New insights into the Immune TME of adult-type diffuse gliomas

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Purpose of review
Adult-type diffuse gliomas are highly heterogeneous tumors. Bulk transcriptome analyses suggested that the composition of the tumor microenvironment (TME) corresponds to genetic and clinical features. In this review, we highlight novel findings on the intratumoral heterogeneity of IDH-wildtype and IDH-mutant gliomas characterized at single-cell resolution, and emphasize the mechanisms shaping the immune TME and therapeutic implications.

Recent findings
Emergent evidence indicates that in addition to genetic drivers, epigenetic mechanisms and microenvironmental factors influence the glioma subtypes. Interactions between glioma and immune cells contribute to immune evasion, particularly in aggressive tumors. Spatial and temporal heterogeneity of malignant and immune cell subpopulations is high in recurrent gliomas. IDH-wildtype and IDH-mutant tumors display distinctive changes in their myeloid and lymphoid compartments, and D-2HG produced by IDH-mutant cells impacts the immune TME.

Summary
The comprehensive dissection of the intratumoral ecosystem of human gliomas using single-cell and spatial transcriptomic approaches advances our understanding of the mechanisms underlying the immunosuppressed state of the TME, supports the prognostic value of tumor-associated macrophages and microglial cells, and sheds light on novel therapeutic options.

Keywords
adult-type diffuse gliomas, D-2-hydroxyglutarate, IDH mutation, immune tumor microenvironment, intratumoral heterogeneity, single-cell and spatial transcriptomics

INTRODUCTION

Adult-type diffuse gliomas are brain tumors with aggressive behavior characterized by cell migration into the brain parenchyma, thereby precluding curative surgical resection. Survival and quality of life of patients remain dismal with current standard of care consisting of surgery followed by adjuvant radiation and chemotherapy. In the current classification (WHO CNS5), isocitrate deshydrogenase (IDH1/2) mutations and 1p/19q codeletion along with histology define three major categories of adult diffuse gliomas: glioblastoma grade IV (IDH-wildtype); astrocytoma grade 2–4 (IDH-mutant without 1p/19q-codeletion); and oligodendroglioma grade 2–3 (IDH-mutant and 1p/19q-codeleted) [1] (Fig. 1). Of these, glioblastomas are the most aggressive tumors with patients having a median overall survival of 15 months. Patients with low-grade IDH-mutant gliomas have a more favourable prognosis, but these tumors invariably progress, recur as higher grades, and become resistant to therapy. It is increasingly recognized that the tumor microenvironment (TME) is a key factor of tumor progression and response to immunotherapies. Here we discuss the latest findings regarding the intratumoral heterogeneity of gliomas, with focus on the composition of the immune TME, highlight therapeutic implications, and provide research perspectives.

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KEY POINTS

- High intratumoral heterogeneity and environmental stimuli define aggressive and recurrent gliomas.
- Dynamic competition of resident and infiltrating macrophages occurs during glioma progression.
- Distinctive changes in the immune TME are linked to the IDH mutation status.
- Cell-extrinsic D-2HG impinges upon the function of immune cells.

INTRATUMORAL HETEROGENEITY OF IDH-WILDTYPE GLIOMAS

Bulk transcriptome profiling of The Cancer Genome Atlas (TCGA) glioma cohort suggested four tumor subtypes: proneural, neural, classical, and mesenchymal, characterized by defined genetic drivers [2]. Deconvolution analyses of the immune cell composition of these tumors, revealed that the mesenchymal subtype, which exhibits the worst prognosis, is enriched in neutrophils and tumor-associated macrophages (TAMs) [3]. This enrichment involves NF1 deficiency in malignant cells, which promotes chemoattraction of TAMs [3]. Longitudinal analyses showed that recurrent tumors increase the TAM population whereas temozolomide-related hypermutation correlates with enrichment of CD8+ T cells [3]. However,

