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Feature Review

Challenges in glioblastoma research: focus on the tumor microenvironment

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Glioblastoma (GBM) is the most deadly type of malignant brain tumor, despite extensive molecular analyses of GBM cells. In recent years, the tumor microenvironment (TME) has been recognized as an important player and therapeutic target in GBM. However, there is a need for a full and integrated understanding of the different cellular and molecular components involved in the GBM TME and their interactions for the development of more efficient therapies. In this review, we provide a comprehensive report of the GBM TME, which assembles the contributions of physicians and translational researchers working on brain tumor pathology and therapy in France. We propose a holistic view of the subject by delineating the specific features of the GBM TME at the cellular, molecular, and therapeutic levels.

Introduction

GBM is the most common type of malignant primary brain tumor, with an incidence of two to five cases per 100 000 people per year in the USA and Europe [1]. The prognosis for GBM remains poor, with a median survival of ~15 months for patients who receive standard therapy [2] and less than 1 year in population-based studies [1]. GBM is characterized by wild-type forms of *IDH1/2* [3], high proliferation rates, core necrosis, microvascular proliferation, and tumor infiltration. GBM treatment involves surgical resection and radiotherapy together with concomitant chemotherapy with temozolomide (TMZ), also known as the Stupp protocol, a treatment that has not evolved since 2005 [4]. Resistance to treatment is mainly caused by tumor cells escaping resection and/or invading normal brain parenchyma. To date, the mechanisms underlying GBM development remain incompletely understood and current experimental systems only partially recapitulate GBM properties.

The genetics of GBM have been extensively studied [5]. Recent DNA-sequencing techniques at the single cell level have documented the coexistence of different genetic subclones, while single cell RNA sequencing has unveiled different transcriptomic profiles [6]. However, this tumor cell-centric view may have underestimated the role of the TME, which has led to only incremental improvements in the therapeutic response. The TME includes components from the tumor niche as

Highlights

The tumor cell-centric view has underestimated the role of the tumor microenvironment (TME) and failed to translate into therapies that outperform Stupp treatment for glioblastomas (GBMs).

The tumor environment includes components from not only the tumor niche (TME), but also the tumor organismal milieu (macroenvironment).

The TME comprises several compartments, with intricate cellular and molecular interactions both within and between each compartment.

Neurons are recently investigated players in GBM development, which they regulate via GBM cell-neuron synapses.

Many experimental GBM models have been developed, including *in vivo/ex vivo* models in several species as well as 3D *in vitro* models.

Attempts to increase the complexity of 3D models have done so by introducing selected components of the TME.

The various therapeutic approaches in use or developed still require improvement by better defining the combination of the most promising targets within the TME.

Current therapies might also modify the TME and modulate the therapeutic response.

well as the tumor organismal milieu. A consensus review [7] listed the challenges that need to be addressed in neuro-oncology, including research on the brain TME, thereby establishing a close link with neuroscience. The TME controls both tumor growth and invasion in GBM, thus opening new possibilities for therapeutic intervention [8].

However, knowledge of the microenvironmental cues that impact GBM cell properties is sparse. The GBM microenvironment is complex, with intermingling immune cells, mainly represented by myeloid cells/microglia (one of the most abundant cellular constituents of the TME), blood vessels, astrocytes, and extracellular matrix (ECM), which varies across different tumor areas [9]. These interactions have been studied at the cellular and molecular level, such as the role of vascular endothelial growth factor (VEGF) and VEGF receptors (VEGFRs), guidance factors, and positive or negative cues between GBM cells and blood vessels [10]. More recently, an intricate interaction with neurons has been documented [11], but how all of these different elements are spatially and causally integrated is unknown.

The GBM immune landscape has also been explored and compared with brain metastasis using a combination of approaches [12]. The drawback of this work is its purely descriptive nature and the lack of information on how microglia or monocyte-derived macrophages spatially interact with the other components of the TME. Future work should investigate: (i) molecular events (and their dynamics) between tumor cells, neurons, vascular, and immune compartments; (ii) tumor regional heterogeneity; (iii) external constraints and cues regulating the TME and their impact on tumor development; and (iv) the impact of therapeutic interventions in the complex brain tumor environment (Figure 1). Given that both physicians and translational researchers working on brain tumor pathology and therapy, we provide here an extensive review that delineates the specific features of the GBM TME at the cellular, molecular, and therapeutic levels.

Glioblastoma stem cells

Cellular and molecular aspects

The concept of glioblastoma stem cells (GSCs) has provided new insights into GBM pathophysiology. This heterogeneous tumor cell population, which can be quiescent or proliferating, is essential for promoting tumor growth and heterogeneity. Although GSCs were first thought to be rare and confined to specific niches, recent studies point to a more widespread and complex distribution within tumor tissue [13].

Accumulating evidence suggests that GSCs correspond to a cell functional state rather than to a lineage, a novel and provocative concept further reinforced by the demonstration that GBM cells can reversibly adopt or lose stem-like properties in response to TME cues [13]. As such, tumor cells evolve on a continuous undifferentiated–differentiated axis, with epigenetic regulations underlying cell interconversions between these phenotypes [13–16]. Interactions with surrounding normal cells, such as subventricular zone (SVZ) cells of the neural stem cell niche, endothelial cells in the perivascular or hypoxic niche, or with tumor-activated macrophages in the immune niche, are instrumental to promote and maintain a stemness phenotype. GSCs from the SVZ display higher tumorigenicity features than those isolated from other locations [17]. Accordingly, patients with GBM cells in intimate contact with the SVZ exhibit increased multifocal recurrence and a substantial reduction in survival [18]. The crosstalk between GSCs and their non-cancerous neighbors is bidirectional and instrumental for shaping the TME and promoting therapy resistance. GSCs actively participate in perivascular niche formation mainly by the secretion of chemoattractant and pro-angiogenic factors promoting endothelial cell (EC) proliferation and recruitment of their progenitors [19]. GSCs can also transdifferentiate into vascular/pericyte lineages and ECs [20]. Similarly, GSCs contribute to immune cell infiltration and the formation of a protumorigenic

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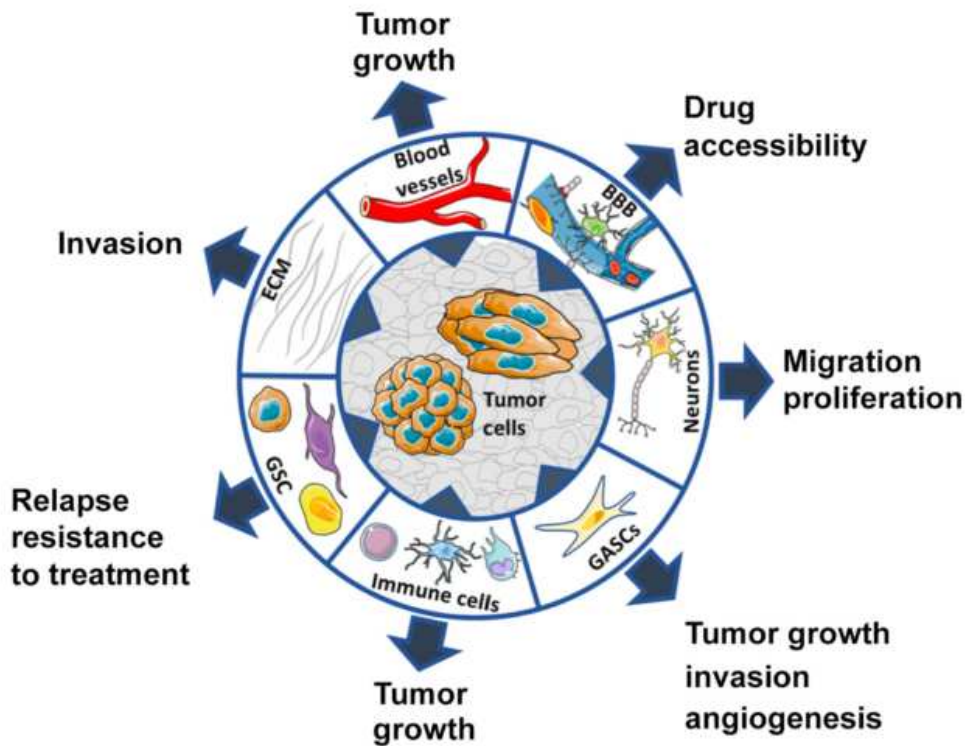
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Trends in Cancer

