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REVIEW

Necrotic reshaping of the glioma microenvironment drives disease progression

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Abstract

Glioblastoma is the most common primary brain tumor and has a dismal prognosis. The development of central necrosis represents a tipping point in the evolution of these tumors that foreshadows aggressive expansion, swiftly leading to mortality. The onset of necrosis, severe hypoxia and associated radial glioma expansion correlates with dramatic tumor microenvironment (TME) alterations that accelerate tumor growth. In the past, most have concluded that hypoxia and necrosis must arise due to "cancer outgrowing its blood supply" when rapid tumor growth outpaces metabolic supply, leading to diffusion-limited hypoxia. However, growing evidence suggests that microscopic intravascular thrombosis driven by the neoplastic overexpression of pro-coagulants attenuates glioma blood supply (perfusion-limited hypoxia), leading to TME restructuring that includes breakdown of the blood–brain barrier, immunosuppressive immune cell accumulation, microvascular hyperproliferation, glioma stem cell enrichment and tumor cell migration outward. Cumulatively, these adaptations result in rapid tumor expansion, resistance to therapeutic interventions and clinical progression. To inform future translational investigations, the complex interplay among environmental cues and myriad cell types that contribute to this aggressive phenotype requires better understanding. This review focuses on contributions from intratumoral thrombosis, the effects of hypoxia and necrosis, the adaptive and innate immune responses, and the current state of targeted therapeutic interventions.

Keywords Glioblastoma · Necrosis · Tumor-associated macrophages

Introduction

Glioblastoma (IDH-wild type, WHO grade 4) is the most frequent malignant brain tumor and has a dismal prognosis. The 5-year survival rate is only 5.6% and the median survival interval is 15 months from initial diagnosis [173]. By definition, glioblastoma is a high grade, infiltrating astrocytic glioma with one or more of the following features: (1) necrosis, (2) microvascular proliferation, or (3) the presence of specific genetic alterations (*EGFR* amplification, *TERT* promoter mutation, or the +7/−10 cytogenetic signature) [230]. Historically, the histologic presence of necrosis was the first recognized feature linked to poor prognosis among diffuse gliomas and it remained the sole criterion for establishing the diagnosis of glioblastoma as grade 4 for decades. Even today, it is recognized that nearly all patients with glioblastoma die after a brief period of accelerated tumor expansion following the onset of necrosis.

In fact, necrosis is a criterion of malignancy in many tumor types, highlighting its fundamental association with rapid growth and poor patient prognosis [28, 197]. The prevailing dogma passed along to explain the relationship of malignancy and necrosis has been that "cancer outgrows its blood supply," as metabolic demands exceed supply during the rapid and uncontrolled cell division and tumor expansion (*diffusion-limited* hypoxia). While this explanation has been superficially satisfying, it has never been supported by evidence, it is counterintuitive on deeper inspection, and its perpetuation has precluded serious investigations into more plausible mechanisms that link malignant behavior to necrosis in a manner that might shed light towards potential therapies.

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Several studies seem to contradict the malignant necrosis dogma and suggest that cancers grow in a manner that actively compromises their blood supply, leading to necrosis and augmented growth due to hypoxia- and tumor microenvironment (TME)-mediated mechanisms. This theory holds that tumors grow in a manner that attenuates local blood flow, leading to *perfusion-limited* hypoxia and necrosis. There is strong evidence to suggest that microscopic intravascular thrombosis within a tumor, most likely driven by the neoplastic overexpression of pro-coagulants, initiates or propagates hypoxia and necrosis that in turn causes TME restructuring in a manner that favors accelerated growth [71, 150, 199, 234, 241]. The spatial distribution of thrombosis in and around foci of necrosis is highly suggestive of an intimate relationship between the two, with thrombosis potentially causing necrotic development. Microscopic thrombosis can be identified in nearly all glioblastomas but is rarely found in lower grade gliomas without necrosis, which are characterized by sheet-like diffuse infiltration and grow more slowly (Fig. 1). The small number of diffusely infiltrative astrocytic gliomas that have thrombosis, but not necrosis, are also associated with poor prognosis, suggesting that it is a precursor to the development of necrosis and higher grade behavior [241].

Molecular genetic alterations driving progression among the diffuse gliomas are well characterized and have elucidated several molecular subtypes based on genomic alterations, epigenetic signatures or transcriptional class [1, 25, 27, 32, 153, 172, 181, 254]. Transcriptional classification has identified three robust subtypes among the IDH-wild-type GBMs (proneural, classical and mesenchymal) that appear to have distinct TME properties. Proneural (PN) tumors are enriched for *PDGFRA, CDK4* and *SOX2* amplification and display increased PI3K/AKT signaling [22, 181]. Despite PDGF signaling correlating with immune modulation in other solid tumors, glioblastoma displays a strictly proliferative association with PDGF expression [9]. Mesenchymal (MES) tumors contain inactivating mutations in *NF1*, increased MAPK signaling, and are enriched for endothelial markers and inflammatory infiltrates, especially the macrophage component [22, 170, 259]. Regarding pro-angiogenic signals, MES upregulate ADAM9 that enhances chemotactic factor shedding from tumor cells, cleaves the extracellular matrix (ECM) promoting invasion, and releases angiogenic factors from endothelial cells promoting

