate (Morantz *et al.*, 1979), making them important considerations for tumour biology and therapy. These innate immune cells are meant to participate in tumour surveillance and eradication, but they become compromised by glioblastoma and exploited in the process. In this review,

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we discuss the evidence demonstrating that GAMMs are subverted in glioblastoma. We consider the use and limitations of glioblastoma models, the strategies developed to modify or ablate GAMMs, and the translatability of these approaches to treat the human condition.

# Characteristics of glioblastoma-associated microglia and macrophages

In the healthy individual, microglia and macrophages are the main innate immune cells of the CNS where their primary goal is to maintain homeostasis (Ousman and Kubes, 2012; Michell-Robinson *et al.*, 2015). Microglia and some populations of CNS macrophages originate from precursors in the embryonic yolk sac during development (Ginhoux *et al.*, 2010; Gomez Perdiguero *et al.*, 2015), while monocytes can migrate into the CNS to become macrophages in adulthood following neurological injury. Under threat of infection, the protective roles of microglia and macrophages become apparent where these cells adopt a pro-inflammatory state and secrete inflammatory cytokines (Hanisch, 2002) to mount cytotoxic responses against microbes. They also phagocytose pathogens and dead cells (Chan *et al.*, 2001; Sierra *et al.*, 2013) and participate in tumour surveillance (Jaiswal *et al.*, 2010). Pro-inflammatory microglia and macrophages may be replaced by those that are anti-inflammatory under pathological conditions to promote tissue remodelling, repair, and angiogenesis (Martinez *et al.*, 2009; Rawji *et al.*, 2016), features thought to be supportive of tumour progression (Kennedy *et al.*, 2013; Wei *et al.*, 2013; Hambardzumyan *et al.*, 2016).

Although over-simplified, macrophages have been designated as M1- or M2-polarized cells that correspond to pro-inflammatory and anti-inflammatory responses, respectively (Mills *et al.*, 2000; Gordon, 2003; Martinez *et al.*, 2008). This designation arose from observations on peripheral macrophages exposed to infectious pathogens *in vitro* (Mackaness, 1962; Nathan *et al.*, 1983; Stein *et al.*, 1992; Michelucci *et al.*, 2009) and the use of very specific M1- and M2-inducers (Gordon, 2003). Early studies classified GAMMs together because they are histologically indistinguishable from each other and they both expressed M2 markers such as CD163 and CD204 (Komohara *et al.*, 2008; Prosniak *et al.*, 2013; Sielska *et al.*, 2013). However, multiple attempts since to categorize GAMMs as M2 have failed to establish a robust separation (Szulzewsky *et al.*, 2015; Gabrusiewicz *et al.*, 2016; Mignogna *et al.*, 2016). States such as M2a, M2b, and M2c (Mantovani *et al.*, 2004) were proposed to better fit the intermixed phenotypes GAMMs were displaying, but even then there was little mutual exclusivity between categories (Szulzewsky *et al.*, 2015). This is not surprising because the M1 and M2 definition is based on response to infection, not cancer, and *in vitro* observations of polarity largely do not correspond to *in vivo* observations (Hambardzumyan *et al.*, 2016). Also, unlike T cells and the Th1/Th2 system that the M1/M2 system mirrored, microglia and macrophages do not expand clonally and thus do not give rise to comparably distinct subsets (Martinez and Gordon, 2014). Lastly, resident microglia are molecularly dissimilar to peripherally-recruited macrophages (London *et al.*, 2013; Goldmann *et al.*, 2016). Newer studies are beginning to view GAMMs as separate entities that cannot be conveniently classified into one polarization state (Szulzewsky *et al.*, 2015; Gabrusiewicz *et al.*, 2016). It is perhaps more useful to classify GAMMs as grossly pro-inflammatory/anti-tumour or anti-inflammatory/pro-tumour, although this is still an over-simplification.

The source of GAMMs includes brain-intrinsic microglia that become activated in response to tumour growth, and infiltration of systemic monocytes that mature into macrophages. Flow cytometric studies of human glioblastoma tissue demonstrated that there were more $CD11b^{+}/CD45^{bright}$ (monocyte-derived macrophage) than $CD11b^{+}/CD45^{dim}$ (microglial) cells (Parney *et al.*, 2009). In animal models, human glioma xenografts are highly infiltrated with peripherally-recruited macrophages (Zhou *et al.*, 2015). Furthermore, gliomas in mouse bone marrow chimeras generated with head-protected total body irradiation (to preserve microglia) did not become infiltrated by peripheral monocytes/macrophages until the late exponential growth phase of tumour development (Muller *et al.*, 2015). The $CX3CR1^{GFP/wt}CCR2^{RFP/wt}$ knock-in mouse model has been used to distinguish between microglia ($CX3CR1^{+}$) and peripherally-derived monocytes/macrophages ($CCR2^{+}$) (Saederup *et al.*, 2010). After injecting syngeneic glioma cells into the brains of $CX3CR1^{GFP/wt}CCR2^{RFP/wt}$ mice, both microglia and monocytes/macrophages were found; interestingly, their functions may differ because electrophysiological measurements in brain slices showed inward rectifying currents in microglia, implying a state of immunosuppression relative to macrophages, which had outward rectifying currents (Richter *et al.*, 2014). A caveat of using this knock-in model is that CCR2 is also expressed by T cells and natural killer (NK) cells while subsets of monocytes and macrophages are CCR2 protein-negative (Saederup *et al.*, 2010; Goldmann *et al.*, 2016). Furthermore, following monocyte differentiation into macrophages, CCR2 expression may be downregulated or lost altogether (Fantuzzi *et al.*, 1999). In the future, use of the recently identified microglia-specific transcriptional regulator, Sall1 (Buttgereit *et al.*, 2016), may aid in defining the roles of microglia and macrophages.