Figure 1. Schematic of the interaction of glioblastoma (GBM) cells with their microenvironment and functional consequences for tumor progression and response to treatment. Different representations of GBM tumor cells (sphere/stem versus adherent/mesenchymal) at the center of the cartoon are indicative of tumor cell heterogeneity. Abbreviations: BBB, blood-brain barrier; ECM, extracellular matrix; GASC, glioma-associated stromal cell; GSC, glioblastoma stem cell (GSC)-specific niches.

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inflammatory environment through the secretion of cytokines and growth factors [21]. In this context, GSCs induce tumor-associated macrophages (TAMs) to adopt an immuno-suppressive phenotype, whereas TAMs secrete factors contributing to GSC maintenance [22]. Therefore, the TME appears to be a key regulator of stemness, whereas GSCs affect the microenvironment. This complex dynamic is instrumental for tumor aggressiveness and, thus, its targeting could improve therapeutic strategies.

Therapeutic implications

GSCs are resistant to current therapies and have a crucial role in tumor recurrence. Indeed, tunneling nanotubes (TNTs), thin membranous open-ended channels connecting distant cells, provide alternative means of connection between GSCs, allowing mitochondrial transfer. GSCs from the metabolic area in the infiltrative zone (FLAIR) defined by MRI spectroscopy in patients treated for GBM have more TNT connections compared with GSCs from the non-metabolic area [23]. Moreover, radiotherapy can stimulate TNT formation and mitochondria transfer in GSCs from the metabolic area (Box 1). These results show the importance of plasticity in GBM cells and metabolic heterogeneity in response to radiotherapy [24]. Scientists have since tried to develop strategies directed at their elimination either by cytotoxic agents [25] or by repressing their stemness properties [26,27].

Box 1. Metabolism in GBM

Primitive brain cancers and brain cells share an affinity for glucose and glutamine [145]. Aberrant glucose and glutamine metabolism is a major metabolic hub stimulating GBM growth and inducing chemoresistance [146]. Numerous studies have shown that GBMs are metabolically plastic and can adapt to nutrient shortage by switching from one carbon source to another, or to alternative energy sources [147]. Furthermore, the microenvironment has been shown to be implicated in metabolic plasticity and reprogramming [148]. Glucose/glutamine interconnection in GBM could be reminiscent of the astrocyte–neuron metabolic interplay in which glutamate to glutamine conversion and reciprocal reaction fuels the tricarboxylic acid (TCA). By contrast, lactate produced in glycolytic cells can be released and then taken up by surrounding cancer cells for making amino acids, nucleic acids and fatty acids through anaplerosis [149]. The microenvironment is one of the major actors in cancer metabolism plasticity, which determines most glioma features, such as migration, cell survival, cell death, EMT, dormancy, and stemness. Mitochondria have a central role in ATP production and redox balance, but are also involved in many cellular processes, such as proliferation and cell death. Thus, changes induced by metabolism affect organelle functions and several cellular processes. Cancer development progression and treatment resistance in GBM is linked to distinct subsets of GSCs [150]. Metabolically, GSCs have a higher plasticity than their normal counterparts (neural stem cells) and their differentiation in proliferating GBM appears to be under the control of several metabolic enzymes, with non-canonical PKM2 activity being crucial in this process [151]. As reported above, original studies have shown that GSCs mainly depend on glycolysis, but recent articles have demonstrated that these cells might prefer mitochondrial oxidative metabolism. The slow-cycling GSCs appear to rely on both glycolysis and oxidative phosphorylation depending on the environmental situation and give rise to metabolically distinct GBM subpopulations [152]. GSC maintenance is also under the control of a methyl donor metabolism, nicotinamide *N*-methyltransferase (NNMT), implicated in epigenetic regulation [15].

GBM vascular compartment**Cellular and molecular aspects**

GBM are highly vascularized tumors, in which the endothelium not only fuels tumor growth with oxygen and necessary nutrients, but also provides a protective microenvironment. As such, ECs are active components of tumor development. The formation of new blood vessels in brain tumors leads to disorganized, convoluted, and leaky vascular networks, suggesting abnormal angiogenesis in the tumor. Pro-angiogenic factors are produced by tumor cells, cancer-derived stromal cells, and inflammatory cells [28], leading to the idea that the TME provides an aberrant vascular bed [29]. In the brain tumor ecosystem, metabolic, hypoxic, and inflammatory stressors modulate the production rate and nature of pro-angiogenic factors. Notably, cells within the hypoxic environment, often at the periphery of the necrotic core, release aberrant levels of VEGF, among other pro-angiogenic paracrine factors, which might also trigger enhanced vascular permeability, exacerbated vascular bed inflammation, and edema [29]. In line with this finding, tumor-derived ECs maintain their identity while gaining mesenchymal characteristics, which promotes tumor proliferation, migration, and permeability [30]. Furthermore, tumor-induced angiogenesis might rely on the recruitment of bone marrow-derived endothelial progenitor cells, or other stem cells [31]. Hypoxia also increases local DNA methylation disorder and shifted cell states, which reflects an epigenetically facilitated adaptive stress response [32].

Vasculature is also rerouted for the benefit of the tumor through co-option of pre-existing vessels. This migration of tumor cells toward and along pre-existing vessels provides cancer cells with privileged access to nutrients and oxygen, as well as a trail for invasion [33]. More atypical features, such as vascular mimicry (i.e., the propensity of tumor cells to simulate an endothelial phenotype), might provide additional complexity to the brain TME [34]. This interdependency between cancer cells and vasculature might be even more plastic and dynamic than initially thought, following the discovery of new modalities involved in building a dedicated tumor vasculature, including processes such as *trans*-differentiation by the commitment of GSCs to endothelial and pericyte lineages [35]. This process might be associated with the so-called ‘perivascular niche’, where GSCs and ECs are in close contact [36]. Single cell sequencing of CD31-expressing ECs led identified five clusters when comparing the tumor core to the tumor periphery. Three clusters are present in the tumor core and two in the periphery. Expression profiles of blood–brain barrier (BBB)-associated transporters indicate that the BBB is partially preserved [37]. The variety of

ECs in GBM is further enhanced by the finding that tumor-derived ECs (TDECs), which arise from GBM cell *trans*-differentiation, and tumor-associated vessel ECs (TAVs) are molecularly distinct, although heterogeneity is observed within each group [38].