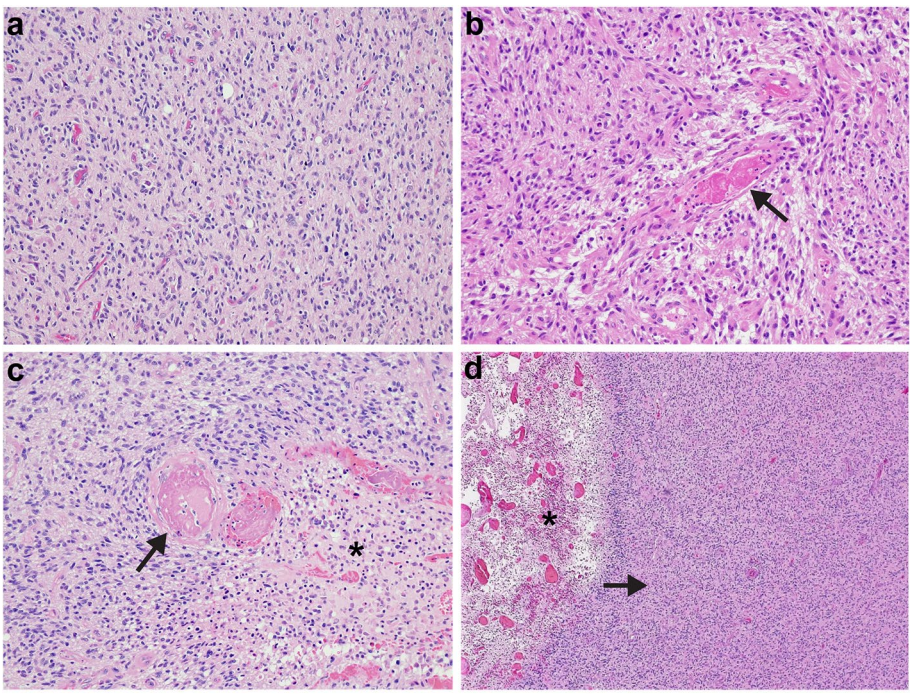

Fig. 1 Histopathology of glioma progression (H&E staining). Diffusely infiltrating astrocytic tumor without necrosis (histologic grade 3) shows a pattern of sheet-like infiltration by individual tumor cells within the brain parenchyma (**a**). The presence of intravascular thrombosis (arrow) within a diffuse glioma signals transition to the development of hypoxia, necrosis, and rapid progression. Note the perivascular clearing of neuropil, representing the initial stages of BBB breakdown, activation of perivascular TAMs, parenchymal disruption, and outward migration of glioma cells (**b**; see also Fig. 3 right panel). At later stages, the presence of intravascular thrombosis (arrow) is spatially associated with adjacent tumor necrosis (**c**; asterisk). As the necrotic focus enlarges (asterisk), it becomes surrounded by palisading cells that migrate radially outward in three dimensions (**d**; arrow indicates direction of movement; see also Fig. 2d, boxed region for corresponding MRI region, and Fig. 5)

microvascular hyperproliferation [178]. MES tumor cells also display increased MMP14 membrane localization that promotes ECM cleavage, endothelial and tumor cell invasion and contributes to the vascular abnormalities commonly seen in solid tumors [51]. Classical (CL) tumors are characterized by *EGFR* mutation and amplification, NOTCH pathway activation, and downregulation of both apoptotic and MAPK signaling pathways [22]. EGFR activation in combination with PTEN loss enhances VEGF expression to support angiogenesis [186], as well as CCL2 secretion that enriches TAM infiltration [4].

Despite transcriptional class differences, all glioblastoma subsets display accelerated progression following the onset of necrosis, indicating that it may be a shared final common pathway that represents an abrupt turning point towards rapid expansion [93, 202]. Of interest, cellular proliferation rates are not a prognostic factor once necrosis develops, indicating that other factors influence survival to a greater extent [19]. Most likely, accelerated growth of glioblastoma is due at least to some extent to hypoxia-induced expansion encouraged through TME dynamics [79]. There is no doubt that glioblastomas are highly heterogeneous, as recognized by the now outdated term "multiforme". In addition to glioma cells of variable morphologies, differentiation states and stem-like features, glioblastomas also contain tumor-associated macrophages (TAMs), a variety of other immune cells, florid angiogenesis, entrapped native neural elements and reactive glia [15, 54, 107, 153, 191, 245]. TAMs consist of activated resident brain microglia and bone marrow derived monocytes (BMDMs), which differentiate into macrophages upon extravasation into the brain parenchyma. While TME restructuring following necrosis in glioblastoma appears to be an initiator of rapid tumor growth, appropriate animal models to establish the causal relationship between necrosis, TME alterations, and radial expansion are lacking. Indeed, many orthotopic patient-derived xenograft (PDX) mouse models do not develop necrosis [103, 132]. A recent study postulates that this arises in part from defective cross species chemokine signaling [40]. This review assesses the stages of TME-related changes that occur during disease progression in glioblastoma, highlighting the role of hypoxia and necrosis in modulating the immune response.

Thrombosis

The blood–brain barrier (BBB), comprised of brain microvascular endothelial cells, astrocytes, pericytes, oligodendrocytes and unique basement membrane, represents one of the most controlled vascular networks of any organ and its deterioration marks a dramatic change in disease progression among patients with diffuse gliomas. The BBB is largely intact in non-necrotic, lower grade diffuse gliomas and corresponds to the absence of contrast enhancement on MR imaging [255]. The enhancement pattern that becomes apparent in high-grade gliomas represents contrast agent seeping through the BBB and being retained in the brain tumor parenchyma (Fig. 2) [96]. Initial stages of contrast enhancement are often subtle and patchy and can be noted before the onset of necrosis. This likely represents the first stages of vascular pathology and barrier compromise (corresponding with endothelial hypertrophy) yet precedes the onset of severe hypoxia and necrosis that is associated with more extensive vascular proliferation (Fig. 3). Prior work has suggested that microscopic intravascular thrombosis arises at this early stage of glioblastoma progression and is responsible for initiating or propagating hypoxia. The classic MRI features of glioblastoma, with central necrosis surrounded by a rim of intense contrast enhancement and enveloped by T2

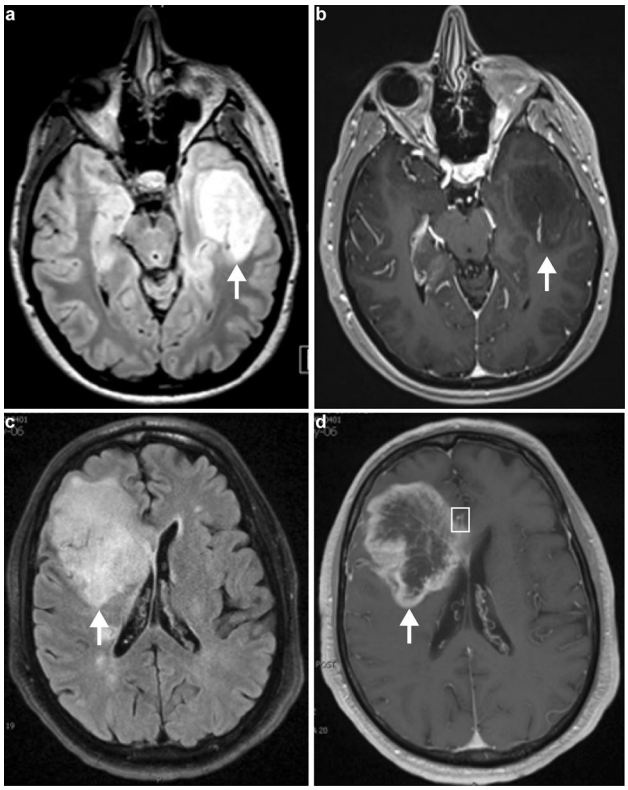

Fig. 2 MRI images typical of glioma progression. Fluid-attenuated inversion recovery (FLAIR) (**a**, **c**) and T1 post-contrast (**b**, **d**) MRI. The outline of this histologic grade 3 diffusely infiltrative glioma is noted on FLAIR images (**a**, arrow). The tumor does not demonstrate central necrosis or contrast enhancement on post-contrast images (**b**, arrow, corresponding to histology in Fig. 1a). After the onset of hypoxia and necrosis, MRI demonstrates the tumor outline on FLAIR images (**c**, arrow), while T1 post-contrast images show the classic features glioblastoma, with a prominent region of central necrosis surrounded by a rim of intense contrast enhancement (**d**, arrow). The region of the box (**d**) corresponds to the histology in Fig. 1d, with the border region moving radially outward away from the tumor center

