THE STATE-OF-ART IN IMMUNO-ONCOLOGY, WHAT TO DO WITH GLIOBLASTOMA?

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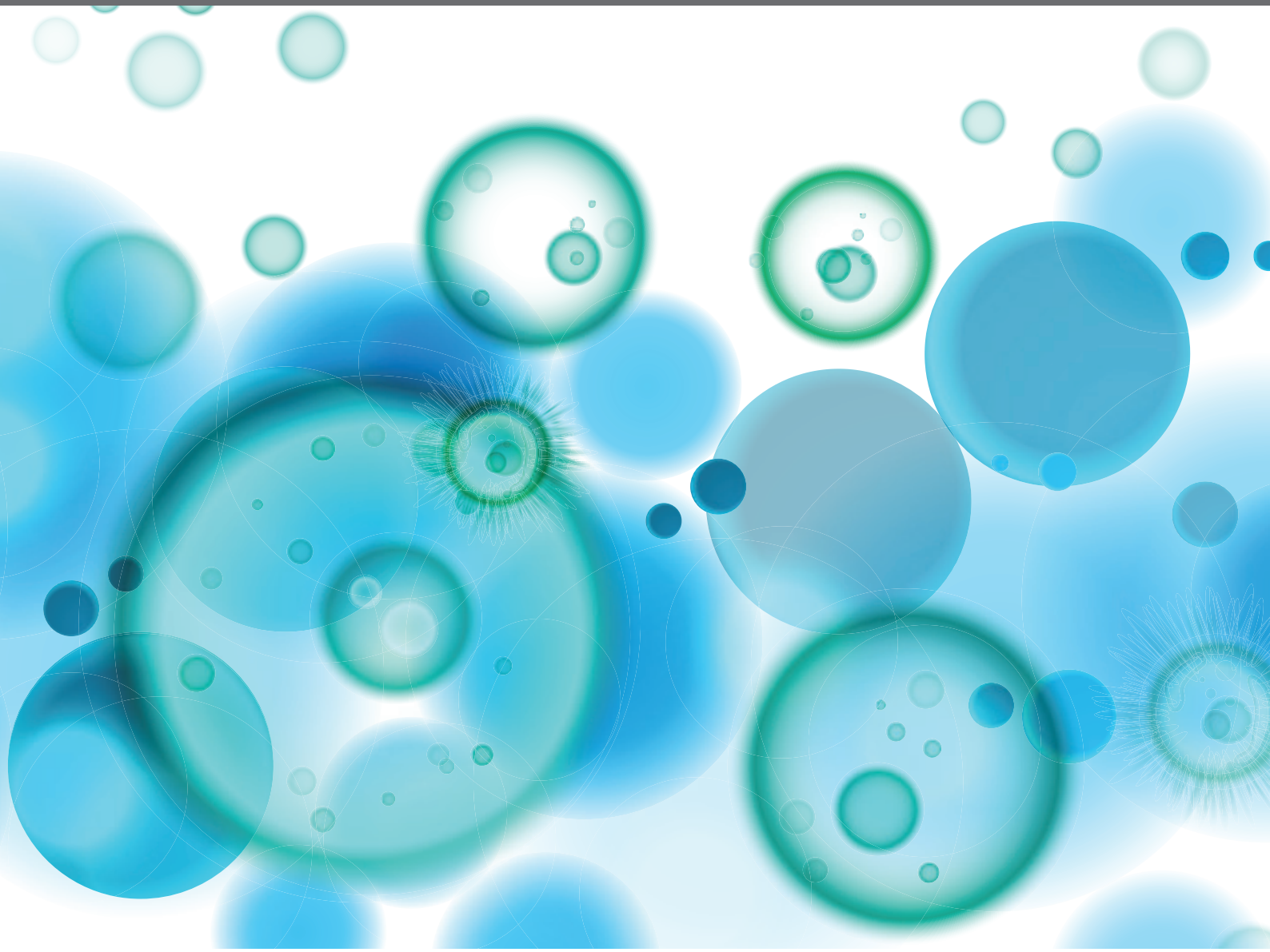

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THE STATE-OF-ART IN IMMUNO-ONCOLOGY, WHAT TO DO WITH GLIOBLASTOMA?

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EDITORIAL
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Editorial: The State-of-Art in Immuno-Oncology, What to Do With Glioblastoma?

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Keywords: glioblastoma, immunotherapy, tumor microenvironment, CAR T cell therapy, multi-target

Editorial on the Research Topic

The State-of-Art in Immuno-Oncology, What to Do With Glioblastoma?

Gliomas are the most common primary tumors of the central nervous system, originating in the brain which is one of the immunologically privileged organs. As high-grade glioma accounts for 80% of malignant brain tumors, glioblastomas (GBM) are characterized by high inter- and intra-tumoral heterogeneity and immunosuppressive tumor microenvironment. At present, the conventional treatment of GBM in a clinical context is surgical resection, combined with systematic radio- and chemo-therapy. But the median survival time of GBM patients is still not ideal. Therefore, an urgent need for finding new and more effective treatment strategies remains.

In recent years, some novel therapeutic strategies, such as immunotherapies represented by checkpoint inhibitors/antibodies and cell therapies exemplified by chimeric antigen receptor (CAR) engineered T cells, give new hope for current GBM patients. These novel therapeutics targeted T cells aiming to improve their infiltration and enhance the activity of cytotoxic T cells. However, many factors in the tumor microenvironment can prevent T cell infiltration and induce T cells to the exhausted subtype, such as tumor-associated macrophages that form immune barriers and metabolites secreted by tumor cells. Hence, elucidating the characteristics of the GBM immune microenvironment is crucial for immunotherapies for GBM.

The Research Topic "The State-of-Art in Immuno-oncology, what to do with Glioblastoma?" focuses on every aspect of immunology in GBM, aiming to clarify the immune landscape of GBM and bridge the immunology and therapeutics of GBM.

Yuanhao Chang et al. reported that a gene named Glutaredoxin (GLRX), which is a vital gene maintaining cell redox balance and playing a regulatory role in the progression of many malignant tumors, could be highly expressed in high-grade gliomas, and become an independent prognostic predictor of GBM patients. Mechanically, by employing the single-cell RNA sequencing of GBM samples, and bioinformatic analysis of CGGA and TCGA online databases, the authors demonstrated the GLRX was specifically expressed in M0 macrophages, and positively correlated with the complexity of tumor microenvironment and the infiltrated immune cells, indicating that GLRX was a key regulator of glioma immune microenvironment formation. Therefore, therapeutics targeting the cell redox regulation are expected to enhance the effect of glioma immunotherapy.

It is well-known that GBM is a malignant tumor with a high recurrence rate. Resistance to various therapeutics often occurs in recurrent GBM, with the remodeling of the immunosuppressive microenvironment. Weilun Fu et al. presented a high-dimensional view of the complex immune

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microenvironment in primary and recurrent GBM by mass cytometry (CyTOF). They demonstrated that glioma-associated macrophages (GAMs) accounted for a large proportion of GBM tumors, exhibiting great inter- and intra-tumoral heterogeneity. GAMs and other immune cells such as exhausted T cells, infiltrating Tregs, and nonfunctional NK cells contributed to the immunosuppressive characteristics. Primary and recurrent GBM showed a similar immune microenvironment, but the proportion of GAMs decreased from 59.05% in primary GBM to 27.87% in recurrent GBM. These results above suggest to us that more specific and comprehensive therapeutic strategies are urgently needed to treat the recurrent GBM.

Recently, CAR T cell therapy directed at tumor-specific targets has achieved great effects in the treatment of a variety of tumors. But the response to CAR T therapy in GBM is debatable. Long Li et al. reviewed the current status and prospects of CAR T immunotherapy in GBM treatment and found that CAR T cells targeting IL-13RA2, EGFRvIII, and HER2 showed significant clinical efficacy and safety in phase 1 and 2 clinical trials conducted in patients with GBM. However, its efficacy is still limited by the blood-brain barrier, high tumoral heterogeneity, and antigen escape. Therefore, the combined therapeutic strategy of conventional therapy and multi-target CAR T cells is more appropriate for GBM treatment. A review on glioma immunotherapy by Boyuan Huang et al. presented the same viewpoints. Meanwhile, they pointed out that combined therapeutics such as vaccine research and development, immune checkpoint blocking, and CAR T cell targeting have the prospect of glioma immunotherapy.

Recent evidence has indicated exosomes and metabolites from glioma cells could remold the immune-microenvironments within the tumor community. The lipid and cholesterol metabolic by-pathway could reinforce such an effect. The future battlefield will be a multi-target approach both at glioma cells and immune cells. Alternative therapeutic avenues including innovative targets and drug and effective antibody delivery methods will shed new light on GBM therapy.

AUTHOR CONTRIBUTIONS

All authors have contributed to the manuscript and approved the final version for publishment.

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ORIGINAL RESEARCH
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Single-Cell Atlas Reveals Complexity of the Immunosuppressive Microenvironment of Initial and Recurrent Glioblastoma

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The Glioblastoma (GBM) immune microenvironment plays a critical role in tumor development, progression, and prognosis. A comprehensive understanding of the intricate milieu and its interactions remains unclear, and single-cell analysis is crucially needed. Leveraging mass cytometry (CyTOF), we analyzed immunocytes from 13 initial and three recurrent GBM samples and their matched peripheral blood mononuclear cells (pPBMCs). Using a panel of 30 markers, we provide a high-dimensional view of the complex GBM immune microenvironment. Hematoxylin and eosin staining and polychromatic immunofluorescence were used for verification of the key findings. In the initial and recurrent GBMs, glioma-associated microglia/macrophages (GAMs) constituted 59.05 and 27.87% of the immunocytes, respectively; programmed cell death-ligand 1 (PD-L1), T cell immunoglobulin domain and mucin domain-3 (TIM-3), lymphocyte activation gene-3 (LAG-3), interleukin-10 (IL-10) and transforming growth factor-β (TGFβ) demonstrated different expression levels in the GAMs among the patients. GAMs could be subdivided into different subgroups with different phenotypes. Both the exhausted T cell and regulatory T (Treg) cell percentages were significantly higher in tumors than in pPBMCs. The natural killer (NK) cells that infiltrated into the tumor lesions expressed higher levels of CXC chemokine receptor 3 (CXCR3), as these cells expressed lower levels of interferon-γ (IFNγ). The immune microenvironment in the initial and recurrent GBMs displayed similar suppressive changes. Our study confirmed that GAMs, as the dominant infiltrating immunocytes, present great inter- and intra-tumoral heterogeneity and that GAMs, increased exhausted T cells, infiltrating Tregs, and nonfunctional NK cells contribute to local immune suppressive characteristics. Recurrent GBMs share similar immune signatures with the initial GBMs except the proportion of GAMs decreases.

Keywords: glioblastoma, recurrent glioblastoma, CyTOF, immune profiling, microenvironment

INTRODUCTION

Glioblastoma (GBM) is the most common and aggressive primary brain tumor (1). Because of their malignant growth and invasion into the brain parenchyma coupled with resistance to therapy, GBMs are among the deadliest of all tumors (2). A recent study demonstrated that through hijacking the immune system, GBM cells limit the efficacy of conventional therapies (3). GBM cells secrete numerous factors that promote tumor infiltration of a range of immunocytes (4, 5). Locally produced factors and their crosstalk with the extracellular matrix drive and reprogram infiltrating immune cells to acquire distinct functional phenotypes (6). These infiltrating immune cells have been shown to engage in reciprocal interactions with neoplastic tumor cells to play a crucial role in tumor growth, metastasis, and response to treatment (7).

Glioma-associated microglia/macrophages and tumor-infiltrating lymphocytes constitute the major infiltrating immune cell population (8, 9). Microglia and peripheral macrophages, which extensively infiltrate GBMs, are collectively termed GAMs (10); tumor-infiltrating lymphocytes mostly comprise CD4+ T cells, CD8+ T cells, and regulatory T cells (Tregs) (4). Current checkpoint blockade therapies mainly function to rescue T cells from exhaustion or to deplete Tregs, while GAM-targeted treatments may improve the prognosis of GBM patients as immunotherapeutic interventions (11). Dissecting the details of immune cells, particularly regarding GAMs and T cell function and distribution at tumor sites, might lead to novel strategies to further strengthen anti-tumor immunity.

Compared with initial GBM, recurrent GBM is thought to exhibit different clinical features, molecular subtypes and gene alterations (12, 13). Preliminary data suggest that neoadjuvant anti-programmed cell death protein 1 (PD-1) immunotherapy promotes a survival benefit with intra-tumoral and systemic immune responses for patients with recurrent GBM (14). While several trials utilizing anti-PD-1 or anti-programmed cell death-ligand 1 (PD-L1) are currently ongoing in patients with initial GBM (15), whether the specific features of recurrent GBM create unique immune changes and exhibit differences in their immune microenvironment remain unclear.

Developing immunotherapies that are effective against initial or recurrent GBM requires combinatorial strategies that target multiple aspects of immune tolerance (16). Realizing this potential requires a comprehensive understanding of the GBM immune microenvironment. In this study, we applied a single-cell level technology mass cytometry (CyTOF) (17) to capture immunocyte populations in situ to determine their roles in both the microenvironment and the peripheral blood and to address the cellular and molecular complexity of the immunosuppressive microenvironment in initial and recurrent GBM. Our data provide a detailed dissection of GBM immune cell types, revealing inter- and intra- tumoral heterogeneity of GAMs and T cell exhaustion in GBM lesions. These observations will facilitate a better understanding of the complexity of the immunosuppressive microenvironment of initial and recurrent GBM and will benefit in designing patient-specific immunotherapy.

MATERIALS AND METHODS

Human Specimens and Ethics Statements

Blood and GBM tissues were obtained from GBM patients undergoing craniotomy surgery at Beijing Tiantan Hospital (Beijing, China) from 2018.7 to 2018.10 after informed consent was provided. All cases were confirmed by histopathology. Healthy donor peripheral blood was taken from healthy volunteers after obtaining informed consent. None of the patients or healthy donors used glucocorticoids before sampling. This research was approved by the Institutional Review Board (IRB) and Ethics Committee of Beijing Tiantan Hospital (Beijing, China). Written informed consent was obtained from all patients and healthy donors.

GBM Tissue Single-Cell Dissociation

Glioblastoma tissues were washed with ice-cold Dulbecco's phosphate-buffered saline (DPBS, without Mg^{2+} and Ca^{2+}, catalog no. D8537, Sigma-Aldrich, St. Louis, MO, United States) immediately after the operation. Briefly, the samples were dissociated using type IV collagenase (catalog no. 17104019, GIBCO, Gaithersburg, MD, United States) for 10 min at 37°C. Then, the samples were washed with Dulbecco's Modified Eagle's Medium (DMEM, catalog no. D5796, Sigma-Aldrich, St. Louis, MO, United States) and centrifuged (4 min at 300 *g*, 18°C, minimal braking). The samples were then filtered through a 70 mm cell strainer with DPBS and washed with red blood cell (RBC) lysis buffer (catalog no. 555899, BD Biosciences, Franklin Lakes, NJ, United States). The dissociated cell suspension was then washed once with DPBS. The cell pellet was resuspended in 1 mL of staining buffer (DPBS containing 5% fetal bovine serum, FBS; catalog no. 0500, ScienCell, Carlsbad, CA, United States) and washed one more time.

Blood Single-Cell Dissociation

Fresh blood samples were collected into ethylenediaminetetraacetic acid (EDTA) anticoagulation tubes and then centrifuged (5 min at 800 *g* with minimal braking) to remove plasma. Then, the samples were transferred into SepMate PBMC isolation tubes containing Ficoll (catalog no. 86450, STEMCELL Technologies, Vancouver, Canada) and centrifuged (10 min at 1200 *g*, minimal braking). The cells were washed with RBC lysis buffer and then washed twice with staining buffer.

Mass Cytometry

A panel of 30 antibodies designed to distinguish a broad range of immune cells was used. Antibodies were either purchased in a preconjugated form from Fluidigm (South San Francisco, CA, United States) or purchased from Biolegend (San Diego, CA, United States) in a purified form and conjugated in-house using the Maxpar X8 Multimetal Labeling Kit (catalog no. 201300, Fluidigm, South San Francisco, CA, United States) according to the manufacturer's instructions. The antibodies and reporter isotopes are included in **Supplementary Table S1**.

Briefly, cell samples were rapidly rewarmed and then washed and stained with cell surface antibodies for 30 min on ice. Subsequently, the samples were permeabilized at 4°C overnight and stained with intracellular antibodies for 30 min on ice. The antibody-labeled samples were washed and incubated in 0.125 nM intercalator-Ir (catalog no. 201192B, Fluidigm, South San Francisco, CA, United States) diluted in phosphate-buffered saline (PBS, catalog no. 806544, Sigma-Aldrich, St. Louis, MO, United States) containing 2% formaldehyde and stored at 4°C until mass cytometry examination. Before acquisition, the samples were washed with deionized water and then resuspended at a concentration of 1×10^6 cells/mL in deionized water containing a 1:20 dilution of EQ Four Element Beads (catalog no. 201078, Fluidigm, South San Francisco, CA, United States). The samples were then examined by CyTOF2 mass cytometry (Fluidigm, South San Francisco, CA, United States).

