



Circumventing vascular barriers for effective immunotherapy in brain tumors – focus on glioblastoma

Commentary

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Abstract

Blood vessels play a fundamental and unique role in brain tumor pathogenesis, including by mediating interactions with the peripheral immune system. Despite this intimate connection, endogenous immune surveillance and multiple modalities of immunotherapy have thus far exerted relatively little impact on disease progression and patient survival in high-grade brain tumors, such as glioblastoma (GBM). This applies to both adults and children, where complex vascular processes have emerged as possible actionable targets beyond anti-angiogenesis. Indeed, vascular responses in GBM include angiogenic, non-angiogenic (cooption, vasectasia), and angiocrine interactions mediated by soluble factors and extracellular vesicles (EVs). It is still poorly understood why immune cells are excluded from the GBM tumor microenvironment and what barriers may operate at the immune-vascular interface which could be modified to improve immunotherapy outcomes. The emerging research directions include efforts to overcome the immune cell exclusion, defining molecular hallmarks of treatment susceptibility in subsets of patients, assessing different immune effectors, and rational temporal scheduling of immunotherapy administration relative to the effects of cytoreductive treatments. It is suggested that experimental insights into the interplay between vascular and immune cell compartments may serve as hypothesis-generating material for future clinical studies in GBM.

Keywords

angiogenesis • glioblastoma • cancer • tumour vasculature • immunotherapy • NK cells • blood brain barrier • extracellular vesicles • vasectasia • Rab27

1. Introduction – The unmet clinical needs in high-grade brain tumors

With rapid progress in molecular diagnosis and classification of brain malignancies, their landscape is becoming increasingly complex, as does the evidence-based disease management [1-3]. While in many instances these advances have improved cancer control and prognosis, high-grade astrocytic brain tumors, such as grade IV glioma, including glioblastoma (GBM), remain stubbornly incurable with debilitating symptoms and uniformly poor outcomes in both adults [4] and children [5].

In this regard, adult GBM represents an especially stark example of a mismatch between the accumulating wealth of biological information, intensity of research, and investment in clinical trials vis-à-vis stagnant and depressingly poor survival statistics [4]. Indeed, despite aggressive standards of care including gross total resection followed by adjuvant chemoradiation, primarily with temozolomide (TMZ), the median overall survival of GBM patients has long remained in the neighbourhood of fifteen months post diagnosis [4]. Arguably, these dismal outcomes are largely attributable to the lack of effective maintenance therapy, as the initial treatment leads to a dramatic reduction of the tumor burden, often with no radiologically detectable disease [4]. Sadly,

this response is quickly followed by inevitable tumor relapse associated with discernible molecular drift of cancer cells [6,7] and the onset of therapy resistance [4]. Some of these events can be recapitulated using animal models [8], albeit with considerable diversity of detailed findings [9] and a limited impact on clinical translation thus far [10,11].

This impasse is further exacerbated by the absence of established standards of care for recurrent GBM, with tentative second-line options that may include additional surgeries, anti-angiogenesis with bevacizumab, tumor treating fields (TTF), palliation [4], experimental therapies with targeted agents [12], or different forms of experimental immunotherapy [13]. These approaches have yet to meaningfully impact the overall survival in GBM patients [4] pointing to a dire need for new biological and therapeutic paradigms.

2. The emerging biological complexity of glioblastoma

The striking absence of curative options in GBM, as mentioned, stands in contrast to the explosion of biological studies aiming at deeper molecular and biological understanding of these

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complex tumors. Consequently, much has been learnt over the past two decades, and as a consequence, the landscape of GBM has changed dramatically [3,7,14], resulting in re-definition of the disease, both diagnostically and pathogenetically [4]. Thus, GBM is presently understood to be a cluster of grade IV gliomas expressing wild-type IDH1 and distinctive morphological and clinical features [4]. In approximately 95% of cases, GBM arises without any discernible precursor lesion amidst rapidly progressing clinical manifestations [15]. Molecularly, GBM diagnosis encompasses three transcriptionally distinguishable molecular disease subtypes, including proneural (PN), classical (CL), and mesenchymal (MES) tumors, each associated with a different repertoire of oncogenic driver mutations, as exemplified by genomic and transcriptional alterations in *PDGFRA*, *EGFR/EGFR*, and *NF1* genes, respectively, along with changes in chromatin methylation patterns, and other hallmarks [4].

