Glioblastoma Therapy in the Age of Molecular Medicine

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1.   Abstract

Glioblastoma (GBM) is the most common and fatal primary malignant brain tumor. Despite advances in the understanding of the biology of gliomas, little has changed in the treatment of these tumors in the past decade. After phase III clinical trials showed no benefit for the use of Bevacizumab in newly diagnosed patients, there is renewed search for new anti-angiogenic drugs, as well as immunotherapeutic approaches, including checkpoint inhibitors, Chimeric Antigen Receptor T cells and intracerebral CpG-oligodeoxynucleotides. The emerging role of infiltrating microglia and macrophages, and of metabolic alterations is also being taken into account in pre-clinical research and drug development. In this review, we discuss progress in the search for new therapeutic strategies, in particular approaches focusing on the tumor microenvironment.

2.   Keywords: Glioblastoma, Cancer Therapy, Immunotherapy, Microglia, Angiogenesis, Cancer Metabolism.

3.   Glioblastoma's heterogeneity and the search for new therapeutics

GBM is the most common and deadliest primary malignant tumor of the central nervous system (CNS), with an incidence rate of 3.20 cases per 100,000 habitants and a 5-year survival rate of 5.5% in the United States (US) [1].

According to the World Health Organization (WHO) classification, these tumors are grade IV diffuse astrocytic tumors presenting nuclear atypia, cellular pleomorphism and intense mitotic activity associated with necrosis and microvascular proliferation [2, 3]. This traditional approach to the diagnosis of the CNS tumors was based mostly on histopathological criteria established in the 1920's by the classification system developed by Bailey and Cushing [4]. In accordance with this criteria, tumors should be classified following the histological similarity to the tissues of supposed origin and highlighted by specific microscopic features using lineage specific phenotypic markers [2]. This histological classification and the grading system have been officially used for almost a century because they succeeded in correlating with prognosis and have remained relatively helpful in determining which treatment should be dispensed to the patients, despite some incongruities. However, the great heterogeneity of those tumors often leads to multiple types of diagnosis, reflected by the inter-observer divergences on the final classification [5].

Given these challenges and the increasing knowledge about glioma's biology, in 2016, WHO changed the Central Nervous System (CNS) Tumors Classification to a molecular categorization based on genetic and epigenetic features [3]. Nevertheless, most of the changes were in the diagnosis of low-grade gliomas, now mostly based on genetic alterations rather than morphological status:

Gliomas bearing the 1p/19q codeletion should be classified as oligodendrogliomas [6, 7]. Diffuse astrocytomas bearing the Isocitrate Dehydrogenase (IDH) mutations but lacking the 1p/19q codeletion should be classified merely as astrocytoma. Astrocytoma diagnosis also relies on the presence of two other mutations, at the TP53 gene and ATRX [3]. Both are mutually exclusive to 1p19q codeletion and, if they are present in IDH-mutant gliomas, the diagnosis of oligodendroglioma is immediately excluded [8-10].

The GBMs are the class that has changed the least in this new classification: their diagnosis remains mostly histological but IDH-wild-type tumors correspond to clinically-defined primary or *de novo* GBM, and those bearing the mutant IDH isoform are designated as secondary GBM, meaning they progressed from low-grade gliomas to GBM [11].

The origin of GBMs is still a matter of debate. Initially, it was suggested that astrocytes, the only cells known to replicate in the adult brain, would suffer oncogenic alterations and give rise to GBM [12]. Other hypothesis suggests that these tumors may derive from precursors, such as Neuronal or oligodendrocyte Progenitor Cells (NPCs or OPCs) [13, 14], but the cellular heterogeneity of GBM makes it extremely hard to precisely identify its cell of origin. More recently, glioma stem cells (GSCs) with self-renewal ability and capability to form tumors *in vivo* with the same characteristics from the primary tumor were also identified [15, 16]. Although those are tumor-propagating cells (and not tumor-initiating), their presence has been associated with heterogeneity of these tumors, since they can divide and differentiate asymmetrically and be more resistant to conventional chemotherapy [17]. In GBM we can distinguish between intertumoral and intratumoral heterogeneity. The first refers to the distinct genetic alterations that occur in individual tumors, allowing molecular subgroup classification. On the other hand, intratumoral heterogeneity refers to the diversity within individual tumors. It is related both to the diversity of tumor cellular phenotypes that compose the tumor mass [18, 20-22] and to the other cellular entities recruited to the tumor microenvironment, such as microglia/macrophages and endothelial cells [23-25].

Currently, standard therapy for newly diagnosed GBM patients consists of: maximal safe surgical resection + Standard or Hypofractionated brain radiotherapy (RT) + concurrent and adjuvant temozolomide (TMZ) + Alternating Electric Field Therapy (or TTFields), even for elderly patients [26-28]. The mechanisms of this treatment are further explained in **Box 1**. Despite this aggressive treatment, virtually all patients experience tumor recurrence [29-32]. The basis for GBM drug resistance is further explained in **Box 2**, where we review recent strategies attempting to overcome this issue. Another important fact is that in order for being effective against gliomas, drugs or cell therapies need to effectively cross the BBB. The need to overcome the BBB is a main obstacle that halted the development of numerous treatments. In **Box 3** we review some recent and innovative delivery strategies.

Despite all efforts over the last decade to develop improved treatment for GBM patients through the discovery of new chemotherapeutic agents or new approaches, their success is markedly lower than several therapies applied to other cancers, such as breast and gastrointestinal tumors. While there is still a long way to go in the battle against GBM, many scientific advances have been made in recent years leading to new discoveries that can hopefully change the prognostic of GBM patients. They will be revised in the following sections.

4.   Immunotherapy: The next frontier?

The immune system is an essential component for tumor development and progression, especially in highly heterogeneous tumors as GBM. [33-36]. The immunosuppression is currently recognized as a cancer hallmark present in the Escape phase, where specific cells and molecules, such as M2 macrophages, regulatory T-cells, myeloid-derived suppressor cells and immune checkpoints are present [37, 38]. The GBMs' microenvironment is characterized by an important immunosuppressive infiltrate [33-36]. Several immunotherapies have already been approved by regulatory agencies in the United States, such as cancer vaccines, immune checkpoint inhibitors, bi-specific

T-cell engager monoclonal antibodies, oncolytic virus and adoptive cell transfer therapies [39], summarized in **Figure 1**.

Immune checkpoint inhibitors

Immune checkpoints are negative immunological regulators. The most studied immune checkpoint receptors are cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death protein 1 (PD-1) [40-42]. Over the last years, there was a marked increase in the development of clinical trials based on the blockade of immune checkpoints. A few of them were well succeeded and the Federal Drug Administration (FDA) approved their use for several types of cancer. This is the case of Ipilimumab (a CTLA-4 inhibitor); Pembrolizumab and Nivolumab (PD-1 inhibitors); Atezolizumab, Avelumab and Durvalumab (PD-L1 inhibitors). There are currently nineteen recruiting trials, five active clinical trials of PD-1/PD-L1 in GBM and one completed Phase 2 clinical trial of neoadjuvant Nivolumab. All of these have no published results as yet.

Notwithstanding, a retrospective study showed no Overall Survival (OS) or Progression-free Survival (PFS) benefit in using Nivolumab in patients with recurrent GBM after anti-angiogenic treatment with neutralizing antibodies against VEGF (Bevacizumab) [43]. Nevertheless, initial results from the Checkmate 143 Clinical Trial (NCT02017717) demonstrated the safety of Nivolumab and Iplimumab in GBM patients. In addition, a low response rate to PD-1 inhibitors for patients with High-Grade Gliomas (HGG) was also verified, although in this study a small number of patients clearly benefited from the treatment, having good partial responses and stable disease with increased PFS [44].