CyTOF Data Analysis

Data were obtained in the form of .fcs files. The addition of EQ Four Element Beads allowed us to use the MATLAB-based normalization technique using bead intensities as previously described (18). The CyTOF data were analyzed on Cytobank[1]. Cell types were identified based on the following parameters: T cells, CD45+CD3+; B cells, CD45+CD19+; natural killer (NK) cells, CD45+CD3-CD16+CD56+ (19); monocytes, CD45+CD14+CD16+ (20); macrophages or microglia cells, CD45+CD11b+CD68+ (21); Tregs, CD45+CD4+CD25+CD127- (22); naïve CD4+ T cells, CD45+CD45RA+CCR7+CD4+ (23); and naïve CD8+ T cells, CD45+CD45RA+CCR7+CD8+ (23). Monocytes and macrophages constitute mononuclear phagocytes (24) (**Supplementary Table S2**). Manual gating was applied to mark cell types as previously reported (25). Data were analyzed using viSNE (26) algorithms on the indicated gated cells. Then, automatic cluster gate functionality was used for the hierarchical cluster analysis. The number of events to be sampled was set by the maximum available cell numbers in the smallest sample to avoid skewing the data toward larger samples. Heatmaps of marker expression and relative marker expression were generated by R software (version 3.4.0).

Heatmap Data Normalization

For **Figure 3A**, we compared the indicated factor expression level of GAMs to that of each paired patient peripheral blood mononuclear cells (pPBMC) sample (1:1 comparison). The relative factor expression level of GAMs was obtained by calculating the log10-normalized value of the ratio of the mean expression level of the factor in GAMs over its expression level in the paired pPBMC sample. For each factor i in patient j, the formula is summarized as follows:

$$Relative\ expression\ level\ E_{ij} = log10(\overline{E_{GAM_{ij}}}/\overline{E_{pPBMC_{ij}}})$$

E is the relative expression level.

[1]www.cytobank.org

For **Figures 3C**, **4B**, **5G**, the log10-scaled values were first used to normalize the mean value of each marker, and min-max normalization was then used to obtain the final normalized values in the heatmap. The min-max normalization formula is as follows:

$$z = \frac{x - min(x)}{\max(x) - min(x)}$$

Z is the final normalized value, x is log10-scaled value; and min and max are the minimum and maximum log10-scaled values, respectively.

Histology and Immunofluorescence Staining

Glioblastoma samples were fixed overnight in 4% formalin (4°C) and embedded in paraffin blocks for paraffin sections. Hematoxylin and eosin (H&E) staining was performed as previously described (27). For immunofluorescence, paraffin sections (3 μm) were washed twice 15 min in PBS (catalog no. 806544, Sigma-Aldrich, St. Louis, MO, United States), permeabilized in 0.2%–0.5% Triton X-100 (catalog no. T8200-100, Solarbio, Beijing, China) and blocked in 5% normal donkey serum (catalog no. 017-000-001, Jackson Lab, West Grove, PA, United States) for 1 h and stained with primary antibody overnight. Primary antibody were detected using fluorescent-conjected second antibodies (catalog no. PV-6000, ZSGB-BIO, Beijing, China). Sections were mounted with fluorescence mounting medium (catalog no. S3023, Dako, Glostrup, Denmark). As previously described (28), the Opal 4-Color Manual IHC Kit (catalog no. NEL810001KT, Perkin Elmer, Waltham, MA, United States) was used for the analysis of formalin-fixed paraffin-embedded GBM sections according to the manufacturer's protocol. Fluorescent images were acquired on a Zeiss LSM880 NLO microscope and Zeiss Axio Scope A1 was used to obtain H&E images. Primary antibodies were: anti-CD45 (catalog no. AB40763, Abcam, Cambridge, MA, United States), anti-CD68 (catalog no. AB955, Abcam, Cambridge, MA, United States), anti- tumor necrosis factor α (TNFα) (catalog no. 60291-1-Ig, Proteintech, Rosemont, IL, United States) and anti-indoleamine-pyrrole 2, 3-dioxygenase (IDO) (catalog no. 86630S, CST, Danvers, MA, United States).

Statistics

For CyTOF experiments, 13 initial GBM samples and nine paired blood samples, three recurrent GBM samples and three paired blood samples, and eight healthy donor blood samples were analyzed. For comparison of the nine initial GBM tissues and paired pPBMCs, the Wilcoxon matched-pairs signed rank test was used. Additionally, for the initial GBM tissues samples, recurrent GBM tissue samples and hPBMCs, the Mann–Whitney test was used to analyze each cell subset. Statistical analysis was performed using GraphPad Prism 7.00 software. *P*-values less than 0.05 were considered statistically significant.

RESULTS

Single-Cell Profiling of the GBM Immune Microenvironment

We obtained 13 initial GBM tumor tissues, nine of which had paired pPBMC samples. The blood samples of the other 4 initial cases didn't pass quality control for CyTOF test. We also obtained three additional recurrent GBM tumor tissues and three paired pPBMC samples. The baseline characteristics of all patients are summarized in **Table 1**. Eight healthy donors provided peripheral blood samples (hPBMCs) as a control.

Approximately 37000 CD45+ cells were detected on average per sample. We simultaneously mapped the immune compartments of GBM lesions and pPBMCs. We also compared pPBMCs with hPBMCs to distinguish changes in GBM circulating immunity (**Figure 1A**). The initial gating strategies for the single living cells are provided in **Figure 1B**. The dimensionality reduction tool viSNE (26) was employed to convert high-dimensional CyTOF data from each sample into a two-dimensional map (**Figure 1C**).

Mononuclear Phagocytes and T Lymphocytes Dominate the Initial GBM Microenvironment

We analyzed the distributions of the different immune cell lineages that accumulated in initial GBM lesions and pPBMCs across patients. The most abundant immune cells in initial GBM lesions were mononuclear phagocytes (59.05%) and T lymphocytes (16.39%). Compared with that in pPBMCs, the proportion of mononuclear phagocytes at the tumor site was significantly increased ($p < 0.01$), while the proportion of T cells was significantly decreased ($p < 0.01$) (**Figures 2A,B**). The remaining CD45+ cells constituted immunocytes that could not be defined by markers in this panel.

TABLE 1 | Basic characteristics of all 16 patients.

Variable	Initial GBM (*N* = 13)	Recurrent GBM (*N* = 3)
Age-mean, years (range)	55.5 (31 – 74)	45.5 (36 – 63)
Male	10 (76.9%)	1 (33.3%)
Female	3 (23.1%)	2 (66.6%)
IDH1		
Mutation	4 (30.8%)	1 (33.3%)
Wild type	9 (69.2%)	2 (66.6%)
IDH2		
Mutation	0 (0%)	0 (0%)
Wild type	13 (100%)	3 (100%)
TERT promoter		
C228T	3 (23.1%)	1 (33.3%)
C250T	4 (30.8%)	0 (0%)
Wild type	6 (46.2%)	2 (66.6%)

N, number; IDH, isocitrate dehydrogenase; TERT, telomerase reverse transcriptase.

To investigate changes in the circulating immunity of GBM patients, we also compared PBMCs from GBM patients and healthy donors. The results showed a diminished T cell fraction in GBM patient peripheral blood compared to that in healthy donors ($p < 0.01$), while the proportions of NK cells and B cells were similar across all samples (**Figures 2A,B**).

Diversity of GAM Subsets in GBM Lesions

Previous studies showed the extensive infiltration of gliomas with microglia and peripheral macrophages (29), collectively termed GAMs. In the current study, GAMs were the most enriched population in GBM lesions. They showed inter-tumoral heterogeneity, as immune checkpoints PD-L1, lymphocyte activation gene-3 (LAG-3) and T cell immunoglobulin domain and mucin domain-3 (TIM-3), immunosuppressive cytokines interleukin-10 (IL-10) and transforming growth factor-β (TGFβ), tumor necrosis factor-α (TNFα) and vascular endothelial growth factor (VEGF) were expressed at various levels among patients (**Figure 3A**).

We performed a hierarchical cluster analysis of the GAM subpopulations using automatic cluster gate functionality to fully capture the heterogeneity of the GAM compartment. The GAMs were identified based on the expression of protein markers, including CD45, CD68, and CD11b, and then subdivided into 13 subgroups according to the surface markers (**Figure 3B**).

Regarding the expression of the immune checkpoint and cytokines among each subset, GAM phenotypes showed substantial intra-tumoral diversity. One group involving cluster M-6 displayed higher HLA-DR and CD68 expression levels and lower CD11b expression levels than the other groups, suggesting that these GAM cells were mature (29). This cluster was characterized by high expression of the immune checkpoint marker PD-L1. By expressing PD-L1 on the cell surface, GAMs may promote T cell apoptosis (30). Additionally, M-10 was also recognized in the mature group, but PD-L1 was expressed at lower levels than M-6 in this group (**Supplementary Figure S1**A). Among the GAM subsets, PD-L1 was frequently expressed, while certain immune checkpoints, such as TIM-3 and LAG-3, were seldom expressed (**Figure 3C**). At the single-cell level, surprisingly, the viSNE map showed that a certain GAM subgroup (M-1) could coexpress anti-tumor (TNFα) and pro-tumor (IDO) markers, while PD-L1 was also highly expressed in this subgroup (**Figures 3C,D**). We revealed that mononuclear macrophage infiltration in GBM lesions using H&E staining (**Supplementary Figure S1**B). Polychromatic immunofluorescence verified the finding that anti-tumor (TNFα) and pro-tumor (IDO) markers were co-expressed in certain GAM subgroups (**Figure 3E**).

We merged GAMs from all initial patients for analysis and found similar trends as the representative patient. GAMs can be divided into subgroups of different phenotypes (**Supplementary Figure S1C**). Anti-tumor and pro-tumor markers were shown to be co-expressed in certain subgroups (**Supplementary Figures S1D,E**).

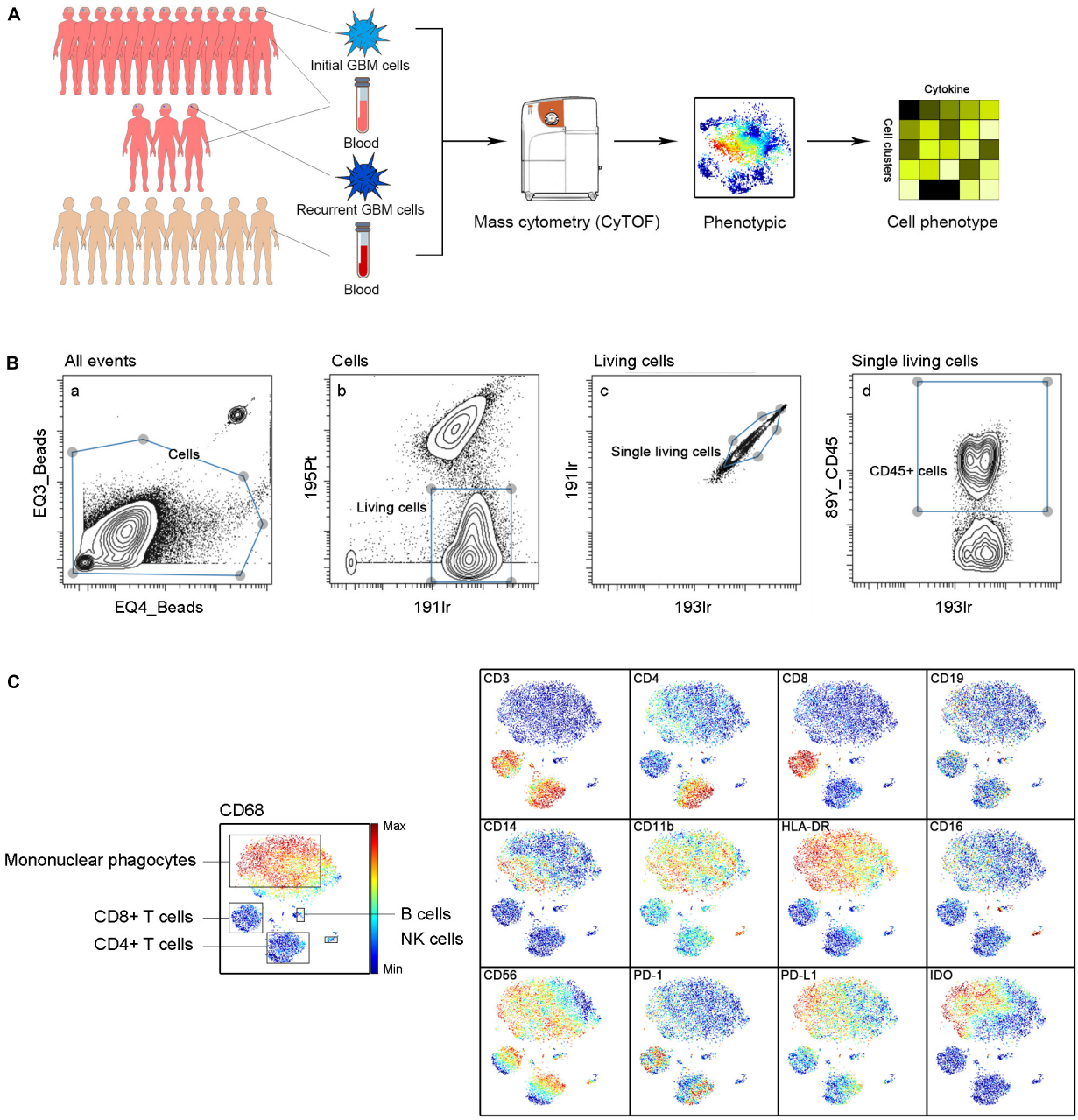

FIGURE 1 | Immunosuppressive microenvironment of initial and recurrent GBM using CyTOF. **(A)** Schematic for defining the immune composition of GBMs. Initial or recurrent tumor tissues and pPBMCs were collected from patients, and hPBMCs were collected from healthy donors. Samples were processed and stained with antibodies conjugated to metal isotopes. CyTOF single-cell data were used to identify the immune features of patients. **(B)** All ungated events were sequentially gated in Cytobank to identify living single cells. (a) 151Eu EQ3 and 153Eu EQ4 beads were used to identify cells. (b) Living cells were identified by gating cells negative for 195Pt. (c) 191Ir and 193Ir were used to obtain living single cells from (b). (d) CD45+ cells were obtained from living single cells. **(C)** ViSNE analysis of immune cells from samples indicated by the relative expression of CyTOF markers for a representative patient; the cell populations are also indicated (left).

T Cells Are Exhausted and Tregs Are Increased in Initial GBM Lesions

Specifically, compared to those in pPBMCs, the Treg proportions in the tumor lesions were significantly increased across all patients (p = 0.0508) (**Figure 4A** and **Supplementary Figure S2A**). PD-1+, TIM-3+ or LAG-3+ T cells are recognized as exhausted subsets (31, 32). Compared to that in pPBMCs, the proportions of exhausted CD4+ and CD8+ T cells were distinctly higher at the tumor sites (**Figure 4A**).

According to the surface markers, the T cells were subdivided into 16 subgroups. The expression profiles of the T cell clusters were visualized in a heatmap (**Figure 4B**), and heterogeneity in

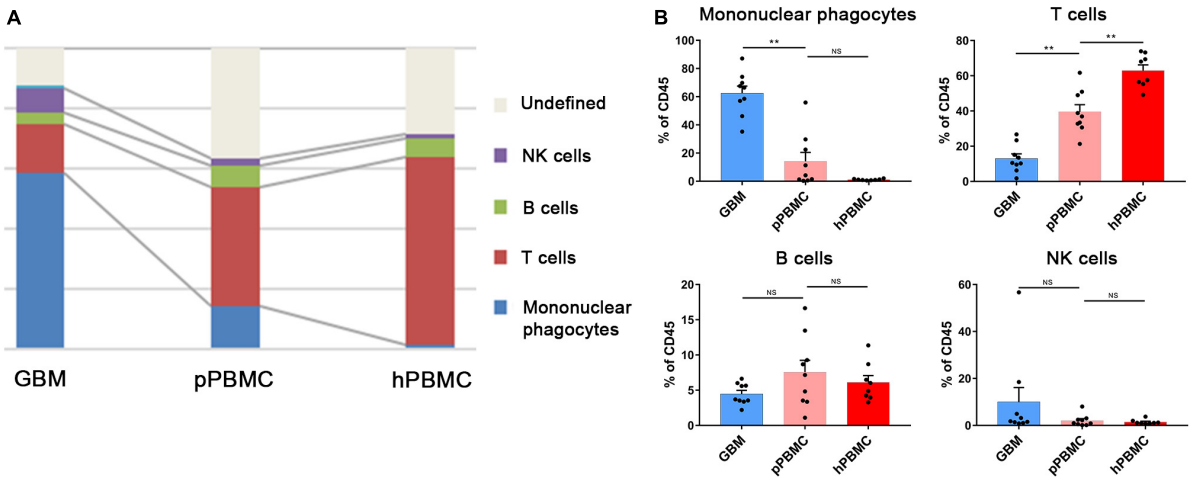

FIGURE 2 | Immunosuppressive changes in the initial GBM microenvironment and circulating immunity. **(A)** Composition of the CD45+ compartment showing the average frequencies of major immune lineages for each tissue. **(B)** Bar plots showing the frequencies for each initial patient and pPBMC sample (by Wilcoxon matched-pairs signed rank test) and the frequencies for each pPBMC and hPBMC sample (by the Mann–Whitney test). Bar plots show the mean with SEM (NS, no significance; $^{**}p < 0.01$).

the immune-related marker levels was assessed at the single-cell level using viSNE maps (**Figure 4C**). This approach led to the identification of six CD4+ phenotypes, six CD8+ phenotypes, one CD4+/CD8+ double-positive phenotype, one naïve CD4+ phenotype, one naïve CD8+ phenotype and one Treg phenotype.