Future work should better define the different processes involved in vascularizing and perfusing tumors and determine how they dynamically coexist during tumor evolution and in response to treatment. The molecular circuitry driving the adaptability of ECs in hostile conditions remains an open question.

Therapeutic implications

Several clinical studies involving VEGF/VEGFR inhibition have been reported, with mixed outcomes (75 clinical trials, of which 53 involved bevacizumab). Most anti-angiogenic therapies (e.g., bevacizumab, cilengitide, or cediranib) failed to improve the overall survival (OS) and quality of life of patients [39]. There are several possible reasons for this limited efficacy, including VEGF-independent angiogenesis, vessel co-option, and inefficient antiangiogenic factor delivery to the tumor [40]. Alternatively, VEGF is secreted and transported within extracellular vesicles, where it is inefficiently targeted by bevacizumab [41–44].

Efforts have been made to develop novel therapies based on anti-angiogenic factors that target angiogenesis through different mechanisms of action. Recently, regorafenib, an inhibitor of several kinases involved in neoangiogenesis regulation, showed promising results when used as monotherapy for recurrent GBM [45]. Alternative anti-angiogenic therapeutic approaches rely on the targeting of vasculogenesis and vessel co-option through alteration of the CXCR4/CXCL12 axis [46]. As such, the CXCR4 inhibitor plerixafor was evaluated in a Phase 1/2 trial upon continuous infusion during chemoradiation for patients with newly diagnosed GBM. This treatment was well tolerated by patients and was associated with decreasing blood volume in the irradiation field. Other factors involved in vessel co-option included angiopoietin-2, IL8, EGFRvIII, MDGP/FABP3, IRE1 α , CDC42, and Ephrin-B2. The inhibition of angiopoietin-1 and -2 by trebananib for patients with recurrent GBM was well tolerated but did not improve patient survival alone or in combination with bevacizumab [47]. Regarding the targeting of EGFRvIII, the first results of a specific vaccine called rindopepimut were disappointing [48] but new drugs are under investigation [49]. Finally, the MET pathway was explored to disrupt the angiogenic process in GBM. This pathway was suspected to be involved in angiogenesis independent of VEGF and represented an interesting approach alone or in combination with a VEGF inhibitor. However, onartuzumab, a MET inhibitor, used in combination with bevacizumab, yielded inconclusive results [50]. The tyrosine kinase inhibitor cabozantinib was also tested as monotherapy for patients with recurrent GBM who had either benefited or not from previous anti-angiogenic therapy [51,52]. Although it failed in patients who already received bevacizumab, it was associated with a response rate ranging from 14% to 17% in patients naïve to anti-angiogenic therapy, thus opening future opportunities for clinical development.

More recently, combinations with immunotherapy have been proposed. These therapies generally fit into the following categories: targeted molecular inhibitors, vaccine-based therapies, viral therapies, and adoptive T cell therapies. Bevacizumab is the only anti-angiogenic agent that has so far been trialed with immunotherapy in GBM. Bevacizumab has the theoretical advantage of indirectly promoting an immune response by reducing the use of corticosteroids. However, there have not yet been any successful Phase 3 trials in immunotherapy and anti-VEGF combinatorial treatments for GBM. The two best-studied combinations are immune checkpoint inhibitors (ICIs) with bevacizumab and vaccine-based therapies with bevacizumab, such as the Durvalumab (NCT02336165) and Gliovac studies (NCT01903330). Yet, targeting the GBM

TME using a combinatorial strategy (anti-angiogenics and immunotherapy) remains an appealing therapeutic approach.

GBM and the blood–brain barrier

Cellular and molecular aspects

The BBB is an anatomic and biochemical barrier with multiple proteins controlling the transport of various molecules across its cellular and acellular structures. Several mechanisms of transport are described based on molecular weight, liposolubility, electric charge of cargo molecules, or interactions with proteins in circulation or the BBB. Blood pressure is also involved in modulating the traffic of molecules across the BBB. Small lipophilic molecules use passive transcellular and paracellular pathways between and across vascular ECs. Larger or hydrophilic molecules are transported by transcytosis or specific receptor-mediated transport. The efflux pump system is involved in expelling harmful molecules out to the bloodstream [53,54]. In GBM, the BBB is partly disrupted, and the term ‘blood–tumor barrier’ is preferred. However, the BBB remains intact in the bed around the tumor, where invasive cells involved in drug resistance and recurrence are located [55,56].

Therapeutic implications

Overall, antitumor drugs cross the BBB inadequately. The most effective drugs used in neuro-oncology are TMZ and nitrosoureas, with an efficacy for crossing the BBB of 15–30%. Although the dose reaching the tumor cells is limited, increasing the delivered dose can expose the patient to systemic toxicity. Multiple strategies are under development to disrupt, bypass, or overcome the BBB obstacle, such as increasing local or systemic delivery and increasing drug penetration by drug or BBB modulation. Increasing drug delivery relies mainly on high-dose intravenous doses or intra-arterial chemotherapies with modest results in GBM. Another approach to overcome the BBB relies either on the use of carrier agents to promote drug penetration or BBB modulation using drugs or physical interventions [57]. A Phase 2 clinical trial testing TMZ and liposomal doxorubicin showed promising results, with four out of 22 patients with recurrent GBM showing a response [58]. Finally, transient BBB disruption using chemical/physical agents (e.g., hyperosmotic agents or ultrasound) appears a promising approach and is being investigated in GBM clinical trials [55]. Nevertheless, the BBB remains a major factor involved in the chemoresistance of GBM cells.

The GBM neuronal compartment

Cellular and molecular aspects

The effects of neuronal activity and synaptic signaling on GBM dynamics have been studied by grafting the tumor in the visual cortex and controlling light exposure or by optogenetically increasing neuronal activity, resulting in modulation of the number of dividing tumor cells [59]. Neuronal activity-driven GBM growth is first mediated by secreted factors, such as brain-derived neurotrophic factor (BDNF) or the synaptic adhesion molecule Neuroligin-3 [60]. Neuroligin-3 acts on GBM cells, triggers its own secretion (autocrine effect), and favors cell division through the PI3K-Akt-mTOR pathway. The expression of functional receptors for several neurotransmitters in GBM cells constitutes another level of exposure to the neuronal environment. For instance, GBM cells express fully functional dopamine receptors, including the D2 and D4 subtypes [61], the blockade of which affects GBM growth. Glutamatergic excitatory synaptic signaling is a major microenvironmental driver of GBM growth. Extracellular glutamate concentration increases up to 100-fold both in gliomas and their vicinity in humans [62] and mouse models (Box 1). A single dose of sulfasalazine reduces brain glutamate concentration by 20–50%, in correlation with the expression of the cysteine–glutamate antiporter (SXC; SLC7A11) [63]. Direct bona fide synapses from neurons to glioma cells have been documented in animal models and human tissue,

especially in astrocytomas and GBM [11,64]. It was reported that 10–16% of glioma cells contained these synapses, linking axons of pyramidal cells with microtubules of glioma cells, and mainly comprised postsynaptic glutamatergic AMPA receptors, with high rates of edited GluR2 subunits, which allowed for Ca^{2+} permeability. Glutamatergic signaling led to the division and migration of tumor cells [11,64]. GABA also regulates GBM cell proliferation [65]. Glioma cells express ionotropic GABA receptors with a different status according to their state of malignancy, with functional receptors identified on low-grade glioma cells, whereas nonfunctional receptors mostly formed by Theta subunits are expressed by GBM cells [66]. GABA signaling also modulates GBM growth by regulating chloride ion fluxes [67]. However, pharmacological approaches targeting those pathways have not yet translated into efficient clinical strategies.