Overall, monocyte-derived macrophages and CNS-intrinsic microglia are both represented within glioblastoma multiforme specimens in patients and in models. This has implications for therapy since both CNS-penetrating drugs to target microglia, and peripherally acting medications to affect monocytes, are necessary to affect GAMMs. However, much remains to be investigated as microglia and macrophages, and their pro- and anti-inflammatory subsets, may have different functions in glioblastoma

biology at specific phases of tumour evolution. Lineage-tracing experiments coupled with functional studies in multiple models, and substantiation of findings in human glioblastoma tissue are needed to fully characterize the origin, functions and selective manipulation of GAMMs to improve prognosis for patients with this disease.

A multitude of evidence shows glioblastoma to exert significant influence on microglia/macrophages to suppress their innate anti-tumour functions (Rosales and Roque, 1997; Chicoine *et al.*, 2007; Galarneau *et al.*, 2007; Hwang *et al.*, 2009; Mora *et al.*, 2009; Zhang *et al.*, 2009; Brantley *et al.*, 2010; Wei *et al.*, 2013; Hambardzumyan *et al.*, 2016). When co-cultured with patient-derived glioblastoma stem cells, microglia/macrophages from non-tumour epilepsy brain specimens decreased the proliferation of tumour cells whereas GAMMs permitted tumour growth. Examination of the cytokine profile of epilepsy microglia/macrophage-conditioned media compared to GAMM-conditioned media demonstrated a pro-inflammatory and anti-inflammatory profile, respectively (Sarkar *et al.*, 2014). The time at which microglia/macrophages become immunosuppressed during the course of tumour evolution remains controversial. In a syngeneic murine glioma model, upregulation of anti-inflammatory profiles in GAMMs occurred in the final stages of tumour progression (Kennedy *et al.*, 2009). However, most studies find that microglia/macrophage function is almost immediately altered upon exposure to glioblastoma and its secretome (Dranoff, 2004; Rolle *et al.*, 2012). In addition to innate immune suppression, adaptive immunity is also stifled in glioblastoma (Fig. 1).

Lack of clarity about the timing of microglia/macrophage compromise in glioblastoma is due to differences in the models used, differences between when and how microglia and macrophages are individually affected, and when and how GAMMs are assessed during tumour evolution. Spontaneous mouse models of glioma are best suited to address these questions since tumorigenesis and development are uninterrupted by external manipulation, mirroring the situation in the human disease. Nevertheless, it appears the strategies glioblastoma employs to recruit, immunosuppress, and exploit the invasive and angiogenic capabilities of microglia/macrophages eventually results in a hostile takeover. These strategies (Table 1) and the models used to study them will be explored below and we will discuss strategies to overcome the compromise of GAMMs for therapeutic gain.

## Models used to study glioblastoma-associated microglia and macrophages

Both *in vitro* and *in vivo* models have been developed to study and characterize GAMMs. Cellular models *in vitro* include primary GAMM cultures from GBM tissue, isolation of peripheral monocytes/macrophages, and immortalized cell lines. Primary GAMM cultures are obtained by dissociation of fresh GBM tissue followed by Percoll® density centrifugation and/or cell sorting with the pan-macrophage/microglia surface marker CD11b. These techniques require a large quantity of fresh tumour tissue in order to yield enough GAMMs for experimentation because many cells are lost during processing. In addition, resulting cell cultures are impure because both density centrifugation-derived and CD11b-sorted cultures can contain astrocytes and non-microglia/macrophage cells including activated T cells (McFarland *et al.*, 1992), NK cells (Zhang *et al.*, 2016), and neutrophils (Singhal *et al.*, 2016). The dissociation and isolation process may also alter the activation state of GAMMs. To avoid the labours of obtaining GAMMs, peripheral monocytes/macrophages can be used as substitutes. Peripheral monocytes/macrophages can be easily obtained from human donor blood and from the blood, bone marrow or peritoneum of mice. One common method used obtain peripheral monocytes/macrophages uses cell sorting for the monocyte/macrophage surface protein CD14. Isolated monocytes can be cultured with granulocyte-macrophage colony-stimulating factor (GM-CSF) (Wu *et al.*, 2010; Komohara *et al.*, 2012; Xu *et al.*, 2014; de Vrij *et al.*, 2015) or colony-stimulating factor-1 (CSF-1) (Zhang *et al.*, 2008) to induce a macrophage phenotype. Results derived from cells manipulated by GM-CSF and CSF-1 must be interpreted with caution because these factors can sway macrophages towards pro- and anti-inflammatory phenotypes, respectively (Nakagawa *et al.*, 2007; Komohara *et al.*, 2012; Xu *et al.*, 2014). Furthermore, the translatability of results obtained using peripheral cells may be limited because the transcriptomic signatures of peripheral monocytes/macrophages and CNS microglia and macrophages differ (Beutner *et al.*, 2013; Hickman *et al.*, 2013; Goldmann *et al.*, 2016). Immortalized cell lines such as HMO6 (human) and BV-2 (murine) are used to model macrophages and microglia (Carson *et al.*, 2008). An important limitation of immortalized cell lines is the presence of oncogenes that affect gene expression and phenotype (Righi *et al.*, 1989; Horvath *et al.*, 2008; Stansley *et al.*, 2012). Overall, besides the aforementioned limitations, studies using cells cultured from patients have the additional confounder that the microglia and macrophages are no longer in the tumour microenvironment that imprints their *in vivo* characteristics.