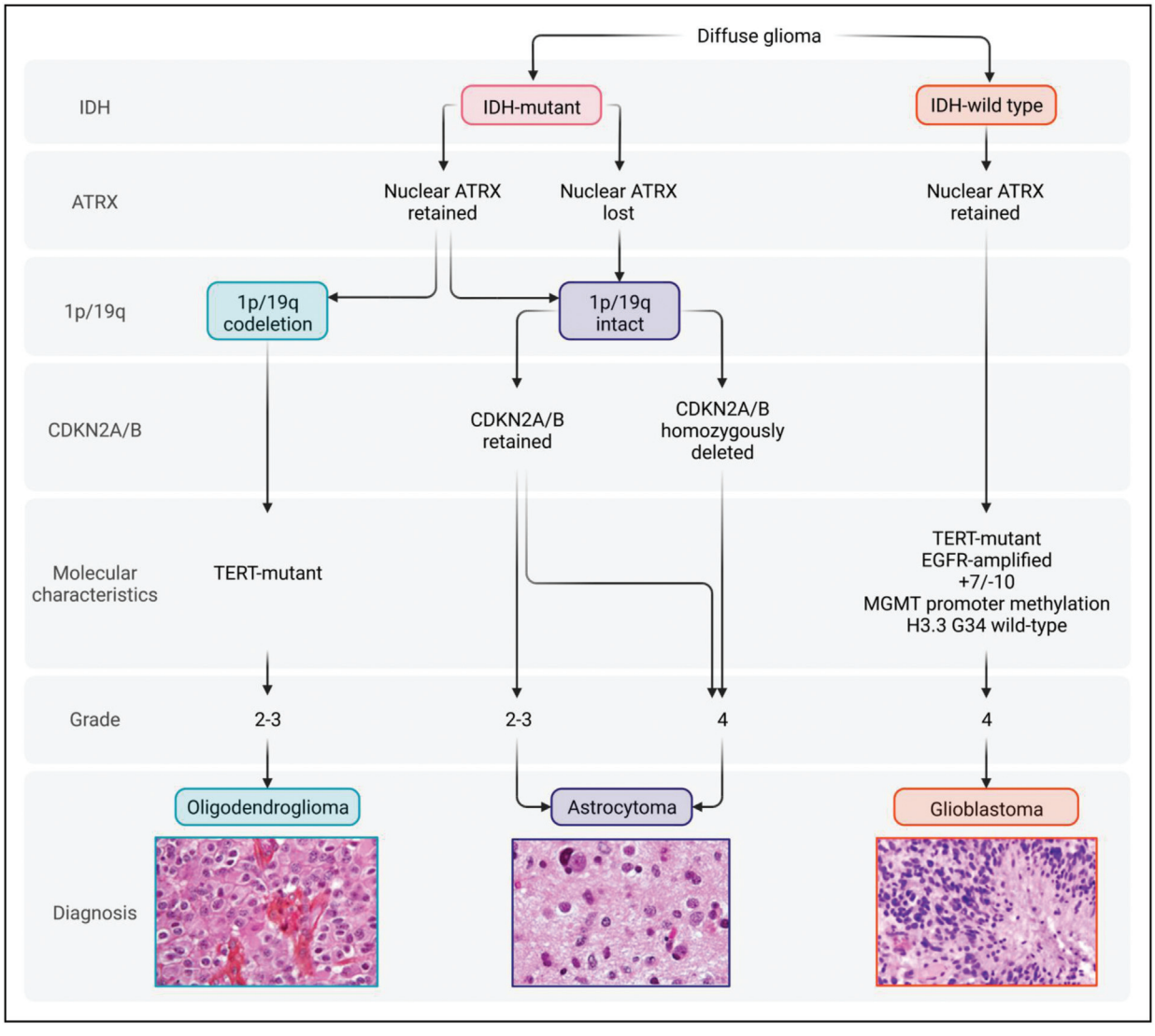

FIGURE 1. Adult-type diffuse glioma classification (WHO CNS5). The main genetic alterations of IDH-wildtype and IDH-mutant tumors and their corresponding histological appearance are indicated. IDH, isocitrate dehydrogenase.

these findings await confirmation, as it is possible that hypermutation might correlate with enrichment of CD8+ T cells in specific subpopulations (e.g. pediatric patients with CMMRD) rather than in temozolomide-related contexts. Previous bulk RNA-seq studies suggested that transition from proneural to mesenchymal subtype occurs with disease recurrence and resistance to treatment. However, it was not until the advent of powerful single-cell RNA sequencing (scRNA-seq) that a more accurate assessment of the intratumoral heterogeneity of gliomas, including malignant and immune cells, has been enabled.

It turned out that four cellular malignant states coexist in a given tumor: neural, progenitor-like (NPC-like) oligodendrocyte progenitor-like (OPC-like), astrocyte-like (AC-like), and mesenchymal-like (MES-like) [4] (Fig. 2a). These states, with the exception of MES-like are reminiscent of neurodevelopmental programs as they express astrocytic, oligodendroglial, and stem progenitor cell signatures to some extent. Importantly, it was shown that in addition to genetic drivers, the predominance of one state over the others defines the tumor subtype [4]. Evidence supporting dynamic interconversion between these states was provided in lineage-tracing experiments using a genetic mouse model and patient-derived xenografts, in which one single cell gives rise to the four archetypal subtypes [4].