Limited T cell infiltration and activation in the immunosuppressive microenvironment as well as the paucity of validated biomarkers might explain the difficulties encountered in GBM. Strategies to overcome these complications may lay on the combination of therapies, such as immunotherapy with stereotactic radiosurgery or local chemotherapy [45].

Intracerebral CpG-ODN: a promise that went wrong?

Oligodeoxynucleotides containing CpG motifs (CpG-ODN) derived from bacterial DNA bind to intracellular Toll-like receptor 9 (TLR9) and act as potent activators of innate and adaptive immune systems, triggering a lymphocyte (Th1) response [46]. CpG-ODNs have already been successfully used in diverse experimental models of viral infections, allergies and cancer. Preclinical studies showed that CpG-1826 prolongs survival of mice with gliomas, by inducing tumor cell apoptosis and stimulation of the immune response [47]. Subcutaneous vaccination with tumor cell lysate plus CpG-2006 increased mice survival [48]. The use of multiple low-dose intratumoral CpG-ODN, to avoid toxicity, could lead to tumor eradication and induce long-term remission (longer than 3 months), with an important role of NK cell activation in this process [49]. In addition, radiosensitizing effects that result in inhibition of tumor growth and increased survival have been attributed to CpG-28 and CpG-107 [50-52]. A role in potentiating chemotherapy has also been recently described, using the combination of cyclophosphamide/CpG-1826 and TMZ/CpG-28 [53, 54].

Finally, the delivery mode of CpG-ODN is relevant, since TLR9 is located intracellularly. Both the uptake and immunopotency have been enhanced using carbon nanotubes conjugated with CpG [55]. Multifunctional lipid nanocapsules loaded with paclitaxel and CpG-1826 locally injected via Convention Enhanced Delivery (CED) also increased OS in glioma-bearing mice [56].

There are so far 3 clinical trials using CpG-ODN on GBM patients, which stated that CpG-28 is well tolerated at doses up to 20 mg per injection [57], with only modest activity on the 6-month PFS [58] and no improvement in OS [59]. Despite the relative success in animal studies, CpG-ODN results in patients have been disappointing. Among conceivable causes of failure, authors point out the delivery method used and the possibly variable TLR9 expression among tumors. Given this data, the improvement in the delivery method of CpG-28 and its association with immune checkpoint inhibitors might lead to more effective outcomes.

Rindopepimut®: the *EGFRvIII mutation vaccine*

Cancer vaccines are another modality of immunotherapy designed to strengthen the patient anti-tumor immune response. Therapeutic vaccination can be done with tumor cell vaccines (autologous and allogenic), dendritic cell vaccines and protein/peptide-based vaccines [60]. It mobilizes various immune effector mechanisms to attack and destroy tumor cells while sparing non-tumor cells [60]. EGFRvIII is a tumor-specific EGFR mutation frequently observed in GBM [61, 62], consisting of an in-frame deletion of 801 bp from the extracellular ligand-binding domain (exons 2-7) that removes amino acids 6-273, inserts a new glycine at the fusion junction [63] and encodes a constitutively active tyrosine kinase associated with tumor aggressiveness and treatment resistance [64, 65].

EGFRvIII is an ideal candidate for a peptide-based vaccine due to its tumor-specific nature and high immunogenicity. Rindopepimut®, the EGFRvIII-targeting vaccine developed by Celldex Therapeutics, is composed of 14 peptides conjugated to the immunogenic carrier protein keyhole limpet hemocyanin and proved to be efficient in preclinical murine models, but in some cases the treatment failured due to the outgrowth of EGFRvIII-negative cells [66].

Clinical trials of Rindopepimut® in GBM patients (ACTIVATE, ACT II/III and ReACT) had impressive results which led to Breakthrough Therapy Designation by the FDA in February 2015, despite pending results from the final Phase III ACT IV trial in newly diagnosed GBM patients [67-69]. However, the ACT IV phase III trial was terminated earlier for lack of benefit. Final analysis showed no OS benefit and a slight increase in side-effects [70]. In a small group of patients submitted to second surgery during the trial, almost 60% had lost EGFRvIII expression (57% in the Rindopepimut® arm and 59% in the placebo arm), raising the question of the importance of GBM's heterogeneity when choosing an immunotherapeutic target. Given that this was the first phase III trial of Rindopepimut®, further studies will be needed to evaluate which subgroups of patients will likely benefit from this vaccine. Moreover, it will be important to establish the role of the highly

immunosupressive GBM microenvironment in the absence of response to the vaccine and other combination approaches to increase the efficacy of immunotherapy in GBM.

Chimeric Antigen Receptor (CAR)-T adoptive cell therapy

There is also the adoptive immunotherapy, which consists of artificially activating patient's lymphocytes or dendritic cells to elicit anti-tumor response. Adoptive immunotherapy has gained increasing attention in the last years, as it obviates the multiple steps involved in the stimulation of a primary anti-tumor immune response, while being potentially faster and more effective than immune checkpoint blockade and vaccines. CAR constructs for redirecting T cells have been the most successful form for redirected adoptive immunotherapy. They consist of an extracellular antigen-recognition domain (usually an antibody single chain variable segment - scFv) linked to the intracellular signaling domain of the T cell receptor (TCR) and co-stimulatory domains. This allows engineered T cells to target an antigen independently from MHC exposure, provided that the antigen is a tumor-specific one to avoid off-tumor/on-target effects [71-73].

Since the EGFRvIII mutation is a tumor-specific antigen in GBM patients and is also expressed in treatment resistant GSCs [61, 62], a first-in-human study using peripherally infused EGFRvIII CAR-T cells for recurrent GBM patients was recently reported [76]. CAR-T cells were able to infiltrate the tumor mass, but induced alterations in the tumor microenvironment, such as loss of antigen expression in tumor cells and greatly increased both PD-L1 expression and regulatory FoxP3^{+}T cell infiltration [76].

CAR-T-Cells for other tumor-associated antigens such as EphA2, HER2 and IL-13 Receptor α2 (IL-13Rα2) are also being tested [76-80]. A Phase I clinical trial using IL13BBζ–CAR-T cells (targeting IL-13Rα2) administered post-surgery inside the resection cavity and intraventricularly in patients with recurrent GBM reported a first-in-human unprecedented transient complete response. Intracavitary doses induced local disease regression and intraventricular doses induced regression of

distant metastasis (even spinal metastasis), with outstanding improvement in the quality of life (including discontinuation of glucocorticoids and return to normal life activities) [81].

Therefore, the use of CAR-T cells as an immunotherapeutic approach seems promising for GBM, especially when locally administered. Apparently, this modality of treatment is able to somehow overcome the highly immunosuppressive and complex GBM microenvironment, the major barrier to successful immunotherapy. The next years will determine if the use of intracerebral and intraventricular CAR-T cells is indeed safe and feasible and if this strategy induces sustained responses.

Oncolytic virus therapy

Cancer immunotherapy using oncolytic viruses has gained attention. These viruses possess the ability of infecting and killing tumor cells without causing damage to normal tissues [82]. A potential candidate to this new therapeutic strategy is the rodent H-1 protoparvovirus (H-1PV), the smallest among all oncolytic viruses and nonpathogenic for humans [83]. In addition, in glioma-bearing immunocompetent models, proinflammatory cytokine release and upregulated CD80 expression were detected in antigen-presenting cells, including microglia [84]. Recently, a phase I GBM trial showed that patients treated with parvovirus increased tumor infiltrated lymphocytes and extended median survival [85].