Murine models are also commonly used to examine GAMMs. Mice that develop spontaneous gliomas can be achieved after modification of genes implicated in tumorogenesis (Aguzzi *et al.*, 1995). For example, some of the transgenic mouse models available express mutant epidermal growth factor receptor (EGFR) in glial cells on a tumour suppressor-deficient genetic background (Holland *et al.*, 1998), or conditionally express TP53 (Zhu *et al.*, 2005) or phosphatase and tensin homolog (PTEN) (Kwon *et al.*, 2008) alleles. However, the genetic alterations required to spontaneously generate tumours can interfere with lymphopoiesis and clonal expansion, processes necessary for host immune function (Sughrue *et al.*, 2009). Also,

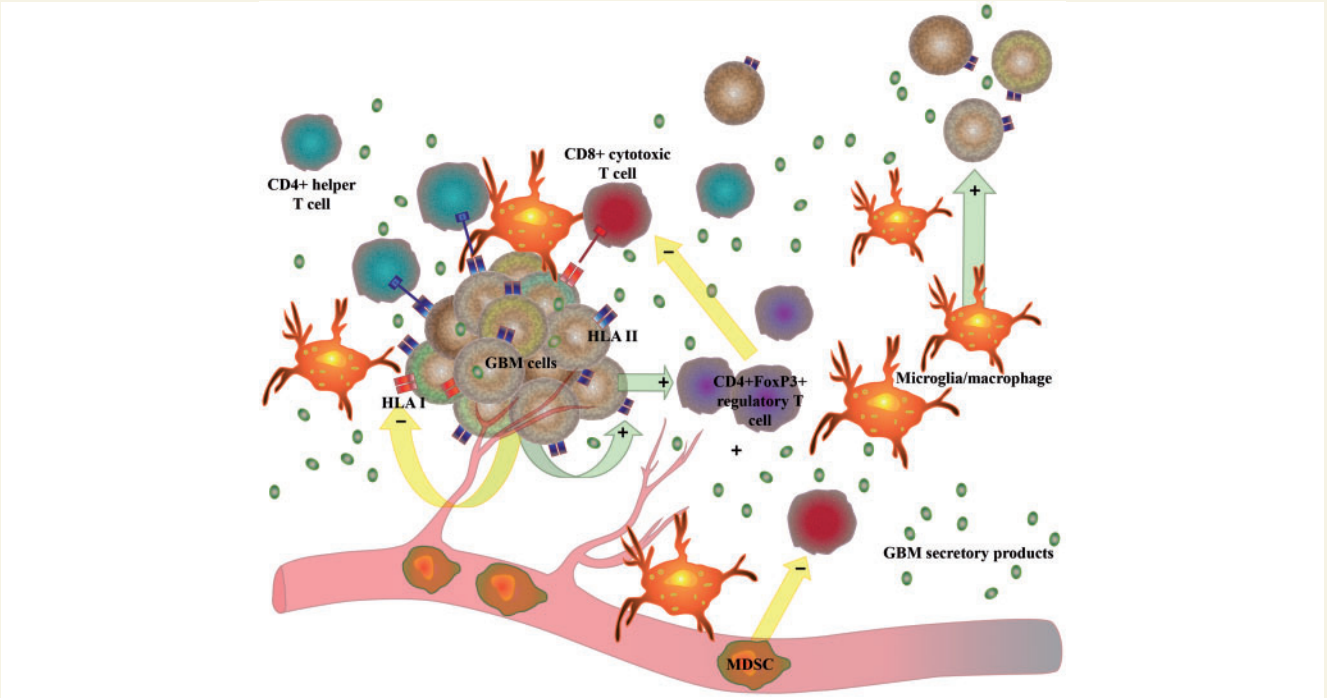


**Figure 1 The immune landscape created by glioblastoma.** Microglia and macrophages are co-opted by glioblastoma and its secretory products to enhance tumour progression. HLA I antigens are downregulated while HLA II antigens are upregulated, enhancing the response of CD4+ helper T cells, which do not inhibit tumour growth. The regulatory T cell subset which suppresses T cell activation is also upregulated. Glioblastoma (GBM) patients exhibit lymphopenia and the ability of their circulating monocytes to differentiate are hindered. Myeloid-derived suppressor cells are also upregulated. HLA I = human leukocyte antigen class I; MDSC = myeloid-derived suppressor cell.

**Table 1 Factors promoting glioblastoma progression**

| Function | Factor or axis | Reference |
|---|---|---|
| GAM/M recruitment | CCL2 | Platten *et al.* (2003) |
| | CCL7 | Okada *et al.* (2009) |
| | CSF-1 | Alterman *et al.* (1994); Komohara *et al.* (2008); da Fonseca *et al.* (2013) |
| | CX3CL1/CX3CR1 | Held-Feindt *et al.* (2010) |
| | CXCL12/CXCR4 | Rempel *et al.* (2000); Wang *et al.* (2012) |
| | Ecrg4 | Lee *et al.* (2015) |
| | POSTN | Zhou *et al.* (2015) |
| | VEGF | Forstreuter *et al.* (2002); Johansson *et al.* (2002) |
| Immunosuppression of GAM/Ms | IL-6 | Zhang *et al.* (2012) |
| | MIC-1 | Shnaper *et al.* (2009) |
| | MIF | Ghoochani *et al.* (2016) |
| | STAT3 | Penuelas *et al.* (2009); Lin *et al.* (2014); Peixoto *et al.* (2016) |
| | TGF-β | Paulus *et al.* (1995) |
| GAM/M enhancement of angiogenesis | CXCL2 | Brandenburg *et al.* (2016) |
| | IGFBP1 | Nijaguna *et al.* (2015) |
| | IL-6 | Chen *et al.* (2014) |
| | VEGF | Brandenburg *et al.* (2016) |
| GAM/M enhancement of invasion | CSF-1/CSF-1R | Yamaguchi *et al.* (2006); Coniglio *et al.* (2012) |
| | MMPs | Zhao *et al.* (2012); Munaut *et al.* (2003); Guo *et al.* (2005); Coniglio and Segall (2013) |
| | Pyk2 | Rolon-Reyes *et al.* (2015) |
| | TβIIR | Wesolowska *et al.* (2008) |