Underneath this inter-patient diversity, individual GBM lesions consist of complex cellular ecosystems comprising equilibria of cancer cells with PN, CL, MES, or mixed phenotypes, as revealed by single-cell sequencing studies [16]. Functionally, GBM cell populations include cells with increased tumor-initiating potential [17], often referred to as glioma stem cells (GSCs), which in different tumor subdomains may adopt either PN-like or MES-like transcriptional profiles [18,19]. Single cell analyses also led to the notion that GBM cells, rather than being rigidly defined, exist in fluid transitory molecular states with a tendency to adopt transcriptional biases and tumor initiating properties that are licensed, at least to some extent, by the nature of their underlying oncogenic transformation events and the boundaries of cellular lineage plasticity [20].

In addition to their individual traits, GBM cells function within integrated cellular populations. For example, experimental studies revealed that GSC-initiated lesions in mice may form networks where the cells remain physically connected with each other by cytoplasmic bridges known as tumor microtubes (TMs) [21], or else remain linked by cellular junctions [22] and other structures allowing the intercellular exchange of molecular content. TMs, for example, are suggested to possess the ability to propagate calcium fluxes from 'pacemaker-like' cancer cell subsets across the GBM cellular web, thereby coordinating growth and therapeutic responses [23]. GSCs [24] and GBM cells [25] may also communicate through swapping cellular fragments known as extracellular vesicles (EVs), containing mutant oncoproteins, such as epidermal growth factor (EGFR), and its oncogenic variant III (EGFRvIII) [25-27], along with corresponding mRNA [28], DNA, [29] and other cargo. Indeed, the landscape, molecular content, and biological activity of GBM EVs are programmed by oncogenic transformation [30,31], epigenetic factors, and tumor microenvironment (e.g., hypoxia) [32], and they have been implicated in cell-cell communication and interactions with surrounding host cells over both short and long distances [33].

3. The impact of vascular microenvironment on glioblastoma biology

The signature trait of GBM is the deep penetration and subversion of normal brain tissues. GBM cells exhibit infiltrative growth patterns, rapidly invading multiple and often remote regions of the brain [4]. Conversely, approximately 30-40% of the primary tumor mass in GBM is composed of host cells, including microglia, reactive astrocytes, nerves [34] and the vasculature, collectively recruited and altered to form a complex tumor microenvironment (TME) [35].

Amidst this heterogeneous cellular ecosystem, blood vessels play a particularly intriguing and still poorly understood functional role. Thus, GBM vasculature arises on the background of a dense cerebral microvascular network, the distinctive feature of which is a cluster of microanatomical, molecular, and functional features that protect brain parenchyma from the content of the circulating blood, a property known as the blood-brain barrier (BBB). Elements of BBB include endothelial cell molecular apparatus enriched in ABC transporters, inter-endothelial junctions securing a continued vascular lining, including tight junctions mediated by claudin 5 and other proteins, vascular basement membrane, mural cells, and astrocytic foot processes, all of which ensure a highly regulated and restrictive molecular passage in both directions [36].

In GBM blood vessels are transformed to exhibit multiple abnormal features. Those include diagnostically relevant microvascular proliferation [37], structural anomalies in the vascular architecture [38], microvascular occlusive thrombosis resulting in hypoperfusion and hypoxia, with associated regions of pseudopalisading necrosis of the tumor mass and other alterations [39-42]. Moreover, extensive vascular remodelling [43], dysmorphia, and poor functionality [44] of the GBM-associated vasculature is coupled with pronounced blood vessel hyperpermeability to plasma and radiologically evident edema [44], features often attributed to the elevated expression of vascular endothelial growth factor (VEGF) [45], a potent mediator of trans-endothelial transport [46], angiogenesis and immunosuppression [47].