Several studies have shown that CD4+/CD8+ double-positive T cells (DPTs) are more than just a developmental stage (33). At the single-cell level, surprisingly, the viSNE map showed that in initial GBMs, DPTs were the major source of IL-10, IDO and TGFβ (**Figure 4D**). Importantly, the DPTs at the tumor sites expressed higher levels of PD-1, LAG-3, and TIM-3 than the CD4+ T cells, CD8+ T cells, Tregs, and naïve T cells (**Figure 4E**).

NK Cells Are Not Cytolytic in GBM Lesions

Strikingly, NK cell proportions were not significantly increased at the tumor site compared with those in the peripheral blood of patients. The NK cells that infiltrated into the tumor lesions expressed higher levels of CXC chemokine receptor 3 (CXCR3) ($p < 0.05$) (**Figure 4F**), a molecule reported to be required for NK cell infiltration (34), than those in peripheral blood. However, the NK cells that remained at the tumor site were no longer cytolytic, as these cells expressed lower levels of interferon-γ (IFNγ) ($p < 0.05$) (**Figure 4F**). Moreover, NK cells infiltrated into recurrent GBMs presented similar characteristics (**Supplementary Figures S2B,C**).

Recurrent GBMs Share Similar Immune Signatures With Initial GBMs

Compared to that in initial GBM tissues, the proportion of GAMs in the recurrent GBMs was decreased (59.05% vs. 27.87%, $p < 0.05$). Furthermore, the proportion of undefined CD45+ immune cells was changed (5.18% vs. 58.26%, $p < 0.05$). The undefined immune cells were regarded as CD45+ infiltrating immune cells but could not be defined as specific immune cells by the present panel which included 30 antibodies. Concluding that the number of these cells expanded is difficult because their identification was not possible. The proportions of the immunocyte subgroups (T cells, B cells, and NK cells) in the recurrent GBM samples were similar to those in the initial GBM samples (**Figure 5A** and **Supplementary Figure S3**A).

The undefined CD45+ cells in the recurrent GBMs expressed higher levels of the immune checkpoint protein IDO than those in the initial GBMs (**Figure 5B**). In the viSNE map of recurrent GBM, the undefined immune cells, which were located close to or mixed with GAMs, expressed IDO, CD56, and CD11b at levels similar to those in GAMs but expressed less HLA-DR than GAMs (**Figures 5B,C**).

Compared with the initial GBMs, the recurrent GBMs displayed similar suppressive immune changes. Exhausted T cell proportions were not significantly between the initial and recurrent GBMs (**Supplementary Figure S3**B). DPTs in the recurrent GBMs also expressed higher levels of the immune checkpoint proteins PD-1, LAG-3, and TIM-3 than the CD4+ T cell, CD8+ T cell, naïve T cell, and Treg subgroups (**Figure 5D**).

Glioma-associated microglia/macrophages in the recurrent GBM were subdivided into 13 subgroups based on the surface markers (**Figure 5E**). Similar to those in initial GBMs, one of the GAM subpopulations (rM-1) could co-express anti-tumor (TNFα) and pro-tumor (IDO) markers, while PD-L1 was also highly expressed in the recurrent GAMs (**Figures 5E,F**).

DISCUSSION

The GBM immune microenvironment plays a critical role in tumor development, progression, and prognosis. A comprehensive understanding of the intricate milieu and its interactions remains unclear, and single-cell analysis

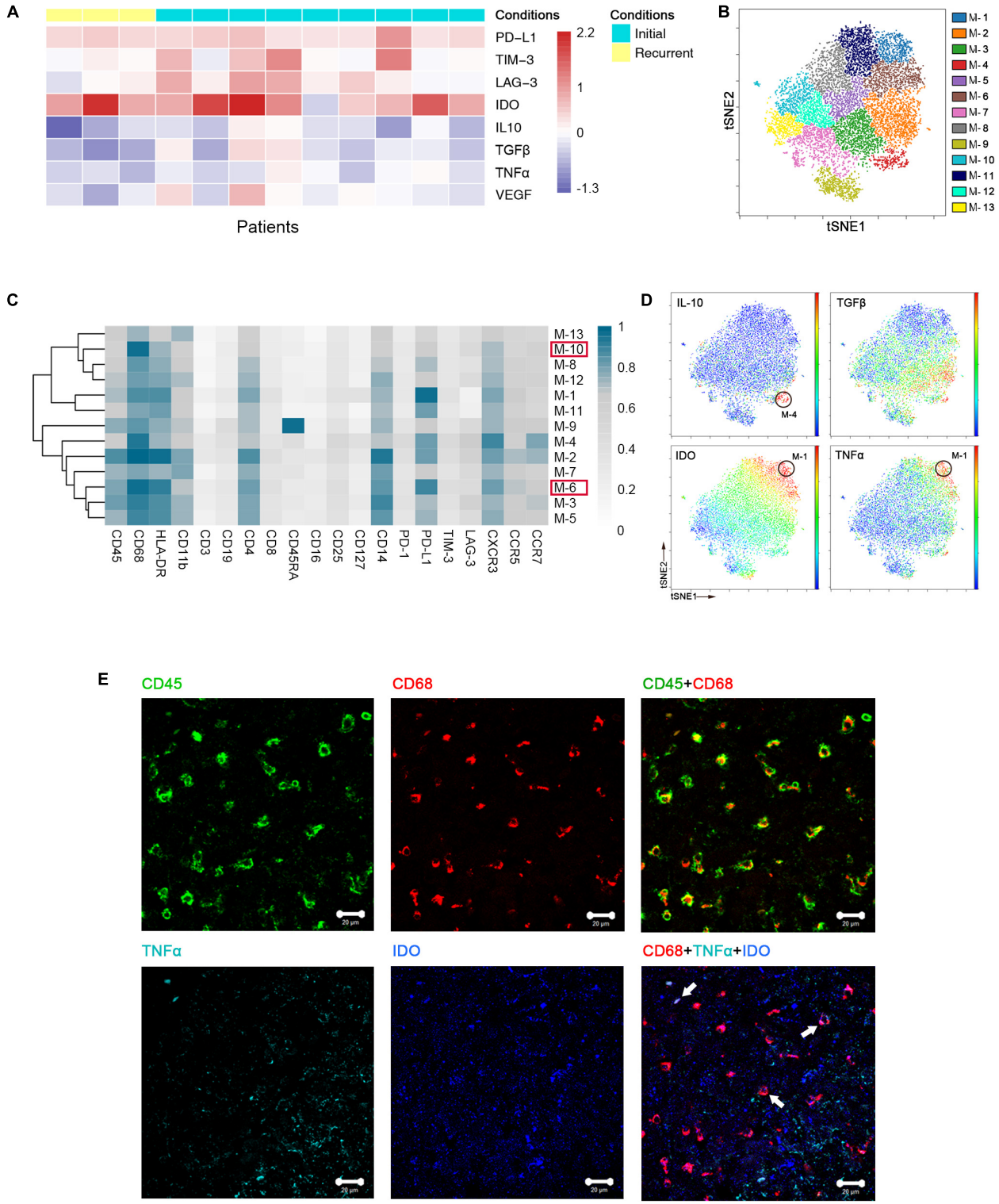

FIGURE 3 | Heterogeneous characterization of GAM phenotypes in GBM. **(A)** Heatmap showing relative marker expression levels in 3 recurrent and 9 initial GBM cases. The relative marker expression levels were determined by the ratios of the indicated marker expression levels of GAMs at the tumor site to those of mononuclear phagocytes in pPBMCs. **(B)** ViSNE map, colored by clusters, displaying 13 GAM subgroups from a representative patient. **(C)** Heatmap showing the normalized expression of the indicated markers for 13 GAM clusters identified from the representative patient. **(D)** Normalized expression of the indicated markers on the viSNE map. **(E)** Representative GBM tissue stained for CD68 (red), CD45 (green), IDO (blue), and TNFα (cyan). Polychromatic immunofluorescence of CD45 and CD68 (upper) indicated that most CD45+ immunocytes in GBM were CD68+ cells. Co-staining of CD68, IDO, and TNFα (lower) demonstrated that GAMs could co-express TNFα and IDO (Arrows).

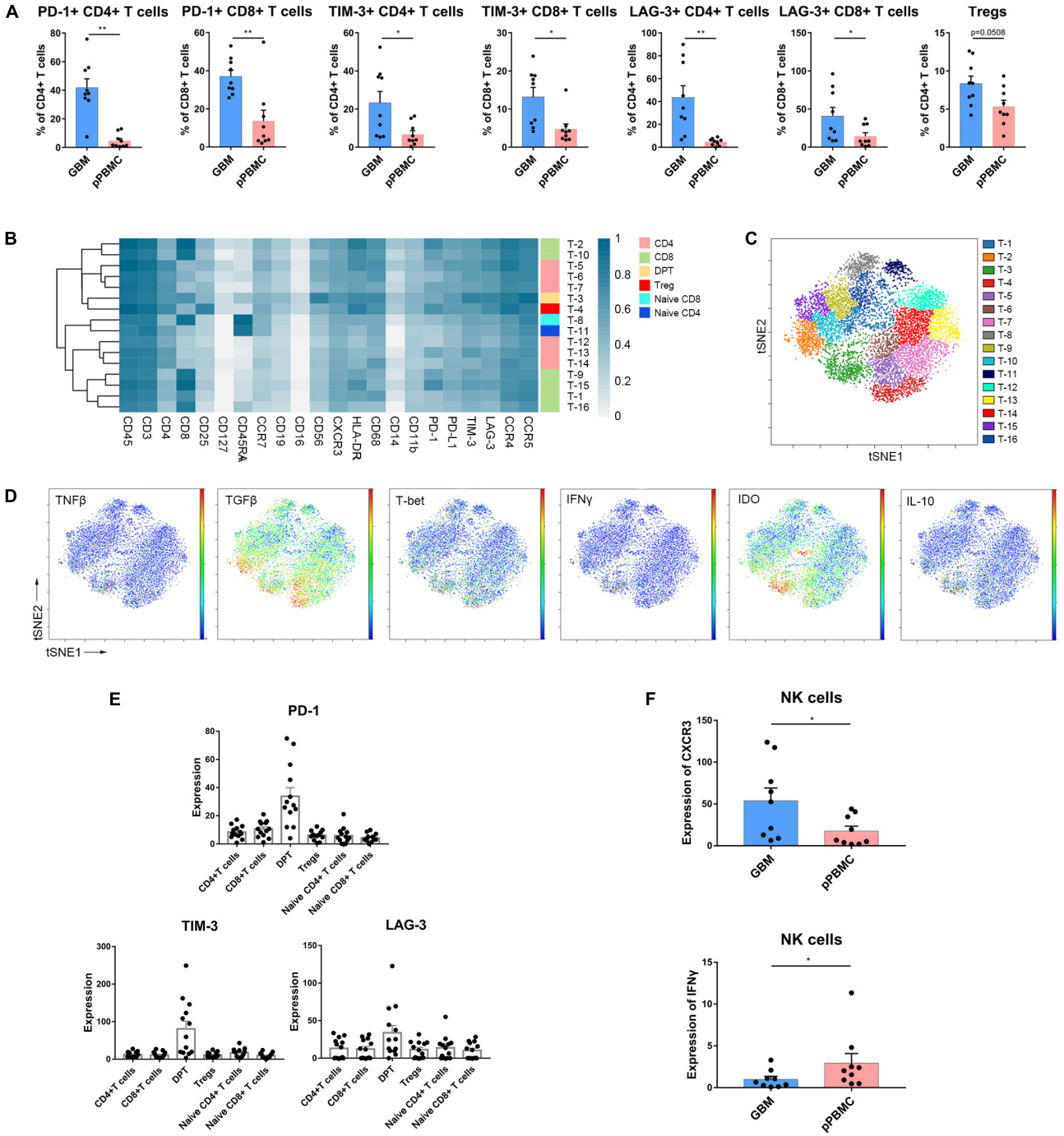

FIGURE 4 | Exhausted T cell compartment in GBM. **(A)** Bar plots showing the frequencies of T cell subgroups across tumor sites and pPBMCs from patients with initial GBM (by Wilcoxon matched-pairs signed rank test). Bar plots show the mean with SEM (*$p < 0.05$, **$p < 0.01$). **(B)** Heatmap showing the normalized expression of markers for the 16 T cell clusters identified from a representative patient. **(C)** ViSNE map, colored by clusters, displaying T cell subgroups from the representative patient. **(D)** Normalized expression of the indicated markers on tumor T cells shown by viSNE plot. **(E)** Bar pots of PD-1, LAG-3, and TIM-3 expression in T cell subsets across all patients with initial GBM. Bar plots show the mean with SEM. **(F)** Bar plots demonstrating CXCR3 and IFNγ expression in NK cells across tissue samples from initial GBM patients and the paired pPBMCs (by the Wilcoxon matched-pairs signed rank test). Bar plots show the mean with SEM (*$p < 0.05$).

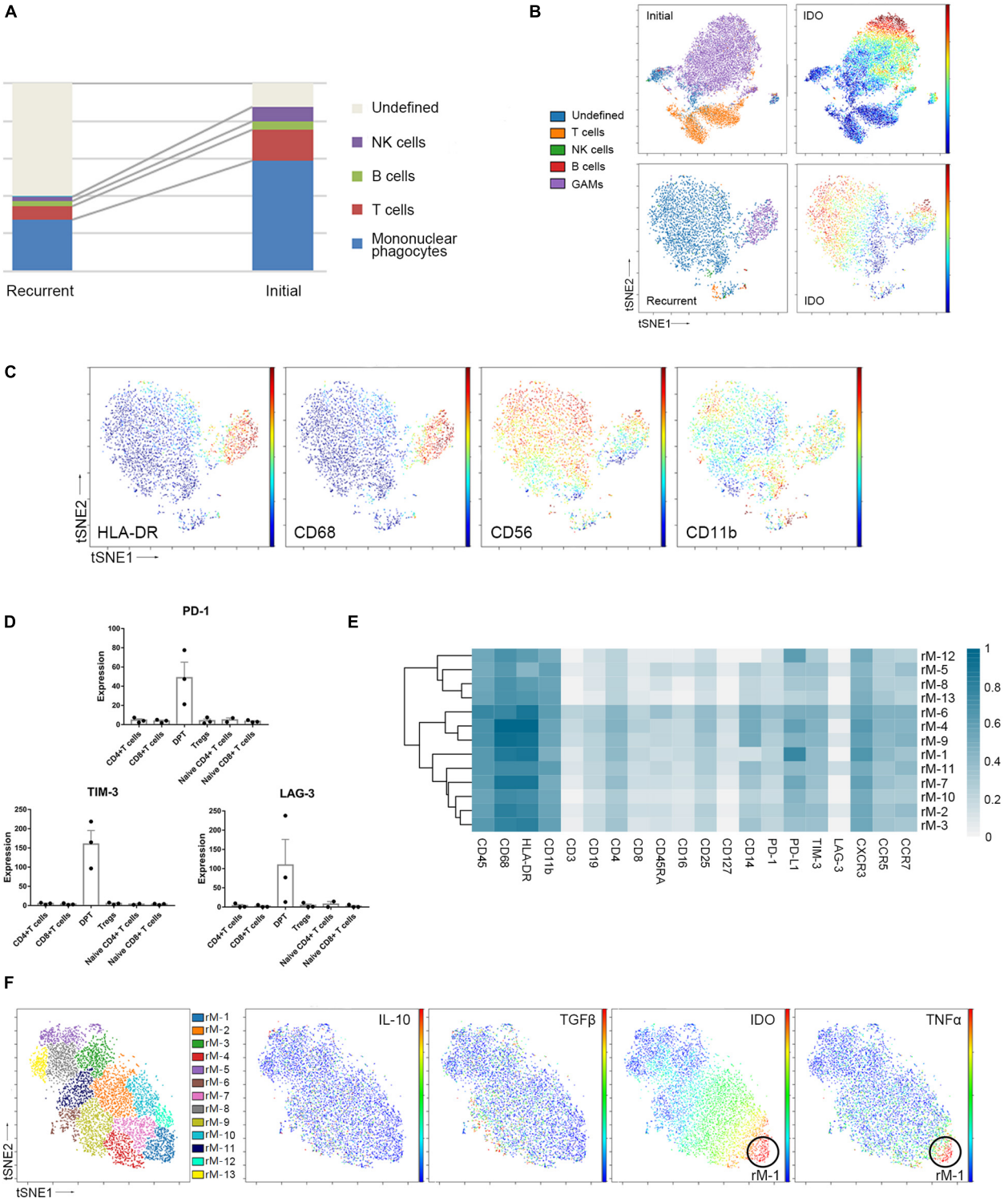

FIGURE 5 | Recurrent and initial GBMs share similar immune signatures. **(A)** The frequencies of recurrent and initial GBM immunocytes. Composition of the CD45+ compartment showing the average frequencies of major immune lineages for each tissue. **(B)** ViSNE maps of representative patients with initial and recurrent GBM, colored by immunocyte subsets (left), displaying the expression level of IDO in undefined CD45+ cells (right). **(C)** ViSNE maps from the representative recurrent patient displaying expression levels of the indicated markers in undefined CD45+ cells. **(D)** Bar pots of PD-1, LAG-3 and TIM-3 expression in T cell subsets across all patients with recurrent GBM. Bar plots show the mean with SEM. **(E)** Heatmap showing the normalized expression of markers from the panel of 13 GAM clusters identified from a representative recurrent patient. **(F)** ViSNE map, colored by clusters, displaying GAM subgroups and the normalized expression of the indicated markers from the representative recurrent patient.

is crucially needed. With CyTOF approach, we aimed to analyze infiltrating immune cells from initial and recurrent GBM surgical tissues, both of which were coupled with their paired pPBMCs. Using a panel of 30 markers, we provide a single-cell view of the complex GBM immune microenvironment. Our study confirmed that GAMs, as the dominant infiltrating immune cell population, exhibit substantial inter- and intra-tumoral heterogeneity in the GBM immune microenvironment (**Figures 2**, **3**), and increased proportions of exhausted T cell subpopulations and Tregs substantially contribute to local immune suppressive characteristics (**Figure 4**). Recurrent and initial GBMs were shown to share similar immune signatures except that the proportion of GAMs was decreased in the recurrent GBM samples compared with the initial GBM samples (**Figure 5** and **Supplementary Figure S3**).

