16 Glioblastoma: To Target the Tumor Cell or the Microenvironment?

STEVEN DE VLEESCHOUWER[1] • GABRIELE BERGERS[2]

[1]Department of Neurosurgery, University Hospitals Leuven, Leuven, Belgium;
[2]VIB-KU Leuven Center for Cancer Biology, Campus Gasthuisberg, Leuven, Belgium

Author for correspondence: Steven De Vleeschouwer, Department of Neurosurgery, University Hospitals Leuven, Belgium.
E-mail: Steven.devleeschouwer@uzleuven.be

Abstract: Solid cancers develop in dynamically modified microenvironments in which they seem to hijack resident and infiltrating nontumor cells, and exploit existing extracellular matrices and interstitial fluids for their own benefit. Glioblastoma (GBM), the most malignant intrinsic glial brain tumor, hardly colonizes niches outside the central nervous system (CNS). It seems to need the unique composition of cranial microenvironment for growth and invasion as the incidence of extracranial metastasis of GBM is as low as 0.5%. Different nontumor cells (both infiltrating and resident), structures and substances constitute a semi-protected environment, partially behind the well-known blood–brain barrier, benefitting from the relatively immune privileged state of the CNS. This imposes a particular challenge on researchers and clinicians who try to tackle this disease and desire to penetrate efficiently into this shielded environment to weaken the GBM cells and cut them off from the Hinterland they are addicted to. In this chapter, we focus on how GBM interacts with the different components of its tumor microenvironment (TME), how we can target this TME as a useful contribution to the existing treatments, how we could make further progress in our understanding

and interaction with this environment as a crucial step toward a better disease control in the future, and what efforts have already been taken thus far.

Key words: Immune cell infiltration; Tissue matrix; Tumor heterogeneity; Tumor microenvironment; Vascularization

Introduction

For many decades, cancer has been regarded and therapeutically approached as a chaotic aggregate of uncontrolled cancer cells, escaping any type of internal cell cycle control due to driving and bystander (passenger) genetic alterations as elegantly summarized by Vogelstein et al. (1). Referring to the often used, illustrative metaphor of a cancer cell as an encyclopedia in which variable amounts and types of information have been incorrectly copied and translated, any given cancer cell can be described based on the number of pages missing (deletions), the number of double pages (amplifications), or the typos in the body of the encyclopedia's text (mutations). This abnormal genetic information leads to "druggable" protein modifications in the cell in conventional chemotherapy (2) or more recently in the logic of the emerging immunotherapies, to "neoantigens" (neoepitopes) in case of nonsynonymous mutations (3, 4). Although undisputable on the cancer cell level, this one-dimensional interpretation of cancer strongly neglected the complex interplay of the cancer cell with its environment in the cancerous organ. Especially, this tumor microenvironment (TME) starts to become crucial for a better understanding of pivotal concepts like intratumor heterogeneity (5), organ specificity of a cancer and its metastases (6), or its hostility against conventional and emerging treatments, causing chemoresistance and radioresistance (7) or immune escape, (8). Glioblastoma (GBM), the most common and aggressive brain tumor arising from glial cells in the central nervous system (CNS), either *de novo* as primary GBM, or from preexisting low-grade astrocytomas as secondary GBM (9), thrives in a highly specific and poorly accessible microenvironment rendering this fatal tumor notoriously hard to treat. Tackling this, TME is increasingly being recognized as a promising, novel asset in the anticancer armamentarium to diminish or overcome therapy resistance and (selectively) break down the different layers of protection the tumor creates to escape from its ultimate destruction. In this regard, therapies aiming to interfere with the protective TME might be ideally designed to combine conventional and upcoming cytotoxic agents and treatments in general. This chapter presents an overview of the TME composition and interactions in GBM, the ways to interrogate, study, and mimic, or ultimately even modulate this environment in order to improve the chances for tumor control and destruction.

The Heterogeneic Nature of Glioblastoma Cells

GBM cells are considered to be of glial, astrocytic origin (9). Although the stochastic tumor model, which states that all tumor cells have comparable proliferative, migrating, and infiltrating properties, and arise at random from

genetically impaired glial cells, is increasingly being challenged by the hierarchical tumor model in which cancer stem cells (CSCs) are the only ones with true self-renewal and multipotent differentiating properties (10), it remains very hard to define uniform CSC markers - in GBM. Based on gene cluster analysis of abnormalities in PDGFRA, IDH1, EGFR, and NF1, four molecular subtypes of GBM have been characterized; theoretically, every GBM can be stratified into proneural/neural–classical–mesenchymal subtypes (11). Using global DNA methylation profiling techniques like the Illumina 450k methylation array, Sturm et al. introduced an epigenetic classifier of six pediatric and adult GBM subtypes with distinct mutational patterns (12). Again, it should be stressed that even this meticulous and elegant molecular dissection of the GBM cancer cell holds a substantial risk of underestimating the true heterogeneity of this tumor, as already in 2013, Sottoriva et al. demonstrated that more than one molecular subtype can be found in one individual tumor depending on the tumor quadrant the sample had been harvested from (13). Particularly because of the highly invasive nature of GBM, we should realize that interrogating cells harvested from the tumor bulk might neglect the increasing genetic difference between founder cells in the tumor and infiltrating cells in the surrounding brain as spatially adjacent cells in the tumor are more likely to be closely related in terms of number and type of genetic alterations as compared to mutually distant cells within the same tumor (14). Needless to say that, without a more generalizable concept behind this heterogeneity, any attempt for personalized medicine against a validated target within a given subtype will result in only partially hitting the tumor with inevitable recurrence or outgrowth as a direct consequence. In this regard, more conventional treatments like temozolomide (TMZ) and radiotherapy have already proven to modify the predominant genetic signature of the surviving tumor cells (15), as well as the TME (16).