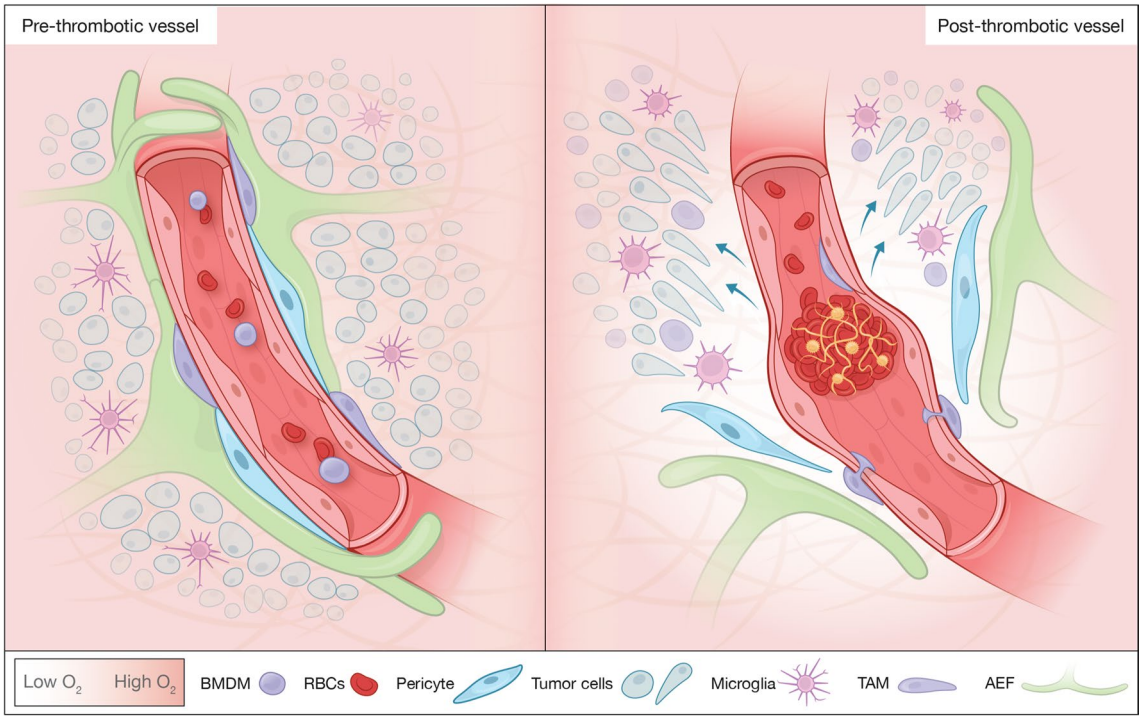

Fig. 3 Vascular pathology disrupts the blood–brain barrier, limits perfusion, and reshapes the tumor microenvironment. Astrocyte end feet, pericytes, and endothelial cells in pre-thrombotic vessels form an intact blood–brain barrier (left) and provide a relatively immune privileged environment within the central nervous system. When intact, this barrier largely excludes circulating immune cells from the early tumor microenvironment (TME). Following thrombosis (right), astrocytes and pericytes detach from vascular endothelial cells, which also develop leaky junctions, permitting circulating BMDM to traverse the vascular wall and enter the TME. Vascular disruption reduces perfusion and leads to focal hypoxia, driving cells radially out from the area of nutrient deprivation

signal intensity, are noted later, once there is substantial loss of BBB integrity and extensive microvascular proliferation in and around the contrast-enhancing component (Fig. 2).

The link between cancer and thrombotic events is not new. Trousseau recognized well over a century ago that cancer patients exhibit significant systemic dysregulation of coagulation, resulting in frequent peripheral deep venous thromboses and embolic events [58, 150, 246]. This same tendency towards thrombosis is present within the neoplasm, where the causative pro-coagulants are highly expressed [210, 239]. Many investigations have focused on tissue factor (TF), the body's most potent pro-coagulant, as the primary mediator of systemic coagulopathy [208, 209]. Notably, TF is significantly upregulated in gliomas and its levels correlate with tumor grade [203, 204]. Factors associated with malignant behavior in gliomas, such as EGFR overexpression, PTEN loss and hypoxia-induced early growth response gene (Egr)-1, have all been shown to upregulate TF expression by gliomas [203, 204, 241, 248]. The vascular leakiness that is noted by neuroimaging at early stages of malignant progression would allow circulating coagulation factors, including TF's primary downstream effector, factor VIIa, to encounter TF. Interestingly, IDH mutant gliomas show significantly reduced TF levels as compared to IDH-wild-type gliomas, potentially related to their slower rate of malignant progression [249, 250]. Conversely, higher grade gliomas display dysfunctional coagulation/fibrinolysis regulatory pathways supporting local coagulation events within the tumor [266]. Increased levels of coagulation are also likely due to thrombin-protease-activated receptor 1 (PAR1) signaling, which is similarly upregulated in glioblastoma [60, 71, 126]. PAR1 localizes to astrocyte end feet where its binding to thrombin leads to a wide variety of downstream effects, including neuroinflammation and vascular pathology [71]. Thrombin-mediated PAR1 cleavage actives the G-protein-coupled receptor leading to Rho and phospholipase C activation and adenylyl cyclase inactivation [20, 30] and promotes VEGF secretion [99] while eliciting an immunosuppressive response [215]. Local VEGF accumulation around the BBB induces pericyte detachment, basement membrane degradation, vessel enlargement and leakiness, perpetuating the cycle of vascular pathology [237, 238]. During this process, glioblastomas also upregulate podoplanin, which enhances local platelet aggregation and has been implicated in systemic thrombosis through its cell surface expression by circulating glioblastoma cells [180, 199, 234]. Podoplanin binds C-type lectin-like receptor (CLEC)-2 on circulating platelets and induces clotting

[180]. Concurrently, the emerging hypoxia upregulates plasminogen activator inhibitor 1 (PAI-1) and fibronectin in the perinecrotic niche and surrounding occluded vasculature, generating local pro-coagulant environments [207].

