Modeling the interaction between the microenvironment and tumor cells in brain tumors

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Abstract

Despite multimodal treatment and improved knowledge of tumor biology, malignant brain tumors are still associated with low survival rates or severe long-term sequelae. As opposed to other cancers, the limited progress in the development of immunotherapies relates in part to the difficulties of an accurate reproduction of the specificities of the brain microenvironment with current preclinical models. Once thought to be characterized by a very limited immune response, the central nervous system is now referred to as an immunologically distinct rather than "privileged" site. The cellular interactions among microglia (the main brain resident macrophage population), recruited tumor-associated macrophages, stromal cells, glial cells, neurons and cancer cells and their implication in tumor growth or behavior are starting to be documented, but there are many questions that current models have not answered yet. The role of the blood-brain barrier as well as the impact of the different components of the extracellular matrix are also crucial points to consider in brain cancer development. Another layer of complexity has to be added because tumor microenvironment is influenced by the tumor type or even by the tumor molecular subtype as well as the spatial heterogeneity inside the tumor. Here, we focus on brain microenvironment features elicited by the impact of tumor biology. Furthermore, we discuss the limits of current preclinical models, and how complementary models, such as humanized animals and organoids, will allow further investigations on the mechanisms related to cancer development as well as more efficient drug testing in order to build new treatment strategies including immunotherapy for brain cancers in the near future.

Introduction

Brain tumors have proved challenging to treat, mainly because of their anatomical and biological characteristics. The most widely used classification and grading of brain tumors is that of the World Health Organization (WHO)(1) (see Figure 1 for a simplified summary). Primary brain tumors are significantly different in children and in adults in terms of incidence, histology, biology and prognosis(2). The majority of primary brain tumors in adults are high-grade gliomas (HGGs) or meningioma, mainly occurring in the cerebral cortex, and often associated to high mortality rates(2). Embryonal tumors, e.g. medulloblastoma, followed by low-grade gliomas (LGG) are the most frequent brain tumors in children. They are usually associated with good overall survival rates, although severe long term sequelae have been described for long term survivors(3). In children, HGGs are biologically different from those seen in adults, but also characterized by low survival rates, some being currently considered as incurable like diffuse midline glioma (DMG) associated with Histone 3(H3) K27M mutation.

Their localization often hampers complete surgery, and tumor exposure to systemic treatments might be reduced by the blood-tumor barrier (BTB) which can be different from the normal blood-brain barrier (BBB). Furthermore, brain tumors have very specific microenvironmental features associated with complex developmental characteristics. As a whole, these features may explain the dismal results of otherwise commonly used conventional anticancer treatments. Indeed, the success rate of new therapies in brain tumors is the lowest among major pathologies(4), and most of the time associated with only a slight increase in survival. Nevertheless, for specific entities, often driven by a single molecular alteration, spectacular responses have been observed creating new and more ambitious expectations. This is the case of effective therapies with mutation-specific small molecule inhibitors in the treatment of *BRAF* V600E mutated gliomas(5), or targeting *NTRK* fusions(6), which have been found at significant frequencies in central nervous system (CNS) tumors.

Several studies have underlined the major role of the immune system and tumor microenvironment (TME) over the entire process of cancer development. Results of immunotherapies have been less impressive for brain tumors than for other tumor types. Of note, the primary brain tumors that are the most sensitive to immune checkpoint inhibition are the ones associated with constitutional mismatch repair deficiency (CMMRD)(7,8). CMMRD is a cancer predisposition syndrome resulting from biallelic germline mutations in mismatch repair genes, and it is characterized by high mutation and neoantigen loads, thus likely explaining the susceptibility to T-cell-related immunotherapies.

The unique BBB/BTB that may restrict the access to the tumor site to immune cells, the specific features of the extracellular matrix (ECM), the resident microglia and the overall immunosuppressive microenvironment, likely play a major role in brain cancer development and progression, and might also explain the scarce response of brain tumors to common treatments including immunotherapies. The comprehension of their spatial-related features and tumor-promoting roles, through the use of relevant preclinical models, is essential for the identification of potential new therapeutic targets.

This review will first summarize our knowledge about the different components of the TME and how they may be targeted, then revisit the current existing types of preclinical models, and finally propose more pertinent alternatives to explore specific areas of the interaction of brain tumors in their complex stroma.

Blood-brain barrier and tumor vascularity

The BBB is a specialized structure composed of endothelial cells connected by tight junctions (TJs), surrounded by a specialized basal lamina that is shared with pericytes and astrocytic foot processes. Those elements are sparsely interconnected by neuronal endings and microglia, which can contribute to regulate and maintain the BBB integrity in case of injuries(9). The BBB is a selective barrier between the cerebral parenchyma and the systemic circulation and its function is to maintain brain homeostasis by protecting the brain from infection and toxic molecules; however, in cancer treatment, it represents a major obstacle for the delivery of many therapeutic agents. The BBB also limits the entry of immune cells and immune mediators into the CNS supporting the concept of immune privileged brain(10).

The importance of a healthy BBB for brain development and functioning is clearly stressed by the examples of rare human monogenic neurological disorders characterized by a genetic defect in BBB-associated cells, highlighting a link between BBB dysfunction and neurodegeneration. Mutations in the genes encoding TJ proteins, such as occludin and junctional adhesion molecule-C, are responsible for altered brain growth, intracranial hemorrhage and calcifications as well as abnormal gyration(11,12). Several BBB alterations have also been observed in many sporadic neurodegenerative disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS)(13).

During cancer development, the Blood-Tumor-Barrier (BTB), characterized by reduced junctional proteins, aberrant pericyte distribution and scarce astrocyte foot processes and neuronal connections, allows the leakage of blood components and cells, aberrant transport and clearance of molecules into the brain parenchyma, including contrast agents, and conversely leakage of brain tumor components such as DNA or microRNA(14,15). This is responsible for higher drug penetration, for the leakage of T cell subpopulations and peripheral monocytes found in the TME of some brain cancer types, and, indirectly, for the detection of circulating brain tumor markers (e.g. tumor cell-free DNA from gliomas or even tumor cells in medulloblastomas)(16). As brain tumors progress, the tumor vasculature becomes increasingly heterogeneous, with different features in the tumor core when compared with the periphery and the brain parenchyma, and the blood vessel supply is increased through the co-option of existing vessels and creating new ones (angiogenesis), hypoxia being the main trigger for this phenomenon(17,18).

Interestingly, BTB structure varies according to cancer types, and, within the same histology, according to molecular features, as shown for pediatric medulloblastoma(19) (Figure 2). The extensively fenestrated BTB that characterizes Wingless-related integration site (WNT)-driven medulloblastomas might be one of the reasons explaining higher levels of accumulated chemotherapeutic agents into the tumor, associated with better response rates of this disease. In contrast, the Sonic hedgehog (SHH)-driven subtype presents an intact BTB and poorer penetration of fluorescent dextrans. The identification of these differences in the BTB structure among different medulloblastoma subtypes has important consequences for the choice of existing therapeutics, and for the design of new strategies. Indeed, in WNT-driven medulloblastomas, the use of larger molecules and/or small-molecule inhibitors with usually limited BBB penetration might be considered, as well as immunotherapeutic approaches with the purpose of increasing the trafficking of immune cells into the brain and modulate those already present in the TME. These alternative treatments would aim to reduce long-term side effects of current treatment, for example by decreasing radiotherapy doses at the expense of

less toxic but equally effective chemotherapies. At the same time, applying treatments capable of transiently disrupting the BBB in non-WNT-driven medulloblastoma and other brain cancers, therefore increasing their vessel patency and porosity, might be necessary to improve drug delivery(20,21).