As the largest intra-tumoral immune cell population, GAMs interact with tumor cells, express a variety of immunosuppressive cytokines and play an emerging role in tumor progression and the regulation of anti-tumor immune responses (35, 36). Taking advantage of CyTOF technology, we used surface markers to demonstrate inter- and intra-tumoral heterogeneity in the GAM population, which was also shown to play diverse roles in gliomagenesis, as immune checkpoints, immunosuppressive cytokines, TNFα and VEGF were differentially expressed in the GAMs among the patients (**Figure 3A**). Meanwhile, GAM subpopulations showed different phenotypic patterns presenting different predominant immune checkpoints and immunosuppressive cytokines (**Figures 3B,C**). Moreover, anti-tumor cytokines and immunosuppressive cytokines or immune checkpoints may be expressed simultaneously in the same GAM subpopulation (**Figures 3D,E**). Substantial diversity and specificity are characteristic of GAMs, and dissecting the heterogeneity and specific roles of each intra-tumoral GAM subset may be of critical importance for successfully targeting the immunosuppressive GAM population in a clinical setting and for the individual design of future immunotherapies (37).

CD8+ T cells that are specific for tumor-associated antigens can engage tumor cells in an antigen-specific manner, and these cells drive anti-tumor immunity by secreting effector cytokines, releasing cytotoxic molecules and inducing apoptosis in tumor cells (38). In addition to CD8+ T cells, IFNγ-expressing CD4+ T cells, and NK cells have potent anti-tumor effects in the immune microenvironment (34). The infiltration of tumors by T cells is generally interpreted as a sign of immune recognition, and there is a growing effort to reactivate dysfunctional T cells at such tumor sites (39). In our study, we found that T cell populations exhibited complex diversity based on their surface markers. The markers PD-1, LAG-3, TIM-3, and IDO are highly expressed in some T cell subgroups. Immune checkpoint (PD-1, LAG-3 and TIM-3)-positive CD4+ and CD8+ T cells cannot exert an anti-tumor effect and are regarded as nonfunctional or exhausted subsets. Although the GBM microenvironment was infiltrated with CD4+ T cells, CD8+ T cells, and NK cells, the proportions of nonfunctional immune cell subpopulations and Tregs increased, while whole T cell numbers were reduced at the tumor site. DPTs at tumor sites secrete more IL-10, IDO, and TGFβ and express higher levels of immune checkpoints PD-1, LAG-3, and TIM-3 than Tregs, CD4+ T cells and CD8+ T cells. However, they also secrete T-bet and TNFβ; thus, the DPTs in the context of the GBM microenvironment might play a dual role in the immune response. Tregs inhibit the proliferation of any cytokine-secreting effector T cells and are potent suppressors of the adaptive immune response (40). NK cells showed a high infiltration ability in GBM lesions, but they did not show a strong cytolytic ability according to their surface receptors and secreted cytokines. Our results indicated that the intrinsic capacity of intra-tumoral effector T cells and NK cells was impaired, which suggests that in addition to increasing their quantity, approaches that simultaneously promote the anti-tumor quality and eliminate the pro-tumor ability of these cells will benefit clinical efforts to reactivate intra-tumoral immune cells.

Little is known about how the microenvironment changes in recurrent GBM. Only 20–30% of recurrent GBM cases are accessible for surgical treatment (41). Even less is known about changes in the immune environment and the immunogenicity of recurrent tumors (42). Mohme et al. (43) used flow cytometry and cytokine assays to profile tumor-infiltrating lymphocytes and blood lymphocytes from GBM patients. The study showed that the tumor-infiltrating lymphocytes of recurrent GBMs exhibited restricted T cell receptor repertoire clonality and a more activated memory phenotype than those of initial GBMs (43). Using CyTOF method, the current study demonstrated that on a single-cell basis, the recurrent and initial GBMs shared similar immune signatures; however, the proportion of GAMs in the recurrent GBMs was decreased compared with that in initial GBMs (**Supplementary Figure S3**). Interestingly, in the recurrent GBM samples, the proportion of undefined CD45+ immune cells was significantly increased and overwhelmed the proportion of GAMs (**Figure 5A**). Furthermore, the undefined immune cell subset in recurrent GBM might partially functionally resemble GAMs in its presentation of strong immunosuppressive features, but more research is needed on this topic. Because most immunotherapies are first applied to recurrent GBMs, comparing the immune landscapes of initial and recurrent GBMs will help predict the efficiency of immunotherapy applications in treatment-naïve GBM patients.

Our study has some limitations. Deciphering the immune environment of GBMs and determining the association between the GBM immune microenvironment and patient prognosis requires more cases. We acknowledge that the small number of recurrence cases may result in a lack of sufficient power to identify immune microenvironment differences between initial and recurrent cases. We performed clustering analysis to study immune environment heterogeneity. We must admit that in our study, clustering in CyTOF analyses can change depending on the parameters selected, and the number of clusters is not absolute. Some of our findings should be further explored by single-cell RNA sequencing, particularly regarding the changes in undefined immune cells in recurrent GBM. Ideally, dissecting

the features of recurrent GBM should incorporate paired samples and their corresponding initial and recurrent GBM samples from a single patient.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

This research was approved by the Institutional Review Board (IRB) and Ethics Committee of Beijing Tiantan Hospital (Beijing, China). Written informed consent was obtained from all patients and healthy donors.

AUTHOR CONTRIBUTIONS

YC, JZ, DC, JaW, and SW conceived and designed the study. WF and WW analyzed and interpreted the data. HL, YJ, RH, JeW, and ZY participated in sample collection and data acquisition. All authors participated in drafting the manuscript, read and approved the final version of the manuscript, and gave their consent for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00835/full#supplementary-material

REFERENCES

1. Ricard D, Idbaih A, Ducray F, Lahutte M, Hoang-Xuan K, Delattre JY. Primary brain tumours in adults. *Lancet*. (2012) 379:1984–96. doi: 10.1016/S0140-6736(11)61346-9
2. Stupp R, Taillibert S, Kanner A, Read W, Steinberg D, Lhermitte B, et al. Effect of tumor-treating fields plus maintenance temozolomide vs maintenance temozolomide alone on survival in patients with glioblastoma: a randomized clinical trial. *JAMA*. (2017) 318:2306–16. doi: 10.1001/jama.2017.18718
3. Cheng W, Ren X, Zhang C, Cai J, Liu Y, Han S, et al. Bioinformatic profiling identifies an immune-related risk signature for glioblastoma. *Neurology*. (2016) 86:2226–34. doi: 10.1212/WNL.0000000000002770
4. Fecci PE, Mitchell DA, Whitesides JF, Xie W, Friedman AH, Archer GE, et al. Increased regulatory T-cell fraction amidst a diminished CD4 compartment explains cellular immune defects in patients with malignant glioma. *Cancer Res*. (2006) 66:3294–302. doi: 10.1158/0008-5472.CAN-05-3773
5. Alexiou GA, Vartholomatos G, Karamoutsios A, Batistatou A, Kyritsis AP, Voulgaris S. Circulating progenitor cells: a comparison of patients with glioblastoma or meningioma. *Acta Neurol Belg*. (2013) 113:7–11. doi: 10.1007/s13760-012-0097-y
6. Gieryng A, Pszczolkowska D, Walentynowicz KA, Rajan WD, Kaminska B. Immune microenvironment of gliomas. *Lab Invest*. (2017) 97:498–518. doi: 10.1038/labinvest.2017.19
7. Lohr J, Ratliff T, Huppertz A, Ge Y, Dictus C, Ahmadi R, et al. Effector T-cell infiltration positively impacts survival of glioblastoma patients and is impaired by tumor-derived TGF-beta. *Clin Cancer Res*. (2011) 17:4296–308. doi: 10.1158/1078-0432.CCR-10-2557
8. Roesch S, Rapp C, Dettling S, Herold-Mende C. When immune cells turn bad-tumor-associated microglia/macrophages in glioma. *Int J Mol Sci*. (2018) 19:E436. doi: 10.3390/ijms19020436
9. Fujimura T, Kambayashi Y, Fujisawa Y, Hidaka T, Aiba S. Tumor-associated macrophages: therapeutic targets for skin cancer. *Front Oncol*. (2018) 8:3. doi: 10.3389/fonc.2018.00003
10. Hussain SF, Yang D, Suki D, Aldape K, Grimm E, Heimberger AB. The role of human glioma-infiltrating microglia/macrophages in mediating antitumor immune responses. *Neuro Oncol*. (2006) 8:261–79. doi: 10.1215/15228517-2006-008
11. Bloch O, Crane C, Kaur R, Safaee M, Rutkowski M, Parsa A. Gliomas promote immunosuppression through induction of B7-H1 expression in tumor-associated macrophages. *Clin. Cancer Res*. (2013) 19:3165–75. doi: 10.1158/1078-0432.CCR-12-3314
12. Li R, Chen X, You Y, Wang X, Liu Y, Hu Q, et al. Comprehensive portrait of recurrent glioblastoma multiforme in molecular and clinical characteristics. *Oncotarget*. (2015) 6:30968–74. doi: 10.18632/oncotarget.5038
13. Hu H, Mu Q, Bao Z, Chen Y, Liu Y, Chen J, et al. Mutational landscape of secondary glioblastoma guides MET-targeted trial in brain tumor. *Cell*. (2018) 175:1665–78.e18. doi: 10.1016/j.cell.2018.09.038
14. Cloughesy TF, Mochizuki AY, Orpilla JR, Hugo W, Lee AH, Davidson TB, et al. Neoadjuvant anti-PD-1 immunotherapy promotes a survival benefit with intratumoral and systemic immune responses in recurrent glioblastoma. *Nat Med*. (2019) 25:477–86. doi: 10.1038/s41591-018-0337-7
15. Boussiotis VA, Charest A. Immunotherapies for malignant glioma. *Oncogene*. (2018) 37:1121–41. doi: 10.1038/s41388-017-0024-z
16. Jackson CM, Lim M, Drake CG. Immunotherapy for brain cancer: recent progress and future promise. *Clin. Cancer Res*. (2014) 20:3651–9. doi: 10.1158/1078-0432.CCR-13-2057
17. Simoni Y, Mhy C, Li S, Fehlings M, Newell EW. Mass cytometry: a powerful tool for dissecting the immune landscape. *Curr Opin Immunol*. (2018) 51:187–96. doi: 10.1016/j.coi.2018.03.023
18. Finck R, Simonds EF, Jager A, Krishnaswamy S, Sachs K, Fantl W, et al. Normalization of mass cytometry data with bead standards. *Cytometry A*. (2013) 83:483–94. doi: 10.1002/cyto.a.22271
19. Poli A, Kmiecik J, Domingues O, Hentges F, Blery M, Chekenya M, et al. NK cells in central nervous system disorders. *J Immunol*. (2013) 190:5355–62. doi: 10.4049/jimmunol.1203401
20. Ziegler-Heitbrock L, Ancuta P, Crowe S, Dalod M, Grau V, Hart DN, et al. Nomenclature of monocytes and dendritic cells in blood. *Blood*. (2010) 116:e74–80. doi: 10.1182/blood-2010-02-258558
21. Lu-Emerson C, Snuderl M, Kirkpatrick ND, Goveia J, Davidson C, Huang Y, et al. Increase in tumor-associated macrophages after antiangiogenic therapy is associated with poor survival among patients with recurrent glioblastoma. *Neuro Oncol*. (2013) 15:1079–87. doi: 10.1093/neuonc/not082
22. Lamble AJ, Dietz M, Laderas T, McWeeney S, Lind EF. Integrated functional and mass spectrometry-based flow cytometric phenotyping to describe the

immune microenvironment in acute myeloid leukemia. *J Immunol Methods*. (2018) 453:44–52. doi: 10.1016/j.jim.2017.11.010
23. Sommershof A, Aichinger H, Engler H, Adenauer H, Catani C, Boneberg EM, et al. Substantial reduction of naive and regulatory T cells following traumatic stress. *Brain Behav Immun*. (2009) 23:1117–24. doi: 10.1016/j.bbi.2009.07.003
24. Jardine L, Wiscombe S, Reynolds G, McDonald D, Fuller A, Green K, et al. Lipopolysaccharide inhalation recruits monocytes and dendritic cell subsets to the alveolar airspace. *Nat Commun*. (2019) 10:1999. doi: 10.1038/s41467-019-09913-4
25. Korin B, Ben-Shaanan TL, Schiller M, Dubovik T, Azulay-Debby H, Boshnak NT, et al. High-dimensional, single-cell characterization of the brain's immune compartment. *Nat Neurosci*. (2017) 20:1300–9. doi: 10.1038/nn.4610
26. Davis KL, Tadmor MD, Simonds EF, Levine JH, Bendall SC, Shenfeld DK, et al. viSNE enables visualization of high dimensional single-cell data and reveals phenotypic heterogeneity of leukemia. *Nat Biotechnol*. (2013) 31:545–52. doi: 10.1038/nbt.2594
27. Li D, Fu J, Du M, Zhang H, Li L, Cen J, et al. Hepatocellular carcinoma repression by TNFα-mediated synergistic lethal effect of mitosis defect-induced senescence and cell death sensitization. *Hepatology (Baltimore, MD)*. (2016) 64:1105–20. doi: 10.1002/hep.28637
28. Li W, Yang L, He Q, Hu C, Zhu L, Ma X, et al. A homeostatic Arid1a-dependent permissive chromatin state licenses hepatocyte responsiveness to liver-injury-associated YAP signaling. *Cell Stem Cell*. (2019) 25:54–68.e5. doi: 10.1016/j.stem.2019.06.008
29. Kratochvill F, Murray P, Natoli G. Macrophages and cancer: from mechanisms to therapeutic implications. *Trends Immunol*. (2015) 36:229–39. doi: 10.1016/j.it.2015.02.004
30. Gabrusiewicz K, Li X, Wei J, Hashimoto Y, Marisetty AL, Ott M, et al. Glioblastoma stem cell-derived exosomes induce M2 macrophages and PD-L1 expression on human monocytes. *Oncoimmunology*. (2018) 7:e1412909. doi: 10.1080/2162402X.2017.1412909
31. Kurachi M. CD8(+) T cell exhaustion. *Semin Immunopathol*. (2019) 41:327–37. doi: 10.1007/s00281-019-00744-5
32. Graves M, CelliMarchett G, van Zyl B, Tang D, Vilain RE, van der Westhuizen A, et al. Monitoring patient response to pembrolizumab with peripheral blood exhaustion marker profiles. *Front Med (Lausanne)*. (2019) 6:113. doi: 10.3389/fmed.2019.00113
33. Overgaard NH, Jung JW, Steptoe RJ, Wells JW. CD4+/CD8+ double-positive T cells: more than just a developmental stage? *J Leukoc Biol*. (2015) 97:31–8. doi: 10.1189/jlb.1RU0814-382
34. Wendel M, Galani IE, Suri-Payer E, Cerwenka A. Natural killer cell accumulation in tumors is dependent on IFN-gamma and CXCR3 ligands. *Cancer Res*. (2008) 68:8437–45. doi: 10.1158/0008-5472.CAN-08-1440
35. Chen Z, Feng X, Herting CJ, Garcia VA, Nie K, Pong WW, et al. Cellular and molecular identity of tumor-associated macrophages in glioblastoma. *Cancer Res*. (2017) 77:2266–78. doi: 10.1158/0008-5472.CAN-16-2310
36. Darmanis S, Sloan SA, Croote D, Mignardi M, Chernikova S, Samghababi P, et al. Single-cell RNA-seq analysis of infiltrating neoplastic cells at the migrating front of human glioblastoma. *Cell Rep*. (2017) 21:1399–410. doi: 10.1016/j.celrep.2017.10.030
37. Poon CC, Gordon PMK, Liu K, Yang R, Sarkar S, Mirzaei R, et al. Differential microglia and macrophage profiles in human IDH-mutant and -wild type glioblastoma. *Oncotarget*. (2019) 10:3129–43. doi: 10.18632/oncotarget.26863
38. Speiser DE, Ho PC, Verdeil G. Regulatory circuits of T cell function in cancer. *Nat Rev Immunol*. (2016) 16:599–611. doi: 10.1038/nri.2016.80
39. O'Rourke DM, Nasrallah MP, Desai A, Melenhorst JJ, Mansfield K, Jjd M, et al. A single dose of peripherally infused EGFRvIII-directed CAR T cells mediates antigen loss and induces adaptive resistance in patients with recurrent glioblastoma. *Sci Transl Med*. (2017) 9:eaaa0984. doi: 10.1126/scitranslmed.aaa0984
40. Colombo MP, Piconese S. Regulatory-T-cell inhibition versus depletion: the right choice in cancer immunotherapy. *Nat Rev Cancer*. (2007) 7:880–7. doi: 10.1038/nrc2250
41. Weller M, Cloughesy T, Perry JR, Wick W. Standards of care for treatment of recurrent glioblastoma–are we there yet? *Neuro Oncol*. (2013) 15:4–27. doi: 10.1093/neuonc/nos273
42. Campos B, Olsen LR, Urup T, Poulsen HS. A comprehensive profile of recurrent glioblastoma. *Oncogene*. (2016) 35:5819–25. doi: 10.1038/onc.2016.85
43. Mohme M, Schliffke S, Maire CL, Runger A, Glau L, Mende KC, et al. Immunophenotyping of newly diagnosed and recurrent glioblastoma defines distinct immune exhaustion profiles in peripheral and tumor-infiltrating lymphocytes. *Clin Cancer Res*. (2018) 24:4187–200. doi: 10.1158/1078-0432.CCR-17-2617

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ORIGINAL RESEARCH
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Identification of Immune-Related Genes Contributing to the Development of Glioblastoma Using Weighted Gene Co-expression Network Analysis

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Background: The tumor microenvironment (TME) of human glioblastoma (GBM) exhibits considerable immune cell infiltration, and such cell types have been shown to be widely involved in the development of GBM. Here, weighted correlation network analysis (WGCNA) was performed on publicly available datasets to identify immune-related molecules that may contribute to the progression of GBM and thus be exploited as potential therapeutic targets.