While these features may suggest a breach in BBB, the passage of macromolecules, drugs and cells from the blood stream into the TME of GBM is markedly impeded by the remaining elements of the BBB [48], along with vascular and physical tumor tissue characteristics known as the brain tumor barrier (BTB) [44]. Moreover, while GBM-associated edema responds to treatment with VEGF inhibitors [45] these agents, of which bevacizumab is among the most extensively tested examples [49], do not abrogate tumor vasculature [50] or radically prolong patient survival [49].

These observations suggest that the relationship between GBM progression and resulting responses of the vasculature is not reducible to VEGF-dependent stimulation of tumor

angiogenesis. Indeed, several different mechanisms have been suggested as drivers and modifiers of GBM neovascularization, including angiogenic responses to tumor hypoxia [37], cooption of preexisting vessels by migrating cancer cells [43,51,52], vasculogenic mimicry of a subset of cancer cells [53], trans-differentiation of GSCs to pericyte-like cells [54], or non-angiogenic vascular growth processes exemplified by EGFR-driven vasectasia [27], and possibly several others [55]. These molecularly and functionally distinct vascular responses are associated with a multiplicity of endothelial cell phenotypes, some of which markedly extend beyond the natural heterogeneity of the normal brain endothelium [56] and exhibit some cancer-specific features, as revealed by recent single cell sequencing studies [27,57].

It should also be noted that the functional role of the vasculature in GBM progression is far more complex than that of a conduit for blood supplying oxygen and nutrients. For example, blood vessels are an important element of the vascular niche required for the maintenance of GSCs [58], and, according to some reports, constitute an alternative support system for GSCs deprived of the niche effects afforded by their surrounding bulk (non-GSC) cancer cells [59,60,61]. Blood vessels also provide important tracks for perivascular GSC invasion [54], and their paracrine (angiocrine) activity may elicit multiple cancer cell responses [62]. Examples of such a paracrine role include the ability of endothelial cell-derived EVs to modulate the phenotype of proneural GSCs, rendering them mesenchymal-like and more invasive [63].

An important role of the endothelial cell compartment in cancer also encompasses immunoregulation. For example, the milieu of VEGF-enriched vascular microenvironment activates several immunosuppressive mechanisms, including recruitment of myeloid-derived immunosuppressive cells (MDSCs), altered maturation of dendritic cells (DCs), and others [47]. Importantly, the repertoire of endothelial adhesion molecules [64] and their contribution to the vascular barrier function [44] emerges as a potentially critical element in regulating the interaction between tumor blood vessels and immune cells in the circulation, with profound implications for the prospects of effective immunotherapy [64].

4. The unfulfilled promise of immunotherapy in glioblastoma

The advent of immunotherapy has been transformational for many aspects of cancer care [65]. The profound consequences of the introduction of immune checkpoint inhibitors (ICIs) were recognized by Nobel Prize for James Allison and Tasuku Honjo in 2018 [66] amidst proliferation of clinical approvals for PD1, PD-L1, and CTL4 inhibitors [66]. In quick succession, the chimeric antigen receptor expressing cytotoxic T (CAR T) cells have also entered the clinical scene [67] epitomizing both the

initial success and the excitement about harnessing the forces of the immune system for anticancer therapy. Encouragingly, ICIs are especially effective in combination with antiangiogenic inhibitors of the VEGF pathway, possibly due to both vascular and anti-immunosuppressive effects of these agents [64]. Moreover, advantages were found in combining ICIs with chemotherapy, likely due to aspects of immunomodulation, xenogenization, and tumor debulking, the latter resulting in a more favourable ratio of immune effectors to their target cancer cells [10,68,69]. However, while the effects of immunotherapy have in some cases been dramatic (even curative), they were hardly universal, as large subsets of cancer patients and patients with specific cancer types do not benefit from the administration of these potent agents for reasons that remain to be fully understood and therapeutically confronted [65].

