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.

Department of Clinical Neurosciences, University of Calgary, Calgary, Alberta, Canada

Correspondence to: John J. P. Kelly
Department of Clinical Neurosciences,
Foothills Medical Centre 1403 29th Street NW Calgary,
AB, CA T2N 2T9,
Canada
E-mail: jjkelly@ucalgary.ca

Keywords: glioma; glioblastoma; microglia; macrophages; immunotherapy

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,
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., 2013), 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+/CD45bright (monocyte-derived macrophage) than CD11b+/CD45dim (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 CX3CR1GFP/wtCCR2RFP/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 CX3CR1GFP/wtCCR2RFP/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 down-regulated or lost altogether (Fantuzzi et al., 1999). In the future, use of the recently identified microglia-specific transcriptional regulator, Sall1 (Buttgereit et al., 1999), 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 tumorigenesis (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

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

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 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; MIF = macrophage inhibitory cytokine 1; MMPs = matrix metalloproteinases; POSTN = periostin; Pyk2 = proline rich tyrosine kinase 2; STAT3 = signal transducer and activator of transcription 3; TGF-β = transforming growth factor-beta; TjiiIR = 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 (Sztamari 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 xenographs that peristin 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., 2012b)] decreased MMP9 expression and microglial migration towards tumour cells while having no effect on macrophages (Jacobs et al., 2012a). 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...
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-γ, tumour necrosis factor (TNF)-α, 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-α, NFKB, 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-α 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 neuralglial-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 EGF 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 intravitral microscopy and GL261-bearing mice to show that GAMMs adopted highly motile phenotypes in the perivascular area compared to other areas of the tumour,
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 ganciclovir to transgenic CD11b-negative simple 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 microglial/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.

**Funding**

We thank the Alberta Innovates - Health Solutions and Alberta Cancer Foundation Team program for support of operating funds. C.C.P. is supported by a Queen Elizabeth II Graduate Scholarship and studentships from the University of Calgary Faculty of Medicine.

**References**


Muller A, Brandenburg S, Turkowski K, Muller S, Vajkoczy P. Resident microglia, and not peripheral macrophages, are the main source of brain tumor mononuclear cells. Int J Cancer 2013; 137: 278–88.