This switching model argues for a dynamic plasticity of four different cell states, and contrasts with two other scRNA-seq studies supporting the cancer stem cell (CSC) hypothesis, in which a cellular hierarchy prevails [5,6▪,7▪]. Indeed, a signature of quiescent (nonproliferative) CSCs was identified, which differs from the transcriptional signatures of the four archetypal cellular states [6▪]. Importantly, chemotherapy exerts selection pressure on CSCs, and may account for therapy resistance to antimitotic drugs and temozolomide [6▪,7▪], thus emphasizing the need to target the right cells. Regardless of the cell of origin and the defined genetic drivers, the question remains about the factors that influence the plasticity and outcomes of glioblastoma cells.

Multiomics analyses of glioma cells at single-cell resolution revealed that intratumoral epigenetic diversity (but not genomic alterations alone) accounts for adaptive changes to environmental stimuli such as hypoxia and irradiation, leading to cell-state transitions [8▪,9▪▪]. Additional characterization of glioblastomas by spatially resolved transcriptomics showed that inflammation and hypoxia, as well as changes in metabolic activity and the neural environment contribute to the transcriptional heterogeneity that characterizes the four cellular archetypes [11▪]. In particular, expression of potassium channels and metabotropic glutamate receptors are important for the transition between

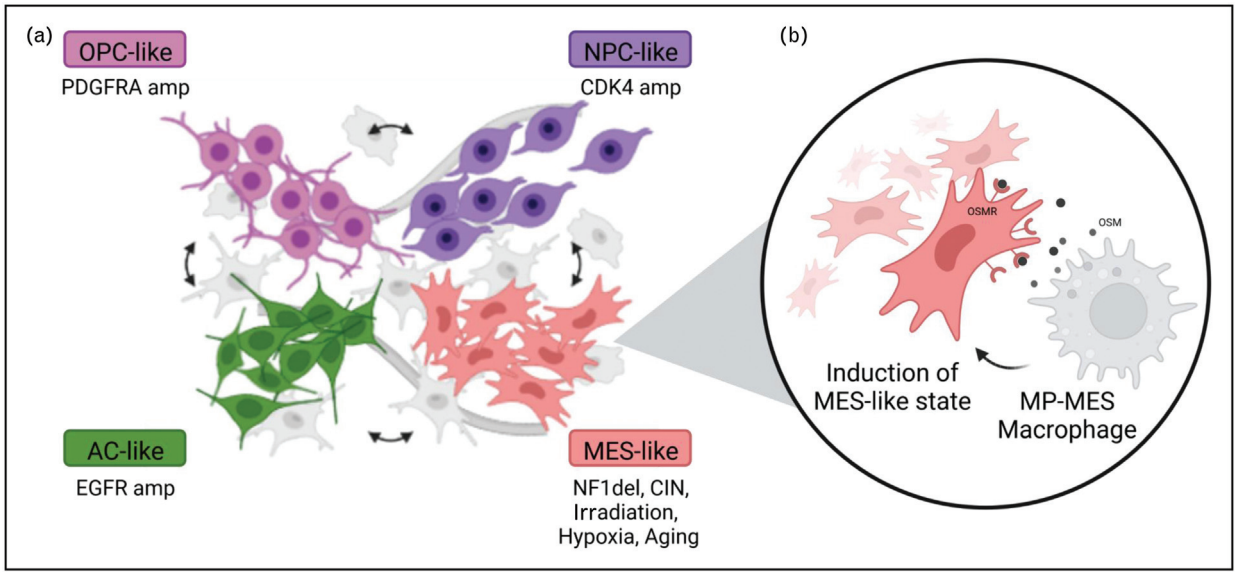

FIGURE 2. Intratumoral heterogeneity of glioma cells and immune-evasion mechanisms in the mesenchymal-like subtype. (a) The four cellular archetypes present in a given glioma, and their corresponding genetic drivers are indicated. Additional factors influencing the proportion of the MES-like state such as chromosome instability (CIN), hypoxia, irradiation, and a senescent environment are also indicated. (b) Induction of MES-like glioma cells by MES-like macrophages. MES, mesenchymal-like.