Methods: WGCNA was used to identify highly correlated gene clusters in Chinese Glioma Genome Atlas glioma dataset. Immune-related genes in significant modules were subsequently validated in the Cancer Genome Atlas (TCGA) and Rembrandt databases, and impact on GBM development was examined in migration and vascular mimicry assays *in vitro* and in an orthotopic xenograft model (GL261 luciferase-GFP cells) in mice.

Results: WGCNA yielded 14 significant modules, one of which (black) contained genes involved in immune response and extracellular matrix formation. The intersection of these genes with a GO immune-related gene set yielded 47 immune-related genes, five of which exhibited increased expression and association with worse prognosis in GBM. One of these genes, *TREM1*, was highly expressed in areas of pseudopalisading cells around necrosis and associated with other proteins induced in angiogenesis/hypoxia. In macrophages induced from THP1 cells, *TREM1* expression levels were increased under hypoxic conditions and associated with markers of macrophage M2 polarization. *TREM1* siRNA knockdown in induced macrophages reduced their ability to promote migration and vascular mimicry in GBM cells *in vitro*, and treatment of mice with LP-17 peptide, which blocks TREM1, inhibited growth of GL261 orthotopic xenografts. Finally, blocking

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the cytokine receptor for CSF1 in induced macrophages also impeded their potential to promote tumor migration and vascular mimicry in GBM cells.

Conclusions: Our results demonstrated that TREM1 could be used as a novel immunotherapy target for glioma patients.

Keywords: glioblastoma, macrophages, tumor microenvironment, M2 polarization, bioinformatics, TCGA

INTRODUCTION

Glioblastoma (GBM) is one of the most deadly types of malignant solid tumor. Despite considerable effort toward the molecular understanding and treatment of the disease, the patient survival rate remains dismally low. The 5-year survival rate of 6.8% is especially low for GBM relative to all tumor types (1). A compounding problem for the incidence of GBM is the increasing longevity of the human population worldwide. In the United States, the incidence of GBM is estimated to be 3.22 per 100,000 individuals (1). However, the incidence of glioma rises rapidly with increasing age, reaching a peak incidence of 15.29 per 100,000 individuals in the elderly between the ages of 75 and 84 (1). Therefore, the development of effective treatment strategies to prevent the progression of GBM and improve the quality of life for patients is urgently needed.

In recent years, a molecular classification scheme adopted by the World Health Organization (WHO) has provided insight into the response of GBMs to current treatment strategies (2, 3). GBMs are now categorized as one of four molecular subtypes with variants in isocitrate dehydrogenase genes (IDH) generally appearing in cases that exhibit better overall survival. Although genetic changes reveal the precise molecular pathways corrupted during the development of individual GBMs, the biology of the brain poses additional challenges for treatment; it is a critical organ with an extremely rich blood supply, a complete blood-brain barrier (BBB), and a parenchyma lacking immune cells. Such features constitute the tumor microenvironment (TME) of GBM, which is increasingly becoming a therapeutic target of interest, in part due to the role immune cells play in tumor development.

In the last decade, checkpoint blockade immunotherapy has shown remarkable success in treating a variety of tumors, including advanced melanoma (4), non-small-cell lung cancer (NSCLC) (5), and Hodgkin's lymphoma (6). A series of clinical trials investigating the efficacy of checkpoint inhibitors in GBM showed that only a small subset of patients (8%) demonstrated objective responses (7). One possible explanation for this result is the lower tumor mutational burden of GBM (8) and the low level of T-cell infiltration (9). However, a more rigorous understanding of the biology of other immune cell types, such as tumor-associated macrophages (TAMs), which promote or inhibit the progression of GBM through the secretion of multiple cytokines (10, 11), might also provide new therapeutic targets of interest.

In this study, we performed weighted correlation network analysis (WGCNA), which identifies/generates highly correlated gene clusters by summarizing such clusters using module clustering or the identification of intramodular hub genes (12), to specifically identify immune-related genes associated with the development and/or prognosis of GBM from publicly available datasets, namely The Cancer Genome Atlas (TCGA), the Chinese Glioma Genome Atlas (CGGA), and Rembrandt. The analysis yielded a gene called *TREM1*. Inhibition of *TREM1* reduced migration and vascular mimicry *in vitro*, and tumor growth *in vivo*, possibly through decreased release of the cytokine CSF1. Thus, targeting TREM1 might be of therapeutic value in the treatment of human GBM.

MATERIALS AND METHODS

Microarray Data

Microarray data for human gliomas were downloaded from The Cancer Genome Atlas (TCGA, http://cancergenome.nih.gov/abouttcga) (13), the Chinese Glioma Genome Atlas (CGGA, http://www.cgga.org.cn/) (14), and the Rembrandt brain cancer dataset (http://www.betastasis.com/glioma/rembrandt/) (15). These datasets include whole-genome expression profiles and corresponding clinical information of the patients.

The CGGA expression dataset was collected using the Agilent Whole Human Genome Microarray platform and includes data from a total of 301 glioma samples. All probe sets were mapped to gene symbols according to the probe annotation files of the GPL4133 platform, and gene expression values were $\log_2$ transformed. The TCGA and Rembrandt databases have been previously described.

Weighted Gene Co-expression Networks and Their Modules

WGCNA is a freely accessible R software package (version R 3.4.3) developed for the construction of weighted gene co-expression networks. Rather than focusing only on differential gene expression, WGCNA uses information from the genome to identify a set of genes of interest and converts the associations of thousands of genes with phenotypes into associations between several gene sets and phenotypes, eliminating the problem of multiple hypothesis test correction. The parameter β is a soft-thresholding power parameter that strengthens strong correlations and penalizes weak correlations between genes. A hierarchical clustering tree was constructed, with different branches of the tree representing different gene modules. The adjacency matrix was transformed into a topological overlap matrix (TOM). Genes were divided into different gene modules based on the TOM-based dissimilarity measure.

Module Genetic Analysis and Sub-network Analysis

Gene ontology (GO) (16) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (17) analyses were used to explore the biological function of the module with the highest correlation with clinical traits and to screen hub genes. The STRING database (https://string-db.org/) (18) is currently the largest database of protein interactions. All genes in the selected module were first analyzed by GO and KEGG pathway enrichment analysis using DAVID web tools (https://david.ncifcrf.gov/home.jsp) (19). A plug-in for Cytoscape (20), MCODE (21), determines the hub gene and extracts sub-networks based on the degree of connectivity of genes to surrounding genes in the network. Hub genes were defined as those with gene significance (GS) > 0.3 and module membership (MM) > 0.8.

Cell Culture and Induction of THP-1 Cell Differentiation

Human GBM cell lines U87MG and LN229 and the mouse GBM cell line GL261 were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China). The human monocyte leukemia cell line (THP-1) was a kind gift from Professor Yuan Guo, Department of General Medicine, Shandong University. U87MG, LN229, GL261, and THP1 were cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific; Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific). THP-1 cells were treated with 200 nM phorbol-12-myristate-13-acetate (PMA; Sigma-Aldrich; St. Louis, MO, USA) for 24 h to allow for differentiation into macrophages in six-well plates. All cells were maintained at 37°C in a cell incubator containing 5% CO_2.

Gene Silencing

RNA interference (RNAi) technology was used to knock down the expression of target genes. Small interfering RNAs (siRNA) were synthesized (GenePharma; Shanghai, China) and transfected into cells using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's protocol. Knockdown efficiency was evaluated 48 h after transfection using RT-qPCR and Western blotting. Sequences of the siRNA *(n = 2)* that generated efficient knockdown are the following:

si-*TREM1* 1# 5′-GGAUCAUACUAGAAGACUATT-3′; si-*TREM1* 2# 5′-GGUCAUUUGUACCCUAGGCTT-3′; si-Control: 5′-UUCUCCGAACGUGUCACGUTT-3′.

Real-Time Quantitative PCR (RT-qPCR)

Total RNA was isolated from GBM cells using the RNA-Quick Purification Kit (Shanghai YiShan Biotechnology; Shanghai, China) according to the manufacturer's protocol. Reverse transcription was conducted using the ReverTra Ace qPCR RT Master Mix Kit (FSQ-101, TOYOBO; Osaka, Japan), and cDNA was used as the template in real-time fluorescence quantification. RT-qPCR was performed with the hot start reaction mix SYBR Green Master (Roche; Basel, Switzerland) on a Real-Time PCR Detection System (Roche 480II). Independent experiments were conducted in triplicate, and ACTB served as an internal control. The following primers were used:

TREM-1: F 5′-TTTGTTTCCCAGTCTGTGTGC-3′, R 5′-TCCCCTATTCTCCATCACCACT-3′; ACTB: F 5′-CATG TACGTTGCTATCCAGGC-3′, R 5′- CTCCTTAATGTCACGC ACGAT-3′; CD206: F 5′-CGAAATGGGTTCCTCTCTGGT-3′, R 5′-TTTATCCACAGCCACGTCCC-3′; CD163: F 5′-GTAG TCTGCTCAAGATACACAGAA-3′, R 5′-GCGTTTTGAGCT CCACTCTG-3′; IL1B: F 5′-TGATGGCTTATTACAGTGGA-3′, R 5′-GGTCGGAGATTCGTAGCTGG-3′; CSF1: F 5′-CTCC AGCCAAGATGTGGTGA-3′, R 5′-TCAGAGTCCTCCCAGG TCAA-3′; CSF2: F 5′-AGCCCTGGGAGCATGTGAAT-3′, R 5′-GCAGCAGTGTCTCTACTCAGG-3′; IL6 F 5′-CCTG AACCTTCCAAAGATGGC-3′, R 5′-TTCACCAGGCAA GTCTCCTCA-3′; CXCL: F 5′-TGTGAAGGTGCAGTTT TGCC-3′, R 5′-GGGGTGGAAAGGTTTGGAGT-3′; TGF-α: F 5′-GTTGTAGCAAACCCTCAAGCTG-3′, R 5′-GAGGTACA GGCCTCTGATG-3′; VEGFA: F 5′-AAAACACAGACTCGCG TTGC-3′, R 5′-CCTCGGCTTGTCACATCTGC-3′.

Western Blotting

Treated cell samples were lysed 30 min in RIPA buffer (Thermo Fisher Scientific) supplemented with the protease inhibitor phenylmethanesulfonyl fluoride (PMSF, Beyotime Biotechnology, Shanghai, China). Protein lysates were separated with 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (0.22 μm, Merck Millipore; Darmstadt, Germany). Membranes were blocked at room temperature for 1 h in Tris-buffered saline with Tween-20 (TBST;10 mM Tris, 150 mM NaCl, and 0.1% Tween 20) containing 5% skim milk powder (Beyotime) and incubated overnight with primary antibody at 4°C, followed the next day by incubation with a secondary antibody conjugated to horseradish peroxidase (HRP) reconstituted in antibody dilution buffer (dilution 1: 5000; Beyotime) for 1 h at room temperature. Specific proteins were visualized with enhanced chemiluminescence (ECL, Millipore; Bedford, MA, USA) according to the manufacturer's protocol. The following primary antibodies were used: rabbit anti-TREM1 (PA5-95477, Thermo Fisher Scientific); rabbit anti-ACTB (20536-1-AP, Proteintech Group, Inc.; Wuhan, China).

Cell Migration Assay

Transwell assays were performed in Transwell chambers (8 μm; Corning Costar; Corning, NY, USA). Cells were cultured in complete medium and supernatant with corresponding treatments (volume ratio: 1:1) for 72 h. Cells (2×10^4) in DMEM medium (200 μL) were then seeded in the top chamber. The lower chamber was filled with medium (600 μL) containing 30% FBS. The chambers were incubated for 24 h. Cells that migrated to the lower surface were fixed with 4% paraformaldehyde (Solarbio; Beijing, China), stained with crystal violet (Solarbio) for 15 min, and counted under bright field microscopy (Leica DMi8; Leica Microsystems, Wetzlar, Germany). Images were acquired from 5 random fields in each well.

Vasculogenic Mimicry (VM) Formation Assay

The VM formation assay was performed as described previously. Briefly, 96-well tissue culture plates were coated with Matrigel (0.1 mL/well; Corning; Bedford, MA, USA) and allowed to polymerize for 0.5 h at 37°C. Cells were cultured in complete medium and supernatant with corresponding treatments (volume ratio:1:1) for 72 h. Cells were resuspended, and 100 μL of suspension was seeded onto Matrigel at 2×10^5 cells/mL and subsequently incubated without serum in 5% CO_2 at 37°C for 6 h. Cultures were photographed using a Leica microscope (Leica DMi8; Leica Microsystems, Wetzlar, Germany).

Orthotopic Xenograft Model

GL261 cells infected with lentivirus expressing luciferase-GFP cells (3×10^5; OBiO Technology; Shanghai, China) were stereotactically implanted into the brains of 6-week-old C57BL/6 mice. After 7 days, tumor size was determined, and animals were divided into the following two groups: Control group, $n = 6$, and LP-17 group, $n = 6$. Mice were administered 50 μg of diluted control peptide (TDSRCVIGLYHPPLQVY) or 50 μg of LP-17 (LQVTDSGLYRCVIYHPP), respectively, by intravenous injection every day (GL Biochem; Shanghai, China). Tumor volume was monitored using bioluminescence imaging (PerkinElmer IVIS Spectrum; Waltham, MA, USA). At the end of the experiment, tumors were dissected and frozen in liquid nitrogen or fixed in formalin for further analysis.

Immunohistochemistry (IHC)

Tumors were removed from sacrificed mice, fixed in 4% paraformaldehyde and embedded in paraffin. Paraffin-embedded samples were sectioned (4 μm) and fixed on glass slides. Epitope retrieval of sections was performed in 10 mmol/L citric acid buffer at pH7.2 heated in a microwave. Slides were subsequently incubated with the primary antibody (rabbit anti-CD11b, dilution 1:200, ab133357, Abcam; Cambridge, UK) at 4°C overnight followed by HRP-conjugated secondary antibody for 1 h at room temperature. Antibodies were detected using the substrate diaminobenzidine (DAB, Beyotime), and slides were counterstained with hematoxylin (Beyotime). Staining degree (scores of 0: negative, 1: light yellow, 2: light brown, and 3: dark brown) and positive ratio (scores of 1: 0–25%, 2: 26–50%, 3: 51–75%, and 4: 76–100%) were used as scoring methods for statistical analysis.

Periodic Acid-Schiff (PAS) Stain

Briefly, slides were deparaffinized, hydrated in distilled water, immersed in PAS solution for 5 min, rinsed 4 times, incubated in Schiff's Solution for 15 min and counterstained with hematoxylin for 2–3 min (Solarbio).

Immunofluorescence Staining

Tissue slices or VM cells were fixed with 4% paraformaldehyde at 4°C for 15 min and incubated in 0.3% Triton X-100 for 15 min. After blocking with 5% goat serum for 30 min, tissue slices or VM cells were incubated with corresponding primary antibodies against TREM1 (1:200), CD11b (1:200), CD68 (1:200), and VEGFR2 (1:200) at 4°C overnight and then incubated with Alexa Fluor 488-conjugated or Alexa Fluor 594-conjugated secondary antibodies (Beyotime) for 2 h. DAPI (Beyotime) was used to stain the nuclei. The immunofluorescent signals were detected by fluorescence microscopy (Leica DMi8; Leica Microsystems, Wetzlar, Germany). The following primary antibodies were used: rabbit anti-CD68 (ab213363, Abcam; Cambridge, UK); rabbit anti-VEGFR2 (26415-1-AP, Proteintech Group, Inc.; Wuhan, China).

Plotting and Statistical Analysis

Each assay was performed at least three times independently. Data analysis was performed using GraphPad Prism 8 software (San Diego, CA, USA). Data were reported as the mean ± SD. The statistical significance of experimental data was evaluated using the Student's *t*-test between two groups and one-way analysis of variance (ANOVA) among more groups. A Chi-square test was used to determine the association between *TREM1* expression and pathological characteristics. The Pearson correlation was applied to evaluate the linear relationship between gene expression levels. In addition, for microarray data in a common database, non-parametric tests were used to detect differences. A log-rank model was used for single-variate survival analysis, whereas a COX regression model was used for multivariate survival analysis. Differences were considered to be significant at the following *p*-values: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

RESULTS

WGCNA Identifies Key Modules in Glioma Expression Data

To find the key modules associated with GBM clinical traits, we performed WGCNA on the CGGA glioma dataset. Clinical sample information includes gender, age, TCGA-subtype, WHO grade, progression-free survival time (PFS), and overall survival (OS). All samples were first clustered using the FlashClust package, and "150" was chosen as the criterion to exclude atypical samples (**Figure 1A**). The soft-thresholding power was set as "5," and a topological matrix with non-scale features (scale-free $R^2 = 0.84$) was obtained (**Figures 1A,B**). The clustering dendrograms of the sample matched the strip chart for clinical features (**Figure 1B**). The topological overlapping heat map depicted the TOM including all genes (**Figure 1C**). The topology matrix was clustered using the dissimilarity between genes and then divided into different modules. We eventually identified 14 modules (**Figure 1D**; non-clustering genes shown in gray). A module and sample trait correlation heatmap was created based on correlations between module eigengenes and clinical traits (**Figure 1D**). Finally, an eigengene adjacency heatmap showed the correlation between different modules (**Figure 1C**). These steps represent the general flow of analysis of expression datasets using WGCNA. Black, green, magenta, tan, and pink modules emerged as the most significant from the analysis.