Other oncolytic viruses demonstrated the ability to induce an effective immune response against tumor invasion. In a mouse GBM model, the intratumoral administration of an oncolytic adenovirus stimulated IFN-γ-producing CD8+ T cells and decreased tumor-infiltrating Treg cell numbers [86]. Moreover, a recombinant polio/rhinovirus chimera also demonstrated immunotherapeutical potential *in vitro* in high-grade GBM [87].

5.   Targeting angiogenesis: Bevacizumab and beyond

Angiogenesis is a complex mechanism of tumor growth in brain tumors due to intricate interactions between endothelial cells and other entities from the GBM microenvironment (particularly tumor cells and macrophages/microglia). GBM vascularization occurs through several alternative mechanisms besides sprouting angiogenesis, such as vascular co-option, vascular mimicry, GSC endothelial differentiation, and vasculogenesis from Bone-Marrow (BM)-derived circulating endothelial precursor cells (CEPC), which are all regulated by complex interactions with the tumor microenvironment [88, 89]. In the hypoxic tumor areas, Vascular Endothelial Growth Factor A (VEGF-A) is upregulated in GBM cells and in infiltrating macrophages, resulting in abnormal microvascular stimulation leading to aberrant highly proliferative, tortuous and leaky vasculature, which further contributes to tumor hypoxia and compromises efficient drug delivery [88-91].

Bevacizumab, an IgG1 monoclonal antibody which neutralizes the effect of VEGF-A, has an inhibitory effect on tumor neovascularization processes. However, Bevacizumab would not inhibit vascular mimicry (when tumor cells acquire genetic and/or phenotypic features of endothelial cells) or the blood supply from pre-existing vasculature [92, 93]. Indeed, histopathological analysis showed that Bevacizumab therapy induces apparent normalization of the vascular structure, decreases microvessel density and improves tumor oxygenation [94].

In 2009, the FDA approved Bevacizumab in monotherapy or combined with chemotherapy for second line treatment of recurrent GBM [29, 30]. Nevertheless, for newly diagnosed GBM patients, adding Bevacizumab to the conventional treatment did not improve OS and was associated with increased side effects. When Bevacizumab was used as first line therapy, patients experienced increased symptom burden, worse quality of life and a more frequent and early decline in neurological function [95]. Given this data, Bevacizumab remains restricted for refractory patients, where it increases PFS, has a corticosteroid sparing effect and improves quality of life, although patients tend to relapse during treatment [96, 97].

GBM recurrence after VEGF blockade is often associated with a phenotypic alteration due to glial-mesenchymal transition of tumor cells [98], apparently triggered by MET (a receptor tyro-

sine kinase of hepatocyte growth factor) phosphorylation. Impairment of VEGF function restores and increases MET activity in a hypoxia-independent manner, resulting in tumor cell invasion and mesenchymal transition through a MET/VEGFR2 complex [99]. Tumor endothelial cells acquire the mesenchymal gene profile without loss of endothelial cell functions, which is defined as endothelial mesenchymal transition (Endo-MT), resulting in enhanced cell proliferation and migration, as well as vessel permeability [100]. These mechanisms are triggered by an axis involving c-MET, ETS-induced 1 and metalloproteinase 14 (MMP14), which also controls VE-cadherin degradation and vascular abnormality. Pharmacological inhibition of c-MET induced vessel normalization in patient tumor-derived endothelial cells. In addition, specific knockout of MET in endothelial cells presented normalized blood vessels, reduced intratumoral hypoxia, which suppressed tumor growth and prolonged survival in GBM-bearing mice after TMZ treatment [100].

Although the anti-angiogenic therapy improves tumor oxygenation, hypoxia persists in the process of tumor resistance to treatment [94]. The hypoxic microenvironment re-induces HIF-1α, resulting in stromal cell-derived factor 1 (SDF-1) and VEGF activation and leading to the migration of pro-angiogenic monocytic cells from the bone marrow, endothelial cells and pericyte progenitors [101]. Another molecule that stimulates angiogenesis in tumor microenvironment by enhancing the VEGF pathways is the placental growth factor (PlGF) [102]. [103] However, no significant clinical activity was observed when the anti-PIGF monoclonal antibody was tested in phase I for patients with recurrent GBM [104].

Even though antiangiogenic therapy has failed to stop GBM progression, the most common strategy to treat recurrent GBM is continuation of Bevacizumab with the addition of a cytotoxic drug [105]. This strategy is still used because lower doses of antiangiogenic therapy normalize tumor microvasculature, which may improve drug delivery and reduce hypoxia [106]. Among the available cytotoxic adjuvants, Lomustine may still have a role in the GBM recurrent setting, since nitrosoureas can easily cross the blood brain barrier [107]. Nevertheless, this effect of chemotherapy is transient and restricted to this "window of normalization" (the time frame where the vascula-

ture is normalized after anti-angiogenic treatment), that is dependent on both treatment dose and duration [108].

An alternative to treat recurrent GBM is the administration of inhibitors of the angiotensin system (ASI) (prescribed as anti-hypertensive drugs) concomitant with cytotoxic-Bevacizumab therapy [109]. These inhibitors block both the angiotensin II receptor I (AT1) signaling and the angiotensin converting enzyme (ACE). The therapy is associated with improved survival in GBM patients receiving standard-of-care therapy [110]. AT1 inhibition with ASI was previously shown to reduce tumor VEGF levels in preclinical glioma models [111]. ASI treatment may also enhance vascular perfusion and reduce edema in the tumor and peritumor brain, improving drug delivery to the tumor, for a fraction of the cost of standard care treatments and with minimal associated risks [108, 112].

Other anti-angiogenic agents could be used as an alternative to the Bevacizumab therapy, such as VEGF receptor tyrosine kinase inhibitors (vatalanib, sorafenib, cediranib, sunitinib and pazopanib), PDGF receptor tyrosine kinases inhibitors (dasatinib, imatinib and tandutinib), protein kinase C inhibitors (enzastaurin), matrix metalloproteinases inhibitors (prinomastat) and proteasome inhibitors (bortezomib) [113-115]. Unfortunately, though many phase I and II studies have been performed, none of these agents has demonstrated to be effective enough to justify a phase III clinical trials [116], as tumors eventually overcome these treatments by the same mechanisms of Bevacizumab resistance.

Tumor cells can evade angiogenic inhibitors through modulation of collateral pathways or co-option of existing normal vasculature to maintain blood perfusion [117]. To overcome the limitations of established antiangiogenic drug therapies, gene therapy was used to target newly formed blood vessels. In a murine model, VB-111, a non-replicating adenovirus 5 carrying a proapoptotic human Fas-chimera transgene (Fas and human TNF receptor 1), was able to specifically act in the expression of the Fas-chimera transgene in angiogenic blood vessels triggering apoptosis of these vessels [118].

Given all the data available, the importance of Bevacizumab cannot be put aside. But the lack of a long-lasting response and the increasing understanding of the mechanisms regulating angiogenesis in the GBM highlight the urge to develop and test new anti-angiogenic drugs that would have a longer window of normalization, or would involve alternative pathways that could be responsible for the resistance to VEGF targeting.

6. Glioma-Associated Macrophages/Microglia (GAMs) as key players in GBM microenvironment

Microglia are the resident immune cells on the brain parenchyma and are involved in many cell events in healthy and diseased CNS comprising chemotaxis, phagocytosis, antigen presentation and cytokine production. Although it has been described that tumor-infiltrating microglia and macrophages compose up to 30% of the tumor mass, those cells do not appear to have antitumor activity. Actually, glioma-associated microglia/macrophages (GAMs) produce pro-tumor factors and contribute to GBM progression [24, 119], view **Figure 2**.