OPC-like and NPC-like tumors, whereas hypoxia leads to genomic instability in MES-like subtype [10▪▪]. Moreover, age-related changes in the neural environment promote enrichment in the MES-like subtype [10▪▪], a finding consistent with the fact that age is the main risk factor for glioblastoma development. Senescence in malignant cells also contributes to the development and heterogeneity of these tumors [11▪,12▪]. Of note, a transcriptional signature of senescence correlated with poor prognosis in human patients, whereas treatments with a senolytic agent improved the survival of mice bearing gliomas [11▪], and efficiently eliminated preirradiated tumors [12▪]. Therefore, targeting of senescent cells appears as a novel therapeutic option.

ROLES OF TAMs IN IMMUNE EVASION AND TUMOR PROGRESSION

In addition to the microenvironment and the genetic drivers, reciprocal crosstalks between malignant cells and TAMs contribute to the aggressive phenotype of MES-like tumors [13▪,14▪]. Serial transplantation experiments of CSCs from MES-like tumors showed that these cells are endowed with immune-evasive properties via demethylation of IRF8, CD73, and PD-L1 [13▪]. This epigenetic immunoediting process leads to the establishment of a myeloid-enriched TME deemed to play immunosuppressive roles. In coculture experiments, TAMs were found to stimulate transcriptional changes responsible for immune-evasiveness cells in CSCs, whereas in glioma-bearing mice, pharmacological elimination of TAMs resulted in increased survival and clearance of immune-evading tumors [13▪]. TAMs can directly induce the MES-like state of glioblastoma cells through a mechanism involving macrophage-secreted oncostatin M (OSM), a well known epithelial-to-mesenchymal transition inducer, which binds the cognate receptor (OSMR) expressed by malignant cells to activate STAT3 signaling [14▪]. Intriguingly, TAMs from MES-like tumors also display a mesenchymal-like phenotype probably induced by ligands produced by MES-like cancer cells that bind cognate receptors expressed by TAMs [14▪] (Fig. 2Bb).

TAM's phenotype and function are determined by ontogeny and environmental cues. Functional specificity or heterogeneity in TAMs has been addressed through scRNA-seq analyses of CD45+ or CD11b+ cells from GL261 tumors and human glioblastomas, which enabled an in-depth characterization of the myeloid compartment [15▪▪,16▪]. New subsets of dendritic cells, monocyte-derived macrophages (MDMs), and border-associated macrophages (BAMs) were uncovered for the first time. Analysis of newly diagnosed and recurrent tumors showed that the myeloid compartment is highly dynamic [15▪▪]. Elegant experiments of GL261 tumors growing in Cx3cr1CreER:R26-YFP mice (to fate-map microglia) and in Ccr2 knockout mice (MDMs recruitment is prevented) demonstrated that brain resident macrophages such as microglia, are outnumbered by MDMs upon recurrence [15▪▪]. Enrichment in pro-inflammatory and proliferative microglial cells has also been reported in high-grade glioblastomas in the contexts of the SETD2 mutation and EGFR overexpression [17,18]. The largest scRNA-seq study to date to characterize myeloid cells in human gliomas confirmed the MES-like phenotype of TAMs and hypoxia subtypes [19▪▪]. Signatures of TAMs were used to interrogate TCGA and scRNA-seq data, and indicated that immunosuppressive MDMs and inflammatory microglial cells correlate with worse and better prognosis, respectively [19▪▪]. This study highlighted the S100A4 protein in myeloid cells as a novel immunotherapy target [19▪▪].

IDENTIFICATION OF KEY LIGAND–RECEPTOR PAIRS

With regard to the composition of infiltrating T cells in IDH-wildtype gliomas, a combined scRNA-seq and T-cell receptor-sequencing analysis identified a subpopulation of CD8+ T cells expressing the inhibitory receptor CD161, which binds to CLEC2D expressed by malignant and myeloid cells to inhibit antitumoral activity [20▪]. Indeed, genetic inactivation of KLRB1 (the gene-encoding CD161) or blockade of CD161 resulted in enhanced killing activity by T cells *in vitro* and improved survival *in vivo* [20▪]. Thus, the authors suggest that targeting the CLEC2D–CD161 axis may synergize PD-1 blockade to enhance the antitumor function of distinct T-cell populations. Further analyses of spatially distinct regions revealed high regional heterogeneity of malignant and immune cells, and highlighted ligand–receptor interactions among glioma, myeloid cells, and T cells [19▪▪]. Similarly, a longitudinal study showed high heterogeneity of genomic alterations, neoantigens, and T-cell clones in recurrent tumors [21▪▪]. The spatiotemporal heterogeneity of the immune infiltrates emphasizes dynamic changes over time and the presence of tumor niches where the proximity (intercellular distances) is critical for immune cell activation/repression.