CCL2 = C-C motif chemokine ligand 2; CCL7 = C-C motif chemokine ligand 7; CSF-1 = colony stimulating factor 1; CSF-1R = colony stimulating factor receptor 1; CX3CL1 = C-X3-C motif chemokine ligand 1; CX3CR1 = C-X3-C motif chemokine receptor 1; CXCL12 = C-X3-C motif chemokine ligand 12; CXCL2 = C-X3-C motif chemokine ligand 2; CXCR4 = C-X3-C motif chemokine receptor 4; Ecrg4 = esophageal cancer-related gene 4; IGFBP1 = insulin-like growth factor-binding protein 1; IL-6 = interleukin-6; MIC-1 = macrophage inhibitory cytokine 1; MIF = macrophage migration inhibitory factor; MMPs = matrix metalloproteinases; POSTN = periostin; Pyk2 = proline rich tyroskine kinase 2; STAT3 = signal transducer and activator of transcription 3; TGF-β = transforming growth factor-beta; TβIIR = TGF-beta type II receptor; VEGF = vascular endothelial growth factor.

a large number of animals are required to generate enough tumours for experimentation (Oh *et al.*, 2014). Thus, although spontaneous tumours in immunocompetent hosts may better represent the human condition (Oh *et al.*, 2014), they are infrequently used or used in conjunction with other mouse models especially in the context of immunological research (Pyonteck *et al.*, 2013; Quail *et al.*, 2016).

Orthotopic models, where tumorigenesis is achieved by surgical implantation of grafts into the brain of recipient animals, have been another common approach to study GAMMs. Human or syngeneic cells can be implanted, but the former necessitates use of an immunodeficient host. There is an obvious danger to interpreting data about immune elements when hosts have abnormal immunity. Implanted human glioma lines can be long-term established lines, such as U87, or they may be patient-derived glioma stem cells that can replicate the original tumour more faithfully (Davis *et al.*, 2016). The most popular syngeneic glioma mouse model involves implantation of glioma 261 (GL261) cells into C57BL/6 mice (Maes and Van Gool, 2011). Its popularity has made it the most well-characterized murine glioma model, heightening its attractiveness to researchers despite its histological resemblance to ependymoblastoma instead of glioblastoma (Ausman *et al.*, 1970; Jacobs *et al.*, 2011). However, GL261 does share some genetic abnormalities in common with glioblastoma such as TP53 alteration (Szatmari *et al.*, 2006).

In sum (Fig. 2), even though murine models are the workhorses of GAMM research and results therein have been translated into clinical trials, the success of those trials has been limited (De Vleeschouwer *et al.*, 2008; Sampson *et al.*, 2008, 2009; Waziri, 2010; Binder *et al.*, 2016). This is likely because the complex immune milieu of the human condition is poorly reflected by murine models, as evidenced by the disparate upregulation of genes associated with immune activation between human and murine GAMMs (Szulzewsky *et al.*, 2016). Whenever possible, human glioblastoma tissue should be included for experimentation to corroborate results.

# Strategies to modulate microglia and macrophage activity in glioblastoma

## Therapies to reduce or exploit the recruitment of tumour promoting glioblastoma-associated microglia/macrophages

Numerous macrophage chemoattractants have been found in human glioblastoma samples, helping to recruit microglia/macrophages into the tumour. The source of these chemoattractants is a matter of debate, but both glioblastoma cells and T cells have been implicated (Leung *et al.*, 1997). The majority of studies view microglia/macrophage recruitment as pro-tumour (Yang *et al.*, 2010). For example, the CX3CL1/CX3CR1 chemokine axis elicited adhesion and migration of primary human GAMMs, and increased the expression of matrix metalloproteinase (MMP) 2, MMP9, and MMP14 (Held-Feindt *et al.*, 2010), enzymes that degrade extracellular matrices and are implicated in tumour invasiveness. Also, it was shown with orthotopic glioma stem cell xenografts that periostin secreted by tumour cells specifically supported the recruitment of anti-inflammatory and consequently pro-tumour monocyte-derived macrophages, a result validated with immunohistochemistry on human glioblastoma tissue, which showed more $CCR2^{+}$ cells in the tumour infiltrate (Zhou *et al.*, 2015).

Another strategy is to block the chemoattractant receptors or ligands. To this end, a phase I/II study investigating plerixafor, a CXCR4 chemokine receptor antibody, in the treatment of newly diagnosed high grade glioma patients is currently recruiting (NCT01977677). Another CXCR4 antagonist boasting more selectivity for this receptor is peptide R (Mercurio *et al.*, 2016). Whereas plerixafor mainly targets the migration of myeloid cells towards glioblastoma, peptide R has additional effects against tumour cell metabolism and proliferation (Mercurio *et al.*, 2016). In a study which showed that different mechanisms were behind microglial and macrophage recruitment, the administration of propentofylline [which reduces tumour necrosis factor receptor superfamily member 19 expression (Jacobs *et al.*, 2012*b*)] decreased MMP9 expression and microglial migration towards tumour cells while having no effect on macrophages (Jacobs *et al.*, 2012*a*). Additionally, C-C motif chemokine ligand 2 (CCL2) is produced by the glioblastoma microenvironment and promotes innate immune cell recruitment (Platten *et al.*, 2003). Older drugs such as minocycline, telmisartan, and zoledronic acid that are used for treating infection, hypertension, and osteoporosis, respectively, have been demonstrated to reduce the synthesis of CCL2 (Salacz *et al.*, 2016). This regimen will soon be administered in a pilot clinical trial in primary glioblastoma (Salacz *et al.*, 2016). Finally, several research groups have attempted to exploit the recruitment of microglia/macrophages by glioblastoma. One tactic is to repurpose recruitment as a vector of drug delivery. For example, the use of murine macrophages as nanoshell carriers into human glioma spheroids for subsequent photothermal ablation has been investigated *in vitro* (Baek *et al.*, 2011).