Histopathological analysis of glioma specimens demonstrated that GBM contains a higher number of infiltrating macrophages compared to lower grade astrocytomas [120]. In the past few years, the activation status of GAMs present in the tumor microenvironment has been considered of prognostic value [121].

Similar to regular macrophages, microglia can acquire an M1 phenotype, that is involved in effective immune response against tumor progression and is characterized by the ability to release pro-inflammatory mediators such as TNF, IL-1, IL-6, CCL2, nitric oxide (NO) and reactive oxygen species (ROS) [122]. However, tumor infiltrating microglia and macrophages mostly also display an M2 phenotype, which possibly contributes to the immunossupressive microenvironment of GBM. These alternatively activated M2 macrophages produce anti-inflammatory and immune suppressive factors, including arginase-1 and CD36, as well as up-regulate the cell surface markers CD204, CD206 and CD163 and anti-inflammatory cytokines, such as IL-10 [123]. In the healthy

brain, resident microglia (also referred to as M0) are considered to possess an attenuated M2 phenotype and contribute to the maintenance of a healthy environment for neuronal function [124]. On the other hand, GAMs were found to express some M1 but mostly M2 markers in human GBM samples [125].

STAT 3 signaling, which is constitutively expressed in GBM [126], is also up-regulated in GAMs [127-129]. The activation of this pathway inhibits M1 macrophage activation, reduces expression of co-stimulatory molecules necessary for antigen-presentation and upregulates immuno-modulatory cytokines IL-6 and IL-10, while down-regulates pro-inflammatory cytokines (IL-2, IL-4, IL-12 and IL-15) [130, 131]. Therefore, STAT 3 inhibition using siRNAs reverses the cytokine profile, leading to GAM activation and inhibition of tumor growth *in vivo* [128]. STAT 3 pharmacological inhibitors penetrate the CNS and have anti-proliferative and pro-apoptotic effect on GBM cells [131, 132]. These inhibitors are capable of reversing the immune tolerant microenvironment by activating microglial cells, increasing lymphocyte-stimulating cytokies (IL-2, IL-4, IL-12, IL-15 and CXCL-10) and upregulating co-stimulatory molecules (CD80 and CD-86), then inducing a TH1-response [132, 133]. There are ongoing phase I and II clinical trials with inhibitors of STAT 3 and its most relevant activator, IL-6, for several malignancies such as head and neck cancer, multiple myeloma and prostate cancer [134]. These trials might also be translated into a new therapeutical option for malignant gliomas.

It has been recently demonstrated that hypoxia increased microglia and macrophage recruitment by chemotaxis as well as the tumor expression of POSTN, a cell-secreted adhesion protein that is capable of promoting tumor progression[135]. However, macrophage motility was attenuated by hypoxia, which corroborates with the idea that GAMs are primarily attracted to hypoxic areas and after they remain imprisoned in these areas [135]. In addition, POSTN expression was enhanced through TGF-β (a hypoxia-inducible growth factor) treatment, resulting in increased recruitment of GAMs to HIF-1α-positive regions [136]. In addition, hypoxia promotes M2-polarization of GAMs and HIF inhibition partially reversed the effects of hypoxia in GAM recruit-

ment and M2 polarization [136]. These findings suggest a connection between the hypoxic environment with GAM enrichment and M2 polarization, key factors in tumor progression that could be targeted to reduce glioma growth and invasiveness. Finally, a recent work showed that the activity of GBM's iNOS was capable of inhibiting microglial caspase-3, which was a crucial step in the transformation of microglial cells in their tumor-promoting phenotype *in vitro* and *in vivo* [137].

Other important pathway in the crosstalk GAM-GBM is the colony stimulating factor 1 (CSF-1), also known as macrophage colony-stimulating factor (M-CSF). CSF-1 is a secreted cytokine that binds to CSF-1R in hematopoietic cells and macrophages stimulating survival, differentiation and proliferation, which have been described to be important in GBM invasion [138, 139]. Furthermore, CSF-1 inhibition can block GBM progression by altering macrophage M2 polarization and increasing survival in preclinical models [121]. In preclinical models, tumors treated with anti-CSF-1 recur in over 50% of mice. CSF-1 inhibition resistance is mediated by the tumor microenvironment, through macrophage production of insulin-like growth factor-1 (IGF-1) and activation of IGF-1 receptor (IGF-1R) in GBM cells. Blockade of IGF-1R or PI3K pathway further prolonged animal survival [140]. Finally, an orally administered CSF-1R inhibitor (PLX3397) is being tested in GBM patients and first results from phase II clinical trials showed that treatment was well tolerated and crossed the blood-brain-barrier but showed no efficacy [141].

The CX3CR1-I249 allele, a recently discovered prognostic marker, is a polymorphism in a chemokine receptor gene associated with cell migration. This variant is associated with prolonged mean OS of GBM patients (23.5 vs 14.1 months) and with reduced tumor infiltration by GAMs [142]. For the first time, a GAM marker has been characterized as an independent, favorable prognostic factor and might be useful in predicting survival in GBM patients.

Another controversy in the literature lies on the differences between microglia and bone-marrow derived macrophages in GBM development. Recent studies using transgenic mice and intra-vital two-photon microscopy gave some insight to this debate: microglial cells and bone-marrow derived macrophages have different transcriptional networks [143] and different spatio-temporal

recruitment, morphology and activation dynamics [144] . Also, recent evidence is pointing to bone-marrow derived macrophages as important players in tumor angiogenesis. Molecules produced by endothelial cells, such as IL-6, could be relevant for attracting and polarizing these cells towards the M2-tumor promoting phenotype through activation of HIF-2α [145]. Once in the perivascular compartment, those cells would be an important source of VEGFa for endothelial cells [91]. Recent observation with intra-vital two photon microscopy in a syngeneic mouse glioma model showed that in early stage tumors there was accumulation of M1-polarized macrophages in the tumor core. While in later stage tumors, those cells accumulated in the perivascular region and were predominantly M2-polarized, VEGFa-secreting [91]. Furthermore, this macrophage-derived VEGFa was responsible for blood vessel dysmorphia observed in GBM. Selective ablation of VEGFa in macrophages could reverse this effect and improve chemotherapy deliver and mice survival [91].

All these findings point to the importance of further studies about the influence of the tumor microenvironment on the response of GAMs and the crosstalk between these and tumor cells, as this can be a promising target for cellular and immunotherapy in GBM.

7.   Targeting Reprogrammed Metabolism: IDH1/2 and beyond.

In order to develop a highly proliferative phenotype, transformed cells experience a metabolic reprogramming, which provides them with required substrates to sustain intense cell division [147]. For many years, glucose was considered the primary energy source for many cancer types, including gliomas, in a process known as the 'Warburg effect' ('aerobic glycolysis') [148]. This effect consists of a shift of the metabolic flux from Oxidative Phosphorylation to fermentation under normal oxygen concentrations [149]. Despite being less efficient, this process confers unique advantages to malignant cells, such as quicker energy gain; acidification of the extracellular microenvironment aiming to facilitating neoplastic invasion; and increased concentrations of glycolytic metabolites and NADH, which will be used to generate aminoacids and fatty acids, ultimately promoting cellular proliferation [149, 150].