In glioblastoma, neoangiogenesis is a hallmark feature of the primary tumor, often associated with BTB disruption in the tumor core(17,22). This latter is mainly due to reduced TJs, non-uniform pericyte vessel coverage and glioma stem cell-derived pericytes(23), which often cause an increased interstitial and intracranial fluid pressure responsible for symptomatic edema. Several groups have shown that glioma stem cells, mostly refractory to cancer treatments, are mainly observed in perivascular, hypoxic and invasive niches(24,25). In these niches, endothelial cells secrete factors, such as nitric oxide, that maintain the tumor stem cell initial pool. Perivascular glioma cells are also known to disrupt the normal interactions between astrocytes and endothelium by physically displacing or eliminating the astrocytic endfoot, thus creating a local breach of the BTB(26). The astrocyte-mediated gliovascular coupling is compromised while glioma cells actively regulate the vessel tone. Indeed, glioma cells might induce vasoconstriction to increase the perivascular space and facilitate perivascular invasion, or shunt additional blood towards the growing satellite tumors cells(26). In contrast, the invasion front of the glioma is often not hypervascular and has an intact BBB. A prototypic tumor in this regard is DMG, which is extremely invasive and has an overall intact vascular structure at diagnosis. Tumors frequently do not significantly enhance after contrast agent injection, except for zones near the tumor core, where hypoxic/necrotic areas are surrounded by ring contrast enhancing parts were the BTB is disrupted. Interestingly, increased enhancement is often observed after radiation therapy, which is likely associated with BBB disruption and could represent a better window for otherwise poorly brain-penetrating agents(14).

Finally, disruption of the BBB has also been shown to result in fibrinogen deposition into the parenchyma in neuroinflammatory and neurodegeneration models, which can alter astrocyte, microglia, and oligodendroglia function(27–29). It is likely that fibrinogen and other blood-derived molecules might also affect the TME and the immune landscape, and the impact of fibrin-targeting immunotherapies should be evaluated in brain cancer treatment(27,30).

Taken together, these observations emphasize the importance of the heterogenous relationships between the different components of the BBB and malignant cells in brain cancer. It is of major importance to precisely understand the mechanisms underlining these specific interactions in order to tailor the use of vascular-targeted therapies and increase drug delivery systems.

Extracellular matrix

The ECM represents 20% of the adult brain mass, and it is mainly composed of glycosaminoglycans (GAGs), including hyaluronic acid (HA), proteoglycans such as lecticans and glycoproteins such as tenascin. Fibrous proteins, such as fibrillary collagen and basement membranes, which are predominant in the ECM of the majority of peripheral soft tissues, are restricted to the vasculature and meninges in the brain. The non-fibrillar nature of the brain ECM contributes to its viscoelastic nature responsible for the high compliance of this organ(31,32).

As a reflection of the unique ECM composition, the brain has specific mechanical properties that influence the embryogenesis, neural stem cell (NSC) and oligodendrocyte progenitor cell (OPC) behavior, homeostasis, and disease development. In the developing brain, cellular movements and organizational changes are instrumental during embryogenesis and as the primitive nervous system forms, requiring spatio-temporally regulated mechanosensitive pathways. In *in vivo* models, the disruption of ECM, ECM receptors or mechanosignaling proteins in neural cells dramatically affects early brain development, thus confirming that mechanical signals shape the developing brain throughout morphogenesis, by controlling cell organization and regulating cell behavior(33,34). ECM components might directly regulate growth factor binding, and affect stem cell properties by modulating cell adhesion, ligand accessibility, and biophysical properties(35). For instance, the stiffness seems to play a role in cell fate, notably influencing functionality, growth and migration of individual neuronal subtypes and OPC proliferation and differentiation in the aging CNS (36–38). In the developed brain, recent findings also demonstrate a correlation between ECM composition and the onset and/or progression of neurodegenerative diseases, such as AD and PD(39). Defining the mechanical microenvironment of these niches and understanding their contribution to cell behavior will enhance the ability to generate more accurate *in vitro* culture conditions to optimize lineage-specific differentiation of distinct CNS cell types. Considering the age-dependent change in brain ECM, with the CNS stiffness increasing over time(38), it is important to take into account that modeling pediatric gliomas might entail mimicking a different TME compared to adult gliomas.

Little is known about the impact of ECM on the regulation of tumor cell niche, angiogenesis, and invasion in brain tumors. Increased production of heparan sulfate proteoglycans as well as an upregulation of tenascin C (TNC) and periostin have been observed in gliomas(40) and posterior fossa ependymomas(41). These macromolecules, mainly concentrated in NSC or vascular niches, promote cancer cell survival and angiogenesis(42,43). In gliomas, while high concentrations of TNC in tumor-associated blood vessels seem to prevent T cell transmigration into the brain parenchyma, periostin, also secreted by glioma stem cells, promotes the recruitment of tumor-associated macrophages (TAMs) in the TME, thus contributing to immune suppression in brain tumors(44,45). In malignant gliomas, TNC-targeting agents (e.g. murine or human anti-TNC monoclonal antibodies, TNC-targeting peptides, RNA interference) and periostin neutralization or knockdown were associated with reduced tumor growth *in vitro* and *in vivo*, thus highlighting the therapeutic relevance for further exploring ECM-targeting molecules in brain cancer (46,47).

Furthermore, ECM stiffness was directly correlated with poor prognosis in gliomas by mechanical testing of fresh biopsies, and it was shown that brain ECM stiffness relates to increasing tumor grade(48). Additionally, the degree of ECM stiffness has been linked with the isocitrate dehydrogenase-1 (IDH1) status, which, whenever mutated, is associated with better patient prognosis(48). Gliomas with wild-type *IDH1* had a higher degree of stiffness, regardless of the tumor grade, while the rare glioblastomas with the mutant form of *IDH1* exhibited a stiffness similar to that of the average low-grade gliomas(49)(Figure 2). This can be explained by the fact that glioblastomas with wild-type *IDH1* usually present a necrotic core, with abnormal vascular structures, which is responsible for an increased signaling through the hypoxia-inducible factor-1α, followed by an upregulation of the expression of TNC and HA(48).

Moreover, during glioma progression, elevated hypoxia levels and the alterations in ECM components can induce neoangiogenesis, which further disrupts the tumor vessels with fluid

leakage and increased tissular interstitial pressure. This elevated pressure together with ECM stiffness has been shown to induce cell migration in glioblastoma through the activation of chemokine receptors, by facilitating the binding between CD44 and hyaluronic acid, and of integrins to their ECM substrates(50). Transcriptional changes in cancer cells have also been described, towards an epithelial–mesenchymal transition phenotype, thus positively influencing tumor invasion and progression(51).

A closer examination of those mechanisms responsible for transcriptional changes and ECM remodeling that enhance tumor invasion should provide critical insights and potentially novel therapeutic targets, especially in invasive gliomas.

Immune microenvironment in the CNS and its targets

Normal and non-tumorous pathologic brain

Historically, the CNS was believed to allow very limited immune response(52,53). This paradigm has been recently partially challenged by the discovery of functional lymphatic vessels in the meninges, the identification of various types of antigen-presenting cells (APCs) and T cells entry through the BBB as well as immunologically relevant populations of immune cells including macrophages residing in the meninges(54–56). Alternative cerebral infiltration routes for immune cells include the meninges and the choroid plexus(57). Based on these observations, it was proposed to refer to the brain as an immunologically distinct rather than "privileged" site.

The use of single-cell RNA-sequencing (scRNA-seq) and high dimensional flow cytometry, in parallel with genetic fate mapping systems, paved the way to a better evaluation of the complexity of CNS immune populations, with the identification of the specificity of each region, from those excluded from systemic immune surveillance (i.e. parenchyma) to those at the border with the periphery, such as meninges and choroid plexus(58,59). Lymphoid cells, such as B- and T-cells as well as innate lymphoid cells, natural killer and natural killer T cells, may be found within the cerebrospinal fluid of the meninges, choroid plexus and the ventricles, while being absent from the brain parenchyma(60).