Upregulation of coagulation factors influences the TME in manners that may be unexpected, distinct from their role in thrombosis. For example, TF, factor VIIa (FVIIa), and factor XIIa (FXIIa), are capable of recruiting TAMs to the TME and inducing an immunosuppressive phenotype [69, 149]. The TF-FVIIa complex can trigger mitogenic, angiogenic, and cell survival signaling, as well as enhance a glioma stem cell (GSC) phenotype in certain contexts [248]. Podoplanin may have effects on dendritic cell mediated immunosuppression by binding to and activating CLEC-2, leading to platelet aggregation, enhancing local and distal thrombotic events and monocyte/macrophage recruitment to the area of vascular pathology, reflecting another mechanistic link between coagulation and tumor progression [146, 195, 199]. The prevention or suppression of early thrombotic events in glial neoplasms represents a strategy to slow down disease progression that results from hypoxia- and necrosis-driven TME changes.

Hypoxia

Hypoxia, a state of low oxygen availability, is a critical mediator of pathologic events, yet remains challenging to model and study within physiologically relevant experimental systems. Establishing hypoxic conditions, maintaining physiological gradients and monitoring of oxygen levels in vivo remain daunting prospects, yet recent advances in positron emission tomography (PET) and two-photon phosphorescence microscopy are encouraging [119, 228, 274]. Nevertheless, fluctuations in oxygen availability have profound effects on homeostasis, as well as disease onset and progression, and therefore must be a central consideration of any serious scientific pursuit of mechanisms relevant to glioma progression [224, 242]. We have suggested that vaso-occlusive mechanisms initiate and propagate the severe hypoxia that is present in nearly all high-grade diffuse gliomas and trigger the adaptive responses that lead to TME restructuring and tumor expansion. Hypoxia-inducible factors (HIFs) 1 and 2 are the predominant cellular oxygen sensors, and are upregulated under hypoxic conditions to activate a transcriptional program conducive to an adaptive response that allows cell survival under these conditions [164]. In the case of malignant gliomas, the response to hypoxia also results in events that favor disease progression (Fig. 4). Nuclear HIFs enhance glycolytic metabolism, cellular migration through a urokinase (uPA)-uPA receptor (uPAR) autocrine loop, and invasion through matrix metalloproteinases (MMPs)-2 and -9 secretion [18, 33, 72, 115, 127, 152, 160, 162, 164, 217, 283]. Enhanced glioma cell HIF expression leads to a distinct survival advantage in hypoxic and necrotic conditions [137, 152, 179, 257, 284] including therapeutic resistance through GSC enrichment [134, 251, 258, 276]. Furthermore, intratumoral HIF and other hypoxia-induced genes correlate with a more aggressive, pro-invasive and highly angiogenic phenotype across many solid tumors including glioblastoma [49, 64, 90, 115, 157, 189, 236, 253, 260, 271].

The adaptive response to hypoxia also influences inflammatory and immune responses following the onset of necrosis. For example, HIF-1α modulates hypoxic T cell

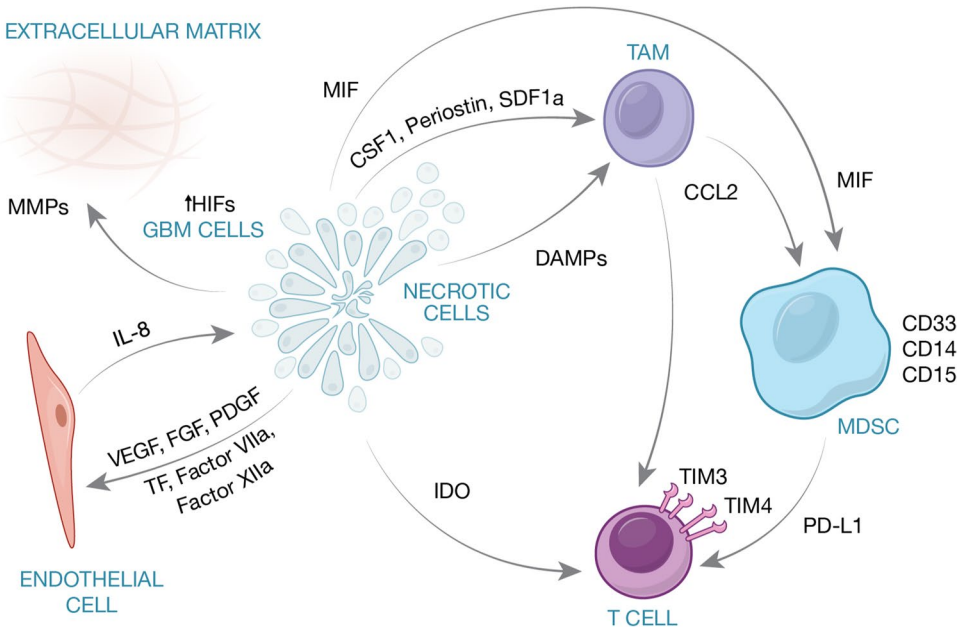

Fig. 4 The hypoxic tumor microenvironment is highly coordinated and dynamic. Glioblastoma cells upregulate HIFs and subsequently stimulate MMPs to remodel the ECM, clotting factors to induce focus thrombosis, angiogenic factors that drive microvascular hyperproliferation, and immunomodulatory factors including damage-associated molecular patterns that form an immunosuppressive environment

metabolism, facilitating Treg recruitment and an immunosuppressive phenotype [158]. Hypoxia increases TF secretion exacerbating focal vascular pathology, and Ras and PI3K-Akt signaling further enhance migration away from the hypoxic region [2, 204]. One study described p21 activated kinase (PAK) 1-dependent autophagy, linking hypoxia to glioblastoma tumorigenesis and radial expansion [62]. Hypoxic glioblastoma cells display not only altered DNA repair machinery, but are increasingly resistant to chemo- and radiation therapies [35, 45, 167]. Others have shown that hypoxia-induced epigenetic changes in histone deacetylase (HDAC) 3 activity and downstream transcription factors CCAAT enhancer binding protein beta (CEBPB) and JUN contribute to temozolomide (TMZ) resistance [66]. Intratumoral hypoxia also induces an inflammatory GSC phenotype that facilitates glioblastoma radial expansion [235]. Thus, acute and sustained hypoxia arising from vaso-occlusion and associated with necrosis has profound effects on disease progression and therapeutic resistance.