To enhance the glycolytic flux, GBM must undergo modifications in protein expression and activity. An elevated glucose transport is guaranteed by increasing the expression of glucose transporters (GLUT1, GLUT3). Elevated glycolytic activity is acquired by expressing unique isoforms of rate-limiting glycolytic enzymes, such as Hexokinase-2, PFKFB3 and PFKFB4, PDK1 and PKM2 [151-153]. Hexokinase-2, which catalyzes the formation of glucose to glucose-6-phosphate (G6P), is not commonly expressed by benign brain tissue and its expression has been correlated with lower GBM patient OS [152]. Knockdown of Hexokinase-2 reduces tumor vasculature and tumor growth in orthotopic xenograft models of GBM, and sensitizes malignant cells to TMZ and RT *in vitro* and *in vivo* [154]. Likewise, PKM2, which catalysis the irreversible phosphorylation of Phosphoenolpyruvate (PEP), is overexpressed in malignant gliomas when compared to normal brain cells [155] or lower-grade gliomas [156]. PFKFBs (PFKFB1, 2, 3 and 4) indirectly regulate the activity of PFK1 by producing and degrading F26BP, a known PFK1's potent activator. Whenever PFK1 is inactive, the addition of one phosphate group to fructose1-phosphate does not occur and the glycolytic flux is reduced [157]. Recently, it was demonstrated that TGFβ-1 increased the glycolytic flux by inducing PFKFB3 expression in GBM [158]. RNAi screening identified PKM2 and PFKFB4 as survival-related kinases in GSC and high PFKFB4 expression was associated with lower survival in GBM patients [159].

Aberrant expression and activity of glycolytic "players" can be therapeutically explored to improve the current therapy of gliomas. Therefore, a number of small-molecule inhibitors has been developed and some of them are currently in phase I/II clinical trial [160]. Lonidamine is a Hexokinase-2 inhibitor, which leads to cellular apoptosis [161] but did not show effective results in phase II trials [162]. Dichloroacetate, a PDK1 inhibitor that increases ROS and mitochondria-dependent apoptosis *in vivo* [163], is currently under phase II trial for GBM (NCT00540176).

As mentioned in **Box 1**, gliomas have point mutations in the Isocitrate Dehydrogenase 1 and 2 genes (IDH1-R132H and IDH2-R172K, respectively) [164]. The wild-type enzyme is responsible for converting isocitrate into α-ketoglutarate (α-KG), the substrate used for the formation of

succinyl-CoA in the Tricarboxylic Acid (TCA) cycle. It has been observed that mutated IDH1-R132H acquires a unique capability of transforming α-KG into 2-hydroxyglutarate (2HG) [165]. The accumulation of 2HG by cells bearing the mutated IDH leads to several consequences, including impairment of epigenetic-related enzymes (mainly DNA demethylases), causing a vast rearrangement of the DNA methylation profile [166, 167]. Considering the importance of IDH/2-HG in the tumorigenesis of gliomas, several research groups developed strategies in order to counteract their activity within transformed cells [168-170]. Some of the studied compounds are already under phase I clinical trial studies, such as: AG-120 (NCT02073994), AG-881 (NCT02481154) and BAY1436032 (NCT02746081).

Although glutamine rather than glucose can be used as an energy source by GBM [171, 172], it is unknown if ketone bodies can be used as well. GBM cells are sensitive to glucose depletion and undergo high oxidative stress in the absence of this substrate, while normal astrocytes maintain their metabolic balance [173]. A number of studies observed higher glycemia as a predictor of lower OS in GBM patients [174, 175]. It has been proposed that a dietetic intervention based on low glycemic nutrients (Ketogenic Metabolic Therapy; KMT), might hold GBM progression and increase patient OS [176]. A recent systematic study reviewing current trials testing KMT in gliomas suggested that KMT is safe, feasible and stand as a potential strategy for combined therapy with current treatments in malignant gliomas [177].

Research on cancer cell metabolism has grown exponentially in the past few years and advances in laboratory techniques allowed the development of more reliable *in vitro* and *in vivo* models [178]. Several potential new metabolic targets are discovered every year and a great number of them are currently under clinical trial and will hopefully, in a near future, improve the care of GBM patients.

8.   Small non-coding RNAs

Non-coding RNAs are a family of RNAs that are not transcribed into proteins and include miRNAs, siRNAs and lncRNAs [179]. They participate in diverse biological processes, including control of gene expression, during development and both physiological and pathological process [179]. miRNAs are the most extensively ncRNAs studied and correspond to a class of small (~22 nucleotides in length), endogenous, evolutionarily conserved RNAs, that regulate gene expression in a post-transcriptional manner by binding to the target mRNAs, thereby repressing their translation or inducing their degradation [180]. Also, miRNAs have become particularly appealing as biomarkers due to their availability and stability in a variety of biological samples (such as blood and saliva) allowing their use as a noninvasive tool for cancer detection and follow-up [181, 182] and as promising therapeutic targets in gliomas [183, 184].

In GBM, oncogenic actions of miRNAs such as miR-10b [185, 186], miR-21 [187] and miR-26a [188] have been correlated with inhibition of key cell cycle inhibitors and pro-apoptotic genes [185] and a reduced median OS in GBM patients [188]. Tumor suppressor miRNAs such as miR-7 [189], miR-34a [190], miR-218 [191] and miR-873 [192] have been described as down-regulated in gliomas when compared to adjacent or normal brain tissue.

miRNAs also participate in several signaling programs of GSCs [193, 194]: miR-125b seems to be involved in the development, migration and replication of GSCs [195]. Ectopic over-expression of microRNA-34a led to inhibition of proliferation in GSCs by targeting Rictor, a component of mTORC2 complex, promoting suppression of AKT and inhibition of WNT/β-catenin signaling pathway [190]. miR-9/9* was also shown to inhibit GSCs differentiation, maintaining their stemness by targeting CAMTA1, a tumor suppressor gene correlated with survival [196]. miR-143 is another miRNA related with GSCs which participate in glucose metabolism. Over-expression of miR-143 has been shown to inhibit glycolysis, promoting differentiation and decreasing the tumorigenic capacity of GSCs *in vivo*, therefore highlighting the important role of this miRNA as a tumor suppressor [197].

Some studies suggest that miRNA expression profiles can be used to distinguish glioma grades and GBM subgroups [198, 199]. Recently, a large scale assessment using 848 human microRNAs, observed that a set of 13 microRNAs (miR-1296, miR-21, miR-155, miR-21*, miR-451, miR-223, miR-210, miR-493, miR-16, miR-22, miR-629*, miR-105, and miR-219-2-3p) had a significant different expression patterns, distinguishing WHO grade II glioma from grade III + IV tumors. A higher expression of miR-21 and miR-210 was also associated with worse OS in GBM patients, suggesting a direct involvement of these miRNAs in glioma development and progression [200]. Visani and co-workers also demonstrated deregulation of three microRNAs (miR‑34a, miR‑101 and miR‑10b) between low and high grade gliomas, with higher expression levels of miR-10b being associated with malignancy, representing a promising target for therapy [201].

Furthermore, siRNAs are promising therapeutic tools due to the possibility of being chemically synthesized and artificially introduced into the cytoplasm of cells to inhibit targeted protein synthesis [202].

Some targets are showing promising results in the pre-clinical setting, such as intratumoral treatment with siRNA targeting HIF-1α, which could reduce 79% of the tumor growth after 50 days of treatment in orthotropic murine models, reducing simultaneously HIF-1α main transcriptional targets, like VEGF, GLUT-1, c-MET and CA-IX. [203]. Cell penetrating peptide-mediated siRNA inhibition of Hsp90 in combination with 17-allylamino-17-demethoxygeldanamycin (17-AAG), an HSP90 inhibitor, reduced GBM tumor growth via akt downregulation [204]. siRNAs anti-Galectin-1 and anti-EGFR were both carried by lipid nanocapsules and administrated intratumorally together with TMZ via CED in an orthotopic GBM mouse model, leading to significantly higher survival [205].