Glioblastoma Needs a Supportive Environment to Develop and Grow

GBM is the most malignant variant of a spectrum of intrinsic brain tumors called gliomas; it is, unfortunately, also the most frequent variant, occurring with an age-adjusted incidence of 0.59 to 3.69 per 10^5 persons per year (17). The standard-of-care (SOC) treatment consists of maximal safe surgery, followed by a combination of radiotherapy and chemotherapy (with TMZ), and results in a median overall survival (OS) of less than 15 months and a 5-year OS of less than 10% (18). To date, the disease is incurable and because of the high case fatality rate and the occurrence in also young and adolescent patients, GBM has the highest number of years of life lost (YLL) in several rankings over the last 10 years, representing a major socioeconomic burden and unmet medical need (19, 20).

Although GBM is rightfully considered as a highly aggressive neoplasm, with a median OS of less than 15 months after full therapy, it virtually only affects the CNS and only very rarely metastasizes toward distant organs (21). It generally exerts its detrimental activity locally at the site of origin, although hematogenous spreading of glial fibrillary acidic protein (GFAP-) positive GBM cells has been reported by Müller et al. (22). Moreover, up to 80% of local recurrences after

contemporary SOC therapy of maximal safe surgery followed by radiochemo-therapy with fractionated, limited field, external beam radiation and TMZ occur within a 2.5 cm margin of the initial resection cavity, although infiltrating GBM cells can easily spread further away throughout the supratentorial CNS (23). This preferential anchoring of the tumor toward its original site of origin somehow contradicts its intrinsic biological aggressiveness but could partially be explained by degressive concentration of infiltrated cells in the periphery, its exclusive hom-ing properties, and the hypothesized systemic immunity preventing it from colo-nizing extracranial tissues (24). The preferential route of infiltration seems to follow axonal tracks or to a lesser extent perivascular spaces (25), again pointing to a well-organized interaction with its direct TME rather than random growth or expansion.

The GBM TME is constituted not only by highly proliferative malignant astrocytoma cells and probably CSCs (26), but sometimes also by impressive quantities of immune cells, both residing and infiltrating, stromal cells, and vascular endothelial cells and pericytes, all creating separate niches within the tumor (27). All these cells are able to interact with each other within the frame of the extracellular matrix (ECM) in which fluids and macromolecules compose the noncellular substrates. Although intratumor heterogeneity as a concept is often restricted to the varying presence of different genetic alterations present in the different tumor cells (1), the true heterogeneity probably far exceeds this level, as many intratumoral niches can be defined based on the relative composition of contributing cell (sub-) types and ECM substances. There is growing evidence that in these niches, different tumor cell types (proliferating, infiltrating, CSC like) and different noncancerous cells (microglia, macrophages, dendritic cells (DCs), lymphocytes) dynamically reshape different parts of the tumor without exactly knowing which cell is the playmaker in which context and background (Figure 1). Microscopically, this results in different microenvironments within the tumor varying from solid tumor cores with densely packed proliferating tumor cells, to necrotic and perinecrotic areas, perivascular areas around vessels with endothelial proliferation, and hypoxic and perihypoxic regions (27). All these regions are ruled by microclimates of cells and molecules, thereby underpinning the need to unmask the tumor as a true organ rather than as a tissue.

Constituents of the Tumor Microenvironment in Glioblastoma

THE GBM VASCULATURE

GBMs are one of the most vascularized tumors with extensive neo-angiogenesis and an abnormal vasculature depicting hyperdilated and leaky vessels as well as vascular glomerular structures in which endothelial cells and pericytes form poorly organized vascular structures, a common hallmark of GBM (28). The pres-ence of various angiogenic factors and chemokines have been reported in gliomas that are mainly expressed by tumor cells or infiltrating immune cells. The vascular abnormalities in GBM are however predominantly attributed to the highly elevated levels of vascular endothelial growth factor (VEGF) which subsequently cause the disruption of the blood–brain barrier (BBB). The BBB is comprised of endothelial cells, pericytes, and astrocytes, forming a neurovascular unit that tightly regulates
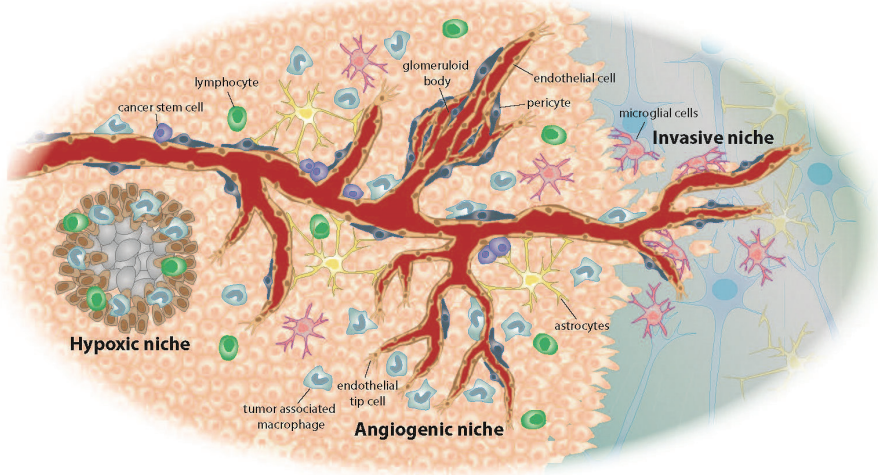
Figure 1 GBM TME niches. Glioblastoma (GBM) and glioma stem cells (GSCs) are embedded in a heterogeneous tumor microenvironment(TME) which not only is composed of diverse stromal cells, including vascular cells, the various infiltrating and resident immune cells, and other nonneoplastic glial cell types, but it is also compartmentalized in anatomically distinct regions, coined tumor niches. These niches can be composed of different cell constituents and look morphologically distinct from each other while the vasculature remains a central part. These niches regulate metabolic needs, immune surveillance, survival, invasion, as well as glioma stem cell maintenance. In the *angiogenic tumor niche* tumor (stem) cells nestle in close juxtaposition with the abnormal angiogenic vasculature while in the *vascular-invasive tumor niche* tumor cells co-opt normal blood vessels to migrate deep into the brain parenchyma. In the *hypoxic tumor niche*, there is either nonfunctional or regressed leading to necrotic areas that are surrounded by a row of hypoxic palisading tumor cells.