Necrosis

While the metabolic stress related to hypoxia contributes to a wide range of adaptive responses, as noted above, the associated development of necrosis also plays a pivotal role in reshaping the local brain tumor microenvironment. Although necrosis has historically been considered an unprogrammed, passive cell death response, work over the last 2 decades has uncovered specific signaling networks that regulate its development [281]. Separating the effects of necrosis and hypoxia may be difficult or impossible, and the classic histopathologic features of glioblastoma, including intratumoral thrombosis, microvascular proliferation, and neoplastic palisade formation around necrosis are intimately related to both (Fig. 5) [18, 191, 265]. Our prior work suggests that intravascular thrombosis causes vaso-occlusion, leading to nutrient deprivation and sustained hypoxia/anoxia that triggers cellular necrosis [202, 204, 241]. How these early hypoxic/necrotic events coordinate TME, reshaping is an area of active research. Necrotic cells are now known to release endogenous damage-associated molecular patterns (DAMPs), capable of recruiting TAMs or damage-associated microglia (DAM) to the TME [21, 50, 87], facilitating

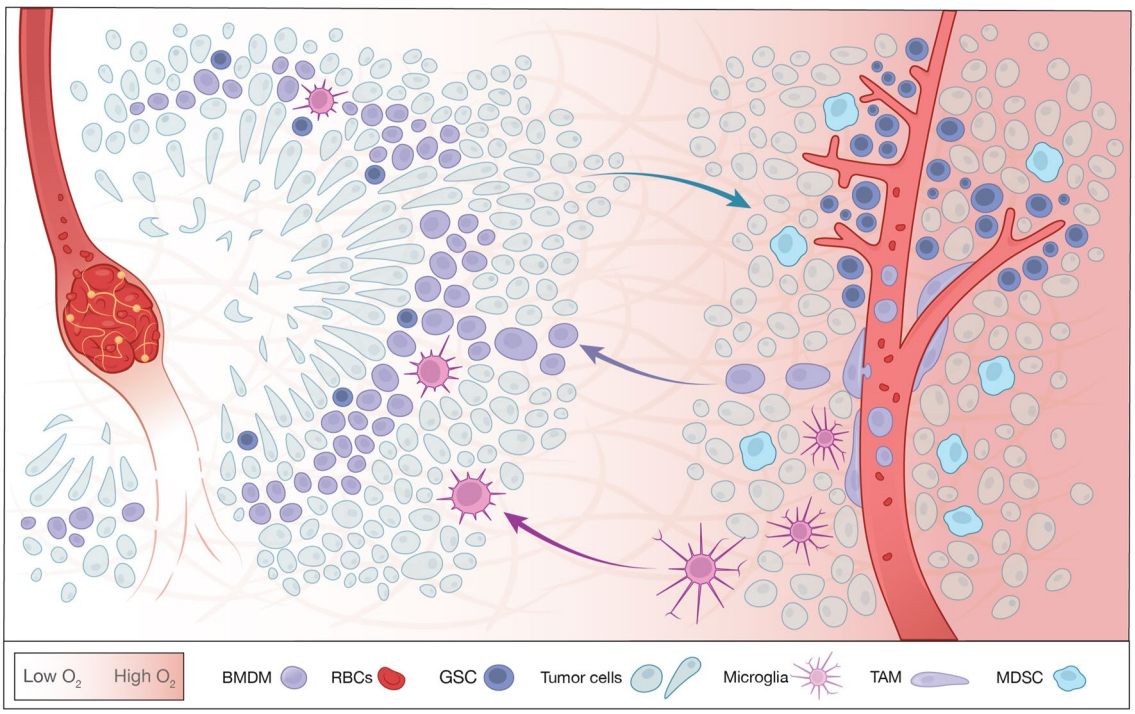

Fig. 5 Necrosis initiates a sterile inflammatory response that promotes glioma progression. Intratumoral thrombosis within the hypoxic/necrotic core forces glioblastoma cells to migrate towards a more hospitable locale. While most surviving perinecrotic glioblastoma cells migrate away from the necrotic core, an enriched stem-like phenotype is found in the highly hypoxic perinecrotic (palisading) niche. Meanwhile, blood–brain barrier disruption allows immune access to the tumor, monocyte influx and macrophage differentiation as those cells migrate towards the necrotic core. Simultaneously this emerging perivascular niche becomes enriched in stem-like cells, budding vessels, and myeloid-derived suppressor cells

disease progression [120, 201] (Fig. 5). DAMP release normally initiates sterile inflammation to drive tissue repair yet when left unchecked can facilitate a chronic inflammatory state resulting in unwanted tissue damage, particularly in ischemia-related injuries [46, 110, 198]. Necrosis-associated DAMPs include adenosine/adenosine triphosphate (ATP) [17, 105, 128], biglycan [6, 200, 213], heparan sulfate [113, 272], heat shock proteins (HSPs) [11, 12, 109, 193, 252], high-mobility group box 1 (HMGB1) [94, 212, 222, 244, 282], hyaluronan (HA) [112, 214, 240], interleukin (IL)-1α [36, 59, 73, 125], IL-33 [31, 163, 216, 220], S100 proteins [42, 91, 92, 135], and versican [95, 121, 264]. Of interest, ATP, HA, HMGB1, IL-1α and S100 proteins are potent DAMPs that are enriched in brain and glioma tissues [10, 21, 73, 92, 94, 128, 182, 192, 221, 229, 247]. Extracellular adenosine binds to adenosine receptors on many immune cells including macrophages, driving initial inflammation, then inducing an M2-like immunosuppressive phenotype [128] and enhancing glioblastoma invasion [182]. HA cleavage from the ECM into small molecular weight fragments engage not only it's canonical receptor, CD44, but also several Toll-like receptors (TLR2 and 4) known to mediate inflammatory responses [240] while simultaneously enhancing glioblastoma invasive capacity [37]. HMGB1 acts through both TLR4 and the receptor for advanced glycation end products (RAGE) to initiate pro-inflammatory cytokine release, recruiting bone marrow-derived monocytes (BMDMs) to sites of injury and contributing to the immunosuppressive TME [94, 101, 232]. Initial IL-1α release from necrotic cells draws neutrophils and BMDM in, followed by a second wave of IL-1α secretion from subsequently activated macrophages, further enhancing the pro-inflammatory microenvironment [59, 125]. S100 proteins also bind RAGE and attract BMDM to the TME, contributing to immune cell reprogramming and at the same time promoting tumor cell proliferation [87, 92, 135]. In addition to the generation of sterile inflammation, necrotic cellular pathology upregulates cell survival pathways to compensate for an increasingly inhospitable environment. Dramatic microenvironmental restructuring following necrosis enriches for distinct cellular subpopulations that thrive under these selective pressures.