## Therapies to manipulate immunosuppressed glioblastoma-associated microglia/macrophages

High expression of many potently anti-inflammatory and immunosuppressive factors such as IL-6 (Zhang *et al.*, 2012) and transforming growth factor (TGF)-β (Paulus

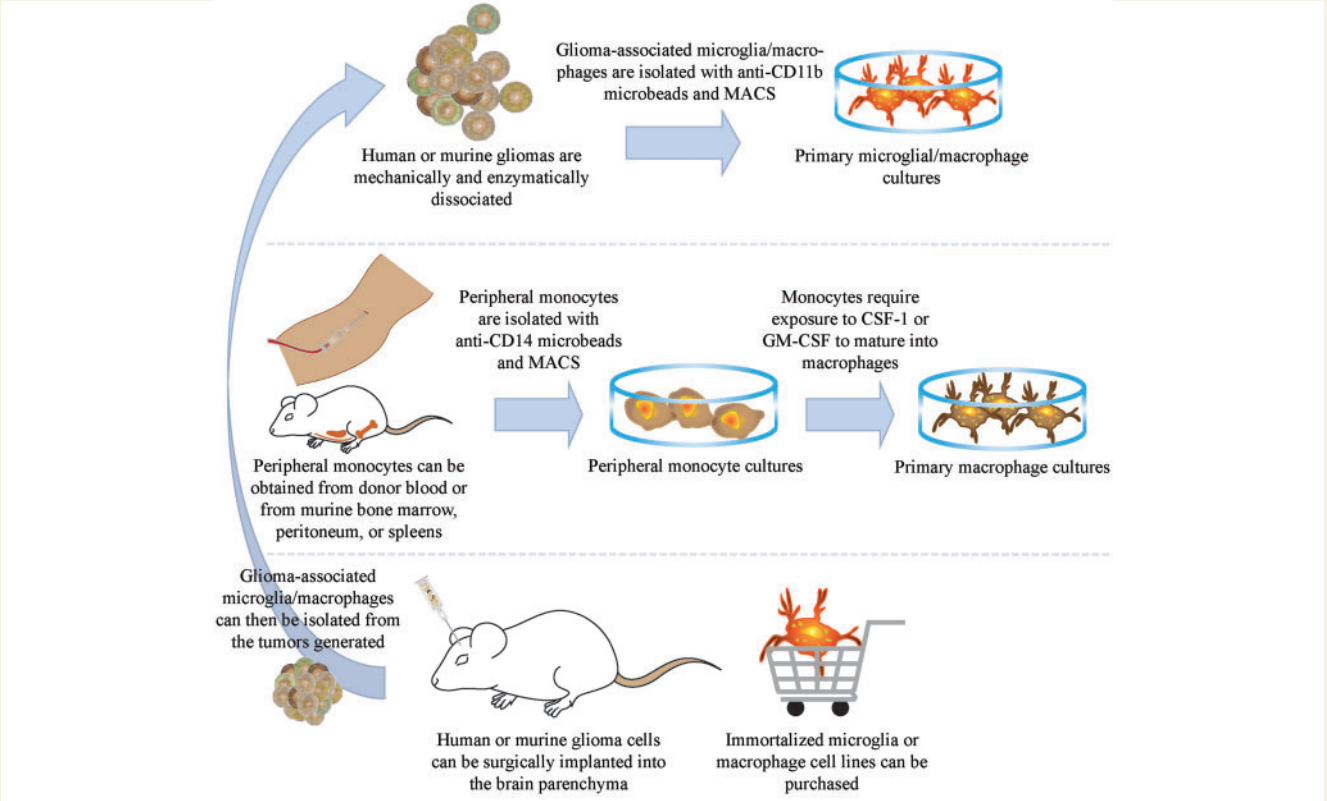


**Figure 2 Models for obtaining glioma-associated microglia/macrophages.** Primary microglial and/or macrophage cultures can be obtained from human or murine tissue. These may originate from CNS or peripheral sources. *In vivo* models commonly involve intracranial implantation of human or murine glioma cells. CSF-1 = colony-stimulating factor 1; GM-CSF = granulocyte macrophage colony-stimulating factor; MACS = magnetic-activated cell sorting.