the transfer of ions and molecules between the blood and the brain (29, 30). A failure in these barrier properties induces vessel permeability with plasma and fluid leaking into the tumor tissue and thereby inducing cerebral edema and interstitial pressure. These changes also compromise vascular function and lead to sluggish blood flow and inconsistent oxygen delivery within GBM. In turn, local hypoxic areas develop that can turn into pseudopallisading necrosis, another hallmark of GBM, when tumor vessels become obstructed. These conditions also attract innate immune cells such as macrophages which elicit proangiogenic and immunosuppressive properties, thereby helping to expand the tumor vasculature to these poorly perfused areas.

Although the GBM vasculature expands mostly by angiogenesis, the proliferation of existing endothelial cells in tumor vessels, and bone marrow–derived vascular progenitors, can also promote neovascularization, albeit to a modest degree (31, 32). More recently, lineage-tracing experiments in mouse GBM models, and genetic mutational analysis of endothelial cells in patient-derived GBM, elicited glioma stem cells (GSCs) as another source of vascular constituents by their ability to transdifferentiate into endothelial cells or pericytes in GBM (33–36). The heterogeneous nature of the GBM vasculature not only affects its

important role as a gatekeeper for potential medicinal products and drugs against the tumor but also changes its adhesive properties affecting cellular adhesion and diapedesis of immune cells. In this respect, it is important to note that VEGF elevation besides generating an abnormal and angiogenic vasculature also thwarts the extravasation of tumor-reactive T cells and fosters an immune-suppressive microenvironment that enables tumors, including GBM to evade host immuno-surveillance (37). VEGF reduces ICAM1 and VCAM1 adhesion molecules in angiogenic vessels and thereby hinders infiltration of immune T-effector cells into the tumor; it also directly inhibits the maturation of DCs and activates antigen-specific regulatory T cells (Tregs). This collectively contributes to the severely immunosuppressive nature of GBM and subsequent rather low CTL infiltration.

THE GLIAL CELL COMPARTMENT

The vast majority of GBMs arise in the supratentorial cerebral hemispheres. There, the tumor cells intermingle with (and overgrow) the local astrocyte, oligodendro-cyte, and neuronal population. To this end, neither much is known about the ultimate fate of these original cell populations at the tumor site, nor do we have a clear view of the interactions between normal stromal cells, neurons, and cancer cells within the tumor and its immediate periphery. The remnants of oligodendro-cyte-like cells and/or (reactive) astrocytes have been identified in many pathologi-cal specimens of resected GBM (38).

Astrocytes, which regulate metabolic and fluid homeostasis as well as vascular blood flow, contact endothelial cells and pericytes with their astrocytic endfeet, covering more than 99% of the cerebrovascular surface in the brain. During gliomagenesis, astrocytoma cells displace normal astrocytes from vessels, thereby disrupting the astrocyte–vascular interactions and regulation of the vascular tone which is sufficient to rupture the BBB (39). In addition, astrocytes surrounding GBM undergo reactive astrogliosis similar to that observed during CNS injury, by which they become proliferative and migratory and produce growth fac-tors, metabolites, and cytokines that promote gliomagenesis. Several paracrine interactions have been described between astrocytes and glioma cell; for example, reactive astrocytes produce connective tissue growth factor (CTGF) that binds to tyrosine kinase receptor type A (TrkA) and integrin beta 1 on CSCs, thereby acti-vating nuclear factor kappa B (NF-κB) and inducing zinc finger E-box binding homeobox 1 (ZEB1), an epithelial–mesenchymal transition (EMT) transcription factor that facilitated tumor cell infiltration (40). To which extent astrocytes and oligodendrocytes contribute to tumor growth is still an ongoing issue of debate, but there appears to be a metabolic symbiosis between stromal and tumor cells based on the differing glycolytic and oxidative (glucose) metabolic flux of the respective cell populations (41, 42).

Although neurons have not been considered as active contributors to tumor propagation or bodily defense, tumor cells like to migrate along axonal trajecto-ries and perivascular spaces. On the molecular level, some enigmatic correlations seem to exist between a higher PDL-1 expression on neurons in the peritumoral adjacent normal brain and a better patient outcome as opposed to the correlation between a higher PDL-1 expression in the tumor and a poor prognosis (43). These observations point to the importance of the precise context depending on which the same biomarkers can predict different biological evolutions. In pediatric GBM

and diffuse intrinsic pontine glioma (DIPG), Venkatesh et al. (44) were able to show that excitatory neuronal activity, through neuroligin-3 synaptic secretion, promotes glioma progression.