Unfortunately, GBM is the case in point for these considerations. In spite of dire needs, initial hopes and efforts aiming at developing effective immunotherapy protocols for GBM, the expected breakthroughs have yet to materialize [70-72]. Even though some of the oncogenic driver proteins in GBM, such as EGFRvIII, possess unique antigenicity and could serve as targets for immunotherapeutics and vaccines, the related clinical efforts failed to produce significant prolongation of patients' survival, largely due to loss of antigen expression on therapy [73]. GBM also possesses a relatively low mutational burden [73]. Nonetheless, some alternative immunotherapy strategies have shown promise, including autologous dendritic cell vaccines (DCVax-L) [74], or the use of CAR T cells in pediatric midline glioma patients [75]. However, robust evidence for major and consistent anticancer activity of immunotherapy is still lacking in high-grade brain tumors [76]. These observations suggest that there must be some features associated with GBM biology that render this particular tumor type unsusceptible to current immunotherapy approaches, and new ideas may be needed [72].

5. Barriers impeding the effective immunotherapy in glioblastoma

While a comprehensive analysis of the mechanisms of immunotherapy resistance in GBM exceeds the scope of this commentary [71,72] it is noteworthy that the immune landscape of these tumors exhibits several thought-provoking features. Among them, perhaps the most striking is the severe scarcity of immune effector cells in the TME, as revealed by single-cell sequencing studies [77], including dearth of both CD4+ and CD8+ T cells, as well as plasma cells, B cells, resting and activated natural killer (NK) cells, and other effector cellular populations [77]. This could be a function of several processes curtailing cellular ingress and activation of the immune response in GBM, such as systemic impairment of the immune cell recruitment into the central nervous system (CNS) [78], residual BBB function of the vascular wall restricting cellular passage [79], BTB effects, [44] and other

factors resulting in immune cell exclusion from the TME. Indeed, while the elevated expression of VEGF in GBM may stimulate trans-endothelial permeability to plasma [46], immune cells may require paracellular mechanisms of brain tumor entry, and those may remain insufficient [80]. These effects are layered upon immune cell exhaustion [81], the immunosuppressive effects [82] of resident myeloid cell populations [35], and the immunologically 'cold' nature of the GBM-associated cellular milieu [83].

Arguably, excluding immune effector cells from the GBM microenvironment is of great functional significance for at least two reasons. First, extreme scarcity of effector immune cells in the TME would result in an overwhelming numerical disadvantage of these cells relative to their cancer cell targets, which, under the best of circumstances, would diminish (if not abrogate) the impact of their mediated intrinsic cytotoxic killing potential [10]. Second, at such low numbers of immune effectors, the immunotherapy agents designed to unblock their activity, such as ICIs, or to target the immunosuppressive microenvironment [84] would unlikely exert a meaningful anticancer effect [10] in GBM. Thus, it stands to reason that, in addition to increasing the cytotoxic activity of immune cells already present in the TME (e.g. by ICIs, cytokine stimulation or CAR expression), for the expected therapeutic effect to occur, the absolute numbers of such immune effectors would need to be markedly increased (by overcoming vascular exclusion), and/or the number of their target cancer cells maximally decreased (e.g. by debulking), or both.

To meet some of these challenges, efforts are underway to improve the efficacy of immunotherapy in GBM [85,86]. Among strategies being explored are the attempts to elicit a more immunostimulatory tumor microenvironment [83], advancing anti-tumor vaccines [87], oncolytic virotherapy [88], improving the penetration of immune cells across the BBB [79,89], therapies involving CAR T cells [90-92], intracranial delivery of immunotherapy [93,94], or targeting intracellular mechanisms of tumor cell-related immune evasion [95].