Therapeutic implications

The fact that glioma stem-like cells can form oncogenic synapses with glutaminergic neurons supports the development of therapies targeting this interaction. Blocking AMPA receptors with the approved antiepileptic drug perampanel resulted in the reduction of glioma proliferation [64] and invasion [11] in animal models, whereas the AMPA antagonist talampanel prolonged the survival of patients [68]. GBM cells also express glutamate metabotropic receptor Grm3. Targeting these receptors by specific inhibitors could constitute an interesting venue for the development of novel therapies [69]. Other potential strategies might include acting directly on glutamine via GLS-mediated glutaminolysis, as reported for SNAP25 [70], repressing glutamate outflow by the Xc system [63], or enhancing glutamate uptake from astrocytes via the upregulation of EAAT2 by the peroxisome proliferator-activated receptor gamma (PPAR γ) [71].

The GBM immune compartment

Cellular and molecular aspects

Immune cell infiltration into GBM is controlled by the release of factors from tumor cells (Box 2), which is regulated by specific pathways in tumor cells, such as metabolic pathways and the unfolded protein response (UPR) (Box 3). Furthermore, autophagy (Box 4) might impact directly on the TME by modifying the immune response. The immune response can be divided into adaptive and innate immunity.

Box 2. The GBM secretome

The secretome corresponds to the material released by cells (proteins and metabolites) into the extracellular space, either using conventional (e.g., the secretory pathway) or unconventional mechanisms. Secreted proteins account for 9–15% of the total human proteome. The GBM microenvironment is impacted by tumor and stromal cell secretion, which are major factors in tumor aggressiveness. Secretome analysis in the human GBM cell line U87 showed high levels of proteins, such as ADAM9, ADAM10, Cathepsin B, Cathepsin L1, Semaphorin7A, Semaphorin3A, and Neuropilin1 [153]. Some of these proteins are proteases, which might help cell detachment and migration. GBMs also secrete cytokines, such as IL1 β , IL6, IL8, or IL18, which are associated with poor prognosis, either through conventional or unconventional routes. Cytokines, such as IL1 β and IL18, are secreted via autophagy-mediated secretion [154]. A secretory reticulophagy route was recently shown to mediate the release of reticulons in a VAMP7-dependent manner and this pathway was increased in cells defective in degradative reticulophagy [155,156]. Therefore, it will be important to characterize unconventional secretion, such as that of endoplasmic reticulum elements, in GBM, its potential effect on tumor development and communication with neurons. For a long time, the functional consequences of the close proximity between GBM cells and neurons were not well understood, but there is now ample evidence to suggest that they participate in a mutually beneficial relationship. Secreted molecules from neurons or the neuronal secretome modulate the GBM microenvironment and impact glioma growth and survival prognosis. Reciprocally, GBM cells produce metabolites that can be secreted into the extracellular space, such as lactate, glutamine, or glutamate. A lactate shuttle has been identified between glycolytic astrocytes and oxidative neurons. The release of glutamate has dual consequences. First, it results in enhanced neuronal activity or hyperexcitability, which sometimes manifests as seizures and can even cause neuronal death to create more space for GB expansion. Second, it has prosurvival effects on GBM cells [157]. This indicates the existence of positive feedback loops because GBMs can enhance neuronal activity by secreting glutamate, thereby fueling their own growth in turn. The characterization of molecules secreted as part of the neuronal secretome likely to impact GB progression opens new avenues for disease treatment and management.

Box 3. The unfolded protein response in GBM

The UPR is an adaptive mechanism triggered in response to endoplasmic reticulum (ER) stress. It is transduced by three sensors: protein kinase R-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 alpha (IRE1 alpha, referred to as IRE1 hereafter), and activating transcription factor 6 (ATF6), and is a key factor in cancer development. GBM cells are exposed to cellular stresses that trigger the UPR. Exploring the role of the UPR showed the involvement of ATF6, IRE1, and PERK in different aspects of GBM pathophysiology [158]. The ATF6 arm of the UPR is involved in GBM resistance to radiotherapy [159]. The UPR also regulates GBM stemness [160] and pharmacological inhibition of PERK limits ER stress-induced GSC death, thus highlighting PERK as an important regulator of GSC growth and differentiation.

IRE1 has been identified as a key regulator of GBM aggressiveness associated with tumor growth, neo-angiogenesis, tumor migration/invasion properties, and proinflammatory processes [158]. Moreover, IRE1 signaling has an active role in tumor immune reshaping, in particular of monocytes/macrophages and neutrophils [161]. IRE1 signaling also contributes to the organization of the tumor architecture, favoring tumor growth, infiltration, and mesenchymal cell phenotypes *in vivo* [162,163]. Antagonistic effects of IRE1 signaling towards XBP1 mRNA non-conventional splicing or RNA degradation were described in tumor invasion, neo-angiogenesis, and the inflammatory response, influencing GBM aggressiveness [161]. Patients with GBM characterized by high IRE1 activity had a poorer prognosis and displayed increased immune, angiogenic, and migration markers. Overall, IRE1 represents an appealing therapeutic target in GBM, and several IRE1 inhibitors are currently available, targeting either its kinase or RNase activities [164]. A recent preclinical study using a mouse model recapitulating the SOC for patients with GBM confirmed the efficacy of such IRE1 inhibitors [165]. Thus, the UPR controls major features related to GB aggressiveness and represents an actionable pathway to improve disease management.

Adaptive immunity

Early studies report the presence of rare infiltrating lymphocytes within tumors, mainly CD4 and CD8 T cells. These cells represent 4–40% of infiltrating immune cells [72]. Such variability was associated with biological heterogeneity, the methods used [e.g., immunohistochemistry (IHC) or fluorescence-activated cell sorting (FACS)], or the characterization performed after *in vitro* expansion [73]. Reduced immune T cell infiltration is attributed to the high immunosuppressive properties displayed by GBM cells, including TGF β secretion [74], and, thus, GBM is defined as an immunologically cold tumor [75]. These initial observations are further explained by the increased

Box 4. Autophagy in GBM

Autophagy is a multistep process of protein/organelle quality control that maintains cell homeostasis under different cellular stress inducers. Autophagy can be associated with either a protumorigenic or tumor-suppressive response depending on various parameters, including the cellular context, type of stimulus, and phase of tumor development. Current data describing a role of macroautophagy (referred to as autophagy hereafter) and chaperone-mediated autophagy (CMA) in TME regulation in GBM are summarized here.

Several studies have demonstrated the link between key players in gliomagenesis and autophagy regulation. Notably, it has been shown that p53, a key tumor suppressor involved in GBM etiology, controls the transcription of two master regulators of mitophagy: PRKN and PINK1 [166]. Interestingly, p53 and PRKN protein levels are inversely correlated according to the grade of gliomas [167] and decreased PRKN microenvironment levels lead to increased tumor progression [168]. The reciprocal relationships between autophagy and the immune system point toward autophagy as an important GB TME modulator. Autophagy promotes tumor progression by increasing the phagocytotic activity of macrophages and by inhibiting inflammasome-mediated responses of microglial cells. It might also foster tumor evolution by either stimulating the differentiation of monocytes into anti-inflammatory M2 macrophages or by delaying monocyte polarization into proinflammatory M1 macrophages [169]. Autophagy has also been shown to decrease tumor activity by inducing an inflammatory response in neutrophils [170].