INFILTRATING AND RESIDING IMMUNE CELLS

A major part of the GBM tumor volume, up to 30 or 40% of the mass, can be made up by immune cells, especially myeloid-derived cells like infiltrating macrophages (45). Although in nonpathological conditions, no substantial amounts of immune cells infiltrate the brain parenchyma, and many of them never trespass the perivascular Virchow Robin spaces where they are held by the glia limitans/BBB, this can rapidly shift in several brain disorders in which inflammation plays a certain role (46). This clearly underscores that the brain and CNS is not absolutely immune privileged as once believed, but that quantity and quality of the immune reaction in the brain is highly contextual (47). Recently, another remarkable anatomical dogma has been challenged since Louveau et al. demonstrated the presence of lymphatics in the wall of (major) dural sinuses thereby providing evidence for a second gateway to (and from) the brain for immune cells (and interstitial fluids), apart from the vascular route (48, 49). In the context of malignant gliomas, making the difference between residing and infiltrating immune cells is difficult; microglia (CD11b+, CD45–) and residing (nonmigratory) DC, are believed to make up only a smal minority of immune cells as compared with their infiltrated counterparts, classically called tumor-associated macrophages (TAMs) (50). Traditionally split up as M1 (pro-inflammatory, anti-tumoral) and M2 (anti-inflammatory, pro-tumoral) subtypes, the full spectrum of TAM is much more diverse (51) and highly dynamic. In an orthotopic mouse model, at early stages of tumor development, M1 TAM infiltrate the microenvironment but a rapid and massive differentiation toward M2 takes place in a more advanced stage of tumorigenesis, possibly corresponding to differences found in human low-grade versus high-grade glioma samples (unpublished own work). Remarkably, these macrophage populations seem to drive stromal and blood vessel architecture in the TME which can be offset (or partially corrected) by knocking down galectin-1 in the TME (52). Chemotactic gradients of substances like GM-CSF, M-CSF, MCP-1, and HGF are responsible for attraction (and retention) of these macrophages. Moreover, the CSF-1 pathway has been elucidated as crucial for M2 macrophage polarization in the TME, culminating in the possibility to re-educate M2 macrophages by the use of CSF1-R inhibition in gliomas (53, 54). The most enigmatic myeloid cell population in the TME and the blood of patients with malignancies, including GBM, are the myeloid-derived suppressor cells (55). To date, no uniform definition based on (lineage-)markers is universally used; this highly versatile cell population might play a key role in the mutual communication between the local immune cell population in the TME and the systemic immunity in the blood and extracranial organs. Recently, Chae et al. (56) elegantly showed that green fluorescent protein (GFP)–labeled monocytes, after undergoing intratumoral immunosuppressive education, can be precursors of MDSC both intratumoral and systemic in the context of glioma bearing mice. This does not only demonstrate the close familiarity of the different myeloid cell populations involved, but also the capacity of the TME to act as a local immune-suppressive factory of immune suppressor cells that can spread back to the systemic immunity after education in the TME.

Tumor-infiltrating lymphocytes (TIL), constitute the smaller, adaptive immunity counterpart of TAM in the TME. Although often outnumbered by TAM, TIL possibly have a more contextual importance for tumor progression, promotion, and ultimate patient prognosis (57). Although conventional CD8+ cytotoxic T cells (CTL) can mediate tumor regression and rejection in several experimental conditions, Tregs seem to infiltrate GBM in untreated conditions. Most of these Tregs are believed to be natural Tregs, thymus-dependent, and active through cellular contact via the so-called checkpoint inhibitors like CTLA-4 and PD-1 (58). Inducible Treg (iTreg), on the contrary, interfere with the local immune reaction predominantly by secreting IL-10 and TGFβ, often called immunosuppressive cytokines (59). Several reports elaborated that TAM may play a role in the induction/attraction of the local Treg compartment in the TME, mainly via CCL22 and to a lesser extent through CCL2 (MCP-1) (60).

THE ECM AND TISSUE MECHANICS IN GB

The ECM is the noncellular component present within all tissues that not only provides essential physical scaffolding for all the cellular constituents but also initiates crucial biochemical and biomechanical cues that are pivotal for tissue morphogenesis, migration, differentiation, and homeostasis. The adult brain consists to about 20% of a uniquely composed ECM that is very distinct and different from the network of fibrous proteins normally found in many peripheral tissues. The brain ECM is almost entirely constituted of a mesh-like scaffold (i.e., perineuronal net [PNN]) of glycosaminoglycans (GAGs), including hyaluronic acid (HA), proteoglycans (e.g., lecticans), and glycoproteins (e.g., tenascins) (61–66). Thereby, long chains of HA project perpendicularly from the neuronal cell membrane at sites where hyaluronan synthases are located (HAS1–HAS3 in mammals) to form the bulk of the matrix. HA chains are bound along their axis by one end of a lectican (a member of the chondroitin sulfate proteoglycan family, including aggrecan, brevican, neurocan, and versican), which are cross-linked to neighboring lecticans at their other end through the glycoprotein tenascin (61, 63, 67, 68). Due to the unique brain properties, neuronal cells of all types contribute to ECM production, maturation, and structure while ECM proteins in many tissues are rather synthesized and deposited by fibroblasts and other mesenchymal cells. For cells, the ECM can provide guidance for preferred migration, invasion of infiltration depending on their nature or an active labyrinth to trap infiltrating immune cells.

During glioma progression, the ECM undergoes deposition and remodeling changing its composition and architecture in part due to the increased and altered production of some of the ECM components in glioma such as tenascin-C (TNC) and HA. Interestingly, in line with these observations, recent studies revealed that lower-grade gliomas (LGGs) and GBMs are progressively stiffer when compared with nontumor gliotic brain tissue (69). This is in agreement with the long-known fact that peripheral tumors are characteristically stiffer than the surrounding normal tissue.

Besides ECM stiffening, there are additional physical changes in the TME that facilitate glioma stiffness, specifically elevated fluid pressure (subsequent to edema), cell compression, and increased tumor cellular contractility. Such PNN alterations have been shown to promote tumor progression through sustained

activation of pro-tumorigenic mechanosignaling pathways, or by providing new "tracks" on which tumor cells can migrate. In addition, these changes obstruct blood vessel integrity, which in turn can influence both the recruitment of inflammatory cells and the permeability of macromolecules, including therapeutic compounds (70–72).