6. Possible role of NK cell therapy in glioblastoma

In addition to antigen-specific anticancer immunity [85], there is also a growing interest in exploring natural killer (NK) cells in the context of GBM immunotherapy [96]. While cytotoxic T cells are directed at non-self features of cancer cells, which is a subject of complex negative regulation, NK cells are programmed to recognize and kill stressed cells, often expressing low levels of major histocompatibility class I (MHC I) antigens, including cancer cells [97]. NK cells may possess a greater ability to penetrate the tumor tissue [96], and are able to trigger cytokine production, recruit other immune cells, and directly induce tumor cell death or dormancy [98]. Notably, NK cells interact with their targets through several activating receptors (NKG2D, NKG2C, DNAM1), which recognize the corresponding ligands on the surface of cancer cells (MICA, MICB, ULBP1-6; HLA-E; or CD122, PVR/CD155, respectively). This recognition initiates a closer physical

contact and triggers the killing process that involves specialized proteins, such as perforin, granzyme A, and granzyme B [97,99]. The effects of NK cells against GBM target cells could be further experimentally enhanced by overexpression of activating cytokines, such as interleukin 21 [100]. In addition, NK cells may also deploy cytotoxic EVs with tumor cell killing properties [101] a property under active exploration in GBM [10]. However, as in the case of T cells, the proportion of endogenous NK cells in human GBM tumor masses is in the range of 1% [77] and consequently, it could be argued, that even if robustly activated *in situ*, these cells would lack numerical advantages required for exerting a measurable tumor growth control [10].

While the web of systemic and local immunosuppression mechanisms represents an important factor in the failure of immune surveillance and immunotherapy in GBM [86], isolated immune effector cells, including NK cells and T cells, are demonstrably proficient at killing their GBM targets *ex vivo* [9,10,102]. Therefore, addressing the scarcity of these cells in the TME *in vivo* is worth considering as an actionable element for future therapeutic studies. Overcoming the vascular barriers characteristic of GBM would likely be part of strategies aiming to increase the presence of immune effectors in the TME.

7. Experimental approaches to circumvent vascular barriers for immunotherapy in glioblastoma

Overcoming vascular barriers to improve delivery of therapeutics into the CNS is a subject of intense worldwide effort, summarized in multiple recently published review articles and updates [103-105]. The emphasis of many of these studies is on means to direct therapeutics from the systemic circulation into the brain microvasculature, and to enhance their trans-endothelial passage to improve their biodistribution in the brain parenchyma [106]. Achieving these objectives is being explored through a range of strategies, including nanomedical formulations (nanoparticles) [84], packaging into extracellular vesicles, using carriers able to recognize molecules enriched on the surface of brain endothelium (TfR, angiopep2, glucose), among other approaches [71]. Moreover, pharmacological inhibitors of claudin 5, the key component of endothelial tight junctions [107], or externally applied physical forces that may transiently disrupt endothelial barrier function, are part of these explorations [106], including MRI-guided focused ultrasound (MRg-FUS) [106]. In addition, alternative routes of drug delivery to circumvent BBB/ BTB, such as intracranial convection-enhanced delivery (CED), or intrathecal injections, have been examined experimentally, as extensively reviewed elsewhere [103,106,108]. As mentioned, these approaches are of great interest in the setting of immune cell delivery into the brain tumor TME [93,94].

Among multiple ongoing efforts to overcome the refractoriness of GBM to immunotherapy [72] reflecting on

specific experimental studies may offer useful examples as to the associated challenges and possible paths forward. In this regard, one important consideration in GBM is the temporal interplay between the cytoreductive backbone therapy and the potential of immune intervention to change the course of the disease, especially at relapse. Thus, in a recent study, patient-derived GSCs with either proneural (PN) or mesenchymal (MES) characteristics were used to generate subcutaneous xenografts in severely immunodeficient (NSG) mice lacking both functional T cells and NK cells. In this setting, the effects of chemotherapy with TMZ were compared between the resulting tumor subtypes [8]. Following the initial exposure to the optimized dose of TMZ, both types of xenografts underwent a complete regression, only to relapse a few weeks later in a form of molecularly divergent and TMZ-non-responsive tumors, expressing either O6-methylguanine-DNA methyltransferase (MGMT), or features of a hypermutant phenotype as mechanisms of drug resistance [8,109]. Strikingly, while this scenario repeatedly occurred in NSG mice, in their SCID counterparts harbouring functional NK cells, only PN-GSC tumors regressed and then relapsed post-temozolomide TMZ, as expected. In contrast, a single dose of the drug permanently eradicated their MES-GSC counterparts, and the mice remained disease-free for several months [10]. Notably, pretreatment of tumor-bearing SCID mice with Asialo-GM1 antibody could reverse the latter responses, due to suppression of NK cell activity. These results collectively suggest that endogenous NK cells could dramatically curtail post-TMZ relapse of MES-GBM tumors.