Despite the promising scenario of ncRNAs therapeutic use, several limitations, including an inadequate delivery of a siRNA to the brain tumor tissue and the stability of these molecules in systemic circulation, have been discussed for RNAi based-therapy in GBMs [202, 206]. Particularly,

more attention should be paid to unpredictable effects, since one miRNA can target multiple mRNAs, and a single mRNA can also be targeted by multiple miRNAs [202].

9. Concluding Remarks

Numerous recent studies increased the understanding of the cellular and molecular events involved in CNS oncogenesis. This led to the major changes in tumor classification made by the WHO in 2016 and to the understanding that tumors with different origins and molecular alterations must not be treated in the same way but instead through personalized therapy. Many promising basic and translational investigations have showed no benefit when translated to patient settings, forcing the scientific community to further concerning and engagement towards unveiling the intricate pathways involved in GBM establishment and progression, but many questions remain to be addressed (see **Outstanding Questions**).

Due to the heterogeneity of GBM, the complex interactions between tumor and parenchymal entities are gaining exponential importance. Unlike many other solid tumors, the GBM microenvironment lacks lymphocyte infiltration while it is abundant in macrophages, which can be pointed as one of the causes of the poor outcome of immunotherapy in these tumors. In this sense, a great deal of the research carried on in the field currently aims at better understanding the role of innate immune cells in the GBM microenvironment, particularly in how those cells are polarized to M2 phenotype after their recruitment and how they interact with other cells from the tumor microenvironment, specially endothelial cells and the few infiltrating lymphocytes. New therapies targeting the myeloid compartment of the tumor microenvironment can hopefully be a viable and effective alternative for treating these tumors as a new line of immunotherapy. Also, targeting the myeloid compartment of the tumor microenvironment can improve the success of immunotherapy with checkpoint inhibitors, by increasing possibility of T cell recruitment and changing the immunossupressive tumor microenvironment.

Also, research in tumor angiogenesis is now evolving from the focus on the VEFG-A/VEGFR2 axis; as targeting this pathway was proven insufficient to properly solve blood vessel dysmorphia and dysfunction in the tumor microenvironment. The search for alternative pro and anti-angiogenic pathways is being further pursued and may also lead to a better understanding on the role of the interaction between macrophages/microglia and endothelial cells, a topic already explored in developmental angiogenesis studies but still largely neglected in the context of GBM and other malignancies.

Finally, recent advances in metabolomics are allowing researchers to see beyond the Warburg Effect in the cancer field. Better understanding the differential expression of distinct enzyme isoforms and the metabolic deviations that occur in tumor cells is helping identifying metabolites with different cellular activities. It can also show what are the crucial steps in the metabolic cascade that should be targeted for inhibiting tumor growth.

10. Acknowledgments

11. Text Boxes:

Box 01 - Molecular Basis of Current Standard Therapy

TMZ is an orally available DNA alkylating agent [207] with radiosensitizing effect [208]. The principal mechanism of action of TMZ is adding methyl groups to N7 and O6 positions of gua-

nine and N3 of adenine in DNA [209]. In addition, it has a cytotoxic effect against malignant cells, resulting in DNA fragmentation and disruption of DNA replication, thus leading to growth suppression that causes apoptotic cell death [210]. However, this DNA methylation can be mended by the O6-methylguanine-DNA methyltransferase (MGMT) repair enzyme, thereby suppressing cytotoxicity [211]. Several patients with GBM show resistance to TMZ due to the expression and methylation status of the MGMT promoter [212]. Patients who have tumors with the MGMT promoter methylation usually experienced improved survival when they were treated with alkylating agents like TMZ, making MGMT methylation a critical biomarker for predicting sensitivity to TMZ therapy [213].

Alternating electric fields therapy (TTFields) is a new modality of anticancer treatment, developed by Dr. Yorum Palti [214-216]. It, consists of electric fields of frequencies between 100 and 300 kHz, capable of inducing violent membrane blebbing, disruption of microtubule spindle elements and mitotic chromosomal order [214]. *In vitro and in vivo* models showed that TTFields can inhibit GBM cell migration, invasion and angiogenesis, making it a promising new therapeutic strategy, with multiple biological effects [217, 218]. Of great importance is the fact that the most common adverse event associated with the device is scalp irritation and contact dermatitis due to hydrogel-induced irritation, which could be overcame with topical corticosteroids and slight shifts of the arrays [219].

Recent clinical trials proved this therapy to be effective both in relapsed patients and in the first line setting [27, 215]. Furthermore, continuation of TFF therapy after first recurrence, associated with a second-line chemotherapy is also associated with an increase in OS (11.8 vs 9.2 months) [220]. Despite being the first new treatment approved for GBM patients in the first-line setting after the Stupp Regimen, the use of TTFields is still under great debate, due to the prohibitive cost of the device, approximately 185,476 Euros per patient per year [221].

Box 02 - The old problem of drug resistance in GBM

One major cause of treatment failure in high grade gliomas continues to be tumor cell resistance to chemotherapeutic agents (multidrug resistance - MDR) [222]. A multifactorial phenomenon which involves several mechanisms that result in evasion of drug-induced cellular damage [223]. The mechanisms of MDR involve deregulation of genes controlling apoptosis, enhanced intracellular drug detoxification, upregulation of DNA repair related genes, activation or overexpression of drug export proteins that reduce levels of drug accumulation in the cell [224].

One category of drivers of the MDR phenotype are the drug efflux proteins that belong to a super family class of molecular pumps known as ATP-binding cassettes transporters (ABC- transporters). Their function is mainly to protect cells from endogenous toxins, xenobiotics and in the transport of many cellular products [225].

High expression of ABC transporters has been shown in the CNS and brain tumors [226]. The transporter P-glycoprotein (P-gp/ ABCB1) is highly expressed on endothelial cells of the blood-brain barrier, while the transporter multidrug resistant-like protein (MRP-1 /ABCC1) and breast cancer associated protein (BRCP/ABCG2) are expressed on lower levels in astrocytes [227]. High levels of expression of multidrug transporters are often observed in drug-naive tumors, even when the tissue of origin exhibits little or no expression of the corresponding gene [228]. This high intrinsic gene expression is likely regulated by signaling pathway components that are involved in malignant transformation. Indeed, recent studies have shown that P-gp over expression in the TMZ resistant GBM cells was caused by an increase in EGFR signaling [229]. Competitive binding assays and specific P-gp inhibition experiments have demonstrated that TMZ is a substrate for P-gp and not MRP-1 [230]. Clinical data suggest that polymorphisms of P-gp have an important role in patient response efficiency to TMZ: tissue samples from patients who exhibited TMZ resistance and tumor recurrence showed increased levels of P-gp expression [231].

The complete profile of substrates for all the ABC transporters associated with MDR is not yet known and despite conflicting information on clinical significance of targeting ABC-transporters, more studies are still underway to fully understand the molecular pathways leading to

their overexpression in tumors. This data would be important in elucidating the mechanisms underlying the resistance of GBM to chemotherapeutic drugs and subsequently establish the basis for selecting better chemotherapeutic agents and re-sensitizing GBM cells to chemotherapy [232]

Box 03 - Overcoming the Blood Brain Barrier: New Delivery strategies for brain tumors.