For interstitial fluid components, several substances in the ECM can modify the free diffusion and as such create environmental niches predisposing to the attraction of different types of infiltrating immune cells based on established gradients (73). In GBM and other malignancies, galectins (74) act as binders (scavengers) of glycosylated cytokines (IFNg) and hamper an efficient anti-tumor immune rejection/response. GBM and their host cells interact with several of these ECM components through abundant secretion of enzymes like hyaluronidase or matrix metalloproteinases. These mechanisms are key for migration and invasion, the preferential way of spreading throughout the brain for GBM tumor cells (75). Some of these ECM components (or the relative lack of it) like fibronectin, have been connected to the low incidence of metastasis of GBM cells outside the CNS (76). Others like tenascin-C have been considered as targets for older monoclonal antibody approaches, some of which had been linked to radioactive OR cytotoxic components (77).

INTERSTITIAL FLUIDS AND SOLUBLE FACTORS

Within the TME, numerous soluble factors, secreted by tumor or stromal cells or extravasated from the intravascular compartments build a dynamic interstitial fluid compartment in which cells and ECM are bathing. Metabolites like lactate, as a result of the typical Warburg effect in tumor metabolism, and adenosine cumulate in hypoxic areas and have a strong impact both on tumor cells and immune cells (78). For the latter, they exert predominantly suppressive effects, for the former they mediate neo-angiogenesis or tumor progression. In spite of their frequently abundant presence in the TME, many of them are unequally distributed throughout the tissue, thereby contributing to the enormous intratumoral heterogeneity in tumors in general and GBM in particular: often this coincides with geographical differences of cell composition in the tumor like has been shown for galectin-1 and TAM (79). These variations in concentrations of soluble factors cause dynamic changes in chemotactic gradients, constantly reshaping the cellular composition of most tumor areas. Many cytokines have been documented in the TME of GBM, with a vast predominance of those especially known to have immunosuppressive effects. Transforming growth factor beta, (TGFβ), originally demonstrated in GBM (80) and interleukin-10, classically depicted as immunosuppressive Th0 cytokine (81) are well-documented examples. The former has been the target of the first-in-kind trabedersen trial in which an antisense oligonucleotide was used to knock down the TGBβ in the GBM TME (82). The latter is a more enigmatic cytokine rather fulfilling a multifaceted mode of action (83) than the purely immunosuppressive effect that is mostly attributed to it. More recently, our group looked at the importance of galectin-1, a key hub molecule in the GBM TME (84) and described a novel approach to selectively modulate it in the TME (85). Galectin-1 (Gal-1) is an evolutionary conserved, β-galactoside-binding lectin of 14.5 kDa, first isolated more than 30 years ago (86). It is a member of the galectin

family, consisting of 15 distinct galectins and characterized by a carbohydrate recognition domain (CRD), responsible for binding glycoproteins and glycolipids (87). Galectin-1 can be found both intra- and extracellularly, as well as at the cell membranes, and exerts its diverse functions through its presence in these different compartments.

Quantitative analysis from computer-assisted immunohistochemical (IHC) assessment revealed that Galectin-1 is being expressed by all subtypes of glial tumors and found a significant higher Gal-1 expression in poor-prognosis high-grade astrocytomas when compared to the clearly lower expression in high-grade glioma patients with better prognosis (88). Moreover, galectin-1 has been identified as a key hub molecule in glioma growth, invasion, and therapy resistance (89) as well as immune escape and suppression (84). Through promotion of the unfolded protein response in glioma cells, Galectin-1 was shown to contribute to the resistance of TMZ (90), an oral alkylating chemotherapeutic drug used in the SOC postoperative treatment of GBM, together with ionizing radiation (18). The latter seems to even increase the levels of galectin-1 expression in GMB rendering a Galectin-1 silencing strategy in GBM even more attractive to restore susceptibility to chemotherapy. In terms of tumor angiogenesis, Thijssen et al. (91) elegantly showed that tumor endothelial cell proliferation and migration relied on the presence of Galectin-1 in the TME and targeted inhibition of Gal-1 expression in Hs683 GBM cells resulted in a decreased VEGF secretion in the culture medium (92). Brain invasion, a problematic hallmark of GBM cells is being promoted by Galectin-1 that is expressed preferentially in tumor cells at the tumor periphery rather than in the core (93). This is perfectly consistent with earlier findings by Rorive et al. (94) and Camby et al. (95), both linking Gal-1 to cell motility and migration. In 2013, we were able to conclude that serum levels of Gal-1 of newly diagnosed GBM patients and recurrent high-grade glioma patients were significantly higher than those in age- and sex-matched healthy volunteers (96), based on the analysis of a prospective data set of 43 healthy controls and 125 patients. This indirectly indicates the important impact of GBM-related production of Gal-1 in the TME, on the systemic level outside the brain.

Apart from a direct promotion of tumor growth, angiogenesis, invasion, and therapy resistance, galectin-1 also indirectly stimulates GBM tumor promotion through impairment of the patient's immune system. Multiple modes of interaction, both with the innate and adaptive arms of immunity have been described and extensively been reviewed (84). These mechanisms include, but are not restricted to: the apoptosis induction of activated T cells, promotion of Th2 type of immune responses while blunting Th1 and Th17 responses, modulation of T-cell proliferation, modulation of T-cell receptor signaling, modulation of the cytokine balance, regulation of T-cell adhesion and trafficking, control of Treg function, modulation of DC tolerogenicity, and macrophage function. All of these lead to a state of immune suppression and immune evasion. As a direct consequence of all these abundant, galectin-1-mediated mechanisms, it was a logical step to investigate the immunological potentials and benefits of a targeted inhibition of galectin-1 gene expression in tumors. Rubinstein et al. (97) were the first to illustrate an increased T-cell-mediated tumor rejection after silencing galectin-1 expression in mouse melanoma cells. In 2014, for malignant gliomas residing in the CNS, we (79) demonstrated the huge impact of a tumor-derived Gal-1

knockdown (Gal-1-KD) in an orthotopic, syngeneic GL261 brain tumor model, on the local brain tumor immune microenvironment and its beneficial consequences for retarded tumor progression. The observed modifications included a decrease of myeloid cell accumulation and phenotype in the tumor after Gal-1 KD, an altered CCL2 and VEGF mRNA expression in brain infiltrating immune cells, a boost in IFN-g producing tumor-infiltrating CD8+ T cells, an immune-mediated survival benefit of mice, and an impaired tumor angiogenesis. Therefore, an improved outcome with DC-based vaccination could be seen after silencing brain tumor–derived (but not systemic, nontumor derived) Galectin-1 in this mouse model.