*et al.*, 1995) have been found at the mRNA and protein level in human glioblastoma. Notably, expression of IL-10, touted as the most powerful immunosuppressive cytokine (Perng and Lim, 2015), has not been confirmed at the protein level in human glioblastoma although there is high mRNA expression and its secretion has been induced *in vitro* by exposing human GAMM to other immunosuppressive cytokines (Hussain *et al.*, 2006; Wu *et al.*, 2010). In contrast, there is minimal to no expression of interferon-$\gamma$, tumour necrosis factor (TNF)-$\alpha$, IL-2 or IL-12 (Hao *et al.*, 2002; Hussain *et al.*, 2006; Penuelas *et al.*, 2009), powerful pro-inflammatory cytokines. Indeed, an important signalling pathway activated by TNF-$\alpha$, NF$\kappa$B, is downregulated in GAMMs compared to low grade glioma-associated microglia/macrophages, leading to reduced expression of inflammatory Toll-like receptor signalling (Mieczkowski *et al.*, 2015). Phagocytic capacity of GAMMs is reported to be low (Wu *et al.*, 2010). Primary human GAMMs also have downregulated expression of CD40, CD80, and CD86, molecules necessary for antigen presentation to T cells (Hussain *et al.*, 2007). Additionally, glioblastoma induces GAMMs to secrete many of the same anti-inflammatory factors it already produces (Wu *et al.*, 2010). Visualization of the interactions between glioma cells and GAMMs in transgenic mice found that upon contact GAMMs change from a ramified to amoeboid shape (Resende *et al.*, 2015), suggesting that the tumour cells initially activate GAMMs but that this activation does not culminate in the adoption of anti-tumour functions.

Of the cytokines found in human glioblastoma, TGF-β plays a salient immunosuppressive role by inhibiting lymphocyte and microglia activation, proliferation, and antigen presentation (Suzumura *et al.*, 1993; Letterio and Roberts, 1998). Additionally, TGF-β enhances tumorigenicity by promoting vascular endothelial growth factor (VEGF) and MMP9 expression (Watters *et al.*, 2005). Unfortunately, TGF-β modulation has been difficult because of its multiple sources and targets. Despite numerous *in vivo* murine studies demonstrating tumour control with TGF-β inhibition (Uhl *et al.*, 2004; Liu *et al.*, 2007; Ueda *et al.*, 2009; Hulper *et al.*, 2011), trabedersen, a TGF-β inhibitor, was disappointing in phase II trials (Bogdahn *et al.*, 2011).

Recent attention has been paid to signal transducer and activator of transcription 3 (STAT3) (de la Iglesia *et al.*, 2009; Sherry *et al.*, 2009; Zhang *et al.*, 2009; Fujiwara *et al.*, 2011; Luwor *et al.*, 2013; Priester *et al.*, 2013; Peixoto *et al.*, 2016). Not only does this transcription factor reduce CD80, CD86, and MHC II molecules in

GAMMs (Kortylewski *et al.*, 2005), it also is key for glioblastoma growth (Li and Graeber, 2012). STAT3 inhibition via intratumoral injection of short interfering (si)RNA in GL261-bearing mice resulted in activation of GAMMs as indicated by increased TNF-$\alpha$ expression and increased animal survival (Zhang *et al.*, 2009). Currently there is a registered phase I trial investigating WP1066, a STAT3 inhibitor, in recurrent glioblastoma (NCT01904123).

Instead of targeting one cytokine or transcription factor, a promising avenue of GAMM-directed therapy involves swaying the overall immunosuppressed phenotype towards immunostimulation. Amphotericin B has been shown to stimulate previously tumour-permissive human GAMMs to inhibit the growth of human glioblastoma stem cells in culture and *in vivo* in immunodeficient mice (Sarkar *et al.*, 2014). Similarly, polyinosinic-polycytidylic acid [poly (I:C)], a Toll-like receptor 3 agonist, has been shown to induce a strong pro-inflammatory response in primary human GAMMs that leads to the inhibition of tumour growth and invasion (Kees *et al.*, 2012). Used as an immune adjuvant, poly (I:C) has shown promise in phase I/II trials (Butowski *et al.*, 2009; Rosenfeld *et al.*, 2010). The use of NK cells in combination with antibodies directed against neuroglial-2, a transmembrane chondroitin sulphate proteoglycan implicated in glioblastoma progression has also been investigated using primary human glioblastoma xenografts. This combination treatment skewed GAMMs from an anti-inflammatory to pro-inflammatory phenotype that resulted in decreased tumour size, a finding that was abolished when peripherally-recruited macrophages were selectively depleted (Poli *et al.*, 2013), thereby highlighting the disparate response microglia and macrophages can have to the same therapy.

## Therapies to mitigate glioblastoma-associated microglia and macrophage enhancement of glioma invasion

Another major role GAMMs play in tumour promotion is enhancement of glioblastoma invasion through modulating MMPs. Human GAMMs upregulated *MMP2* and *MMP9* mRNA expression in response to exogenously administered CX3CL1 *in vitro* (Held-Feindt *et al.*, 2010). Knockout of MMP14 in GAMM decreased GL261 tumour size (Markovic *et al.*, 2009). Primary human glioma stem cells co-cultured with GAMMs prior to orthotopic implantation in NOD-SCID mice were more invasive than naïve glioma stem cells and this was correlated to the upregulation of MMP9 in the tumour cells themselves (Ye *et al.*, 2012). Unfortunately, translation of MMP-based therapies into clinical trials has not been successful. A phase II trial using marimastat, a broad spectrum MMP inhibitor, showed no additional benefit when it was used with temozolomide in recurrent anaplastic gliomas (Groves *et al.*, 2006). Instead of using MMP inhibitors as anti-glioblastoma therapies, attention has now turned to using MMP levels as biomarkers of prognosis (NCT01493219, NCT00083512).