THE IMMUNE TME IN IDH-MUTANT GLIOMAS

The IDH enzyme catalyses the conversion of isocitrate to α-ketoglutarate (α-KG), whereas IDH1/2

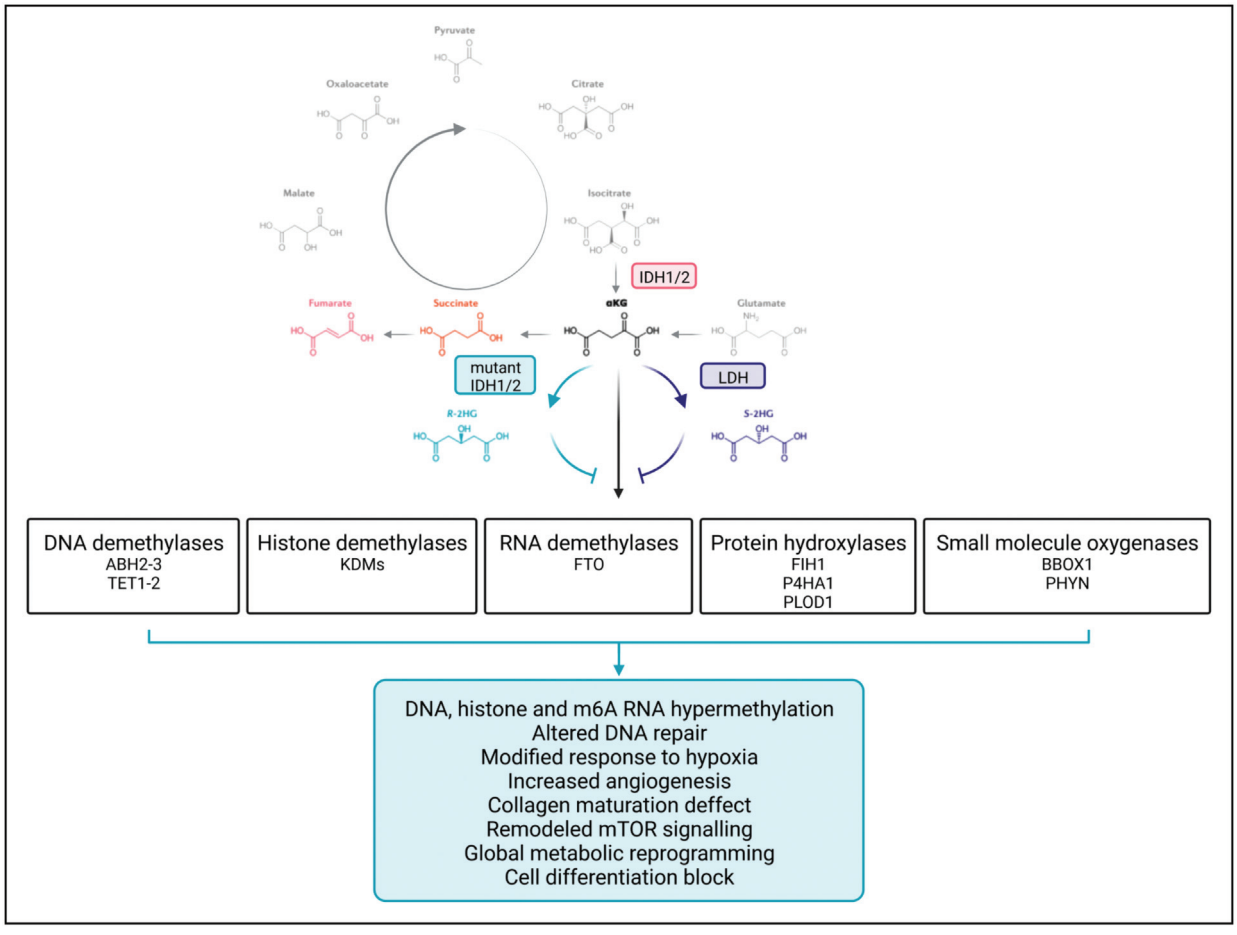

FIGURE 3. Effects of the IDH1/2 mutation. Enzymatic activity of IDH-wildtype produces α-ketoglutarate, whereas neomorphic IDH1/2 mutations produce D-2-hydroxyglutarate (D-2HG). Canonical examples of α-ketoglutarate-dependent enzymes and consequences of their inhibition by high levels of D-2HG are also indicated. IDH, isocitrate dehydrogenase.

mutations, which are frequent in diffuse gliomas, convert α-KG to D-2-hydroxyglutarate (D-2HG) [22] (Fig. 3a). It is believed that such accumulation drives cellular transformation by inhibiting α-KG-dependent dioxygenases [23], ultimately leading to widespread hypermethylation, blocking of cell differentiation and defective collagen maturation [24–28] (Fig. 3b). Moreover, IDH-mutant cells present dysregulation of the metabolic profile and redox state promoting glycolysis and enhancing the production of reactive oxygen species [29]. Strikingly, IDH-mutant, SDH-mutant, and FH-mutant tumors, which accumulate the oncometabolites D-2HG, succinate, and fumarate, respectively, do not only display epigenomic reprogramming but also exhibit a cold immune microenvironment [30].