CMA is a type of autophagy responsible for the lysosomal degradation of cytosolic proteins harboring the KFERQ consensus motif. CMA is constitutively activated in cancer cells [171] and tightly linked to TME regulatory stresses [172]. CMA is important in maintaining aerobic glycolysis in cancer cells and in the catabolism of cell cycle regulatory proteins [173]. Analysis of patient biopsies indicated an increase in the CMA makers LAMP1 and LAMP2A after treatment with TMZ [174]. In addition, CMA has been linked to microglial cell functions in neurodegenerative diseases, providing a potential link to the innate immune response, which might be relevant in GBM [175]. Cellular interactions between perivascular and GB cells are important for tumor progression. CMA-defective pericytes led to a reduction in GB development compared with CMA-competent pericytes [176]. In brief, studies to analyze the role of the various types of autophagy in TME impact on GB development are still needed and could lead to the identification of new therapeutic pipelines.

naïve T cell sequestration in the bone marrow compartment in patients with GBM, resulting in a reduced number of T cells at the periphery and in the tumor site, a phenomenon linked to tumor-induced Sphingosine-1-phosphate receptor 1 (S1P1) loss on the T cell surface [76]. Recently, single-cell analysis of GBM-infiltrating T cells from human glioma unveiled four major T cell clusters, including CD4 (conventional and regulatory), CD8, and cycling T cells [77]. Among the CD8 T cell cluster, one predominant subtype comprised cytotoxic T cells that express not only a cytotoxicity related-gene signature (including perforin-1 and granzymes), but also natural killer (NK) cell-related genes that negatively modulate cytotoxic functions, such as that encoding the inhibitory receptor CD161. This finding opens avenues to target new molecular pathways to transform GBMs from immunologically 'cold' to 'hot' tumors using novel immunotherapy approaches.

Innate immunity

NK cells recognize target cells lacking major histocompatibility complex (MHC) expression. They represent crucial antitumor response agents when cytotoxic T lymphocytes are compromised. NK cells can target GBM cells because these cells express low levels of MHC. GBM cells, including GSCs, are recognized and killed by NK cells *in vitro* [78,79], and high CD56⁺ CD16⁺ activated NK cells are associated with favorable patient outcomes [80]. However, limitations remain to challenging NK cell efficiency in GBM. First, the number of GBM-infiltrating NK cells remains low, representing <1% of all infiltrated immune cells [12]. Second, GBM-infiltrating NK cells harbor a reduced expression of tumor-sensing NK cell-activating receptors, such as Nkp30, NKG2D, DNAX accessory molecule-1 (DNAM1), or surface molecules, including CD317 and CD210. Moreover, GBM tumor cells, including GSCs, are directly responsible for NK cell inactivation by expressing immunosuppressive CD7312 and overexpressing MHC class I [81,82].

The most abundant cells in GBM are tumor-associated macrophages (TAMs). These constitute up to 40% of the tumor mass and correspond to brain-resident microglia and bone marrow-derived myeloid cells. TAMs contribute to tumor heterogeneity and tumor progression. As innate immune cells, TAMs express Toll-like receptor-2, the activation of which leads to the expression of matrix metalloproteinase (MMP)-14, which is necessary for MMP2 release and GBM invasion. TAM infiltration has been associated with poor outcomes in patients with GBM, and in preclinical mouse models of GBM. TAMs also promote GSC proliferation, a mesenchymal phenotype, and resistance to radiotherapy and anti-VEGF therapy [83].

The TME modulates the response to radiotherapy because hypoxia is a major factor for resistance in several tumors, including GBM. Preclinical studies showed that radiation-induced hypoxia prompts an increase in stromal cell-derived factor-1 (SDF1/CXCL12) secretion [84]. This signaling pathway has been targeted in GBM animal models, leading to macrophage exclusion and improvement of local control and survival after radiotherapy [85]. In addition, radiotherapy promotes the expression of tumor antigens along with the translocation of calreticulin to the tumor cell surface, leading to their phagocytosis [86]. This results in cGAS/STING activation and the subsequent induction of interferon β (IFN β) [87]. Interestingly, whereas hypo-fractionated radiotherapy (3 \times 8 Gy) induced an immune cell death through cGAS/STING, 12–18 Gy induced the DNA exonuclease Trex1, thus clearing DNA fragments from the cytosol and inhibiting tumor immunogenicity [88].

Neutrophils, the first line of innate immune defense, are present in the TME as tumor-associated neutrophils (TANs) and can be polarized toward a proinflammatory N1 phenotype in the presence of IFN β or to a protumoral N2 phenotype in the presence of TGF β . Whereas the N1 phenotype inhibits tumor growth and induces the immune response, the N2 phenotype, which is prominent

in GBM, promotes tumor growth, stemness, invasion, and angiogenesis, and suppresses immune responses [89]. Moreover, myeloid cell content changes during tumor evolution and standard-of-care (SOC) therapy. In early-stage tumors, proinflammatory microglia are predominant, whereas in end-stage tumors, anti-inflammatory macrophages and protumorigenic myeloid-derived suppressor cells are present [90]. TMZ decreased myeloid-derived suppressor cells and, when combined with irradiation, intratumoral Granzyme B⁺ CD8⁺ T cells influx into the tumor. Brain meningeal lymphatic vasculature can also be remodeled using ectopic expression of VEGF-C, thus enhancing immune surveillance and T cell-mediated immunity against GBM cells [91].

Therapeutic implications

Four main immunotherapeutic approaches are considered in GBM: (i) immune check point inhibitors (ICIs), (ii) cell, vaccine and dendritic cell (DC) therapies; (iii) oncolytic viruses; and (iv) immune cytokine inhibitors. The main immunotherapy approaches, stimulating the antitumor activities of cytotoxic T cells through inhibition of immune checkpoints, failed to improve the survival of patients with GBM [92]. The current perspectives for ICIs in brain tumors are to refine their administration schedule, to combine ICIs or to test new ICIs (e.g., LAG3, TIM3 or TIGIT inhibitors). ICI efficacy is based on the ability of T cells to infiltrate the tumor and on the development of an environment that is favorable to cytotoxic responses. These conditions are rarely met in GBM because of the abundance of immunosuppressive TAMs. Resistance to ICIs can be circumvented by depleting tumor macrophages with a CSF1R1 receptor inhibitor [93].

One of the most promising cell therapy approaches are chimeric antigen receptor (CAR)-T cells, comprising a modified T cell receptor containing a single-chain variable antibody fragment (scFv) against a tumor-associated antigen. In neuro-oncology, several CAR-T cells are under investigation, targeting EGFRvIII, HER2, and IL13R α 2. First results obtained from Phase 1/2 trials suggested an acceptable safety profile with promising results [94,95]. Recently, a CAR-engineered NK cell that targets both wild-type EGFR and EGFRvIII mutant has been developed, with promising results in mice.