Immune microenvironment

Microglia represent the largest phagocytic cell population in the brain under normal homeostatic conditions. They are unique to the brain and arise from immature yolk sac ($Runx1^{+}$) progenitor cells between embryonic days 8.5 and 9.5 [68, 70, 83]. They are also among the most long-lived brain-resident cells, rivaling post-mitotic neuron life spans [280]. As mentioned, DAMs respond to DAMPs during brain injury [14, 50], representing an early and rapid innate immune response. In some disease states, DAMs play a neuroprotective role and hinder disease progression [120, 154]. However, sustained neuroinflammation and DAM reprogramming can result in neurotoxic events mediated not only by DAMs but also through modulating reactive astrocytes [13, 138]. Upon brain injury, stroke or tumorigenesis, the BBB becomes compromised leading to significant influx of circulating BMDM, as well as microglial activation [41, 168, 183, 262], and distinguishing these cell types and various activation states requires detailed analysis [77, 129]. In addition, TAM derived IL-1b exacerbates BBB defects, enhancing vascular edema and BBB leakiness [88]. These cell lineage determinations become crucial when determining how to counteract disease processes as BMDMs and microglia play differing roles in brain inflammatory responses [29, 55, 277]. A recent study utilizing a mouse model of pediatric high-grade glioma demonstrated that BMDMs, but not microglia are responsible for mediating the intratumoral immune response [206]. In addition, a single-cell RNA sequencing study revealed spatial and functional diversity among infiltrating microglia and BMDMs [130, 169]. These distinct subpopulations require informed consideration when designing therapeutic interventions to effectively target the malignant immune behaviors while preserving neuroprotective responses.

Despite advanced understanding of inflammation following traumatic brain injury and ischemia [46, 110, 262], mechanisms and therapeutic vulnerabilities of the sterile inflammatory response have not been well established in the glioblastoma TME. Following necrosis, TAMs represent the most abundant non-neoplastic cells within glioblastoma, accounting for 30–50% of all cells within the tumor mass (Fig. 6) [39, 79, 80]. TAMs are not passive bystanders, but rather actively promote tumor progression and modulate treatment responses [61, 98, 187, 278]. By the time a malignant brain tumor has developed severe hypoxia and central necrosis, the vast majority (> 80%) of TAMs derive from BMDMs, while the remainder are comprised of microglia [38, 80]. However, not all microglia respond to chemotactic/activating factors leaving residual undifferentiated tumor-associated microglia that appear as web-like immune surveillance cells enriched around the disease periphery [41, 227]. TAM density increases five- to tenfold following necrosis, mainly in hypoxic, perinecrotic zones [48, 156, 259]. Hypoxia induces TAM influx, activation then conversion from an anti-tumor (M1-like) to an immunosuppressive (M2-like) phenotype, promoting tumor progression [38, 39, 86, 169]. A recent TCGA pan-cancer study indicated that glioblastoma has a prominent TAM signature, with a highly immunosuppressive phenotype and suppressed Th1 lymphocytes [243]. Immune response genes are enriched in mesenchymal glioblastomas, indicating genomic background and transcriptional activities influence the TME [54, 111, 254,

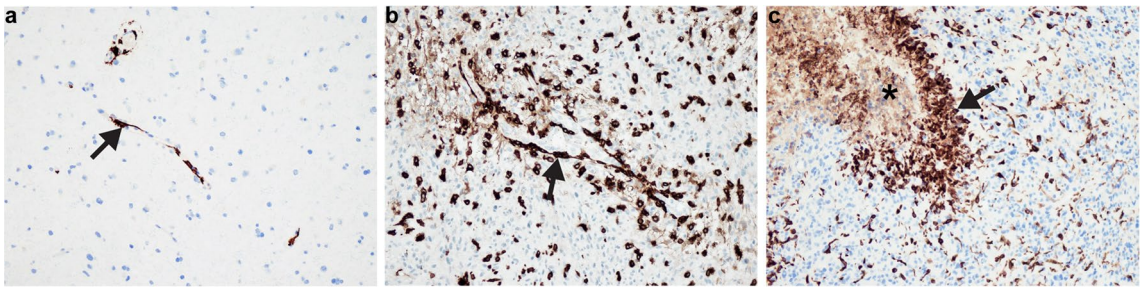

Fig. 6 Enhanced tumor-associated macrophages (TAMs) in glioma progression (CD163 immunohistochemistry). In diffusely infiltrating gliomas that are low grade (histologic grade 2) and have an intact blood–brain barrier (BBB), there is a small population of inactive, flattened, perivascular CD163-positive TAMs (arrows) and only rare CD163-positive cells within the CNS parenchyma (**a**). With glioma progression and the development of hypoxia and BBB breakdown, there is activation and enlargement of the perivascular CD163-positive TAM population (arrow) and a large influx of CD163-positive TAMs from derived from circulating BMDM that traverse the BBB and infiltrate into the brain (**b**, corresponding to histology in Fig. 1b). With the development of necrosis, large numbers of CD163-positive TAMs are noted around necrosis (asterisk) and within the infiltrating component of the glioblastoma (**c**, corresponding to histology in Fig. 1c)

285]. One study found increased immune cell infiltration, including TAMs and lymphocytes, in human mesenchymal glioblastomas compared to proneural and classic subtypes [118], while another found that classical glioblastomas display greater $CD4^+$ and $CD8^+$ T cell infiltration [40]. Analysis of TCGA glioblastoma data showed that allograft inflammatory factor 1 (*AIF1*), the gene encoding ionized calcium binding adaptor molecule 1 (IBA1), was significantly upregulated in mesenchymal glioblastomas compared to others [118]. Distribution within the TME—potentially related to the hypoxia gradient—also alters TAM behavior, as peripheral TAMs display pro-inflammatory signaling, homing circulating BMDMs to the TME [26, 130]. Myeloid-derived suppressor cells (MDSCs) are functionally similar to immunosuppressive TAMs but express specific cell surface markers such as CD33, CD14 and CD15 in humans or CD11b and protein gamma response 1 (Gr1) in mouse models [34, 175]. TAM-secreted CCL2 recruits MDSCs from circulation while GSC-secreted macrophage migration inhibitory factor (MIF) enhances their immunosuppressive activity [3, 34, 175]. The protective role has largely been attributed to enhanced MDSC programmed death-ligand 1 (PD-L1) expression that mitigates $CD4^+$ T cell activity in and around the glioblastoma TME [56]. This active recruitment and reprogramming among immune subpopulations in and around the tumor create an increasingly complex, heterogeneous milieu that we are just beginning to recognize. Future investigations into the temporal and spatial dynamics will enable systematic interventions to reverse the immune privileged tumor state.