In line with these findings, MES-GSCs, but not their PN-GSC counterparts, expressed high levels of NK ligands (MICA, ULBP2-6) [10]. Similar tumor subtype-specificity of NK ligand distribution was also observed in GBM patients as indicated by mining the Cancer Genome Atlas (TCGA). It should be noted that in NK-proficient SCID mice, both PN-GSC and MES-GSC manifested uninhibited engraftment and progression in the absence of TMZ therapy, which suggests that tumor debulking by chemotherapy was a precondition for effective NK cell surveillance. Importantly, these beneficial effects of endogenous NK cells following TMZ tumor debulking were absent when the tumors were injected intracranially and protected from NK cells by the BBB/BBB vascular mechanisms. In this case, however, intracranial injection of exogenous NK cells (NK92MI) resulted, yet again, in a complete post-TMZ tumor resolution and long-term survival. Finally, such curative effects occurred only if NK92MI cell injection was timed in such a way as to coincide with the decline in post-TMZ tumor burden. Delay in the administration of NK92MI cells abrogated their curative effects [10], which could also, to some extent, be recapitulated by NK92MI-derived EVs [110]. Overall, the results of this study illustrate the notion that effective immunotherapy in GBM (by NK cells in this case) may require a simultaneous implementation of several measures to address multiple unique aspects of the disease. This included circumventing

the BBB/BBB by intracranial delivery of NK effector cells, optimization of effector-to-target ratio through timing/dosing of immunotherapy, and consideration given to molecular subtype of cancer cells and their intrinsic sensitivity to specific immune effectors [110]. In the absence of any one of these conditions, the therapy did not work.

While intracranial immunotherapy may offer a workable solution in some instances, expanding the ways to regulate the paracellular traffic of immune cells through modulating the vascular wall in the brain represents another intriguing option [106]. In this regard, a relatively unexplored circuitry of vascular regulation in GBM includes EV-related pathways, which have already been implicated in angiogenic [111] and non-angiogenic vascular growth processes (e.g. vasectasia) [27], and in angiocrine effects of endothelial EVs [63]. These earlier studies motivated our group to explore the vascular characteristics of mouse GBM-like tumors (GL261) [80] in mice deficient or proficient for Rab27 proteins [112], the key effectors in the EV biogenesis pathway [113], also involved in other aspects of intracellular trafficking relevant for the vasculature [114]. Interestingly, while brain tumors in wild-type mice exhibited a characteristic exclusion of immune effectors from the TME, in Rab27-deficient mice, the vasculature became highly dysmorphic, and tumor parenchyma was replete with blood-borne immune cells, which mediated an effective adaptive antitumor response [80]. While these and other experimental observations are not directly applicable in the clinic, they illustrate the fundamental interdependence between the vascular barrier and immune responses in brain tumors. As such, they may carry some hypothesis-generating value for future in-depth and translational studies.

8. Closing comments and future considerations

Applying technological advances to immunotherapy of high-grade brain tumors, including GBM, is ultimately guided by assumptions about the nature of the rate-limiting factors impacting the desired effect. In this regard considering vascular barriers may represent one important frontier. Naturally, this is not the sole factor to be taken into account. It is presently unknown how the molecular oncogenesis of brain tumor subtypes may affect both the vasculature [27,115,116] and the immune responsiveness of brain cancer cells [10]. It is unclear whether different neovascularization processes, such as angiogenesis, vascular co-option, or vasectasia [27] co-exist in GBM and may impact vascular barrier function for immune cells and their regulation.