Vaccination and DCs also present an attractive method for actively targeting GBM. First results using EGFRvIII vaccination reported disappointing results in a Phase 3 trial [48] but personalized approaches, based on patient tumor antigen expression profiles, appear promising despite the complexity of their management [96]. DCs pulsed by tumor-specific peptides, lysates, or antigens can also enhance antitumor T cell responses. Different DC-based vaccinations have been evaluated, showing interesting response rates and excellent safety profiles [97]. However, this approach remains challenging and expensive.

Another immunogenic active approach is the use of oncolytic viruses, which can selectively infect and kill tumor cells. The aim of using these viruses is to turn an immunosuppressive microenvironment into an immune-supporting environment. Various oncolytic viruses are under investigation, including herpes virus, parvovirus, adenovirus, and cytomegalovirus. In a Phase 1/2 trial, an oncolytic H-1 parvovirus was injected before and during surgical resection of recurrent GBM [98]. This virus was able to cross the BBB, spread through the tumor, and trigger an immunogenic stimulus, opening promising perspectives. Similar results were observed in another early-development clinical trial using an oncolytic reovirus, the results of which support the combination of oncolytic viruses and PD1 blockade [99].

Finally, among the different cytokines implicated in the immunosuppressive GBM TME, tumor cell-secreted CSF1 might be involved in the switch of tumor-resident macrophages/microglia

to an immunoinhibitory phenotype. Conversely, TAM depletion by LyG6 antibodies prolonged survival in a GBM mouse model [100], whereas the combination of the arginine-depleting agent ADI-PEG20 with irradiation led to almost-complete tumor eradication in an immunocompetent mouse model by promoting microglial recruitment and M2 to M1 polarization [101]. The first results of phase II trial evaluating the CSF-1 receptor inhibitor alone showed no efficacy [102] but additional studies are ongoing.

Few clinical trials have aimed to optimize irradiation with immunotherapy. In one trial, immunotherapy was added to irradiation in combination with bevacizumab [103]. In another trial, stereotactic 3 × 8Gy re-irradiation in the presence or absence of the anti-PDL1 durvalumab (NCT02866747) was compared. The use of tumor-treating fields (TTFields) as adjuvant treatment after standard radiochemotherapy in patients with *de novo* GBM also increased progression-free survival and OS [104], and presented, beside disrupting mitosis, the capacity to induce immunogenic cell death. In models other than GBM, TTFields increase peritumoral lymphocyte infiltration compared with nontreated animals [105]. The combination of such a device with standard radiochemotherapy is being investigated for *de novo* GBM (Trident EF-32).

GBM-associated stromal cells

Cellular and molecular aspects

The GBM TME also harbors glioma-associated stromal cells (GASCs). These cells have phenotypic and functional properties similar to those of mesenchymal stem cells (MSCs) and cancer-associated fibroblasts (CAFs) [106]. GASCs are present in human low-grade gliomas (LGGs) and GBMs, mostly around blood vessels, but also in tumor tissue. Cultured GASCs have properties similar to MSCs, such as adhesion to plastic, expression of surface antigens characteristic of MSCs (CD73, CD90, and CD105), mesenchymal differentiation potential, and lack of tumorigenesis potential. They also have phenotypic and functional properties in common with CAFs, such as expressing markers FSP1/S100A4 and promoting angiogenesis, invasion, and tumor growth by secreting soluble factors and extracellular vesicles [106]. The origin of GASCs has yet to be determined. They might arise from the *trans*-differentiation of local brain cells, such as astrocytes, pericytes, vascular smooth muscle cells, or ECs. GASCs can also originate from bone marrow-derived MSCs. They are not a homogeneous stromal population, given that GASCs isolated from LGGs and GBMs present different proteomic profiles and cancer cell adhesion capacities [107]. Furthermore, GASCs might also differ between gliomas with the same histological classification. Two peritumoral microenvironments can be found in patients with GBM: a peripheral microenvironment containing GASCs with tumor-promoting and angiogenic properties, and another containing GASCs without such properties [108]. The genetic background of tumor cells could account for these different GASC statuses. GASCs might also have prognostic value in gliomas because the percentage of GASCs in GBMs [109] inversely correlates with overall survival. These cells, which complicate our understanding of the GBM TME, should not be ignored. Thus, additional studies on the impact of GASCs on the response to treatment are urgently required.

Therapeutic implications

Given their tumor-supporting functions, GASCs are promising new targets for glioma treatment. However, additional studies are required to identify candidate target molecules. One way to circumvent this problem is by using the properties of these cells to trap, rather than target, tumor cells [110]. The concept of tumor cell traps, which emerged from the ecological trap strategy, is designed to attract residual brain cancer cells surrounding the surgical cavity into the cavity, where they are trapped within a biomaterial support that can be targeted by treatment, such as stereotactic radiosurgery [111–113]. Molecules or particles with radio-sensitizing and/or chemotherapy characteristics could also be embedded within the biomaterial to improve tumor cell

destruction. Given that GASCs increase the invasiveness of GBM cells, a bacterial cellulose scaffold that was loaded with GASC-conditioned medium was evaluated for use as a trap matrix. The bacterial cellulose membranes released and attracted tumor cells *in vitro* [110]. Once attached to the surface of the membrane, tumor cells were unable to move on, go through, or escape the membrane, even when an attractive medium was present in close proximity [110]. However, further studies are needed to improve the chemotaxis properties of bacterial cellulose membranes to ensure the diffusion of chemoattractants over large distances and at high enough concentrations to trap GBM cells that infiltrate tissues several centimeters away from the resection cavity.

Extracellular matrix in GBM

Cellular and molecular aspects

ECM remodeling and stiffening is associated with a poorer prognosis [114]; however, analysis of ECM components and their spatial distribution in samples from patients with GBM is limited. Given that the brain is a soft tissue, its ECM differs from that of other solid organs. Elasticity of normal brain and brain tumors, measured using shear wave elastography, showed a stiffness increase from 7.3 kPa for normal brain to 11.4–33.1 kPa in GBM of different grades [115]. This observation was confirmed in preclinical models, with heterogeneous GBM tissues becoming softer as hypoxic/necrotic areas develop over time or, conversely, stiffer in zones of dense cell-containing abnormal vascular components [116]. At the molecular level, these differences in tissue stiffness result from modifications in the ECM composition in GBM. Accordingly, increased amounts of hyaluronic acid (HA) are detected in the ECM surrounding the tumor, as a result of locally increased HA synthase 1–3 activity, which promotes ECM remodeling and invasion of GBM cells [117]. Furthermore, tenascins, glycosaminoglycans, laminins, fibronectins, and collagens secreted by tumor cells or by stromal cells also contribute to ECM softening [118].

Mechanical stress has been previously shown to specifically control Rho GTPase [119]. Myosin II appears essential for migration properties by squeezing nuclei upon stiffness [120], suggesting an ECM-induced promotion of glioma invasiveness. *In vitro* biochemical investigations established that the morphology and the migratory behavior of GBM cells were inversely correlated with HA density [121], suggesting that a major requirement for glioma invasion is linked, at least in part, to the interaction between HA and its cognate receptor CD44. This finding also indicates that reduced HA leads to more flexibility [122] and, in turn, could favor the degradation of the surrounding ECM by MMPs, such as MMP2, MMP9, and MMP13, allowing tumor cells to invade surrounding brain tissue [123].