A unique and major aspect of the brain immune environment is its population of resident myeloid cells. At homeostasis, this population is composed almost entirely of microglia, which are the brain's equivalent of tissue-resident macrophages, by far the largest population of immune cells in the CNS, representing 5-15% of adult brain cells(61). Other CNS macrophages include the ones that reside specifically in the meninges, in the choroid plexus or perivascular macrophages associated with blood vessels, and have been called altogether "border-associated myeloid cells" (BAMs)(62). scRNA-seq and mass cytometry have recently shown that microglia and BAMs have different gene expression signatures, with BAMs being characterized by high CD38 and major histocompatibility complex II (MHC-II), thus supporting their role as APCs(59). Finally, $Ly6C^{hi}$ and $Ly6C^{low}$ monocytes as well as dendritic cells (DCs) complete the myeloid compartment of the CNS, mainly in the meninges and the choroid plexus(63). Lastly, an inflammatory brain environment might be responsible for the recruitment of blood circulating monocytes that will give rise to a transient population of inflammatory macrophages that will reside at the site of inflammation and adopt properties specific to the challenge encountered(64).

In mice, microglia arise from yolk sac myeloid progenitors that populate the brain during embryogenesis before the establishment of the BBB, and are maintained through self-renewal capacity without the replacement of circulating monocytes at steady-state(65). After invading the brain parenchyma, these cells acquire a ramified phenotype, mainly under the regulation of transforming growth factor-β (TGF-β), CSF-2, CSF-1R, and its ligands interleukin (IL)34 and CSF-1, through mechanisms involved in migration and differentiation which have not been clarified yet(66,67). Microglia develop in close proximity with neural progenitors, neurons, and other glial cells from early embryonic stages, and they have a major role in CNS development and tissue homeostasis, such as synaptic pruning, interneuron positioning and the regulation of neuronal activity(68,69). During embryogenesis, microglia regulate the neuronal cell number, by phagocytosing progenitor cells and promoting apoptosis of differentiated cells, and migration(70). Before and after birth, they also promote outgrowth or fasciculation of axonal tracts, thus participating at the emergence of connectivity, while after birth, they regulate cell survival in the somatosensory cortex, oligodendrocyte precursors and myelination through the release of the insulin-like growth factor-1 (IGF-1), and are involved in the development of excitatory synapses in the neocortex. Moreover, in later stages of differentiation, they exert key functions in immune surveillance, although, compared to other myeloid cells, they show lower antigen-presenting capacity(59,71).

Microglia's morphology and molecular signatures are quite dynamic, depending on different stimuli from the local microenvironment. Microglia, multiple subsets of BAMs and DCs might coexist in the CNS at steady-state and exhibit specific phenotypic and transcriptional transformations during normal processes of aging, as well as in models of neurodegenerative and neuroinflammatory disorders, such as AD, PD, MS, and various autoimmune pathologies(70,72,73). During aging or neurodegeneration, from a resting state microglia acquire a specific phenotype characterized by thicker, less ramified and motile processes, exhibiting a reduced coverage area in the brain parenchyma, as documented by several studies in mice and humans(74). This activated phenotype is characterized by the increased production of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6(75). In Alzheimer's disease, microglia accumulate near amyloid-beta (Aβ) plaques and might be responsible for an enhanced neurotoxicity(76). This pro-inflammatory phenotype might also cause a progressive decrease in microglial process complexity, which, through microglia phagocytosing of dying neurons' debris, is critical to reduce plaque formation, thus impairing the clearance of Aβ oligomers, the synaptic network and the neuronal repair processes(76–78).

Immune microenvironment in brain tumors

In patients with brain cancers, the BBB, damaged by the inflammatory stimuli and likely by the anti-tumor treatment such as surgery and/or radiotherapy, and often characterized by abnormal vascular sprouting, might allow an influx in the CNS of circulating myeloid and lymphoid cells, usually absent in the brain parenchyma. The predominant cell types of this TME, in both adult and pediatric brain tumors, are the tumor-associated macrophages (TAMs), originating from circulating monocytes in those tumors with a disrupted BTB, and with a minor proportion being microglia-derived (71,79). Macrophages can be localized to distinct locations following their recruitment into the tumor. They can be localized to the advancing tip where they have a role in tumor cell motility, they can be localized to perivascular niches promoting metastasis, or to perinecrotic areas where they are involved in angiogenesis(80).

Recently, lineage tracing approaches and the discovery of specific markers significantly contributed to the discrimination of cell type specific functions between microglia and TAMs

during disease progression(71,81). Interestingly, in both mouse and human tissue models, the presence of TAMs was associated with stage, tumor progression, and lower survival(81,82). This may be due to the fact that, when stimulated and reprogrammed by cancer cells, TAMs are able to secrete immunosuppressive cytokines, including TGF-β and IL-10(83,84). Tumor cells may also produce indolamine 2,3-dioxygenase (IDO), which stimulates the accumulation of regulatory T cells and suppresses T cell activity by depleting tryptophan from the TME(85). Finally, both microglia and TAMs may produce arginase, which, through the depletion of arginine levels, further inhibits T cell proliferation and function in brain tissue(86). This immunosuppression is particularly well documented in the glioblastoma microenvironment(87,88), but can also be found in other brain tumors. For example, glioblastoma cells are capable of activating the aryl hydrocarbon receptor (AHR) in TAMs, which promotes CCR2 and KLF4 expression, as well as NF-kB suppression. AHR can also increase the expression of CD39 in TAMs, which is responsible for $CD8^+$ T cell dysfunction. High expression of both AHR and CD39 has been displayed in malignant gliomas and are associated with poor prognosis, thus becoming two interesting potential therapeutic targets(89). Gliomas cells have also been shown to prevent pro-inflammatory polarization of myeloid cells via CD74 activation through the secretion of the cytokine macrophage migration inhibitory factor (MIF), which is responsible for the inhibition of IFNγ secretion by myeloid cells(90). Notably, the inhibition of the MIF-CD74 signaling through neutralizing antibodies or small interfering RNA was associated with prolonged survival in mouse models, as well as the CSF1-R blockade which was able to alter TAMs polarization and blocked glioma progression(90,91). Mechanisms of immune evasion has also been well described in p53-mutant medulloblastoma, in which p53 negatively regulates the expression of the peptide transporter Tap1 and the aminopeptidase Erap1, which results in the lack of MHC-I expression(92).

An important heterogeneity of the TME has been reported across different histological tumor entities and even within the same histological groups (Figure 2). Among the four main subtypes of glioblastoma, proneural (PN), neural (N), classical (CL) and mesenchymal (MES), defined by their transcriptional profiles(93), a significantly higher percentage of TAMs and microglia has been described in mesenchymal glioblastomas, followed by a higher percentage of tumor infiltrating microglia in the neural group over the classical and proneural subtypes(87). It should also be emphasized that these phenotypes can be simultaneously present in a given tumor due to intratumoral heterogeneity, mesenchymal phenotype being more frequent at the tumor margin while classical phenotype being more frequent in the core of the tumor. Indeed, single -cell analyses performed in human glioblastoma and other brain tumors showed that myeloid cells, mainly microglia and macrophages, are greatly heterogeneous in the TME, with different spatial localization and phenotype(94–96). In glioblastoma, TAMs and microglia preferentially occupy the tumor and peritumoral spaces, respectively. More pro-inflammatory markers were expressed in the tumor periphery (e.g. *IL1A*/*B)*, while more anti-inflammatory (e.g. *IL1RN*, *TGFBI*) and pro-angiogenic (e.g. *VEGFA)* factors were expressed in the tumor core, those latter having a crucial role in enhancing tumor growth, and dissemination via suppression of inflammation, and promoting angiogenesis and extracellular matrix (ECM) remodeling.

Lymphocyte infiltration, which generally constitutes a very low percentage of the total cell number in the glioblastomas, is more important in the mesenchymal subtype. The mesenchymal subtype, commonly associated with a poor prognostic outcome, is enriched in mesenchymal markers (e.g. YKL40, MET,) and co-mutation of the Neurofibromin 1 (*NF1*) and *PTEN* genes, and tends to have the highest immune cell content. At the contrary, classical GBM, characterized by Epidermal Growth Factor Receptor (*EGFR*) amplification, lack of *TP53* mutations, and often homozygous deletion of the Cyclin Dependent Kinase Inhibitor 2A

(*CDKN2A*), has lower immune cell infiltration. In comparison with adult glioblastomas, in DMG single-cell analysis (flow cytometry and RNA-sequencing) showed that the leukocytes predominantly consist of $CD11b^+$ macrophages, with very few $CD3^+$ T lymphocytes(97). DMG is "immune cold" as defined by a lack of CD8 immunoreactivity and an absence of T infiltrating lymphocytes (TILs), while hemispheric gliomas are more infiltrated with TILs(98). However, compared to brain metastasis (e.g. melanoma), T cells are much fewer, particularly in *IDH*-mutated glioblastoma(96), thus mirroring the efficacy of immunotherapies with promising results in patients with melanoma and brain metastasis but with disappointing effects in T cell-excluded glioblastoma(7,99,100).