Glioblastoma exhibits far fewer infiltrating lymphocytes than other solid tumors, consisting largely of Tregs followed by $CD3^+$ T helper cells, other $CD4^+$ T cells and few $CD8^+$ T cells [85, 268]. Importantly, glioblastoma T cell infiltration co-localizes with areas displaying vascular pathology, suggesting that thrombosis, vascular leakiness or angiogenesis may mediate T cell access to the CNS [47, 145]. Tregs respond to glioblastoma secreted CCL2 as well as GSC and dendritic cell (DC) produced indoleamine 2,3-dioxygenase (IDO), and their accumulation inversely correlates with survival [44, 114, 159, 176, 256]. In addition, TAMs upregulate T cell immunoglobulin- and mucin domain-containing molecule (TIM) 3 and TIM4 expression on infiltrating T cells, inducing Treg programming while simultaneously eliminating hypoxia-induced phosphatidylserine (PS) expressing $CD8^+$ T cells in the glioblastoma TME [268, 275]. However, in neurodegeneration and traumatic brain injury, Tregs appear to enhance re-myelination and OPC proliferation while suppressing DAM and $CD8^+$ T cell activity, providing a neuroprotective effect combating disease progression [124, 139, 270]. Given the differing roles that various immune subpopulations play in neurologic disorders, it is essential to properly identify and target those specific immune cells to harness the innate and adaptive immune system to counteract disease progression.

Glioma stem cells

Many recent reviews provide a comprehensive understanding of the history and significance of GSCs and their markers [131, 273]. Single cell RNAseq analysis and lineage tracing experiments reveal substantial inter- and intratumoral heterogeneity and inherent plasticity among GSC subpopulations [15, 53, 76, 107, 165, 245]. While terms and concepts related to GSCs vary considerably in the literature, most studies converge on the conclusion that GSC enrichment correlates with tumor grade, therapeutic resistance and recurrence [63, 131]. Most studies have also indicated that GSCs are enriched in specific biological niches, particularly

in the hypoxic palisading cells around necrosis and within the immediate perivascular region [24, 79, 81, 116, 155]. Thus, establishing mechanistic links between TME enrichment of GSCs is highly relevant to the human disease and may have therapeutic implications. In particular, the perinecrotic niche contains a high density of neoplastic cells that show dramatic upregulation of hypoxia-inducible transcription factors and downstream targets, with a gradually diminishing hypoxic gradient extending beyond this zone [18, 20, 23, 164, 202, 265]. GSCs are enriched within this niche through a combination of hypoxic- and necrotic-driven chemotaxis and GSC phenotype enrichment [7, 18, 84, 102, 117, 134, 217, 226]. In turn, the GSC subpopulation facilitates TAM recruitment and subsequent immunosuppressive conversion along with microvascular hyperplasia surrounding the necrotic zones [63, 225, 253]. Within the perivascular niche, GSCs secrete chemotactic factors, such as VEGF, FGF, and PDGF, that disrupt the BBB and local vasculature; colony-stimulating factor (CSF) 1, periostin and stromal cell-derived factor (SDF) 1a that facilitate BMDM influx into the TME; and IL1 and IL6 that reprogram macrophages into an immunosuppressive phenotype [16, 61, 79, 89, 269, 279, 287]. A recent study found GSC-secreted extracellular vesicles reprogram local endothelial cells and identified potential pro-angiogenic miRNAs [147]. Others have suggested that bone marrow-derived mesenchymal stem cells recruited to the TME directly fuse with perivascular GSCs to drive neoangiogenesis in the expanding glioblastoma [231]. Endothelial cells secrete IL-8, which enhances the GSC phenotype and promotes glioblastoma expansion [155, 219] while also generating a positive feedback loop in which TAMs respond by producing tumor necrosis factor alpha (TNFα) that supports endothelial cell activation and microvascular proliferation [261]. In addition, these tumor-associated endothelial cells protect glioblastomas from radiation therapy [67, 78], chemotherapy [100], and angiogenic blockade [142]. GSCs accumulate within these tumor niches using them as safe havens and represent a critical subpopulation to address when developing future clinical approaches.

GSCs in both the perivascular and perinecrotic niche play a coordinated role in attracting and redirecting circulating monocytes towards the central necrotic region. The BBB disruption that occurs together with vascular pathology not only generates an ideal environment for one subset of GSCs; it also establishes an entry point for recruiting BMDMs into the tumor microenvironment [24, 177, 286]. While some BMDMs will remain in this niche, others proceed through the parenchymal space along the hypoxic gradient into the necrotic core. Upon arrival, tumor infiltrating TAMs again find themselves surrounded by GSCs in the perinecrotic niche, where there is a mutually beneficial relationship in an otherwise inhospitable environment [117, 217, 251]. The specific signaling interplay that enables this directed TAM relocation largely remains a mystery, in part due to difficulty in modeling these unique microenvironmental niches separated by a hypoxic gradient. Understanding this relationship could reveal divergent roles for these GSC subpopulations and enable differential immunotherapeutic based interventions aimed at disrupting complementary homing signals.

Therapeutic interventions

Therapeutic interventions for modulating macrophage activity across cancer types have been the subject of much investigation and review [5, 104, 108, 151, 185]. Here, we highlight recent advances in microenvironmental manipulation within the context of brain disease. Vascular pathology, GSC enrichment and immunosuppressive infiltrating immune cells all contribute to enhanced therapeutic resistance in glioblastoma and serve as rational broad targets for therapy [74, 106, 161, 218, 223, 251].

Glucocorticoids are time-tested immunomodulatory agents that are commonly employed at initial clinical presentation, perioperatively and during radiotherapy for patients with gliomas to diminish reactive edema and improve patient quality of life [52]. However, steroid-related side effects and toxicities necessitate short-term usage and dose de-escalation regimens. Both preclinical and retrospective clinical studies have suggested that corticosteroids may compromise immunotherapeutic efficacies and clinical outcomes [174, 184].