A pro-invasive network of matrix collagens, collagen-interacting proteins, and collagen-processing enzymes is present in mesenchymal tumors and transcriptionally regulated by IRF3. The expression levels of these collagens is significantly correlated with GBM progression and OS [124]. In addition to its fibrous composition, the ECM dynamically controls fluid concentrations and gradients of chemokines within the tumor bulk and in the periphery, via GBM-induced secretion of different enzymes, including hyaluronidases, MMPs, disintegrin, metalloproteinases with thrombospondin sequence (ADAMTs), cathepsins, urokinases (uPAs), and tissue plasminogen activators (TPAs). This leads to chemokine and growth factor accumulation, either by increased production or by the release of pre-adsorbed ligands, such as the CXC family of chemokines or TGF β [125] following ECM degradation, which control GBM invasion and progression. ECM components or ECM-entrapped secreted proteins also have the ability to modify the immune landscape of GBM. For example, M2 TAMs (known to exhibit protumoral functions) are recruited through Integrin $\alpha\beta$ 3 in the tumor by GSC-secreted periostin to promote malignant growth [126]. Tenascin-C impairs T cell infiltration in tumor nodules [127]. Osteopontin overexpressed in GBM microenvironment by immune cells is required for M2 macrophage gene signature maintenance and recruitment to the tumor [128]. However, dynamic changes in brain ECM during

GBM development and progression are not yet extensively characterized or understood. Recently, comprehensive work demonstrated how the compression exerted by GBM on the surrounding brain tissue causes vascular perfusion defects and death of adjacent neurons, likely contributing to extra-tumoral ECM remodeling [129].

Therapeutic implications

Targeting ECM in GBM appears an attractive strategy based on the altered expression of components responsible for many aspects of tumor progression. Hyaluronan is the most studied component [130], with HA oligomers, hyaluronidases, or HA synthesis inhibitors yielding promising results in preclinical studies, despite not yet reaching clinical applications. Among the HA synthesis inhibitors, 4MU induced senescence and decreased CD44 and RHAMM in glioma cells independently of its effect on HA and, thus, is an alternative therapy [131] requiring more investigation. HA was also identified as a therapeutic target in oncolytic adenovirus immunotherapy. ICOVIR17, a conditionally replicating adenovirus expressing soluble hyaluronidase, mediates degradation of HA when directly injected in GBM in mice, resulting in virus spread and antitumor efficacy [132]. Moreover, ICOVIR17 dramatically modified the immune landscape in immunocompetent GBM mice by increasing tumor-infiltrating CD8⁺ T cells and glioma-associated macrophages, and upregulating PDL1 [133]. The combination of ICOVIR17 with anti-PD1 therapy led to increased survival in mice, suggesting strong preclinical evidence in support of a clinical trial.

Other ECM molecules have also been targeted. A brevicin deglycosylated form (dg-Bcan), which accumulates specifically in GBM, could be targeted by a dg-Bcan-targeting peptide (BTP-7) specifically homing to GBM [134], enabling drug delivery with therapeutic effects [135]. Similarly, a tumor-penetrating Tenascin-C binding peptide (PL3 peptide) was used to coat nanoparticles, which then accumulated at Tenascin-C- and NRP1 receptor-positive areas in clinical GBM samples. When loaded with pro-apoptotic factors, these PL3-guided nanoparticles provoked GBM caspase-3-dependent apoptosis and decreased vascularization associated with mouse survival [136]. This finding led to the creation of a bispecific Tenascin-C and fibronectin-targeted peptide (PL1 peptide), which also shows antiglioma activity [137]. Recent development of an antibody–cytokine fusion or immunocytokines, based on an L19 antibody specific for the tumor-associated extra epitope B of fibronectin in fusion with IL12 or TNF α , showed promising results in mouse models. The fusion promoted tumor-infiltrating lymphocytes and increased proinflammatory cytokines within the TME [138], which resulted in L19/TNF α -induced GBM necrosis. This compound is now being tested in a pilot trial in patients with GBM (NCT03779230). Targeting the ECM brings new hope to being able to provide therapeutic alternatives that not only remodel the GBM microenvironment (in particular to enhance anti-tumoral immunity), but also, when used in combination, guide therapeutics inside tumors or promote immune attack.

Concluding remarks

Although no significant improvements in the survival of patients with GBM have been seen in the general population since 2005, two important Phase 3 randomized clinical trials reported positive results in two subgroups of patients with newly diagnosed GBM. First, the EF-14 clinical (NCT00916409) showed that TTFIELDS plus SOC improved progression-free survival and OS in patients with newly diagnosed GBM in good clinical condition (i.e., Karnofsky performance score ≥ 70) after the concurrent radiochemotherapy part of the SOC [104]. Second, the CeTeG/NOA-09 (NCT01149109) clinical trial reported that lomustine added to SOC increased the OS of patients with MGMT-methylated newly diagnosed GBM [139] (a more complete list of current clinical trials is provided in Table S1 in the supplemental information online). Nevertheless, novel therapeutic targets, such as those derived from the study of the TME, must be identified to increase the therapeutic efficacy.

Outstanding questions

What are the molecular events and their dynamics that occur between tumor cells and the nervous, vascular, and immune compartments?

What contributes to tumor regional heterogeneity?

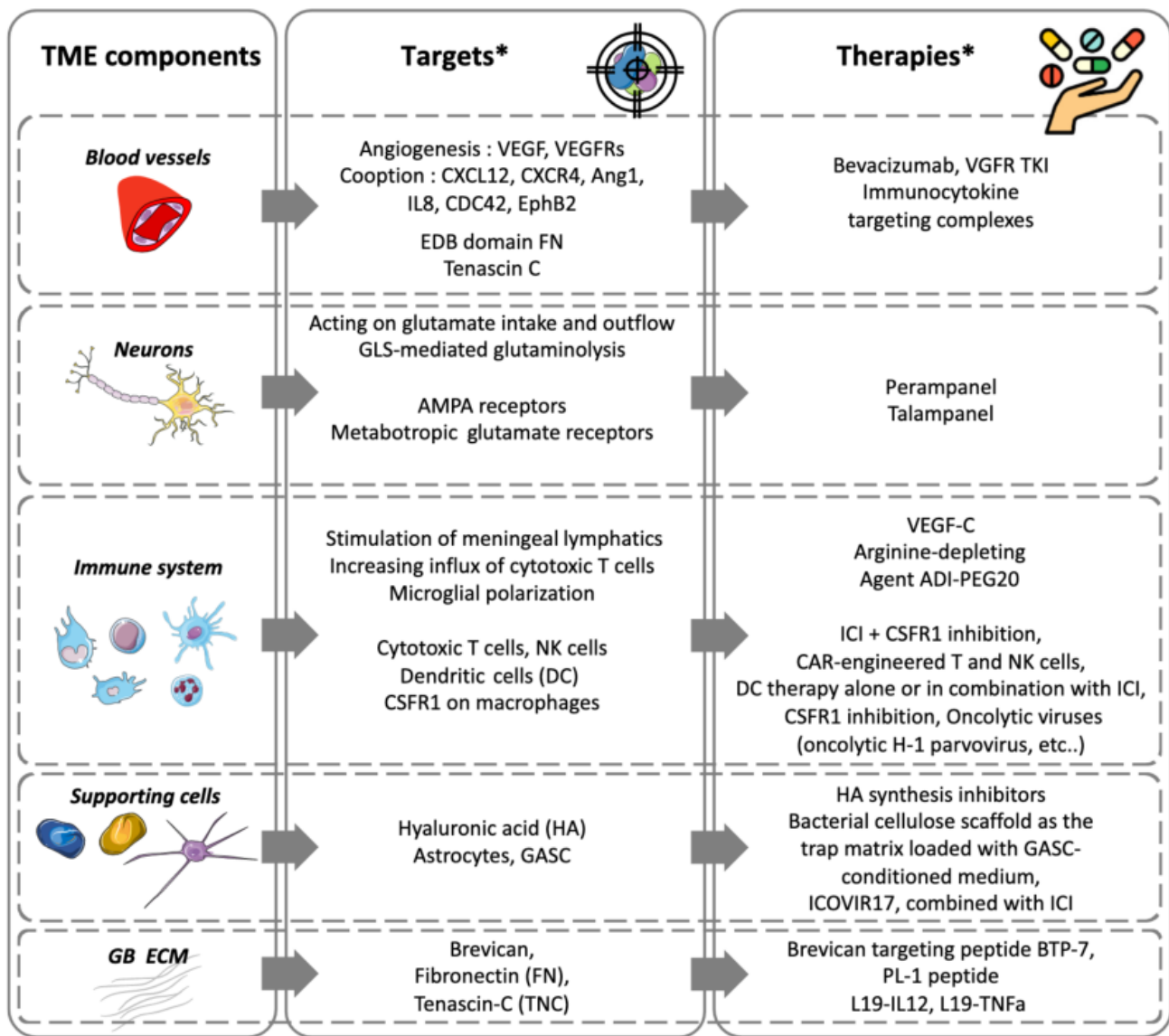
How can 3D GBM models be improved to account for the complexity of the TME?