Seminal studies using scRNA-seq of bulk tumors uncovered essential differences in the tumor architecture of IDH-wildtype and IDH-mutant gliomas [9▪▪,31,32]. On one hand, malignant cells from IDH-mutant tumors follow a hierarchical organization with cycling stem-like cells giving rise to noncycling astrocyte-like and oligodendrocyte-like lineages [9▪▪,31]. On the other hand, high-grade tumors undergo changes in the myeloid compartment with increased abundance of macrophages over microglia [32]. Initial analyses of the immune cell composition using TCGA bulk RNA-seq data, as well as experiments in syngeneic glioma models demonstrated a downregulation of immune-related signaling pathways and chemotaxis factors in IDH-mutant compared with IDH-wildtype gliomas [33,34]. Recent analyses of TCGA and immunohistochemical validations, confirmed a low expression of T-cell markers in IDH-mutant glioma, and revealed significant enrichment of CD4+ naive T cells and a reduction of memory T cells [35]. Low numbers of dendritic cells and immunosuppressive cells, including Tregs (Foxp3+) and TAMs (CD163+) were also shown, particularly in oligodendrogliomas [36]. Additional evaluation of the Chinese Glioma Genome Atlas (CGGA) cohort revealed higher infiltration of natural killer (NK) cells [37]. Moreover, IDH-mutant gliomas exhibit DNA

hypermethylation of the CD274 promoter leading to low expression of the immune ligand PD-L1 [36,38,39].

Two important studies using fluorescence-activated cell sorting followed by RNA-seq or CyTOF analyses of immune cells further confirmed that IDH-wildtype gliomas are more infiltrated by CD8+ and CD4+ T-cell subsets (including Tregs), as well as by MDMs, whereas IDH-mutant tumors display a high proportion of microglial cells and a high monocyte/MDM ratio. NK cells display immature and cytotoxic phenotypes in IDH-wildtype and IDH-mutant gliomas, respectively [40▪▪,41▪▪]. Establishing the differences in the abundance and functionality of the immune cell populations between these tumor types is crucial for the designing of efficient immunotherapeutic strategies.

Although, the IDH-mutated status was suggested to shape the TME, IDH-mutant astrocytomas and oligodendrogliomas differ in some genetic alterations, and exhibit different prognoses. In this regard, evaluation of TCGA and CGGA data indicated that immune infiltration is higher in astrocytomas than oligodendriogliomas [42]. Further analysis of bulk tumors using a combination of scRNA-seq and scATAC-seq approaches revealed a significant overexpression of chemotaxis factors CSF1 and FLT3LG in ATRX-mutated astrocytomas, and upregulation of CD163, a marker of immunosuppressive myeloid cells [43▪▪]. The causal role of the ATRX loss-of-function in shaping the myeloid compartment was confirmed in the SB28 mouse glioma model [43▪▪]. Thus, the effect of this genetic driver is reminiscent of the impact of NF1 deficiency in MES-like glioblastomas and raises the question whether genes affected by the codeletion 1p/19q that characterize IDH-mutant oligodendriogliomas (e.g. CSF1 encoded in 1p and TGFβ in 19q) account for TME changes.

Preclinical studies also explored how D-2HG acting in glioma cells could affect the TME [44,45]. Using a sleeping beauty transposon system to model IDH-mutant astrocytoma, it was shown that ATRX loss enhances DNA damage response via up-regulation of the ATM signaling pathway, which in turn was explained by D-2HG-induced hypermethylation of histone 3 (H3) [44]. The IDH mutation was also associated with hypermethylation of the activating mark H3K4me3 in the promoter region of the gene encoding granulocyte-colony stimulating factor (G-CSF) in CSCs [45]. Hence, CSC production of G-CSF was responsible for an expansion of immature granulocytic myeloid cells infiltrating the TME [45]. These results suggest that compared with IDH-wild type glioma, the overall low level of immune infiltrates in IDH-mutant gliomas involves altered expression of effectors acting on the recruitment or the differentiation of infiltrating immune cells via D-2HG-driven epigenetic alterations in malignant cells. Nevertheless, as this oncometabolite accumulates to millimolar levels in the TME [46,47], it may also affect the phenotypic and functional properties of immune cells.