Bulk and single-cell RNA-sequencing analyses revealed that DMG- and adult GBM-associated microglia/macrophages both express gene programs related to ECM remodeling and angiogenesis, but in DMG they express significantly fewer inflammatory factors(97). Finally, in medulloblastoma, a mild subgroup-specific infiltration of immune cells has also been reported. SHH-driven medulloblastomas displayed stronger signatures of TAMs and T cells, while markers of cytotoxic lymphocytes were enriched in Group 4-medulloblastomas(101,102).

Targeting immune cells in brain tumors?

Based on these evidences and on preclinical results obtained in mouse models, multiple approaches have been tested to either reduce the infiltration of TAMs or shift their immunosuppressive phenotype to an immunogenic one. Note that we are deliberately avoiding to categorize macrophages and microglia in the oversimplified M1 vs M2 dichotomy that cannot reflect the complexity of the macrophage spectrum of activation that is tissue and disease-specific(103,104). These therapeutic strategies focused on molecules and pathways critical for the production, differentiation and function of microglia and macrophages, such as CSF-1 receptor (CSF-1R) and CC-chemokine ligand 2 (CCL2)–CC-chemokine receptor 2 (CCR2) inhibition, CD47 blockade, and CD40 stimulation(91,105–107). Unfortunately they have achieved less promising results than expected so far(108–111). This could be due to the modest penetration of these drugs through the BBB, but it also points to the paucity of models capable to evaluate the interaction between cancer cells and TME in the CNS, and to efficiently test therapeutic drugs and combinations. Lastly, this could be due to the plastic and heterogeneous nature of macrophage populations that might be targeted and affected differently by the treatment.

T-related immune-check point inhibitors have also been explored based on preclinical models(112–114), on acceptable toxicity profile and some activity in patients with brain metastasis of melanoma or non-small-cell lung cancers(99,100,115). Recent studies suggested that immune-check point inhibitors targeting the PD1/PD1 ligand 1 (PDL1) axis and/or cytotoxic T lymphocyte-associated antigen 4 (CTLA4) could have encouraging results in patients with glioblastoma(88,116,117). However, most of the trials did not show significant benefits from these treatments, with the exception of malignancies characterized by a relatively high mutational burden, likely associated with higher numbers of neoantigens, such as those gliomas secondary to chemo/radiotherapy, or with mismatch repair gene mutations(8,118). An increased response rate and improved survival have been reported with the combination of immune-check point inhibitors and radiotherapy in some adult advanced solid tumors(119). The immune modulatory effects of irradiation therapy are multiple: the induction of immunogenic cell death through the exposure of danger-associated molecular patterns followed by the recruitment of brain-resident and circulating immune cells, the increased tumor

immunogenicity by inducing the presentation of neoantigens by upregulation of MHC-I molecules, and the modulation of blood vessels(119). Another example that highlights the benefits of harnessing immune molecules in cancer treatment is based on the resistance to immune rejection of p53-mutant medulloblastoma, due to p53-related low MHC-I expression. Low doses of tumor necrosis factor (TNF) are able to rescue expression of MHC-I as well as of Erap1 and Tap1, required for MHC-I trafficking. In combination with immune checkpoint inhibitors, TNF has been associated with increased tumor response and prolonged survival in this medulloblastoma model(92).

Moreover, chimeric antigen receptor (CAR) T cells have been recently used to treat brain tumors mainly targeting EGFRvIII, human epidermal growth factor receptor 2 (HER2), IL-13 receptor α2 and anti-ganglioside 2, demonstrating that the use of CAR T cells against brain tumors is feasible and might be potentially efficacious(120–122). Safety could not be explored properly since the anti-human GD2 targeting in an immunocompromised mouse does to explore the risk of damaging the numerous GD2 positive normal brain cells. Finally, therapeutic vaccination has been evaluated, with peptide vaccines targeting a single tumor antigen (e.g., EGFRvIII, IDH-R132, wilms tumor (WT1) and survivin) or with dendritic cell vaccines with multiple types of loaded antigens(123–126). Both therapeutic modalities are promising but unproven as yet, lacking, as well as for CAR T cells, stronger clinical data.

Non-hematopoietic cellular microenvironment in brain cancer

Neurons

Neurons are also deeply involved in mechanisms promoting the growth of malignant gliomas, including adult and pediatric glioblastoma, anaplastic oligodendroglioma, and DMG. One of these mechanisms is the cleavage via the ADAM10 sheddase and the secretion of the synaptic adhesion molecule neuroligin-3 (NLGN3)(127). This molecule promotes glioma proliferation through the PI3K-mTOR pathway and upregulates several synapse-related genes in glioma cells. Noteworthy, glioblastoma and DMG growth is prevented in Nlgn3 knockout mice, and ADAM10 inhibitors are capable of blocking glioblastoma development(128).

The interaction among glial cells, neurons and cancer cells is not limited to the release of paracrine factors. Transfer of genetic material has also been documented, mainly through extracellular vesicles (EVs), and exceptionally through cell fusion. EVs secreted by glioma cells are involved in several processes underlying tumor progression, such as facilitating the transport of receptors, signaling molecules, oncogenic genes, and miRNAs, and directly contributing to the modification of the TME(129,130). The glioma ability of modifying gene expression in glial cells and neurons through EVs has been demonstrated by triple transgenic nude mice models in which glioma and non-glioma cell types were labeled with fluorescent proteins, which provided a dynamic picture of their interactions during glioma development(131).

Several studies have shown that increases in neuronal activity promotes glioma proliferation(127,128,132). The mechanisms involved in this vicious circle, in which glioma cells promote network hyperexcitability and these increases in neuronal activity promote tumor growth, are multiple and intricated. The EV-mediated communication may lead to the increase of synaptic activity in neurons, with the effect of further facilitating the neuron and glioma interaction(131). NRLG3 secretion by firing neurons promotes glioma growth(132).

Glioma cells are able to increase the excitability of the peritumoral neuronal network also through the release of glutamate(133), and PIK3CA variants have been shown to selectively

initiate brain hyperactivity through the secretion of proteins, such as GRPC3, capable of triggering synaptogenesis during early tumorigenesis(134). Furthermore, the existence of glutamatergic neuron-glioma synapses has been shown in co-cultures of glioma stem cell and neurons, as well as in murine models. These synapses stimulate glioma invasion and growth, partially via their influence on calcium communication in tumor microtube-connected tumor cell networks(135). In breast-to-brain metastasis, cell growth is stimulated by glutamate release in the context of pseudo-tripartite synapses, with the tumor cells taking the place of astrocytes. This mechanism involves the activation of N-methyl-D-aspartate receptors (NMDARs) by glutamate ligands, and promotes colonization and metastatic tumor growth in the brain(136). Finally, non-synaptic activity-dependent potassium currents are amplified by gap junction-mediated tumor interconnections, forming an electrically coupled network between neurons and cancer cells. *In vivo*, depolarization of glioma membranes promotes proliferation, while blocking electrochemical signaling inhibits the tumor growth and improve mouse survival(132).

Astrocytes

Astrocytes are specialized glial cells, and represent one of the most abundant cell types within the brain(137). In the non-neoplastic brain, they are involved in multiple functions with the main aim of protecting neurons. They build up the micro-architecture of the brain parenchyma, participate to the maintenance of the BBB, store and distribute energetic substrates to neurons, and control the development of neural cells and synaptogenesis(137). Astrocytes respond to all forms of CNS damage and disease with a variety of potential changes in gene expression, cellular structure, and function, commonly referred to as "astrogliosis"(138). Multiple activation states have been described in reactive astrocytes. They are either defined by up-regulation of pathways related to antigen presentation, complement activation and, for some of them, increased neurotoxicity, or by the release of neurotrophic factors and thrombospondins, protecting neurons and synapses(139), although care should be taken again in classifying cell fates in such simple dichotomy.