A) Nanoparticles and Micelles:

Nanoparticles are materials from one to a hundred nanometers, large surface-to-volume rate and capable of crossing the BBB to some extent [233]. Considering the case of brain tumors, where some promising drugs are abandoned during pre-clinical development due to their inability to reach cytotoxic concentrations in the CNS or inability to cross the BBB, these molecules can be of great value [233]. Apart from the nanoparticles, micelles (spherical-shaped aggregates of surfactant molecules, mainly lipids, dispersed in a liquid colloid) are also being loaded with drugs and used to increase BBB penetration.

To this purpose, some studies have focused in improving TMZ delivery and reducing side-effects in GBM *in vivo* models using different strategies (immunoliposomenanocomplex; biotin-functionalized chitosan core covalently conjugated with TMZ) [234, 235]. Micelles carrying flavonoids or traditional chemotherapy (doxorubicin and paclitaxel) showed *in vitro* and *in vivo* anti-GBM activity [236, 237].

Therefore, preclinical studies point nanoparticles and micelles as possible powerful tools to improve drug delivery in GBM, with further studies needed to prove their feasibility.

B) Local infusion and Blood-Brain-Barrier Disruption:

Convection-Enhanced Delivery (CED) is a local delivery technique for facilitating drug diffusion to brain tumors. It allows a direct delivery of high doses by stereotactically inserting catheters into tumor mass or surgical resection cavity [238]. Those catheters are then connected to an infusion pump, which guarantees a positive pressure gradient, to improve drug distribution independently of the agent's molecular weight, allowing infusion of higher volumes [239, 240].

Between 1997 and 2010, there were 14 clinical trials involving conjugated toxins, viruses, liposomes and conventional chemotherapies unable to penetrate BBB. All these studies included a limited number of patients and the results were mostly inconclusive [241]. Despite limited success of those clinical trials, the use of CED for GBM treatment remains relatively promising. Notwithstanding, the choice of agent, cannula design and placement and the cost of the procedure constitute some of the challenges that still need to be overcome.

Furthermore, recent studies have focused on local and temporary BBB disruption, aiming to increase drug concentration in the brain parenchyma and inside the tumor mass. In one of these studies, the MRI-guided laser interstitial thermal therapy was able to induce disruption of peritumoral BBB, mainly between the first 1-3 weeks and decreasing until the 6th week in 14 patients with recurrent GBM., which could open a time window for optimal tumor treatment [242].

12. Figure Legends:

Figure 01. New therapeutic strategies for GBM treatment. (A and B) Current immunotherapy strategies being tested pre-clinically and in Clinical Trials, including adoptive cell-transfer (CAR-T cell therapy) and immune-checkpoint blockade. **(C)** Vaccination strategy targeting EGFRvIII mutation (Rindopepimur©) and anti-angiogenic strategies **(D)**.

Figure 02. Glioma-associated microglia and macrophages infiltrate GBM mass and interact with tumor cells. Confocal microscopy of GBM-injected Swiss mice brain stained for microglia and macrophages with anti-Iba1 (Garcia and Geraldo, unpublished) showing ramified morphology typical of non-activated or "resting" microglial cells, engaged in immuno-surveillance of the CNS **(A)**; and ameboid activated glioblastoma-infiltrating macrophages and microglia **(B)**. **(C-E)** In several injury settings, infiltrating macrophages and microglial cells can acquire different activation phenotypes: classical M1 phenotype was described in infectious diseases as a response to bacterial

LPS for example; alternately, activated M2 cells were described in the context of tissue repair. Currently it is known that microglial cells can shift phenotypes during a single pathological process. Iin the GBM tumor mass, for instance, one can identify microglia and macrophages secreting both M1 (IL-2, IL-4, IL-12 and IL-15) and/or with M2 (IL-10 and TGFβ) cytokines and other growth factors important for tumor growth, such as VEGF.

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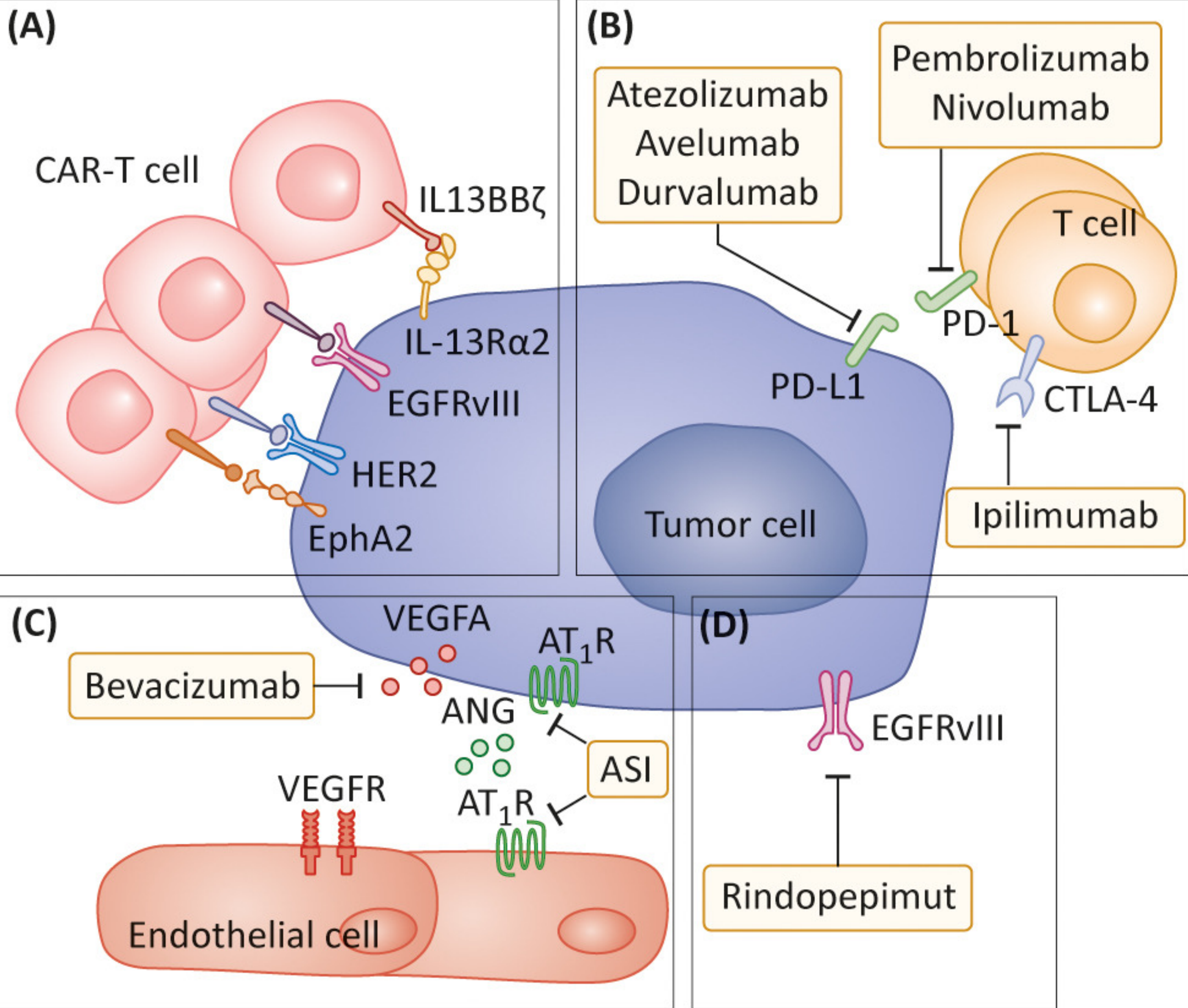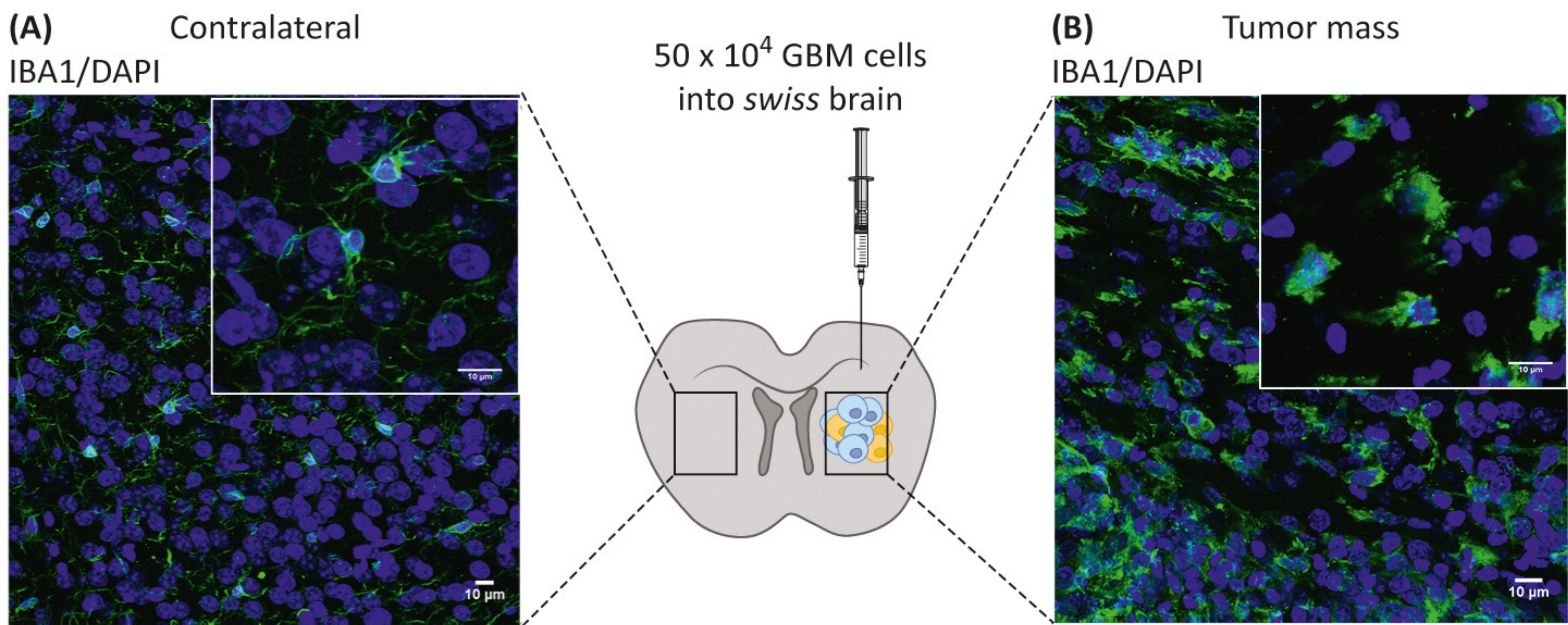
1. **Highlights:**