CELL-EXTRINSIC ROLES OF D-2HG

Recent in-vitro studies provided evidence for the uptake of D-2HG by cells typically residing in the TME, via the sodium-dependent dicarboxylate transporter 3 (SLC13A3) [35] or the glutamate transporter SLC1A1 [48▪] (Fig. 4). Increased D-2HG levels were also found in T cells isolated from acute myeloid leukaemia (AML) patients harbouring IDH2 mutations [49], and in CD11b+ cells from an IDH-mutant mouse model [50▪▪]. Treatments with D-2HG used at nontoxic albeit high concentrations (>5 mmol/l) reduce IL-12 secretion and preclude LPS-induced glycolysis in dendritic cells [51], and prevent LPS-induced activation in murine microglia by affecting the AMPK/mTOR/NF-κB-signaling pathway [52]. In endothelial cells, D-2HG fuels mitochondrial respiration and angiogenesis [48▪].

With respect to cultured T cells, D-2HG promotes a metabolic switch from aerobic glycolysis towards oxidative phosphorylation in activated T cells and favors the growth or differentiation of Tregs [49]. In contrast, in-vivo studies using GL261 cells overexpressing IDH wildtype or IDH mutant showed decreased numbers of Tregs in IDH-mutant gliomas [53] and impaired T-cell activation by reducing proliferation and cytokine production [35]. Because the functional response of immune cells depends on environmental signals and cell–cell interactions, which may be prevented *in vitro*, there is a need to characterize the effects of D-2HG *in vivo*. In this regard, inhibition of the enzymatic function of the IDH mutation increased the CD4+ population and restored the antitumor activity of T cells [35]. Moreover, this therapeutic approach combined with PD-1 inhibition increased overall survival [35,54▪▪].

In addition, recent evidence demonstrated that D-2HG drives an immunosuppressive myeloid state by altering the tryptophan metabolism in MDMs via activation of AHR [55▪]. Pseudotime inference analyses using scRNA-seq data of flow cytometry-purified CD45+ cells from IDH-mutant and IDH-wildtype GL261 gliomas confirmed the high monocyte/MDM ratio previously observed in IDH-mutant human tumors [40▪▪] and further revealed a high monocyte/dendritic cell ratio [56▪]. The authors suggested an immature phenotype of monocyte-derived cells upon D-2HG exposure. However, in-vitro