The role of reactive astrocytes in brain cancer has recently been explored, mainly in high-grade gliomas. A specific transcriptional program has been identified in glioblastoma-associated astrocytes, revealing similarities to fetal astrocytes. This is due to a shift towards the progenitor stage, marked by increased proliferation, Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway activation, and increased PDL-1 expression(140,141). The microglia-astrocyte cross-talk mediates transcriptional re-programming in microglia and myeloid cells towards an anti-inflammatory state, responsible for an immunosuppressive TME characterized by high concentration of IL-10 and TGF-β. The inhibition of the JAK/STAT pathway is able to establish a pro-inflammatory environment by recruiting myeloid cells within the tumor and reverting their phenotype, and reduces tumor growth(141,142). The importance of this pathway has been confirmed in the setting of brain metastases, with the induction of a pro-metastatic program driven by STAT3 in a subpopulation of reactive astrocytes surrounding metastatic lesions(143). In patients with intracranial metastases, active STAT3 in tumor-associated astrocytes correlates with poor survival. In murine models, genetic targeting of STAT3 in reactive astrocytes impairs the viability of brain metastasis, regardless of the primary tumor histology(143).

In human and murine models of breast and lung cancer, carcinoma-astrocyte gap junctions were displayed in case of brain metastasis. Brain metastatic cancer cells were capable of activating the STING pathway and the production of inflammatory cytokines (e.g. interferon-α, TNF) by transferring the second messenger 2'3'-cyclic GMP-AMP (cGAMP) to astrocytes. The

following activation of the STAT1 and NF-κB pathways in cancer cells supported tumor growth and resistance to treatment(144). This paracrine loop can be broken by gap junction directed therapy (e.g. tonabersat, meclofenamate), which has shown encouraging results in xenografts and immunocompetent models(144).

The Cancer Genome Atlas (TCGA) dataset was explored to generate a glioblastoma-associated astrocyte signature based on RNA-sequencing data. A high "astrocyte signature score" correlated with poor prognosis, and was associated with the induction of the expression of periostin and serglycin genes in glioblastoma cells(145). In cell lines and in patient-derived cultures, astrocytes were able to enhance glioblastoma cell growth, and the co-injection of glioblastoma cells and tumor-associated astrocytes in mouse models promoted tumor growth and was responsible for decreased survival rates, compared to the implantation of tumor cells alone or tumor cells with unconditioned glial cells(145).

Finally, a recent work showed that a fraction of tumor cells could *trans*-differentiate into tumor associated astrocytes in a murine model of SHH-activated medulloblastoma. These astrocytes were shown to secrete interleukin-4 (IL-4), which stimulated tumor-associated microglia to produce IGF-1, supporting medulloblastoma growth(146). The description of this multicellular paracrine feedback loop highlights another way that brain tumors can shape the TME in order to favor tumor cell development, and should be explored as a possible target of future therapeutic strategies.

Oligodendroglial lineage cells

Much less is known about the role of oligodendrocytes in brain cancer development. Some studies report an inhibition of astrocytoma growth and proliferation by paracrine signaling via WNT inhibitory factors(147). Recent analysis of the expression profiles of microRNAs (miRNAs) showed that the three most expressed miRNA were related to oligodendrocyte differentiation. Oligodendrocyte lineage cells were increased in the border of glioblastoma, where macrophages and microglia also co-localized. OPCs and macrophages cultures conditioned medium induced stemness and chemo-radioresistance in glioblastoma cells, thus raising the hypothesis of a paracrine role of OPCs, in collaboration with macrophages and microglia, for glioma stem cell niche formation at the tumor border(148,149). Notably, OPCs, as neurons, are a major source of NLGN3, defining a role for OPCs as a key TME cell type contributing to tumor growth(128).

Altogether, these findings suggest that the complex mechanisms used to hijack normal CNS development and plasticity are key features for brain cancer development and, therefore, might become potential therapeutic targets.

Preclinical models to study brain tumor microenvironment

Several *in vitro* and *in vivo* methods have been used to explore the underlying biological mechanisms of brain cancer development and predict the response and/or resistance of cancer cells to different treatments. However, only few of these models can be used to efficiently study the brain TME (Table 1).

In vitro studies, including mouse or human-derived cell lines and primary cells such as tumor stem cells or neurosphere cultures, primary tumor spheroids, three-dimensional (3D) aggregations of several CNS cell types derived from neural progenitor cells (NPCs) without

distinctive cytoarchitecture, have been widely used to identify the genetic and epigenetic changes that contribute to tumor initiation(150,151). Over time, more than 60 cell lines were generated from brain tumors, including HGGs, DMGs, ependymoma, medulloblastoma and atypical teratoid rhabdoid tumors (ATRT)(150). While adherent cultures of brain tumor cells in serum-containing media tend to drift away from the original tumors' genomic features or growth characteristics over time, 3D cultures of brain cancer cells as neurospheres or adherent 2D cultures on laminin-coated surfaces in serum-free conditions better maintain the properties of primary tumors(152,153). Glioma stem cells can be cultured alternatively as monolayer on laminin-coated plates or as neurospheres and, once injected in immunocompromised animals, their growth and differentiation recapitulate the diversity of the initial tumor(152,154). Neurospheres better recapitulate the tumor heterogeneity *in vitro* because they maintain a steady state of glioma stem cells and tumor cells engaged in differentiation, thus making them an attractive choice for proliferation and differentiation studies, although their culture is far more complex than cell lines(155). However, neither cell lines nor neurospheres can appropriately mimic the interaction of cancer cells with the complex TME. Tumoroids or glioma spheroids/organoids derived directly from fresh patient tumor material can be maintained *in vitro* for short periods and retain transiently the tissular heterogeneity of the tumor(151). To build tumoroid models, tumor samples can be cultured as a suspension of all tumor cell types, including non-malignant cells, thus including the entire tumor microenvironment and closely resembling the *in vivo* situation, or as tumor stem cells, then co-cultured with autologous immune cells from the peripheral blood of the same patient, allowing long-term cultures and easier tumor expansion(156). Recent reports have confirmed the feasibility of using tumoroids for different cancer types, including glioblastoma, preserving transiently the stromal and immune microenvironment *in vitro*(156–161).

A method that might bridge the gap between *in vitro* and *in vivo* models is based on organotypic brain slice cultures, first developed in the 1960s(162), and becoming a widely used platform to study synaptic plasticity and memory hypothesis, neurodevelopment as well as neurodegenerative diseases(163). This method provides a useful approach to evaluate the interaction between tumor and normal cells in brain malignancies. Organotypic slice cultures preserve the cytoarchitecture of brain tissue, maintain vascular cells and allow to manipulate and observe directly in the culture dish(164,165). Rodent (rat and mice) normal brain or bearing pre-existing tumor as well as transgenic mice are more broadly used(162,166). Glioma stem cells (GSCs) or tumor spheroids have been co-cultured with the brain slices, and their behaviors are studied over weeks, including mechanisms of infiltration and migration, permitting tracking GSC behavior and their interaction with brain tissue and TME(167). Tumor cells can be injected in a spatially restricted manner, thus enabling the study of specific brain regions. Live-cell imaging can also be performed, which is extremely useful to study the pattern of tumor cells migration (e.g. using both neuronal tracts and blood vessels as a substrate and guide for glioblastoma cells)(168,169), and potentially track the cell lineage reporters. Epifluorescence may need to be enhanced by tissue clearing(166). Another advantage of this model is that multiple brain slices can be generated from one animal, which ensure uniformity while investigating several conditions, and also reducing the number of required animals. The main limitation of this method is the short duration of viable cultures, which is usually no longer than 3 weeks, which could be problematic to study those slower growing human GSC and to test drug response Also, the absence of blood flow impedes the study of the recruitment of immune circulating cells. Furthermore, the slice procedure itself triggers cell death and microglial activation. Yet, it is possible to explore the interaction between microglia and tumor cells deeper into the slice, where such modifications are not observed(167).