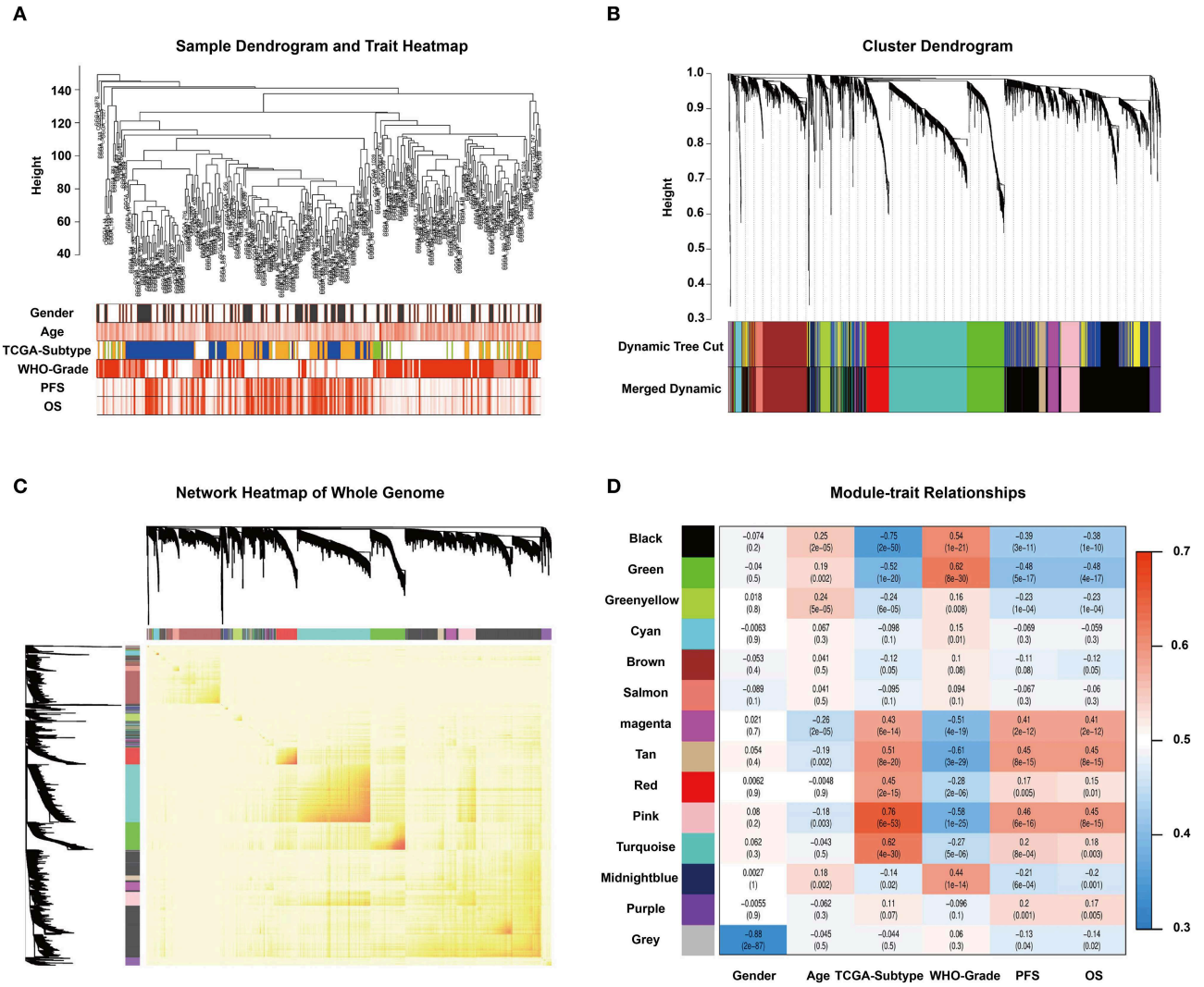

FIGURE 1 | WGCNA and identification of significant modules. **(A)** Sample dendrogram and clinical trait heatmap. The cutoff value of the sample dendrogram is set to 150 to exclude samples with high variability. The samples are clustered according to clinical features (gender, age, TCGA-subtype, WHO grade, PFS, and OS). Gender: white represents male, gray represents female. Age, PFS, and OS: color depth is positively correlated with value. TCGA-subtype: blue represents neural subtype, yellow represents proneural subtype, green represents classical, and white represents mesenchymal. White, pink, and red represent WHO grades II, III, and IV, respectively. **(B)** Cluster dendrogram obtained from transcriptomic data of glioma in the CGGA database with average hierarchical linkage clustering. The color row underneath the dendrogram shows the module assignment determined by Dynamic Tree Cut and Merged Dynamic. Comparison between Dynamic Tree Cut and Merged Dynamic shows that the black and dark blue modules merge into a new black module, which means that the expression characteristics of the genes in the black module are more different. **(C)** Network heatmap of the whole genome. In the heatmap, each row and column corresponds to a gene; light color denotes low topological overlap, and progressively darker red denotes higher topological overlap. Darker squares along the diagonal correspond to modules. The gene dendrogram and module assignment are shown along the left and top. **(D)** Module–trait relationship heatmap. Hierarchical clustering of module eigengenes that summarize the modules found in the clustering analysis. The row represents the module, and the column represents the trait. The values in the box indicate the correlation and *p*-value.

Analysis of Black Module Genes

From the module–trait correlations heatmap, we identified the black module as highly correlated with clinical traits (correlation coefficient = 0.64, $P = 1.1E\text{-}175$; **Figure 2A**). The black module, containing a total of 1,518 genes (**Figure 2A**), was positively correlated with the pathological grade of glioma and negatively correlated with PFS, OS, and TCGA subtypes. To reveal the potential biological functions of the genes within the black module, we conducted GO and KEGG analyses. The GO terms emerging as the most significant were biological process (BP), cellular component (CC), and molecular function (MF) (**Figure 2B**). GO analysis indicated that genes within the black module were mainly involved in immune response, inflammatory response, angiogenesis, cell surface receptor signaling, and

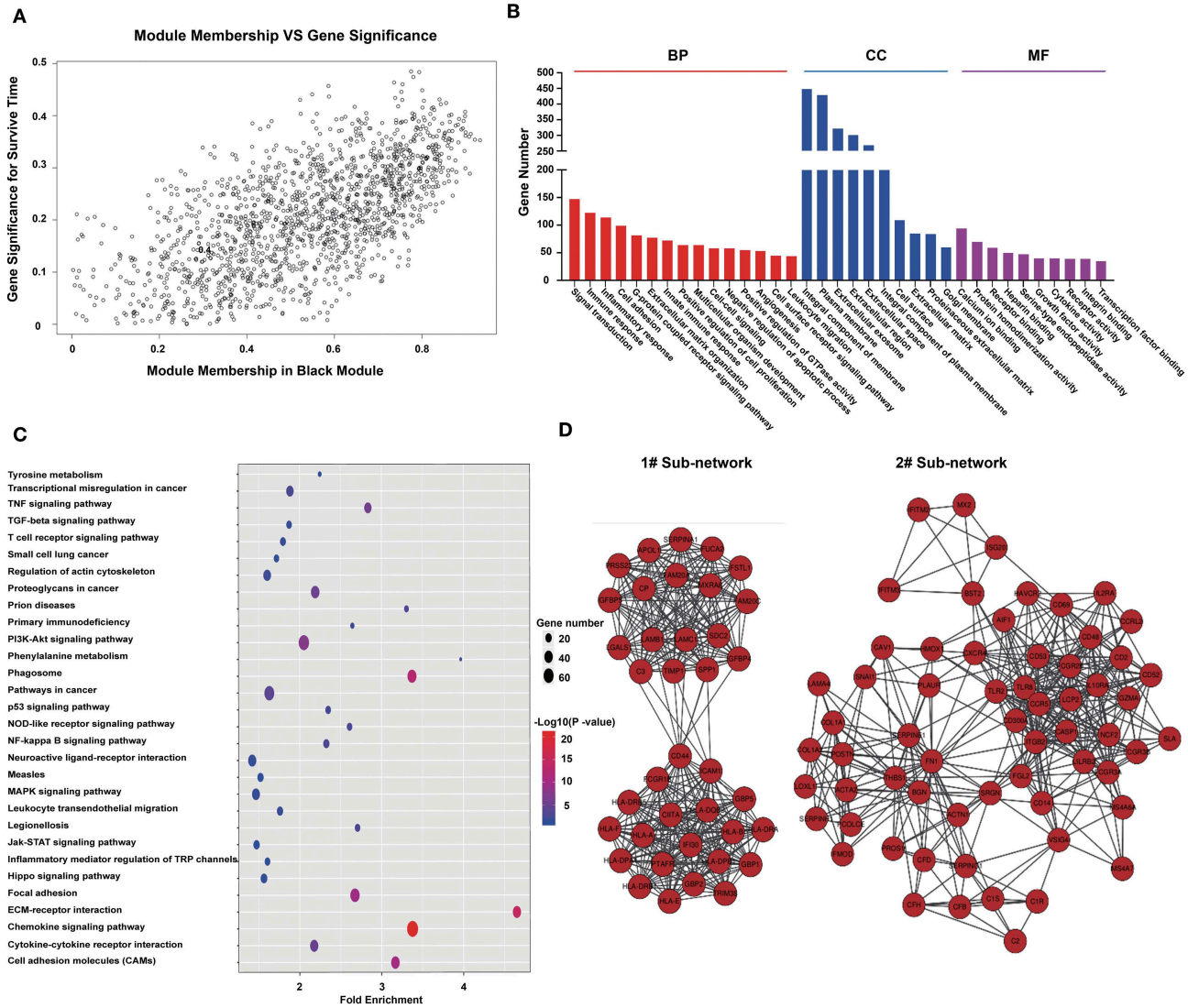

FIGURE 2 | Genes in the black module are involved in immune responses and the formation of extracellular matrix. **(A)** Scatterplot of gene significance for survival time (y-axis) vs. module membership (x-axis) in the black module. The correlation coefficient between them is 0.64 (*p*-value:1.1e-175). **(B)** Histogram of GO analysis for all genes in the black module. MF, CC, and BF respectively represent molecular function, biological process, and cellular component. The FDR values of the corresponding MF items are: 5.71E-07, 1.06E-36, 9.25E-36, 0.089116, 1.8E-18, 0.007131, 6.21E-32, 4.83E-07, 0.007879, 0.001471, 9.37E-11, 2.63E-11, 2.43E-10, 0.002713, and 0.000293; CC items: 1.25E-21, 3.71E-32, 1.72E-07, 8.90E-24, 4.61E-07, 4.83E-14, 6.92E-06, 5.71E-17, 3.35E-11, and 2.63E-06; BF items: 5.64E-05, 7.84E-04, 7.04E-06, 1.82E-15, 1.51E-05, 30E-08, 1.75E-06, 6.34E-04, 0.001701, and 2.39E-09. **(C)** Bubble chart of KEGG pathway analysis for all genes in the black module. **(D)** Protein–protein network constructed by all genes in the black module. Genes were divided into some subnets based on degree of connection. The figure shows the two largest subnetworks. Most of the genes in these subnets are involved in immune responses and the formation of extracellular matrix.

leukocyte migration. KEGG pathway analysis revealed that these genes were involved in cytokine–cytokine interaction, ECM–receptor interaction, PI3K-Akt signaling, cell adhesion, and phagosomes (**Figure 2C**). All genes in the black module were input into String to construct a protein–protein interaction network (**Supplementary Figure 2B**) and then divided into several sub-networks. By setting the module membership (MM) to > 0.8 and the gene significance (GS) to > 0.3, we selected 15 hub genes from the black module: *LAMC1, LANB1, CIITA, SERPINE1, HLA-A, HLA-DBQ, IFI30, CD53, ITGB2, PTAFR, FAM20A, FN1, CCR5, LCP2, and CGR2B*. These core genes are mainly involved in the immune response and the formation of extracellular matrix. The two largest sub-networks are also shown (**Figure 2D**). Genes from the sub-networks are mainly involved in immune reactions, inflammatory reactions, extracellular matrix, and cell adhesion. These results led us to focus on the genes involved in the immune response in subsequent analysis.

Identification of Immune-Related Genes

The genes in the black module were sorted according to their contribution to the clinical traits of the module, and the top 153 genes were selected. The intersection of these genes with GO annotation for immune-related genes (total 148 genes) yielded a list of 47 genes that are functionally involved in immunoreactions and closely related to histopathological grade, TCGA subtype, WHO grade, PFS, and OS (**Figure 3A**). To validate this list of 47 genes, we generated a heatmap using expression profiles and clinical data from the TCGA database (**Figure 3B**). These genes generally exhibited higher expression levels in *IDH* wild-type and mesenchymal molecular subtype GBMs and lower expression levels in low-grade gliomas, astrocytomas, *IDH* mutated GBMs, and neural and pro-neural molecular subtype GBMs (**Figure 3B** and **Supplementary Figures 3A–C**).

Identification of *TREM1* as a Candidate Biomarker for Poor Prognosis

We further characterized these 47 genes based on mRNA expression, survival prognosis, and protein expression using the TCGA GBM data. Immunohistochemistry images of antibody staining in the human protein atlas database were used to verify the protein expression of these genes (https://www.proteinatlas.org/humanproteome/pathology) (22). A group of candidate genes was chosen based on the following three characteristics: 1. mRNA expression levels were higher in GBMs than in non-tumor tissues; 2. high expression of these genes was related to worse prognosis; 3. positive IHC staining increased with increasing pathological grade of glioma (**Supplementary Figures 4A–E**). Genes with these characteristics included *TREM1* (**Figures 4A–C**), *GBP2* (**Figures 4D–F**), *IFITM2* (**Figures 4G–I**), *CIITA* (**Figures 4J–L**), and *TYROBP* (**Figures 4M–O**). Due to the fact that *TREM1* appeared prominently in the black module and little is known concerning a potential role in GBM, we mainly focused on *TREM1* for further analysis in this study.

TREM1 Is Associated With Poor Prognosis in All Databases

To validate *TREM1* as a gene associated with prognosis, we examined molecular features of the gene in samples in the Rembrandt, TCGA, and CGGA databases. In all three databases, the mRNA expression of *TREM1* gradually increased with increasing WHO grade (**Figures 5A,B**). Furthermore, GBM $TREM1^{high}$ signified a worse prognosis than GBM $TREM1^{low}$ ($P = 0.0475$; **Figure 5C**) using the Rembrandt database. Non-G-CIMP-positive and mesenchymal molecular GBM subtype tumors expressed higher levels of *TREM1*. Many studies have demonstrated that non-G-CIMP-positive and mesenchymal molecular subtypes correlate with worse prognosis (**Figures 5D,E**). This result therefore indicated that expression levels of *TREM1*, the G-CIMP state, and GBM molecular subtypes may be linked. The analysis of CGGA data also verified that the expression of *TREM1* was related to gender, age, WHO grade, molecular subtype, and progression-free survival time (PFS) (**Table 1**). Nomograms were constructed to predict the OS of an individual patient based on a Cox proportional hazards regression model (**Supplementary Table 1**).

TABLE 1 | Correlation of *TREM1* expression in human glioma patients with clinicopathological features.

Variable		High TREMI expression	Low TREMI express/On	Chi-square values	*P*-value
Age	≥45	67	58	15.38	<0.0001
	<45	54	120		
Gender	Male	83	97	6.510	0.0107
	Female	38	83		
WHO	II	18	98	52.61	<0.0001
Grade	III	24	33		
	IV	76	48		
TCGA-	Classical	8	15	62.33	<0.0001
subtype	Mesenchymal	86	25		
	Proneural	19	67		
IDH1	WT	95	28	17.13	<0.0001
	Mutation	3	10		
PFS	≥643	34	114	33.27	<0.0001
	<643	82	65		

P-values were determined by chi-square and Fisher's exact tests.

We furthermore examined TREM1 protein expression in images of immunostained GBM samples stored in the Ivy Glioblastoma Atlas Project, which is a foundational resource for exploring the anatomic and genetic basis of GBM at the cellular and molecular levels (23). The areas of GBM samples examined (based on H&E staining) were the leading edge, infiltrating tumor, cellular tumor, microvascular proliferation, and pseudopalisading cells around necrosis. Higher expression of TREM1 appeared in areas of pseudopalisading cells around necrosis than in other regions, suggesting that TREM1 may be closely linked with hypoxia (**Figure 5F**).