Treatment Routes to Reach the Glioblastoma Microenvironment

The hematogenous route appears to be the most accessible path for drugs to the GBM TME but as described above, several restrictions apply to the chemical structure and nature of the compounds to overcome the BBB and egress from the intravascular compartment to the TME to exert its function in a brain tumor (98). Although the BBB does impose evident limitations to drug design, it has been shown that this barrier is not fully intact in GBM, due to the generation of leaky vessels in GBM that can be visualized by conventional MRI imaging in form of strong gadolinium enhancement (99). This, nevertheless, does not detract from the problematic vascular shunting effect that exists in GBM, leading to a poor parenchymal perfusion in the brain tumor in spite of the highly vascularized nature of a GBM (100). As a consequence, many parenterally administered drugs fail to reach the appropriate intratumoral drug concentration for an efficient biological effect.

For several decades, the intraventricular route has been used as an attractive way to administer medicinal compounds directly into the cerebrospinal fluid (CSF) where it can be distributed further throughout the CNS. Although several examples exist to date, many refrain from a universal adoption of this technique given its dose-limiting toxicity and its highly invasive nature with concerns about the reported high rates of infectious complications (101). A comparable approach with one or more catheters directly into the TME or the surrounding brain parenchyma has been coined convection-enhanced delivery (CED). Thereby, a small but active pressure gradient drives the active substance in a soluble form through a microcatheter into the TME. Although perfectly possible and applied for many different types of substances, similar concerns about its invasive nature and side effects limits its full implementation in the clinical arena (102). Moreover, it remains problematic to get a reliable measurement of the isodistribution volumes of a specific drug in the fluid phase throughout the TME and the brain (103).

A more recent approach of drug delivery that gains increasing interest is the nose-to-brain delivery route. Using a transnasal pathway to the CNS, especially the brain, has been studied for more than 30 years with the aspects of anatomical and pharmacodynamic challenges (104). The olfactory region of the nasal mucosa is directly connected to the intracranial forebrain regions and entorhinal circuits via the olfactory receptor neurons, the olfactory filia, nerves, and bulbus (105). To a lesser extent, the same goes for the trigeminal pathway via sensory nerve ends of

the first and second branch of the trigeminal nerve (V1 and V2) in the endonasal respiratory mucosa (106). The presumed predominant mode of transport is paracellular and, as such, it is capable to bypass the BBB, which still is a major issue in the design of new therapies and the development of new pharmaceutical agents (107–109). Transcellular intraneural transport, however, has been described too, mainly for larger molecules (110). Moreover, the nose-to-brain route provides a beneficial biodistribution in which drug concentrations in the brain could largely outnumber systemic availability of the administered active substances. Its theoretical consequence of less frequent and less serious adverse systemic events should translate in a beneficial shift of the therapeutic window. From a patient's perspective, the nose-to-brain route for drug delivery is likely to be much more appealing when compared to other local delivery technology like the invasive CED systems through insertion of brain catheters (111, 112). The noninvasive nature of drug administration and the possibility for repetitive self-administration will lead to a high patient compliance and therapy comfort. The noninvasive character of intranasal delivery avoiding important drawbacks of CED like infections, inflammation, brain edema, wound healing problems, local hemorrhages, and seizures, together with the rapid availability of the drug in the brain environment and the presumed reduced systemic side effects might turn this route into a preferred delivery for the treating physicians too.

The majority of the research regarding the opportunities and pitfalls of nose-to-brain drug delivery has been performed in (small) animal models. Both for non-oncological brain disease (109, 113) and for GBM (85), comprehensive reviews are available in the literature. For neuro-oncological diseases like GBM and CNS lymphoma, mainly the intranasal delivery of several chemotherapeutic agents like methotrexate (114), 5-fluorouracil (115), and the related molecule raltitrexed (116) has been explored in rodent models. A remarkable, common finding in all these studies has been the indication for a preferential drug delivery to the brain (in different grading concentrations at different brain locations) rather than to the systemic circulation. Moreover, in each study, the drug concentrations in the CSF were higher for the intranasal group as compared to the systemic administration. To target Gal-1 in the TME of a malignant intrinsic brain tumor, we aimed to be both selective and inclusive: interference with other galectins should be avoided and reduction of both intracellular and extracellular Gal-1 is mandatory to tackle the full biological repertoire of this key hub molecule in GBM. Therefore, rather than opting for monoclonal antibodies (with a notorious problem to cross the intact BBB), small molecules like Davanat (117) or polypeptides like Anginex (118), we chose to develop an anti-Gal-1 siRNA molecule (siGal-1)-based formulation that could reach the GBM microenvironment upon intranasal delivery to exert its selective biological activity locally at the brain tumor site. Small interfering (si) RNA molecules are double-stranded RNA molecules of 21–25 base pairs that can initiate a sequence-specific mRNA degradation of the target RNA through cytoplasmic interactions with RNAi-induced silencing complex (RISC), resulting in a temporary (reversible) decrease of the protein of interest as nicely reviewed by Agrawal et al. (119). Hashizume et al. (120) reported the results of a study with GRN163, an antisense oligonucleotide targeting telomerase in GBM, which had been delivered intranasally in rats. They reported a rapid distribution in the brainstem through the trigeminal nerve pathway, a significant survival benefit in animals with an established brain tumor and a remarkable tumor tropism within