Matching immune effectors' numerical and functional aspects with the burden of tumor-initiating GSCs and their growth dynamics, including timely disease debulking, may represent an important consideration. It is also important to understand how immune effector cells, be it endogenous, delivered systemically, or administered intracranially (e.g., as CAR T therapy), may gain access to their cancer cell targets, when the latter are

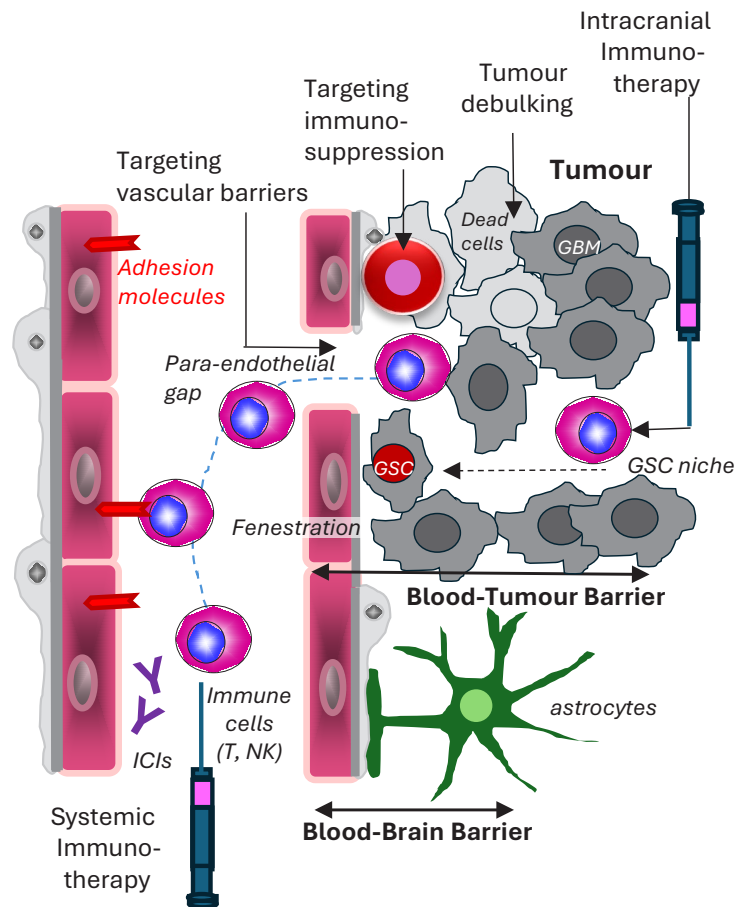


Figure 1. Targeting the immune-vascular interface in glioblastoma. The combined effect of blood-brain barrier (BBB), blood-tumor barrier (BTB), and endothelial anergy contributes to the exclusion of immune cells (T, NK) from the brain tumor microenvironment. Overcoming this scarcity by intracranial delivery of immune cells, modulating vascular wall (para-endothelial gaps), coupled with tumor debulking (to reduce the size of the target cell population) and targeting immunosuppressive mechanisms may collectively improve outcomes of immunotherapy in glioblastoma and other brain tumors (see text)

disseminated throughout the brain, and often lodged at sites adjacent to the vasculature [63]. Can immune cells extravasate at these specific locations? Moreover, the emerging biology of cancer cell webs interconnected through tumor microtubules [21] and other structures [22] may pose new questions about the effects of immune cells in this setting and impact new immunotherapy paradigms. Similarly, the emission by GBM cells of immunosuppressive EVs carrying 'decoy' immune checkpoints [117] may also add to this complexity.

As these questions and findings emerge, they will likely lead to new concepts about mechanisms modulating the vascular-immune interface in GBM and other brain tumors, and new therapeutic approaches may follow. It is not too soon for accelerating innovation in this frightening disease.

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Conflict of interest

The author has no conflict of interest to declare.

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