- Glioblastoma remains an incurable disease with a poor prognosis, despite being among the most

  studied tumors in the past decade

- About a third of the tumor mass is composed of non-neoplasic infiltrating cells, reflecting a very heterogeneous tumor both in terms of pathology and microenvironment.

- The heterogeneous microenvironment turns Glioblastoma into a good candidate to cell and immunotherapies. But it also constitutes a challenge for interpreting treatment-response and developing new strategies.
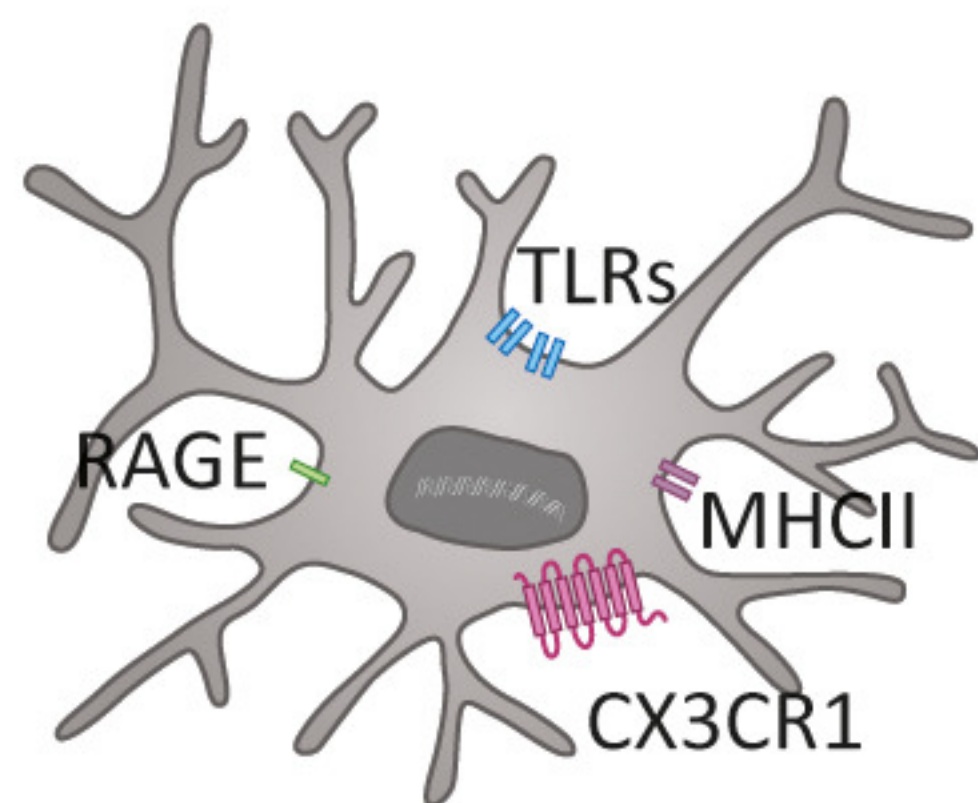
2. Outstanding Questions:

- Will CAR-T cell therapy prove its way and become the first successful immunotherapy for GBM, by overcoming the highly immunosuppressive microenvironment?

- How can we effectively disrupt GBM interaction with infiltrating non-tumoral cells (such as microglia/macrophages and endothelial cells)?

- Is there a role for anti-angiogenic therapy in GBM or will it remain with symptomatic use of VEGF inhibitors?

- Can metabolic alterations be used as biomarkers for distinguishing tumor subtypes or used as therapeutic targets?

(A)

CAR-T cell

IL13BBζ

IL-13Rα2

EGFRvIII

HER2

EphA2

(B)

Atezolizumab
Avelumab
Durvalumab

Pembrolizumab
Nivolumab

T cell

PD-L1

PD-1

CTLA-4

Tumor cell

Ipilimumab

(C)

VEGFA

AT$_1$R

Bevacizumab

ANG

ASI

VEGFR

AT$_1$R

Endothelial cell

(D)

EGFRvIII

Rindopepimut

(A) Contralateral
IBA1/DAPI

10 µm

(B) Tumor mass
IBA1/DAPI

10 µm

50 x 10^4 GBM cells into *swiss* brain

(C) "*Resting*" microglia

TLRs
RAGE
MHCII
CX3CR1

(D) Classical activation M1

IL-15
CXCL-10
TLRs
IL-2
IL-4
IL-12

(E) Alternatively activation M2

IL-10
SR-A1
TGFβ
IL-2
STAT3
CECR1
CX3CR1