For *in vivo* studies, the most common model for brain cancer research is the mouse. The main variations of murine models used in brain cancer studies are orthotopic xenografts of cell lines, Patient-Derived Xenografts (PDXs) and Genetically Engineered Mice Models (GEMMs)(150,170). Orthotopic transplantation of cultured stem cells, patient-derived fresh tissue, isolated cell suspensions, or neurospheres into the immunodeficient mouse brain using stereotaxic surgery is considered as the 'gold standard' method to test tumor development and treatment efficacy(171). Different mouse strains are available, each with its lifespan, tumor engraftment success rate, and sensitivity for chemotherapy and/or radiotherapy(171). In xenografts, histological features, tumor heterogeneity and key molecular characteristics of the primary human cancer are, at least partially, maintained. Although cell line-derived xenografts grow usually quicker than PDXs, both models are complex, time consuming and quite expensive. The use of immunocompromised mice, chosen to ensure a successful tumor engraftment, has the disadvantages of altered tumor immunology and natural microenvironment, thus limiting both the evaluation of the interaction between cancer cells and murine immune microenvironment and the testing of novel immunotherapeutic strategies. A recent complementary *in vivo* model exploited the ''immune-privileged'' developmental time window by injecting human glioblastoma cells into the telencephalic ventricle of wild-type mouse embryos(172). These PDX developed tumors exhibiting complex TME such as functional vasculature, and host immune cell infiltration, and those characteristics persisted in postnatal brains of immune-competent mice. However, differences among mouse strains on murine immune system's features need to be taken into account. In fact, nude mice cannot generate mature lymphocytes but myeloid cells, including microglia, can be at least partially studied, although lacking the interaction with the adaptive immune players. The NSG (NOD scid gamma) mouse is the most severely immunodeficient mouse strain, although microglia are still present, with absent mature lymphocytes and defective myeloid cell maturation(173), thus further limiting their use for research on brain TME in standard PDX models.

Humanized-xenograft models, from immunocompromised mice that could receive human bone marrow transplantation to reconstitute the immune system, might provide a more realistic tumor immune microenvironment (TIME) and could be used for prediction of response to immunotherapies(170). Ideally, the humanized mice would have the same immune system from which the PDX was derived. However, it is very difficult to obtain $CD34^+$ cells from the same patient, and, therefore, an allogeneic immune approach is usually used. Despite their particularly high costs and technical complexity characterized by an intrinsic risk of xenogeneic graft-versus-host responses, they are a step closer to recapitulating human cancers in animals. In brain tumor research, no result has been reported with such models so far.

GEMMs have also largely contributed to the field of cancer research, by developing spontaneous *de novo* tumors generated by adopting key tumor signature mutations in an immune-proficient mouse. These tumors mimic some of the histopathological and specific molecular features of the respective tumor of origin, are able to spontaneously progress toward metastatic disease, but are far from reflecting intratumoural heterogeneity to the same degree observed in their human counterparts. In malignant gliomas, GEMMs have been generated by adopting key glioma signature mutations, alone or in combination, including receptor tyrosine kinase (RTK) genes, *H3F3A, HIST1H3B, TP53, PTEN, RB, NF1* and cell-cycle pathways(174–179). One limitation of these models is that, in many cases, the cell of origin is unknown as well as the timing of tumor initiation. It has been shown, for example, in an ATRT model that tumors develop only when the inactivation of *SMARCB1* tumor suppressor gene occurs at a given timing during embryogenesis(180). A number of different GEMMs have been developed in order to better study the CNS microenvironment. Several models have been used to study

leukocyte migration in neurological diseases, such as knock-out mice for P-selectin, Icam1 and other adhesion molecules(181). Genetic mouse models have also been developed to study BBB function, by targeting those genes involved in TJs formation, efflux and influx transport and transcytosis(182). To study the impact of a reduced amount of pericyte coverage on the blood vessels, Pdgfb and Pdgfrβ deficient mice have also been generated(183). In the oncologic field, GEMMs of pediatric high-grade gliomas helped to describe BBB loss of integrity, and the significant influence of tumor location more than of somatic mutations on BTB structure(184,185). GEMMs setting will offer the opportunity to study other aspects of the CNS TME, such as immune cells and their interaction with cancer cells, in an immunocompetent setting and, interestingly, during early and advanced phases of the tumor development. The limitation of GEMMS reside in the simplification of tumors genotypes and phenotypes and the non-human TME precluding generalization of some of the findings.

To maximize the temporo-spatial resolution of the TME analysis, it is also possible to take advantage of a mouse genetic system termed mosaic analysis with double markers (MADMs)(146,186). The choice of a Cre transgene specifically expressed in the tumor but not in TME might allow to study the recruitment, activation, and hierarchical organization of non-labelled TME cells in relation to green fluorescent protein (GFP)-labeled tumor cells, in the timeframe between early oncogenesis to advanced malignancy. In a medulloblastoma model, relying on the lineage tracing capability of MADMs, the phenomenon of tumor-to-TME trans-differentiation, as well as the anti-inflammatory immune network formed by tumor-associated astrocytes and microglia, could be described(146).

Non-mammalian models also provide great value in exploring brain cancers. Zebrafish models to study glioblastoma have already been built. In zebrafish, patient's tumors can be quite easily transplanted to form PDXs, and genetically modified models have been generated by adding single or combined mutations through recently available CRISPR tools. The transparency of the fish allows direct imaging studies, visualizing tumor cell behaviors and host tissue interactions, including microglia-tumor-cell interactions(187). However, zebrafish-based models present lower conservation of genes, molecular pathways and organ systems with humans, compared with mice.

Future preclinical models to study brain tumor microenvironment

As it stands, the available brain tumor models need to be used in parallel to be able to address different questions. Innovative preclinical models are needed to widen the spectrum of tools allowing an accurate evaluation of the complex interaction among microglia, immune and stromal cells in the TME and cancer cells. 3D organ-like structure formed from organ-specific progenitor represent an exciting new avenue of *in vitro* modeling of brain tumors ("organoids")(156). The "organ-in-a-dish" strategy has been particularly successful for modelling other kind of malignancies and exploring their vulnerabilities(188).

Until recently, it has been challenging to apply this technology to human organs due to the inaccessibility of human progenitor cells until human-induced pluripotent stem cells (hiPSCs) were invented(189). Organ progenitor cells were empirically found to self-organize 3D tissues when they were aggregated and cultivated *in vitro*. PSCs are defined by an unlimited capacity for self-renewal and the ability to differentiate into cells of the three germ layers, namely the mesoderm, ectoderm, and endoderm (189,190). Recently, various differentiation protocols to obtain several organoid types, such as brain, kidney, intestinal, retinal, pancreatic, and liver

organoids, have been published. With that, many complex diseases associated with different organs can now be modelled. However, it has to be taken into account that the transcript levels of organoids are closer to those of fetal stages, thus they cannot be considered as a fully mature organ yet.

IPSCs-derived cerebral organoids have become a tool for modeling several human disorders, such as microcephaly, lissencephaly, Zika virus neuroinfection, AD and autism spectrum disorders(191–193). Upon minimal instructions *in vitro*, cells can self-organize into 3D structures where intrinsic molecular programs are activated to generate neuronal and non-neuronal cell types from different CNS regions. More recently, human cerebral organoids have been efficiently used as a platform for tumor cell transplantation, enabling microscopic observation of brain tumor development, the study of the interaction of tumor cells with normal brain cells and to test therapeutics in order to predict drug response and create a tailored approach for patients with brain cancer(194,195). Whenever combined with genetic manipulations (i.e. viral vectors, electroporation), organoids can also provide an accessible model to document the function of one protein/pathway, and to identify the molecular mechanisms of diseases(196). Brain tumorigenesis can be recapitulated by introducing oncogenic mutations in cerebral organoids via transposon- and CRISPR–Cas9-mediated mutagenesis, which was performed successfully for glioblastoma, medulloblastoma and CNS primitive neuroectodermal tumor (CNS-PNET)-like neoplasms(157,159,160,197–200).