Analysis of reverse-phase protein array data (RPPA; a high-throughput antibody-based technique) from the TCGA GBM dataset yielded proteins significantly associated with TREM1, including IFGBP2, TGM2 VEGFR2, and NDRG1, many of which have also been linked to hypoxia (**Figures 5B,G–I**). IGFBP2 has been reported to exert an oncogenic effect by enhancing invasiveness, angiogenesis, and VM formation and as part of a negative feedback loop with HIF1α in glioma (24–26). It has also been correlated with classic immunosuppressive biomarkers in glioma, such as *CHI3L1, TNFRSF1A, LGALS1, TIMP1, VEGFA, ANXA1*, and *LGALS3* (27). *TGM2* has been reported to be highly expressed in glioma tissues and therefore a possible diagnostic marker for glioma. *TGM2* has been shown to be related to hypoxia and *HIF1α* in malignant pleural mesothelioma and gastric cancer (28). *NDRG1*, a member of the *N-myc* downregulated gene family, is involved in stress and hormone responses, cell growth, and

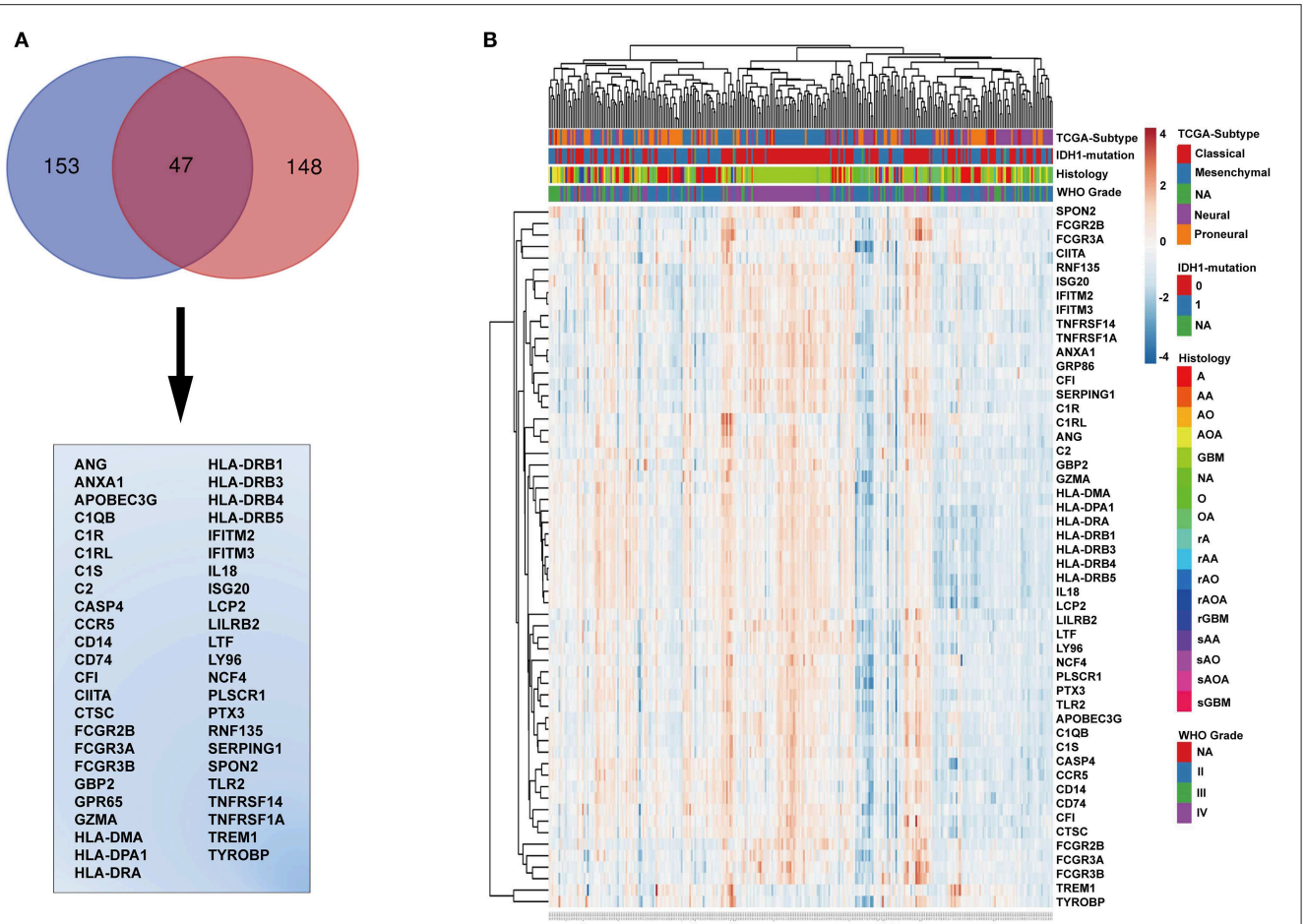

FIGURE 3 | Identification of immune-related genes. **(A)** Venn diagram displaying the intersection between the top 153 genes in the black module and the top 148 immune-related genes. The 47 genes at the intersection of these two groups are listed in the table below. **(B)** Cluster heatmap generated from expression profiles and clinical information of these 47 genes from the CGGA glioma database. IDH mutation, 0, wild type; 1, mutation; NA, unknown; A, astroglioma; AA, anaplastic astrocytoma; AO, anaplastic oligodendroglioma; AOA, anaplastic oligodendroastrocytoma; GBM, glioblastoma multiforme; O, oligodendroglioma; NA, unknown; rA, recurrent astroglioma; rAA, recurrent anaplastic astrocytoma; rAO, recurrent anaplastic oligodendroglioma; rAOA, recurrent anaplastic oligodendroastrocytoma; rGBM, recurrent glioblastoma multiforme; sAA, secondary anaplastic astrocytoma; sAO, secondary anaplastic oligodendroglioma; sAOA, secondary anaplastic oligodendroastrocytoma; sGBM, secondary glioblastoma multiforme.

differentiation, and is regarded as a mesenchymal marker in GBM (29).

As one of the receptors for VEGF, VEGFR2 is a well-recognized marker for hypoxia/angiogenesis. Actually, many clinical trials using monoclonal antibodies (mAB) against the protein have been carried out in an effort to block tumor growth. However, clinical studies using bevacizumab, a humanized mAb that blocks *VEGFA* signaling, did not improve overall survival in patients with GBM (30). GBM often develops resistance to bevacizumab owing to the upregulation of alternative proangiogenic pathways and the induction of tumor cell invasion (31). Moreover, differences in angiogenic responses could originate from inter-individual GBM heterogeneity (32, 33). Although clinical results for inhibitors of VEGFR2 are inconsistent, other strategies for blocking angiogenesis might still hold promise for the treatment of GBM.

TREM1 and Glioma-Associated Macrophages

We next performed Gene Set Enrichment Analysis (GSEA) to obtain functional profiles for molecular signatures involving *TREM1* (34). The top functional profiles were associated with angiogenesis, epithelial-mesenchymal transition, hypoxia, IL6-JAK-STAT3 signaling, TNFα signaling via NF-κb, inflammatory response, IL2-STAT5 signaling, and allograft rejection (**Figures 5C,J**). These results indicated that TREM1 may be induced by hypoxia and participate in angiogenesis, tumor cell migration, and other functions. This prediction has been partially confirmed in a previous work demonstrating that TREM1 was expressed on mature dendritic cells infiltrating the inflamed hypoxic joints of children affected with juvenile idiopathic arthritis. The engagement of TREM-1 elicited DAP12-linked signaling, resulting in ERK-1, Akt, and

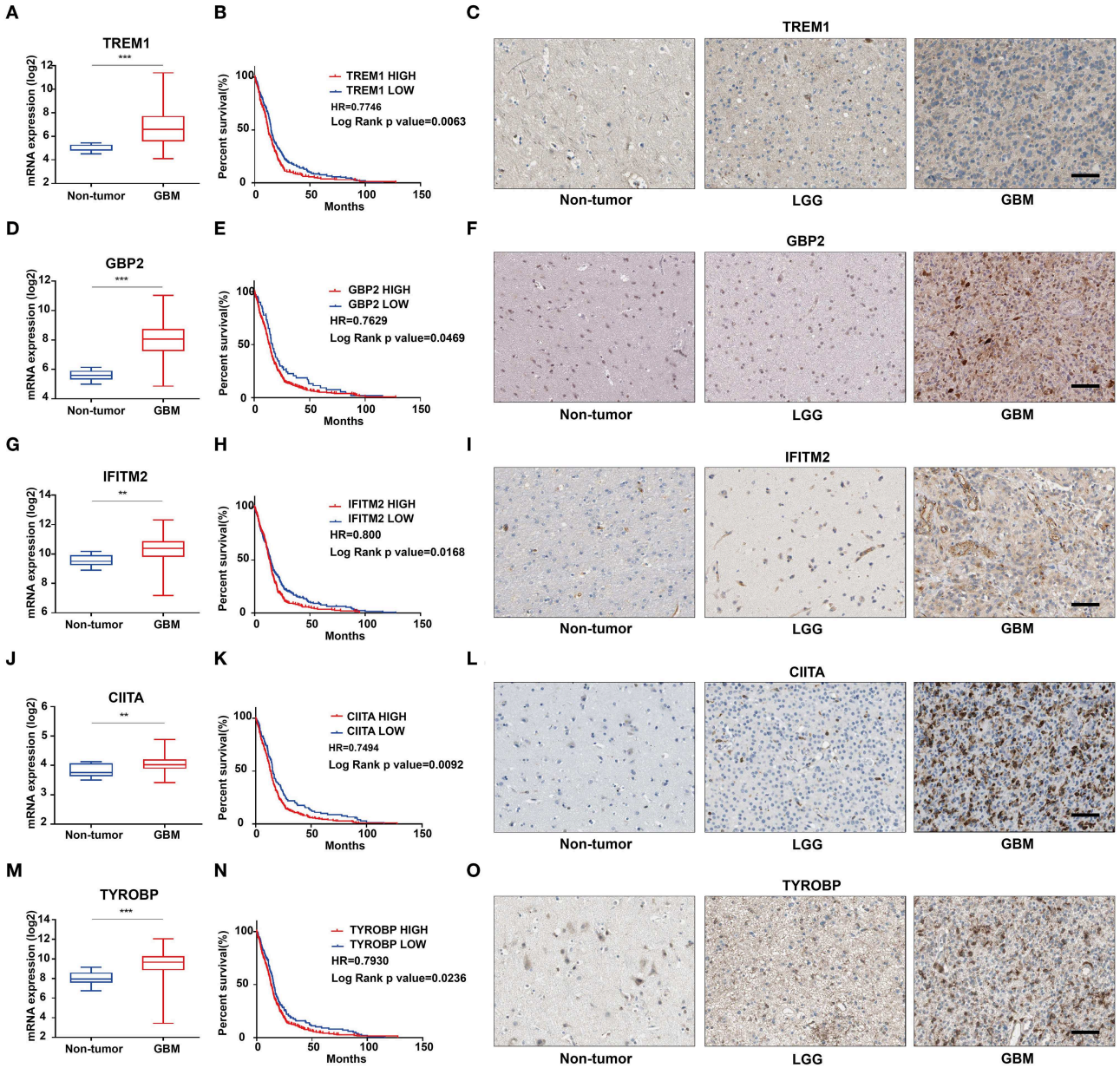

FIGURE 4 | The mRNA expression, survival analysis, and protein expression of TREM1, GBP2, IFITM2, CIITA, and TYROBP using the TCGA data. The three columns consist of images for mRNA levels of genes in non-neoplastic relative to GBM tissue samples, Kaplan-Meier survival curves for patients with $gene^{high}$ vs. $gene^{low}$ tumor samples, and IHC for each gene in non-neoplastic, LGG, and GBM tissue samples from the TCGA database. **(A)** *TREM1* mRNA levels in non-neoplastic relative to GBM tissue samples. **(B)** Kaplan–Meier survival curves for GBM patients with $TREM1^{high}$ and $TREM1^{low}$ tumors. **(C)** IHC of TREM1 in non-neoplastic tissues, LGG and GBM. **(D–F)** GBP2; **(G–I)** IFITM2; **(J–L)** CIITA; **(M–O)** TYROBP. $^{**}P < 0.01$; $^{***}P < 0.001$ compared to non-tumor tissue (scale bar: 100 μm).

IκBα phosphorylation, and pro-inflammatory cytokine and chemokine secretion (35).

Given the vital functions of immune co-stimulatory factors and checkpoint molecules in the regulation of immune processes, we performed correlation analysis to assess the relationship between *TREM1* and several well-known genes in GBM samples. *TREM1* was correlated with *CD40, PDCD1, TNFRSF4, TNFRSF9, CD70*, and *CD86* (**Figure 5K**). These results corroborated a previous study demonstrating that *TREM1* was mainly expressed in tumor-associated macrophages and induced by hypoxia, thus participating in angiogenic and inflammatory responses. Importantly, TREM1 expression was not detectable in GBM cell lines under normoxia or hypoxia, indicating that TREM1 expression originated from cell types other than tumor cells

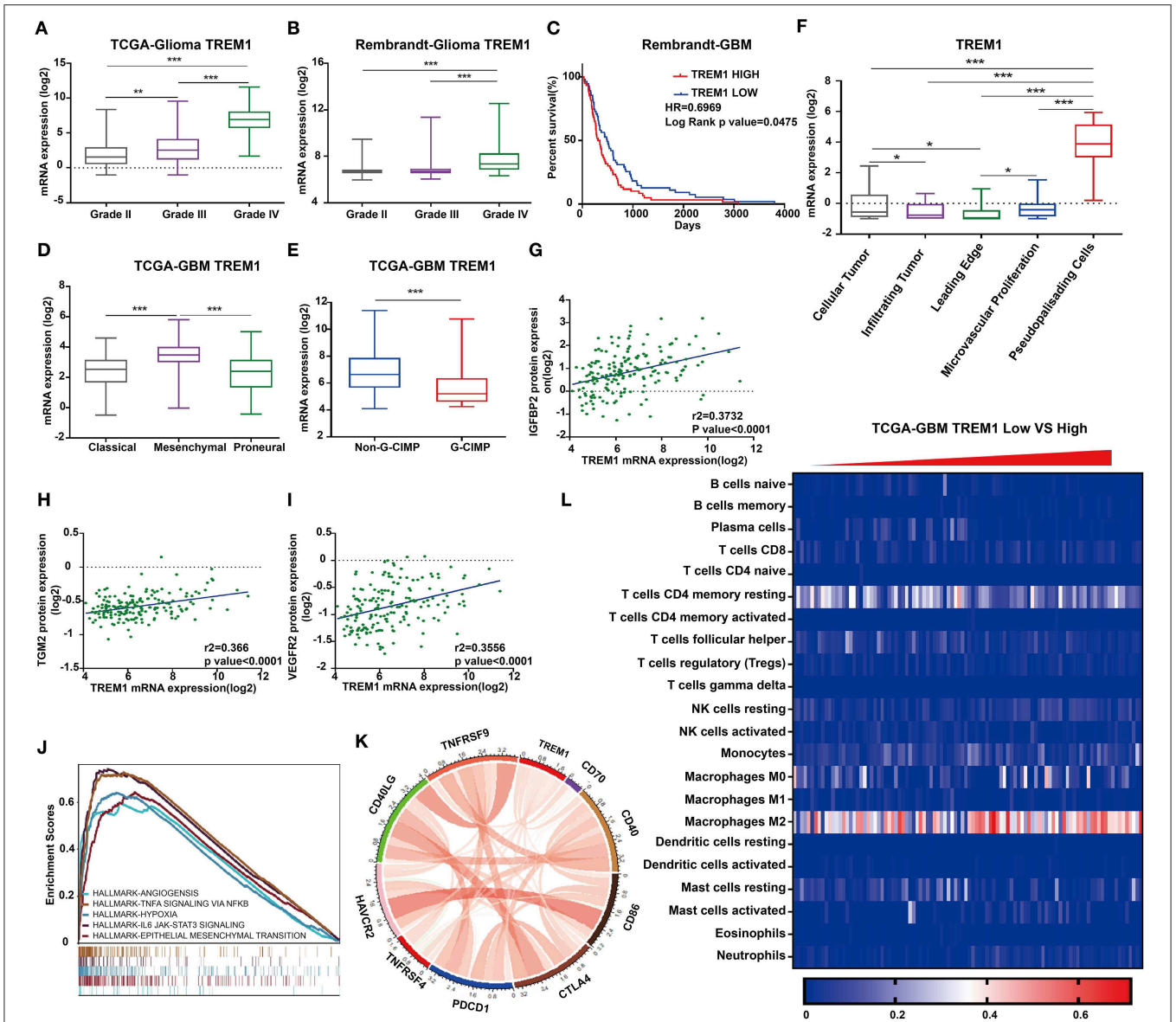

FIGURE 5 | TREM1 is closely related to hypoxia and angiogenesis. *TREM1* mRNA expression profiles based on pathological grade from **(A)** TCGA-Glioma and **(B)**. Rembrandt-Glioma datasets. **(C)** Kaplan–Meier survival curves for GBM patients with *TREM1*^{high} and *TREM1*^{low} tumors using the Rembrandt database. **(D)** *TREM1* mRNA expression levels in different GBM molecular subtypes. **(E)** *TREM1* mRNA expression levels in non-GIMP and GIMP positive samples from the TCGA. **(F)** *TREM1* mRNA expression levels in different pathologic areas/tumor microsections. Corresponding data were obtained from the Ivy Glioblastoma Atlas Project. Scatter plots displaying the correlation between *TREM1* mRNA expression and protein levels of **(G)** IGFBP2, **(H)** TGM2, and **(I)** VEGFR2. **(J)** The results of representative GSEA analysis. **(K)** Chord diagram constructed from data on the correlation between *TREM1* and immune co-stimulatory factor and checkpoint molecules. **(L)** Heatmap generated with *TREM1* mRNA expression levels in multiple immune cells using the TCGA-GBM dataset. Significant difference between the two groups: $^{*}P < 0.05$; $^{**}P < 0.01$;$^{***}P < 0.001$.