Colony-stimulating factor-1 receptor (CSF-1R) is another invasion-associated molecule that has received immense attention. CSF-1R appears crucial for normal microglial function (Imai and Kohsaka, 2002; Erblich *et al.*, 2011; Elmore *et al.*, 2014) while its ligand, CSF-1, increases GAMM density (De *et al.*, 2016). GAMMs secrete epidermal growth factor (EGF) and express CSF-1R, while glioblastoma cells express EGFR and secrete CSF-1 to create a paracrine loop similar to that found in breast and other cancers (Yamaguchi *et al.*, 2006; Coniglio *et al.*, 2012). Pharmacological inhibition of EGFR and blockade of CSF-1R in co-cultures of murine microglia/macrophages and GL261 cells abrogated GAMM enhancement of invasion (Coniglio *et al.*, 2012). *In vivo*, it was shown that administration of PLX3397, a CSF-1R inhibitor, could reduce recruitment of GL261-associated microglia/macrophages and invasion (Coniglio *et al.*, 2012). Similarly, use of another CSF-1R inhibitor, BLZ945, blocked progression of intracranial xenografts of conventional human glioma cells by promoting GAMM anti-tumour gene expression (Pyonteck *et al.*, 2013). Interestingly, this study suggested that GAMM depletion was not achievable with CSF-1R inhibition unlike originally thought (Elmore *et al.*, 2014) since normal microglia/macrophages were depleted instead of GAMMs (Pyonteck *et al.*, 2013). A phase II study investigating the use of PLX3397 in patients with recurrent glioblastoma has recently been completed showing safety but no efficacy (Butowski *et al.*, 2016). CSF-1R inhibition is being tested in several other clinical trials, but it has been shown in transgenic and human glioblastoma xenograft mouse models that resistance to this therapy inevitably arises (Quail *et al.*, 2016).

## Therapies directed at glioblastoma-associated microglial and macrophage-mediated angiogenesis

Interrelated with invasion is angiogenesis. The evidence shows that there is intimate rapport between GAMMs and tumour vasculature. At the mRNA level, GAMMs isolated from GL261 gliomas overexpressed pro-angiogenic molecules such as VEGF and CXCL2 (Brandenburg *et al.*, 2016). Depletion of resident microglia specifically resulted in reduced tumoral vessel counts similar to that observed with total myeloid cell ablation, suggesting that microglia have a more salient role in angiogenesis than monocyte-derived macrophages (Brandenburg *et al.*, 2016). One of the first studies to capture the dynamic relationship between GAMMs and glioma blood vessels used intravital microscopy and GL261-bearing mice to show that GAMMs adopted highly motile phenotypes in the perivascular area compared to other areas of the tumour,