One of the main challenges is recapitulating the complexity of the CNS microenvironment in IPSC-derived organoids models, notably in terms of the vascular system and tissue-resident immune cells. A primitive endothelial network could be reproduced through the addition of previously cultured endothelial cells, engineered to ectopically express human ETS variant 2 (ETV2), to human cerebral organoids during the embryonic body stage(201). These cells formed a complex vascular-like network, which acquired several BBB characteristics, including an increase in the expression of tight junctions and trans-endothelial electrical resistance, and contributed to enhancing the functional maturation of brain organoids(201). Although these vascular-like structures resemble the vasculature in the early prenatal brain and have limited capacity of nutrient delivery into organoids to avoid the formation of a central necrosis, they still provide a platform for starting modeling the neurovascular niche and blood-brain barrier(201,202).

Modeling tissue-resident macrophage differentiation and function, as well as their interaction with normal and tumor cells, without introducing further heterogeneity is a major challenge in brain cancer research. An alteration of gene expression characterized by activation of genes associated with acute inflammatory responses and decreased expression of genes associated with immune function occurs rapidly *ex vivo* and *in vitro*, suggesting that brain-specific signals might be necessary to maintain microglia phenotypes(203). Therefore, it is likely that complex culture conditions, such as co-culture with neurons and astrocytes in 3D or with organoids, will be crucial to accurately model the *in vivo* microglia phenotype. Indeed, IPSC-derived microglia-like cells, co-cultured with cerebral organoids, are able to migrate into the organoid(204). It is noteworthy that, after a mechanical injury of the organoid, the microglia-like cells are capable of modifying their ramified morphology to adopt a round morphology, typical of activated microglia, as observed *in vivo* after CNS injuries(204).
Recently, a protocol that allows the generation of human and murine iPSCs-derived primitive macrophages (iMacs) that closely resemble yolk-sac precursor cells was developed(205). In co-culture with iPSC-derived brain organoids, iMacs further differentiated into microglia-like cells. Murine iMacs also differentiated into functional tissue macrophages in both brain and

lung after transplantation. Noteworthy, in a human model of familial Mediterranean fever, iMacs differentiated from a patient gathered pro-inflammatory characteristics, thus confirming that iMacs are a solid source of tissue-resident macrophages(205).

Together, these studies demonstrate that hPSC-based modeling, combined or not with genome editing, is a powerful platform upon which to build studies on neural lineage dysregulation, microglia, macrophages, other immune cells and stromal cells, and on their role in all phases of human cancer development. The next step will be to generate brain organoids and iMacs from patient-derived iPSC, which would be then co-cultured in order to allow the differentiation of iMacs into microglia-like cells. Patient's lymphocytes can be co-cultured with brain organoids and iMacs, in order to more accurately represent the complex interaction among different immune cell types, and between TME and cancer cells. Patient's tumor cells would be co-cultured with these "immune organoids", and, through scRNA-seq and high dimensional flow cytometry, it will be possible to decipher the molecular pathways of microglia versus other non-malignant cells involved in brain cancer development. By using genome editing tools, those key mechanisms of immune surveillance involving tissue-resident macrophages could be validated, and then targeted by immunotherapies in order to revert their pro-tumoral phenotype (Figure 3).

Concluding remarks

The limited progress made in the treatment of brain tumors, especially concerning immunotherapies, relates, at least partially, to the inaccuracy of preclinical models that have so far failed to consistently reproduce the specificities of the brain microenvironment. It is now well known that microglia and TAMs are key players in brain cancer development, although the mechanisms truly responsible for this interaction are not clear yet. Improved knowledge on this matter will be hopefully achieved through the development of more accurate humanized animal models. In an efficient complementary way, 3D cultures such as IPSC-derived organoids with appropriate TME have enormous potential for further investigations studying these interactions as well as drug sensitivities which may yield new treatment strategies for brain cancers in the next future.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions

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Figure 1. Simplified schema of malignant brain tumors (grade III-IV) as classified by the World Health Organization (WHO). * mainly in pediatric patients; ** mainly in adult population.

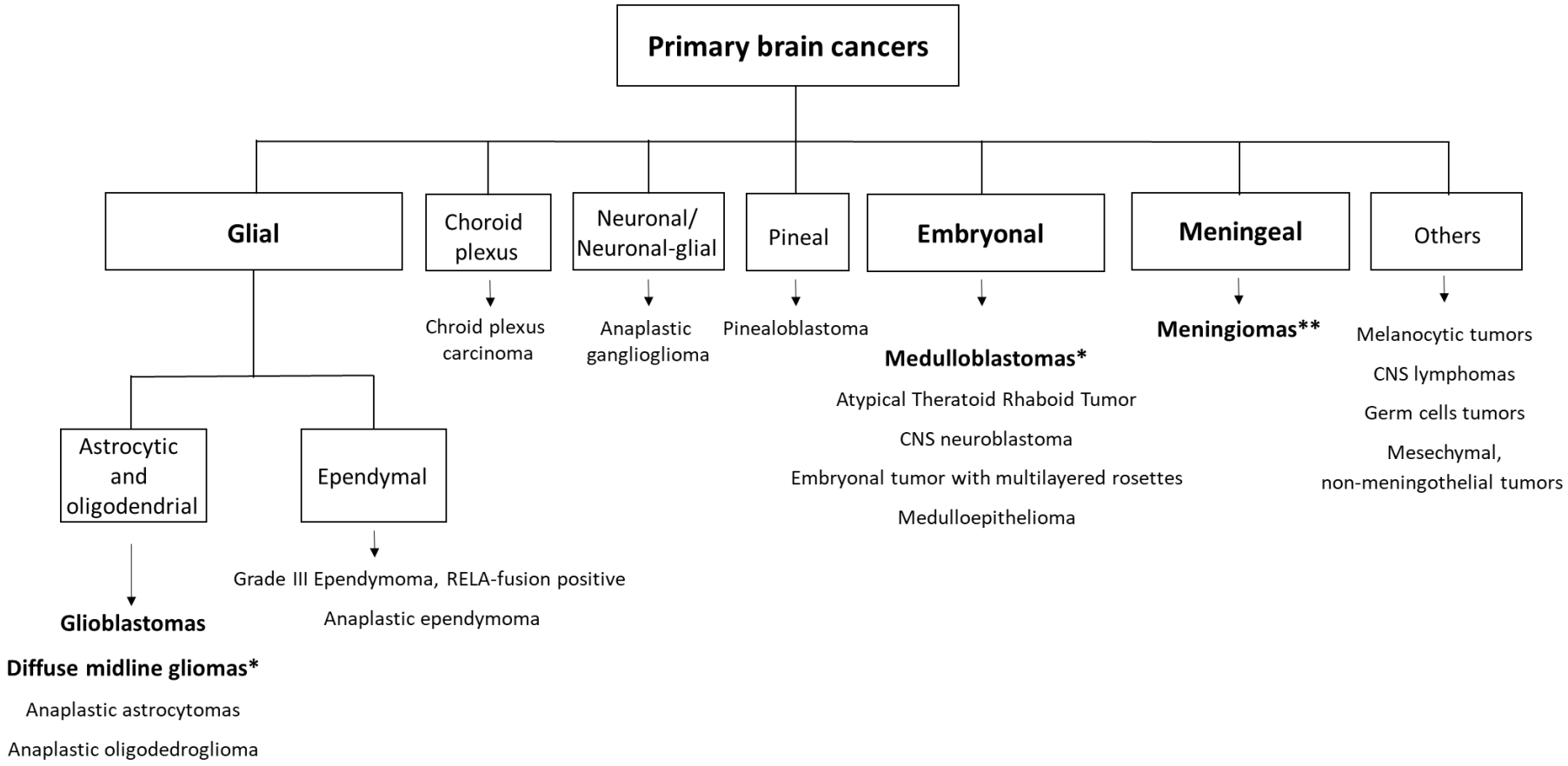

Figure 2. Blood-tumor barrier (A-B), extracellular matrix (C-D) and immune infiltrate (E-G) across the main brain tumor types. The characteristics of the different TME components vary significantly according to tumor histology and its molecular features. SHH-driven medulloblastoma and DMGs present with an intact BTB (panel 1A), while BTB disruption through reduced TJs, non-uniform pericyte vessel coverage and glioma stem cell-derived pericytes is reported in HGGs and WNT-driven medulloblastoma (panel 1B). Panels 1C and D summarize the ECM features in HGGs, according to *IDH1* status. While the rare glioblastomas with the mutant form of *IDH1* exhibit a stiffness similar to that of the average low-grade gliomas (1C), gliomas with wild-type *IDH1* have a higher degree of stiffness, regardless of the tumor grade (1D), with an upregulation of the expression of TNC and HA, and hypersecretion of metalloproteases. Panels E-G show the difference in terms of immune infiltrate among brain tumor types. Mesenchymal HGGs as well as SHH-driven are the most infiltrated with abundant microglia, TAMs and T lymphocytes (1E), followed by proneuronal/neuronal HGGs and group 4 medulloblastoma (1F). At the contrary, classic HGGs and DMGs are characterized by a poor immune infiltrate ("cold tumors"), mostly consisting on microglia (1G).