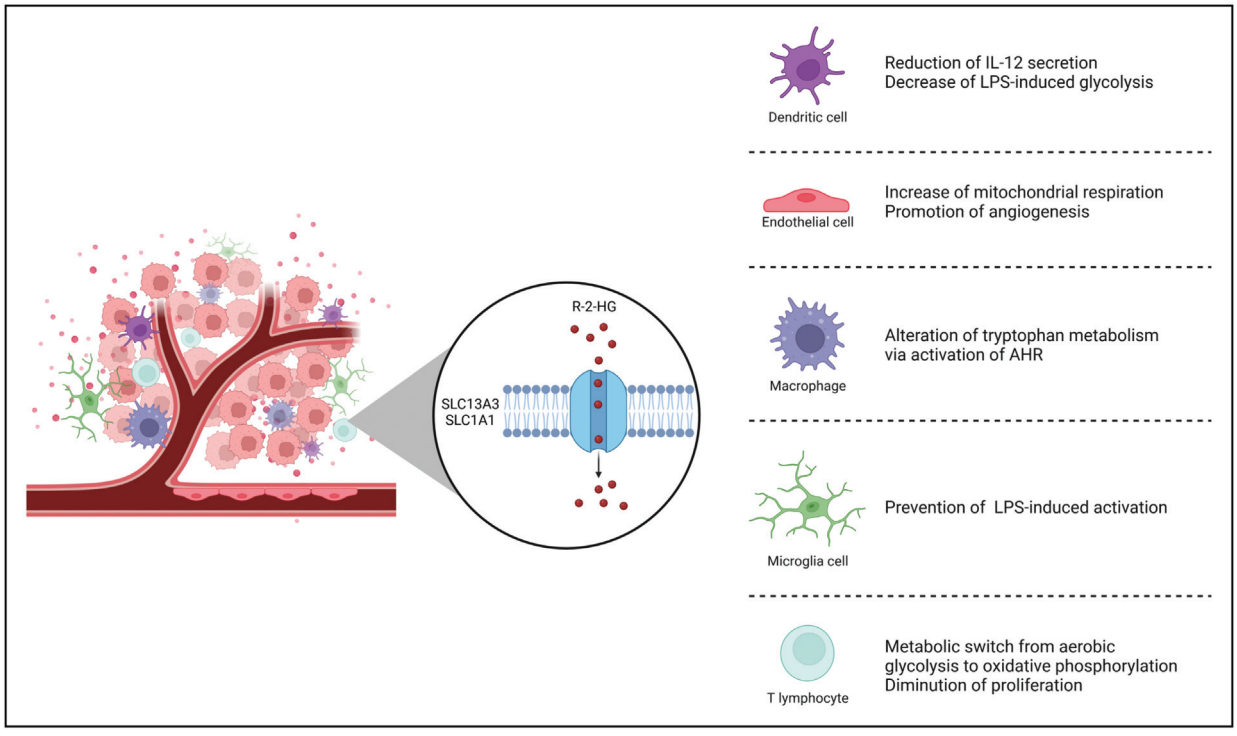

FIGURE 4. Cellular uptake of D-2-hydroxyglutarate. Cell types able to take up D-2HG according to in-vitro studies as well as two of the transporters so far reported are indicated. D-2HG, D-2-hydroxyglutarate.

experiments revealed conflicting results with a previous study showing that neither differentiation, nor antigen presentation of dendritic cells is affected by D-2HG [57]. This further emphasizes the challenges to characterize the effects of D-2HG on immune cell function *in vitro*.

Collectively, these data argue against a simple reduction of immune cell recruitment by chemotactic factors. More investigation is required to specify the roles of D-2HG as immunomodulator of the TME in IDH-mutant gliomas.

CONCLUSION

Although immunotherapy targeting the PD-L1/PD-1 axis has achieved advances in various cancers, phase III clinical trials failed to show efficacy in newly diagnosed and recurrent glioblastomas. The presence of dysfunctional T cells [58,59], as well as suppressive cells such as Tregs and TAMs in the TME may account for this lack of response. The comprehensive characterization of the immune TME at single-cell resolution and experimental evidence in mouse models point to prominent roles of TAMs and their interactions with malignant and T cells during tumor progression. Hence, focus on the myeloid compartment, and the immune checkpoints expressed by these cells is highly encouraged in order to uncover specific mechanisms leading to the immunosuppressive TME.

TAMs do not only offer a prognostic value but also are potential targets for therapies aimed at depleting/repolarizing these cells to a pro-inflammatory state thereby allowing effector T-cell infiltration and activation [60–63]. Nevertheless, targeting the myeloid population should be more specific as MDMs are more abundant in IDH wild-type gliomas and recurrent tumors (regardless of the IDH status) whereas microglial cells are the major population in IDH-mutant gliomas. Moreover, the pro-tumorigenic role of nonparenchymal macrophages, which are located in meninges, perivascular niches, and even within the cerebrospinal fluid, remains unexplored [64,65]. So far, a relatively small number of human gliomas have been profiled for scRNA-seq analysis of the TME. As more data will be generated, a more complete atlas of myeloid cells could help to identify novel subsets that correlate with clinical outcomes. Efforts are currently underway to better characterize TAM subtypes, ligand–receptor pairs, and immune checkpoints expressed by these cells [66]. It is becoming clear that glioblastoma progression requires not only genetic drivers but also microenvironment interactions [9▪▪,10▪▪,11▪,67▪]. While most of the work on immunoevading mechanisms and myeloid interactions has been done in MES-like gliomas [13▪,14▪,18,67▪], the immunomodulatory mechanisms operating in low-grade and IDH-mutant gliomas remain largely unknown.

Differences in the TME of astrocytomas and oligodendriogliomas suggested by bulk RNA-seq studies [36,42,68,69] may be linked to their distinct prognosis and need to be ascertained using scRNA-seq. IDH-mutant tumors are infiltrated by a low number of immune cells. Although results from clinical trials with IDH mutation inhibitors are promising [70], preclinical studies suggest that this approach may be more effective if combined with immunotherapies (checkpoint blockade or IDH1R132H vaccines) [35,54▪▪]. Although cell-extrinsic effects of D-2HG mediate some changes in the TME, the impact of this oncometabolite on the epigenome of immune cells remains unexplored. Hence, these are exciting times to discover additional roles of D-2HG in the TME of IDH-mutant gliomas.

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Conflicts of interest

There are no conflicts of interest.

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