the brain. As fragile siRNA molecules are easily and rapidly degraded in an extra-cellular environment, it is mandatory to load this siRNA in specific (nano-)formulations that can protect and transport it until it reaches its intracellular target. The formulations has several capabilities of which the most important are (i) protecting siRNA from rapid degradation during its journey to the brain, (ii) influencing the mucoadhesive properties (in the nasal mucosa), (iii) facilitating transport through the mucosal and epithelial nasal barriers, (iv) stimulating the perineural (and/or transcellular) transport to the brain parenchyma, (v) promoting tumor tropism, (vi) transfecting the cells and finally, and (vii) getting released from the formulation once it reaches the tumor cell cytoplasm to exert its specific biological activity through interaction with the RISC. Moreover, the excipients used need to be well tolerated and biodegradable. Until now, rather few of the theoretical possibilities have been tested in preclinical animal models of different brain diseases (121–123). To date, we have been able to show a convincing nose-to-brain transport of both siGal-1 formulations based on naïve-chitosan and pegylated-chitosan nanoparticles (NNP and PNP, respectively) in the orthotopic, syngeneic, GL261 brain tumor model, leading to a selective and sequence-specific degradation of Gal-1 in the brain TME (124). This Gal-1 knockdown in the tumor dramatically increased the chemosensitivity to TMZ and showed a promising synergy with anti-PD-1 blocking monoclonal antibodies in the same model (52).

Is It Possible to Capture the Complexity of the Microenvironment in Glioblastoma Models?

Interrogating the TME and respecting all the layers of information (see Table 1) involved, as well as the highly interactive nature of all its constituents, remains a major challenge. Obviously, no *in vitro* cell culture model, whether monolayer, specially designed (125) or neurospheres (126), will faithfully reflect the highly complex interactions of all TME components. Three-dimensional *in vitro* culture models, built on special scaffolds (before being grafted in animals) might be able to mimic key histologic characteristics of GBM (127, 128) but to what extent they also accurately represent the TME remains unknown. The same goes for almost all conventional animal models (129). Heterotopic brain tumor models with GBM cells growing in subcutaneous tissues can never mimic the particular CNS environment of the brain andtherefore, orthotopic brain tumor models should be preferred for this type of research. In that regard, xenograft models, even patient-derived (130), are being created in immune-compromised animals, thereby fully neglecting the impact of the immune system, which accounts for the major supplier of nontumoral cells constructing the GBM TME. Garcia et al. reported in 2014 about an orthotopic xenotransplant model successfully recapitulating the GBM microenvironment (131). Syngeneic orthotopic brain tumor animal models (132) can correct for that shortcoming, although tumor cell biology and immunity differ considerably between species. The ideal model is yet to be built, but will have to combine human tumor cell biology interacting with a human(-ized) immune system (133). Direct on-site interrogation and quick mapping of the GBM TME, for example, during surgery, might be another future track to considerably improve our understanding of its complexity.

TABLE 1	Selected Overview of Components of the Glioblastoma Tumor Microenvironment Targeted by Different Types of Compounds in Varying Phases of Preclinical and Clinical Development			
TME Component	**Target**	**Type of Compound**	**Authors and Reference**	**Year**	**Phase of Development in GB**
Blood vessel components					
VEGF	VEGF-A	mAb (bevacizumab)	Chinot et al. (144), Gilbert et al. (137)	2014	Phase III trial, clinical
VEGF receptor	VEGFR2	Small molecule, TK inhibitor (axitinib)	Duerinck et al. (145)	2016	Randomized, open-label, Phase II, clinical
Pericytes	PDGF	Radioimmunotherapeutic	Behling et al. (146)	2016	Preclinical target validation
Glia limitans	AQP5	siRNA	Yang et al. (147)	2017	Preclinical target validation
Stromal cells					
Microglia	CXCR4	Peptide, ligand-based antagonist	Mercurio et al. (148)	2016	Preclinical PoC
Macrophages	CSF1-R	Small molecule (PLX3397)	Butowski et al. (54)	2016	Phase II trial, clinical
Lymphocytes	PD1	mAb (nivolumab)	Blumenthal (149)	2017	Clinical cohort study
	IL13aR	CAR T-cell	Brown et al. (150, 151)	2017	Phase I + case report, clinical
ECM					
Tenascin and neuropilin 1		Peptide	Kang et al. (152)	2016	Preclinical xenograft model
Hyaluronic acid (HA)	CD44	HA-conjugated liposome nanoparticle	ayward et al. (153)	2016	Preclinical target validation
Fibronectin	neuropilin 1	mAb	Chen et al. (154)	2014	Preclinical target validation
Chondroitin		Humanized bacterial enzyme (chondroitinase ABC)	Jaime-Ramirez et al. (155)	2017	Preclinical xenograft model

TABLE 1	**Selected Overview of Components of the Glioblastoma Tumor Microenvironment Targeted by Different Types of Compounds in Varying Phases of Preclinical and Clinical Development** (Continued)				
TME Component	**Target**	**Type of Compound**	**Authors and Reference**	**Year**	**Phase of Development in GB**
Soluble factors					
TGBβ		Oligonucleotide (Trabedersen)	Bogdahn et al. (82)	2011	Open-label, randomized phase Iib, clinical
Galectin 1		siRNA formulation	Van Woensel et al. (52)	2017	Preclinical rodent model
CCL2/MCP-1		Minocycline, telmisartan and zoledronic acid (drug repurposing)	Salacz et al. (156)	2016	Theoretical concept
Osteopontin		shRNA	Lamour et al. (157)	2015	Preclinical xenograft model

CAR, chimeric antigen receptor; ECM, extracellular matrix; mAb, monoclonal antibody; PoC, proof-of-concept; shRNA, short hairpin RNA; siRNA, small interference RNA; TME, tumor microenvironment.