BTB: blood-tumor barrier; SHH: sonic hedgehog; DMG: diffuse midline glioma; TJ: tight junction; HGG: high-grade glioma; WNT: Wingless-related integration site; *IDH1*: Isocitrate dehydrogenase-1; TNC: tenascin C: HA: hyaluronic acid. TAM: tumor-associated macrophage.

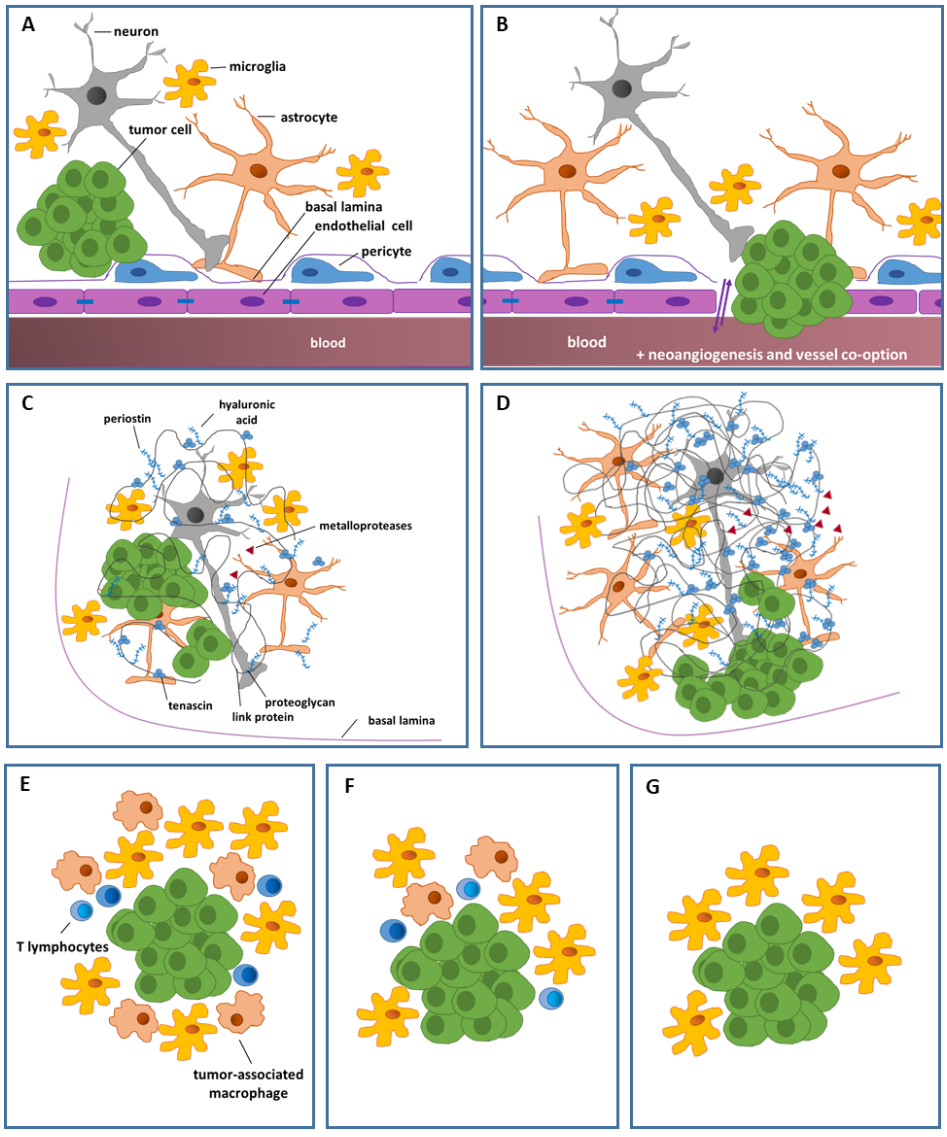

Table 1. Preclinical models to study the interaction between brain microenvironment and cancer cells.

General type of modeling	Model	Method	Advantages	Disadvantages	References
In vitro	**Tumor cell lines, neurospheres or tumoroids**	Patient-derived tumor cells, neurospheres or tumoroids; Co-culture with immune cells	• More predictable and rapid tumor growth • Less expensive • Possible use for large drug screening	• No or altered TME • Changes in gene expression by long-term propagation	*151 - 155*
Ex vivo	**Organoids**	Patient-derived pluripotent stem cells for modelling brain microenvironment; Co-culture/intra-organoid injection of tumor cells or genome editing techniques	• Can mimic microglia, other immune cells and cancer cells interaction • Possible use for large drug screening • Allows to study early phases of cancer development • Less expensive than animal models • Ethical	• Partially altered brain TME (role of the blood-brain barrier) • Technically complicated	*194, 195, 201, 202, 204*
Ex vivo	**Organotypic brain slices**	Brain slices from mice models; Tumor stem cells engraftment or genome editing techniques	• Allows to study tumor infiltration and migration, and to explore early phases of cancer development • Potential tracking of cell lineage reporters • Allows to study the interaction with normal cells	• Short duration of viable cultures • Partially altered microglia function and local inflammation triggered by the slice procedure	*162 - 165* *167 - 169*
In vivo	**Orthotopic Patient-Derived Xenografts (PDXs) in humanized mice models**	Xenograft of patient-derived tumor or tumor cells lines in humanized mice	• Appropriately mimics TME • Provides realistic tumor heterogeneity • Can be used to predict drug response	• Expensive • Technically complicated	*171 - 173*
In vivo	**Genetically Engineered Mice models (GEMMs)**	Use of transgenic mice with introduced brain cancer-specific genetic alterations by genome editing	• Appropriately mimics TME • Can be used to predict drug response • Allows to study early phases of cancer development	• Lack of tumor heterogeneity • Slow and variable tumor development • Expensive	*174 - 179* *180 - 185*
In vivo	**PDXs and Genetically Engineered zebrafish models**	Xenograft of patient-derived tumor or tumor cells lines or introduced brain cancer-specific genetic alterations by genome editing in zebrafish	• Allows to study the dynamic interaction between TME and cancer cells • Possible use for large drug screening • Less expensive than mice models	• More remote from humans than other animal models	*187*

Figure 3. Modeling brain TME in pediatric cancer using IPSC-derived immune organoids. Brain organoids will be generated from patient-derived iPSC (A-B-C) and co-cultured with iMacs in order to allow the differentiation of iMacs into microglia-like cells (B). Patient's tumor cells (★) and lymphocytes (▼) will be co-cultured with brain organoids and iMacs to more accurately represent the complex interaction among different immune cell types, and between TME and cancer cells. Through single-cell RNA-seq and high dimensional flow cytometry, the molecular pathways of microglia versus other non-malignant cells involved in brain cancer development will be explored. By using genome editing tools, those key mechanisms of immune surveillance involving tissue-resident macrophages and other TME components could be validated, and then targeted by immunotherapies (✹).

TME: tumor microenvironment; iPSC: human-induced pluripotent stem cell; iMacs: iPSCs-derived primitive macrophages; PBSC: peripheral blood mononuclear cell; CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats; shRNA: short/small hairpin RNA