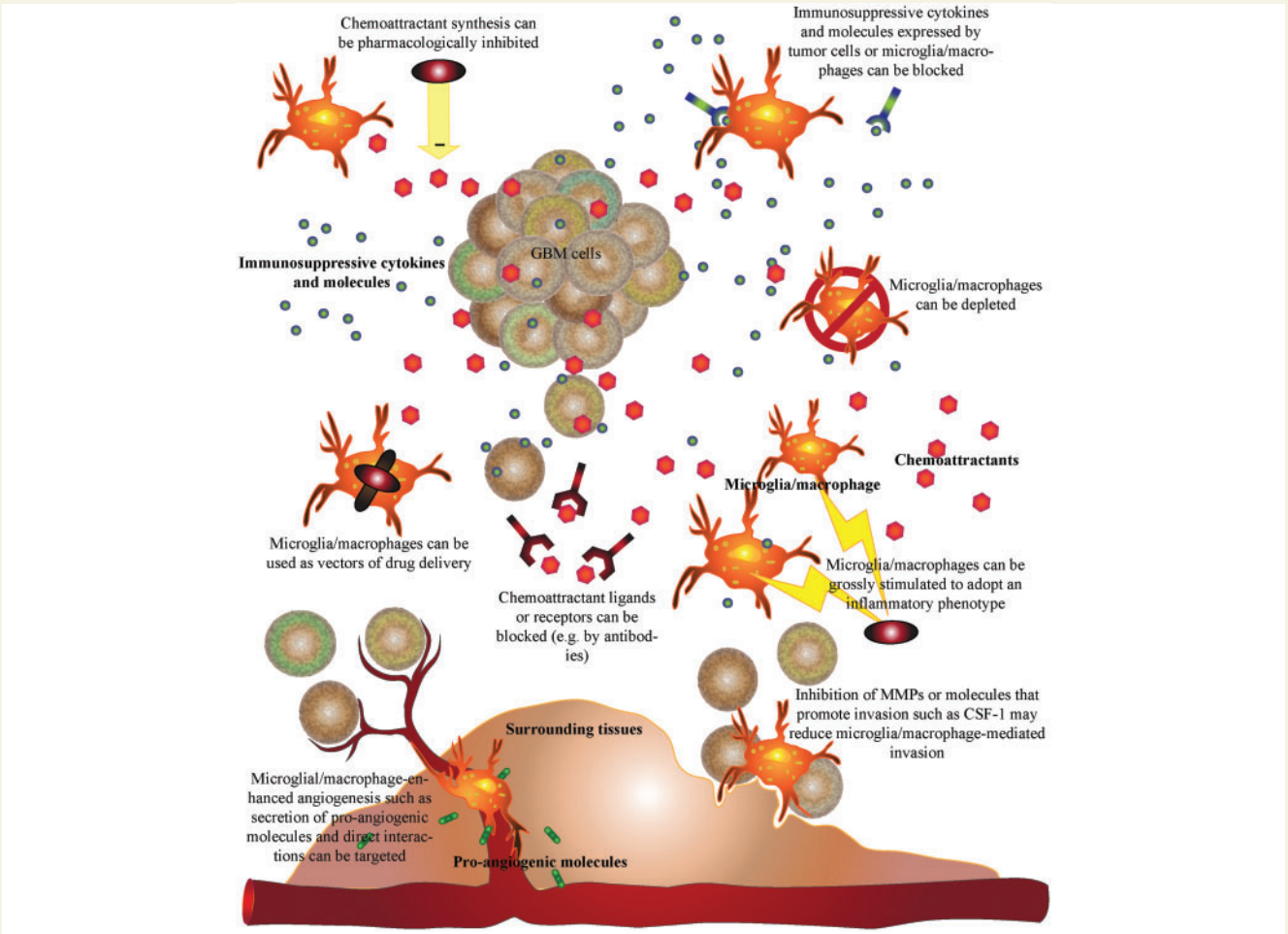


**Figure 3 Strategies to target glioblastoma-associated microglia/macrophage promotion of tumour progression.** As much as there are multiple ways microglia/macrophages enhance the immunosuppression, invasion, and angiogenesis of glioblastoma, there are methods to target these same processes. Exploiting or decreasing the recruitment of microglia/macrophages, decreasing their immunosuppression, mitigating their contribution to invasion and angiogensis, and depleting them altogether are therapeutic strategies. CSF-1 = colony stimulating factor 1; GBM = glioblastoma multiforme; MMPs = matrix matelloproteinases.

signifying increased interaction (Bayerl *et al.*, 2016). A knockout mouse for the receptor for advanced glycation end products (RAGE) was developed to show that IL-6 and VEGF expression was suppressed in GL261-associated microglia/macrophages with RAGE ablation, as was angiogenesis (Chen *et al.*, 2014). In brain autopsy specimens from patients with recurrent glioblastoma, antiangiogenic therapy increased the number of GAMMs, which correlated with poorer overall survival and suggested that GAMMs were contributing to glioblastoma escape from antiangiogenic therapies (Lu-Emerson *et al.*, 2013).

In efforts to overcome the resistance to therapies directed against the VEGF pathway, administration of A2V, a bispecific antibody to VEGF and Ang-2, another pro-angiogenic factor, was investigated in both the GL261 and human glioma stem cell xenograft mouse models. Interestingly, divergent results were observed between the syngeneic and xenograft model, with less tumour growth inhibition and a lack of antivascular effects in the latter. Moreover, when examining the effect of A2V on microglia and macrophages, macrophages were the main cell population to be swayed towards an inflammatory phenotype in the syngeneic model whereas in the xenograft model microglia were chiefly affected (Kloepper *et al.*, 2016). In addition to determining which pro-angiogenic factors have the largest influence on innate immune cells, defining the potentially dissimilar roles microglia and peripherally-recruited macrophages play in angiogenesis promotion will be important for developing appropriately targeted therapies.

## Depletion of glioblastoma-associated microglia and macrophages

Given the many pro-tumour functions of GAMMs (Table 1), their depletion is considered by some to be an anti-glioblastoma treatment (Fig. 3). There are several ways to achieve reduction of GAMMs in murine models.

Administration of gancicyclovir to transgenic CD11b-herpes simplex virus thymidine kinase mice reduces the CD11b+ population (Heppner *et al.*, 2005). Using this depletion method, tumour volume and vascularization in GL261-bearing mice were decreased (Brandenburg *et al.*, 2016). Clodronate-filled liposomes can also selectively deplete GAMMs (Markovic *et al.*, 2005), although it should be noted a peripheral instead of central site of administration may selectively deplete monocyte-recruited macrophages to the exclusion of resident microglia (Poli *et al.*, 2013). In cultured brain slices, injected GL261 cells were less invasive when GAMMs were depleted using directly applied clodronate-filled liposomes but regained their invasive capabilities when GAMMs were restored (Markovic *et al.*, 2005). Another depletion study using the same model attributed invasion to MYD88-driven expression of MMP14 (MT1-MMP) in GAMMs (Markovic *et al.*, 2009). A limitation of these depletion protocols is that microglia/macrophage reduction occurs prior to glioma cell implantation (De *et al.*, 2016). Thus, the translatability of findings is jeopardized because gliomagenesis in the absence of innate immune components is undoubtedly different than when these components are present. *In silico* microglia depletion protocols where microglial cells are programmed to undergo rapid apoptosis in virtual patients based on real patient parameters (Wu *et al.*, 2012) avoids these confounds. This study showed that therapeutic benefit would only be achieved if GAMM-depleting therapies were given early in the disease course when tumour cell burden was still relatively low (Wu *et al.*, 2012).

Considering the many roles GAMMs play in the glioblastoma microenvironment, it is crucial to develop and use models more representative of the human condition to determine which combination of GAMM-directed therapies is the most effective, and to hone the sensitivity and specificity of such therapies to either microglia, macrophages, or both.

# Conclusion

Despite the abundance of preclinical trials conducted to identify novel, effective therapies for glioblastoma, translation into actual clinical benefit has been rare (Rolle *et al.*, 2010; Frosina, 2015; Preusser *et al.*, 2015). *In vitro* and *in vivo* investigations have contributed substantially to our understanding of GAMM biology, but it is still unclear which GAMM-directed therapies hold the most clinical promise. Clarifying the common or dissimilar roles that resident microglia and peripherally-recruited macrophages play in tumour progression and resolving the timeline during which GAMM adopt pro-tumour phenotypes will help to improve GAMM-directed therapies and their potential for incorporation into current treatment regimens. Validating glioblastoma models and intensifying GAMM research will increase our understanding of the immune landscape in glioblastoma. Moreover, simultaneously rejuvenating the compromised adaptive immune cells in glioblastoma will constitute a multi-pronged immune approach to control brain tumour growth. We conclude that engaging immune cells, particularly GAMMs, in the microenvironment of glioblastoma will lead to novel therapies that improve the outcome of patients suffering from this terrible neurological disease.

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