Therapeutic Targeting of the Glioblastoma Microenvironment Components

TARGETING THE VASCULATURE

Due to the substantial inter- and intratumor heterogeneity of GBM cells, an attractive and potentially more effective tactic to overcome the plasticity that is associated with therapeutic GBM resistance may be to target the TME, specifically nonneoplastic components or the molecules they release to support tumor cell growth (Table 1). The first promising TME treatment strategy has been based on abrogating tumor vessel growth by blocking VEGF/VEGFR signaling in GBM. VEGF inhibition with bevacizumab, a humanized monoclonal antibody directed against VEGF-A, resulted in improvements in radiographic response, progression-free survival (PFS), and quality of life of GBM patients which subsequently became the third drug approved by the United States Food and Drug Administration (FDA) for use in recurrent GBM in 2009 (134).

Bevacizumab reduces vasogenic brain edema, and enhances vessel perfusion and subsequent oxygenation concomitant with a decrease in immune suppression which creates conditions for better drug delivery and efficacy (135). Nevertheless, anti-VEGF therapy has benefitted only a subset of GBM patients with transitory improvements in PFS but without improving OS. Interestingly, recent trials revealed that the effects of anti-VEGF therapy maybe dependent on the GBM subtype and thus genetic backbone of these tumors. Two randomized placebo-controlled Phase III trials in newly diagnosed GBM AVAglio (136) and RTOG-0825 (137) reported prolonged PFS, but not OS, with the addition of bevacizumab to radiotherapy plus TMZ. A multivariable analysis, however, revealed that bevacizumab conferred a significant OS advantage versus placebo for patients with proneural isocitrate dehydrogenase 1 (IDH1) wild-type tumors (138). These results, together with the observation that patients who experience enhanced tumor blood perfusion with bevacizumab have a longer survival benefit than those without vascular changes, suggest that subtype stratification of GBM patients with early imaging perfusion markers could help to stratify patients who will benefit the most from bevacizumab (135).

In order to understand the transient improvements of this therapy, it is important to note the inability to finely tune anti-VEGF/VEGFR therapy to create persistent normalization without further vessel pruning. This in turn results in enhanced hypoxic areas and hypoxia-dependent resistance mechanisms which lead to GBM relapse (139). Hypoxia promotes EMT and stem-like properties of tumor cells, upregulates pro-angiogenic and invasive factors, and drives the infiltration and polarization of angiogenic and immune-suppressive myeloid cells (reviewed in (140, 141).

Indeed, radiographic and tissue studies from a subset of patients with recurrent GBM who were treated with bevacizumab or the angiokinase inhibitor cediranib support the results of enhanced tumor invasiveness and immune cell infiltration of TAMs and other CD11b+ myeloid cells observed in GBM mouse model systems (135, 142). Infiltrating innate immune cells including macrophages and neutrophils, have been shown to facilitate resistance to antiangiogenic therapy in various tumor types by rendering tumors nonresponsive to VEGF blockade (139).

TARGETING IMMUNE CELLS

The observation that 30–40% of the cells in gliomas consist of microglia or macrophages has raised the question whether targeting these innate immune cells would provide better efficacy and potentially be useful in combination with other therapies. Indeed, inhibition of the CSF-1 receptor that targets macrophages and microglia, resulted in increased survival and tumor shrinkage in a proneural murine GBM model (53). Intriguingly, the beneficial effects were caused by reeducation of TAMs, rather than their deletion. In contrast to the promising effects in this preclinical GBM model, a recent Phase 2 study of the CSF1-receptor inhibitor PLX3397 in patients with recurrent GBM tissue did not identify any significant improvements, not even in PFS (54). Whether the differing results may be a reflection of GBM subtype-specific responses to blocking CSF1R + myeloid cells,—as observed in recent anti-angiogenic trials-, or a matter of the animal model, remains to be determined. At least, further support for the impact of macrophages and microglia in glioma stems from a recent study in which naïve human macrophages and microglial cells alleviated sphere-forming capacity of glioma-patient-derived stem cells by inducing cell cycle arrest and differentiation while glioma-associated myeloid cells were unable to do so (143). Amphotericin B, a common anti-fungal medication, was able to reprogram myeloid cells and induce an immune-stimulating phenotype that sufficed to impede growth of GSCs *in vitro* and tumor growth *in vivo* (143). Using a different approach with similar effects, therapeutic galectin 1 knockdown in glioma by intranasal delivery of siGal1 RNA enhanced an immunostimulatory environment by reducing Tregs and MDSC and enhancing Th1 properties of macrophages and CTL infiltration (52). Targeting immune cells as part of the TME represents only one approach of all that have been identified in the past and present (144–157) (Table 1)

The studies summarized above highlight some new developments to target signaling cues of host cell constituents in GBM. They also underscore the importance of therapies that promote an immune-stimulatory milieu to enhance the infiltration of cytotoxic T cells into glioma. As some of the drugs targeting those pathways have already been approved for other diseases, this approach may yield an attractive and more effective strategy in combination with standard chemotherapy to sensitize as well as re-sensitize GBM.

Conclusion

Table 1 presents an overview of several approaches to target the GBM TME in their different preclinical or clinical stages of development. Some of the approaches are quite recent and still reside in a phase of early exploratory findings or proof-of-concept in cell cultures or well-defined animal models. Other approaches transcended this stage and have shown preclinical evidence for useful exploitation in upcoming early phase clinical trials. A few substances targeting elements of the microenvironment already passed clinical testing even in randomized controlled trials which up to date have not been able to deliver clinical proof of efficacy in actual GBM treatment strategies. According to the actual state of science and knowledge, it can be anticipated that most, if not all, of these approaches will only

show their full, durable potential if they are used in a rational combination with more conventional surgical, radiotherapeutic, or cytotoxic (chemo-)therapies, especially those that are able to mount an immunogenic cell death in an immune-receptive environment.

Conflict of interest: Steven De Vleeschouwer is co-patent holder of a transnasal siGal-1 formulation (International Patent Classification: A61K9/51 (2006.01).

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