What are the external constraints and cues regulating the TME and what is their impact on tumor development?

What is the impact of therapeutic intervention on a complex brain tumor environment?

Which therapeutic strategies that associate with TME/tumor cell-targeting combinations contribute to an improved outcome of the disease?

Many components of the GBM environment have been reported in the literature, such as vascular and immune components, neurons, and ECM. They interact closely with GSCs or differentiated GBM cells through a network of growth factors, cytokines, and chemokines. [Figure 2](#) summarizes



[* current and potential]

Figure 2. Cellular and molecular actors and potential targets and therapeutic interventions in glioblastoma (GBM). Abbreviations: ADI-PEG20, pegylated arginine deiminase; AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; Ang, angiotensin; CAR, chimeric antigen receptor; CDC42, cell division control protein 42 homolog; CSFR1, colony-stimulating factor receptor-1; CXC, CXC chemokine; CXCR, CXC chemokine receptor; DC, dendritic cell; ECM, extracellular matrix; EDB, extra domain-B; EphB2, ephrin B2; FN, fibronectin; GASC, glioma-associated stromal cell; GB-ECM, glioblastoma-extracellular matrix; GLS, glutaminase; HA, hyaluronic acid; ICI, immune checkpoint inhibitor; IL8, interleukin-8; L19-IL12, L19 antibody-bound interleukin-12; L19-TNFa, L19 antibody-bound TNF α ; NK, natural killer; PL1 peptide, 12-mer targeting peptide selectively interacts with both TNC-C and FN-EDB; TKI, tyrosine kinase inhibitor; TKI, tyrosine kinase inhibitor; VEGF(R), vascular endothelial growth factor (receptor); VEGF, vascular endothelial growth factor.

the cellular and molecular actors and potential targets and therapeutic interventions in GBM. This variety of cell–cell and molecular interactions reflects the heterogenous state of GBMs, of which tumor cell plasticity is a main feature. New technologies [e.g., *in vivo* imaging, artificial intelligence, single cell (sc)RNA sequencing or single nucleus (sn)RNA sequencing, or spatial gene expression] combined with more sophisticated and controlled experimental models are likely to provide relevant novel information to improve our understanding of the GBM TME (Box 5). These might, in turn, contribute to the identification of actionable targets for the development of effective therapeutic strategies (e.g., TME-based tumor traps). Finally, current therapeutic approaches impact the components of the TME through different means, which modulate and modify the therapeutic response. For example, neurosurgical actions alone or in combination with the frequent use of steroid therapy can impact the TME and surrounding brain tissue [140]. Although tumor surgical resection has shown modest effects when used as a stand-alone approach, it can be coupled with local therapy in GBM. Approaches to local drug delivery have included the use of implantable, controlled-release polymer systems [141], delivery into the cerebrospinal fluid (CSF) or a cyst cavity (often using an implanted reservoir [142]), and catheter-based convection-enhanced delivery (CED) [143]. Other therapeutic agents have been investigated (reviewed in [144]). For instance, there are studies using intra-arterial cerebral infusion of anti-angiogenics in relapsed GBM (NCT01269853), opening of the BBB by using a sonocloud-9 device (NCT04614493), infusion of B7-H3 CAR-T in recurrent GBM (NCT04077866), or administration of autologous DCs in primary GBM (NCT04115761).

Although results are limited, these methodologies are promising for targeted local drug diffusion with innovative and effective delivery tools (scaffold or fluidic) for delivery to the tumor and surrounding central nervous system of novel therapeutic agents (CAR-T cells, ICIs, chemo- or targeted therapies). For instance, medical devices, including Ommaya reservoirs, convection-enhanced delivery reservoirs, and *in situ* biodegradable devices, are interesting strategies to increase local drug delivery. A Phase 3 clinical trial reported the efficacy of carmustine wafers in high-grade gliomas, although statistical significance was not reached for GBM [141]. Thus, understanding how to either enhance or minimize these effects for treatment improvement represents a major challenge (see Outstanding questions).

Box 5. Experimental and preclinical GBM models

Improved models of GBM are needed to faithfully recapitulate the landscape of GBM states and TMEs and to contribute to a thorough understanding of the developmental cues yielding GBM heterogeneity. Current models include standard *in vitro* models, rodent implant, or genetically engineered models (GEMMs), and chick or quail, microfluidic or assembloid engineered models.

Rodent implant models for GBM have provided valuable information about various molecular regulations, but are limited. Indeed, syngeneic systems do not recapitulate the genetics of human tumors and xenotransplanted cell lines or patient-derived xenograft (PDX) models lack a fully competent immune system. GEMMs do not recapitulate the genetics of human tumors or their invasive behavior. As an alternative with fewer ethical issues, avian-embryo models have been developed for short-term studies [177]. Microfluidic or bioprinted models are generated with optically clear material-based microchannels networks containing cells and/or matrix to recreate *in vitro* tissue or organ structure and function [178]. Different types of microfluidic device exist, such as the 3D organotypic microfluidic platform [179], the 3D bioprinted tumor vascular model [180] or a microchannel set-up mimicking the GBM microenvironment [181]. These microfluidic-derived models provide some improvement over existing models but have still limitations, such as lacking developmental dynamics, plasticity, and a relevant immune cell component. This complex tissue system maintains the cytoarchitecture of the brain with functional astrocytes and neurons for 2 weeks but lacks functional vessels, a problem also seen in brain/GBM organoids. Micro- and macrovessel engineering has been performed to provide artificial vascularized tissue for functional studies and tissue vascularization. The emergence of organ-on-a-chip technologies has attracted much attention [182]. These technologies aim to create bioactive artificial tissues from cultured cells, often using microfluidic techniques.

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Supplemental information

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