T cell-targeted immunotherapy has become the gold-standard approach to generating anti-tumor immunity in solid tumors. However, the early phase 3 immunotherapy trial targeting programmed cell death protein 1 (PD-1) in glioblastoma failed to improve overall patient survival (NCT02017717), which has been attributed to limited immune access to the TME [196].

Novel preclinical work shows that nanoscale immunoconjugates successfully penetrate the BBB to enhance T cell-targeted immunotherapy and overcome Treg-mediated immunosuppression [65]. Astonishingly, one study even found that anti-PD-1 therapy activated an anti-tumor immune response despite lacking conventional CD8 cytotoxic T cells in the TME [194]. These therapeutic adaptations emphasize the necessity for understanding TME interactions to inform effective clinical interventions.

A recently established macrophage-related gene signature [233], containing both macrophage and glioblastoma expressed genes, predicted therapeutic sensitivity more accurately than the previously published immune response signature [43] or the classical (*EGFR* amplified) signature. Other investigations demonstrated that $CD74^+$ TAMs and MDSCs reduce clinical therapeutic efficacy [123, 267]. Given the unique immunology within glioblastomas,

many interventions have been developed to inhibit TAM influx and/or conversion to an M2-like phenotype. These approaches upregulate IL-12 signaling, disrupt mammalian target of rapamycin (mTOR), colony-stimulating factor 1 receptor (CSF-1R), cyclin-dependent kinase (CDK), or phosphoinositide-3-kinase (PI3K) signaling, yielding mixed results with the most promising demonstrating resensitization to standard of care therapies and increased survival in animal glioblastoma models [8, 97, 133, 136, 140, 141, 144, 188, 190, 191, 263]. Still other studies show promising potential for exploiting the robust immune presence within glioblastoma. For instance, inhibiting proprotein convertases not only reduces immunosuppressive TAM polarization, but re-engages anti-tumoral activity to blunt glioblastoma expansion [205].

Due to treatment resistance inherent in GSC subpopulations, forced differentiation or directly targeting GSC phenotype promoting pathways have a substantial capacity to resensitize glioblastomas to conventional therapeutic approaches and extend time to recurrence. The perinecrotic niche protects GSCs through necrotic-driven DAMP signaling, which when obstructed eliminates these safe havens. This has been supported by the finding that disruption of adenosine signaling was capable of blunting GSC-driven migration and invasion, and that HMGB1 blockade was capable of reducing vascular permeability, neuroinflammation and edema [94, 166, 232]. Another approach seeks to diminish the GSC phenotype, targeting key transcriptional programs along the ERK1/2-SRY-box transcription factor 9 (SOX9), casein kinase (CK)2-signal transducer and activator of transcription (STAT)3, or SOX2-miR-126-3p axes resulting in cellular differentiation, decreased proliferation and invasion, increased apoptosis as well as enhanced susceptibility to radiation and TMZ therapies [75, 82, 143, 148, 211]. In addition, GSCs can give rise to drug refractory recurrent disease necessitating novel second-line therapies. One such study found CDK inhibitor-resistant glioblastomas are sensitive to c-MET/Trk dual inhibition, demonstrating effective sequential intervention modalities [171]. Other approaches exploit GSC-specific metabolism identifying a glycogen synthase kinase (GSK) 3β inhibitor, kenpaullone, and a pyrimidine synthesis inhibitor, 10580, which resensitize tumors to standard of care therapy [57, 122]. Future endeavors will continue to capitalize on these unique disease-related aspects to precisely target neurological and neuroinflammatory malregulation, further emphasizing the importance of understanding these microenvironmental pathways.

Conclusion

The brain TME contains a diversity of cell types, a complex vascular barrier, and unconventional stroma. Combined, these features, along with the access barriers imposed by the skull, make understanding dynamic microenvironmental changes of glioblastoma a challenging process, differing from neoplastic processes in other organs. The state of our current understanding suggests that the TME of diffuse gliomas is dramatically altered with the development of microscopic intravascular thrombosis at an early stage that is responsible for initiating or propagating a cascade that results in rapid disease progression. Glioblastomas display enhanced pro-coagulant activity, stemming from intrinsic genomic drivers (EGFR overexpression, PTEN loss) as well as hypoxia-induced signaling (Egf-1). These coagulant factors (TF, FVIIa, FXIIa) generate focal intravascular coagulation within the TME contributing to central necrosis, BBB disruption, radial progression, immune influx and modulation, which all combine to the advancement of disease. The resultant hypoxic gradient also enhances GSC survival mechanisms while reducing therapeutic efficacy, providing a challenging scenario for clinical intervention.

Prolonged and severe hypoxia cues the onset of necrosis that releases a variety of DAMPs (adenosine, HA, HMGB1, IL-1α, S100 proteins) that initiate sterile inflammation. Perhaps the most substantial TME feature that distinguishes glioblastoma from many other solid tumors and CNS diseases is the massive influx and reprogramming of the innate immune system. While in the past, some have suggested that glioblastoma is an immunologically "cold" tumor, more recent immunohistochemical, flow cytometric and transcriptional analyses have shown that the glioblastoma TME displays an abundance of infiltrating immune cells. Furthermore, hypoxia-induced signaling supports conversion of immune cells from an inflammatory to an immunosuppressive phenotype within the TME, including Treg recruitment, TAM immunomodulation, and MDSC localization to the perivascular niche. At the tumor periphery, MDSCs and DAMs play critical roles in excluding adaptive immune cells from the bulk tumor and represent potential barriers to current T cell focused immunotherapy that are becoming commonplace in other solid tumors.

Current efforts continue to explore spatial, temporal and cell-of-origin related contributions to immunomodulation among microglial and BMDM subpopulations of TAMs. The close spatial and temporal association between TAMs and GSCs in perivascular and perinecrotic niches is worthy of further study for their cooperation in the development of therapeutic resistance, disease progression and recurrence. Given the abundance of TAMs, DAMs, and MDSCs within the TME, the potential for successful

targeted immunotherapies directed at innate immunity is substantial. Other efforts combating GSC enrichment and vascular pathology represent mechanisms to resensitize these tumors to standard of care interventions and could enhance the efficacy of our current clinical options. With better understanding of contributing mechanisms, future combination therapies have potential for improving patient outcomes.

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Declarations

Conflict of interest The authors declare no potential conflicts of interest.

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