(**Supplementary Figures 5D,E**) (36). We subsequently used TIMER, a web server for comprehensive analysis of tumor-infiltrating immune cells (https://cistrome.shinyapps.io/timer/) and found that the mRNA expression levels of *TREM1* were inversely correlated with tumor purity in GBM (37). Specifically, the mRNA expression levels of *TREM1* were negatively correlated with *CD8*-positive T-cell infiltration but positively correlated with neutrophil and dendritic cell infiltration levels (**Figure 5F**).

We also used Cibersort (https://cibersort.stanford.edu/) to further explore the relationship between *TREM1* and immune cell infiltration. Cibersort converts gene expression profile data into relative quantification of immune cells (38). We divided the TCGA GBM data into high- and low-expression groups based on

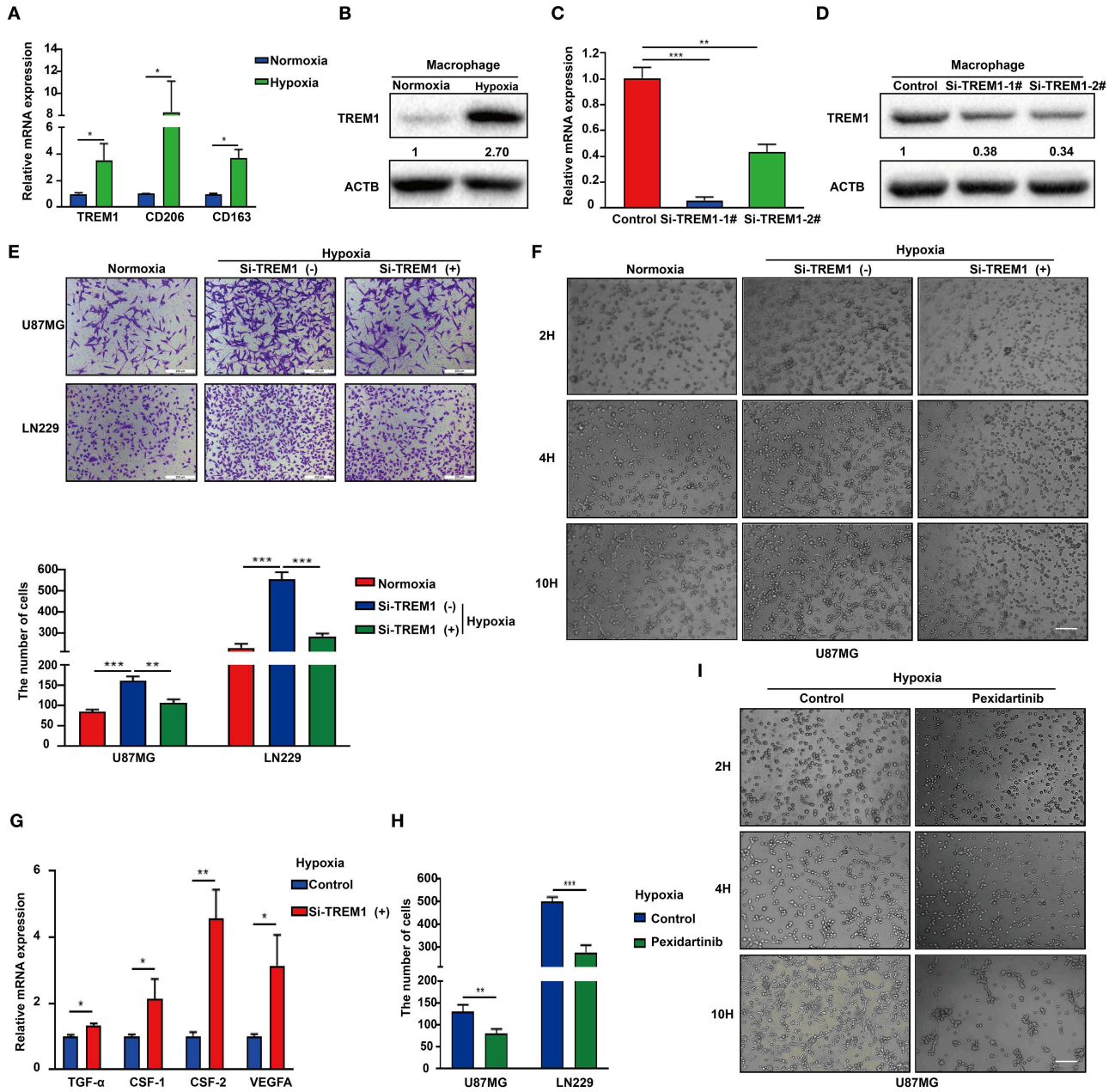

FIGURE 6 | Inhibition of TREM1 suppresses migration and vasculary mimicry formation of GBM cells *in vitro*. **(A)** RT-qPCR results for *TREM1*, *CD206*, and *CD163* in macrophages derived from the THP1 cell line under normoxia and hypoxia. **(B)** Western blot analysis of TREM1 protein levels under normoxia and hypoxia. **(C)** Graphical representation of qRT-PCR after siRNA knockdown of TREM1. *ACTB* was used as an internal reference. **(D)** Western blot to validate the efficiency of si-TREM1 knockdown in macrophages derived from the THP1 cell line. ACTB was used as a protein loading control. **(E)** Representative images of Transwell migration for U87MG and LN229 under normoxia, hypoxia plus control, and hypoxia plus si-TREM1 (scale bar: 200 μm). Statistical results of the invasive ratio with corresponding treatment for 24 h in the Transwell assay. **(F)** Representative images of VM formation assay for U87MG in normoxia, hypoxia plus control, and hypoxia plus si-TREM1. **(G)** RT-qPCR to detect mRNA expression of *TGF-α*, *CSF1*, *CSF2*, and *VEGFA* after knockdown of TREM1 under hypoxia. **(H)** Statistical results of the invasive ratio for U87MG and LN229 in both control and pexidartinib (10 μM) treatment under hypoxia. **(I)** Representative images of VM formation assay for U87MG in both control and pexidartinib (10 μM) groups under hypoxic conditions. Significant difference between the two groups: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

the median expression level of *TREM1*, quantified immune cell populations, and plotted these results in heatmaps (**Figure 5K**). We found that the percentage of M2 macrophages increased significantly in GBM samples with high expression of *TREM1*. Thus, we proposed that high expression of *TREM1* plays a role in promoting the development and progression of gliomas, similar

to the mechanism of M2 macrophages in promoting the disease. We therefore performed a series of experiments *in vivo* and *in vitro* to test this hypothesis.

TREM1 Promotes GBM Cell Migration and VM Formation

Hypoxic necrosis is a major feature in the diagnosis of GBM and is closely related to stem cell maintenance, angiogenesis, energy metabolism, and growth characteristics of tumor cells. Based on the literature and our previous analysis, we hypothesized that *TREM1* may be induced by hypoxia. We also used immunofluorescence to confirm that *TREM1* was mainly found to be expressed in macrophages (**Figure 6A**). *TREM1* expression levels were increased in macrophages cultured under hypoxic conditions relative to those cultured under normoxic conditions. Markers of macrophage polarization, *CD206* and *CD163* (markers of M2 polarization), were also elevated under hypoxic conditions (**Figures 6A,B**).

Supernatants from hypoxic M2 macrophages have been shown to promote the proliferation of pulmonary artery smooth muscle cells (39). Inhibition of GTP cyclohydrolase (GCH1) was discovered to shift the phenotype of TAMs from proangiogenic M2 toward M1, accompanied by a shift in plasma chemokines (40). Host-produced histidine-rich glycoproteins have also been found to inhibit tumor growth and metastasis while improving the effects of chemotherapy by skewing TAM polarization away from M2 to a tumor-inhibiting M1-like phenotype (41). *TREM1* has been shown to act as an inflammatory amplifier, specifically releasing pro-inflammatory chemokines and cytokines or altering the expression of activated cell membrane surfaces upon receipt of external stimuli. Therefore, we suspect that increased *TREM1* may play a role in promoting tumor migration and angiogenesis through the release of certain inflammatory factors. We therefore knocked down *TREM1* with siRNAs in THP1 cells induced to become macrophages and examined their role in promoting biological properties of GBM cells such as migration. Western blot and qRCR analysis were used to verify the knockdown efficiency of *TREM1* (**Figures 6C,D**). After induction of siRNA-treated THP1 cells into macrophages, they were cultured for 24 h under normoxia and hypoxia, and supernatants were collected and mixed 1:1 with culture media containing 10% FBS for incubation with GBM cells.

The results of Transwell and VM formation assays demonstrated that hypoxia-induced macrophages promoted U87 and LN229 tumor cell migration and vascular mimicry but that this effect was significantly reduced after knockdown of *TREM1* (**Figures 6B,E,F**). To explore the molecular mechanism, we compared the changes in expression levels of critical cytokine mRNAs in macrophages under normoxic and hypoxic conditions as well as between control and *TREM1* knockdown groups. Analysis of the intersection of significantly altered genes in these two groups yielded *CSF1* as a common factor potentially involved in *TREM1* (**Figures 6C,G**). To confirm that CSF1 plays a role in promoting GBM cell migration and vascular mimicry, induced macrophages were exposed to pexidartinib (10 μM), an inhibitor of the CSF1 receptor (CSF1R). Supernatants from pexidartinib-treated macrophages relative to controls significantly inhibited cell migration and pathological angiogenesis under hypoxic conditions (**Figures 6D,H,I**). These results indicated that hypoxia can induce upregulation of the expression of *TREM1* in macrophages, thereby promoting GBM progression through the release of CSF-1, which triggers invasion and vascular mimicry in GBM cells.

TREM1 Contributes to GBM Progression *in vivo*

To investigate the effect of *TREM1* on tumor growth *in vivo*, we treated mice bearing orthotopic GBM xenograft with peptides as a control and with LP17, which blocks TREM1. The results from bioluminescence imaging demonstrated that tumor growth was inhibited in animals treated with LP17 relative to controls ($\sim 16.3 \times 10^7$ vs. $\sim 8.2 \times 10^7$ photons/s, control vs. LP17-treated; **Figures 7A,B**). The OS of tumor-bearing animals was enhanced under treatment with LP17 compared to controls (median survival, $>$ 28 days vs. 20.5 days, LP17 and control peptide, respectively, $P < 0.05$) (**Figure 7C**). Immunostaining for CD11b positive cells in the LP17-treated group showed a dramatic decrease compared to the control group (**Figure 7D**). Sections stained with PAS showed that in the LP17 treatment group, vascular mimicry was decreased compared to controls. In summary, these data demonstrate that inhibition of *TREM1* blocked the progression of GBM *in vivo* and may be used as a therapeutic target.

DISCUSSION

The current standard of care for GBM includes surgery, radiotherapy, and chemotherapy (temozolomide, TMZ). New complications arise with each arm of this multi-modal treatment, and tumors recur not long after the primary diagnosis. The most promising approach in recent years for other tumor types has been immunotherapy. Our present work also supports the possibility of interfering with alternative immune cell types typically infiltrating GBM. However, immunotherapy has not proven satisfactory for the clinical treatment of GBM. For example, although immune checkpoint inhibitors, such as anti-programmed cell death (PD)1 antibody, have achieved better prognosis in GBM animal models, a recent clinical trial indicated that PD-1 inhibitors have an objective response rate of only 8% in patients with recurrent GBM (7). In another approach, CAR-T therapy has achieved tremendous success in hematological malignancies. However, CAR-T therapy targeting EGFRvIII, a tumor-specific antigen, has not achieved the desired clinical results in GBM treatment (42).

Several possibilities might account for the reduced efficacy of immunotherapy in GBM treatment. First, the immunocompetent mouse models used to study immunotherapy do not accurately reflect the human GBM TME. The methylcholanthrene-induced GL261 and SMA-560 models are the commonly used orthotopic xenograft models in GBM immunotherapy (27). However, both model types possess a high number of mutations and predict

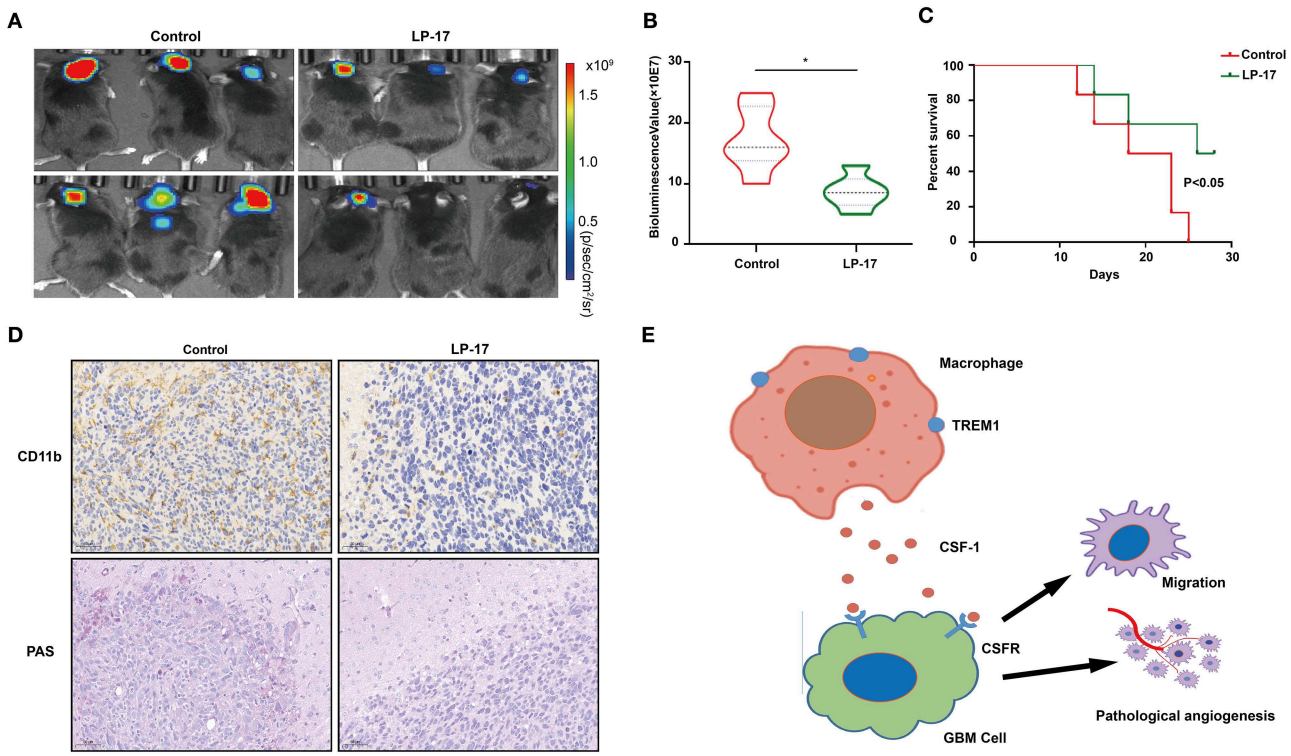

FIGURE 7 | Inhibition of TREM1 suppresses tumor growth *in vivo*. **(A)** Representative bioluminescence images of tumor-bearing mice from control and LP-17 treatment groups at week 3. **(B)** Fluorescence quantitation for xenografts in animals from both groups at week 3. **(C)** Kaplan-Meier survival curves for tumor bearing animals in control and LP-17 treatment groups. A log-rank test was used to assess the statistical significance of the differences. **(D)** Images of IHC staining for CD11b and PAS staining in tumors from each group as indicated (scale bars: 50 μm). **(E)** A hypothetical schematic diagram depicts how TREM1 promotes the development of GBM. In hypoxic conditions, macrophages express increased levels of TREM1 and release more CSF1, which promotes pathological angiogenesis and migration of GBM cells. *$P < 0.05$ compared to control.

neoepitopes and enhanced immune cell infiltration. These features are in contrast to primary GBM samples, which typically exhibit a low tumor mutational load and an immunosuppressive microenvironment (8). Second, immune cell infiltration is significantly less than in other solid tumors, rendering GBM a so-called "cold" tumor. However, immune checkpoint inhibitors still exert anti-GBM effects even though they rely on the recovery of reactive T cells to execute a killing effect (9). Third, although CAR-T therapy generates a significant increase in the number of killer T cells, the presence of the BBB may limit their access to brain tumors, unlike for other solid tumors. Moreover, even after entering the tumor microenvironment, killer T cells may have reduced killing potential due to hypoxic conditions generated because of *IDH* variants and the heterogeneity of *EGFR* mutations (7). Fourth, in addition to PD-L1, PD-1, and CTLA-4, TMEs of GBM may also contain other immunosuppressive factors, such as the A2aR high-affinity adenosine receptor (on lymphocytes and tumor-associated macrophages) or PD-L2 (on macrophages lacking PD-L1 expression). It has been reported that anti-PD-L1 and anti-TIGIT (a novel immune checkpoint inhibitor) combination therapy improved overall survival in GBM patients by increasing effector T cell function and downregulating the number of suppressive Tregs and tumor-infiltrating dendritic cells (43).

In addition, both immune checkpoint inhibitors and CAR-T therapy rely on killer T cells, but the GBM TME exhibits mass macrophage infiltration, which includes phenotypically suppressive CD163+ M2 to undifferentiated M0 macrophages, particularly in the mesenchymal molecular GBM subtype (30). Our laboratory has reported that hypoxic glioma-derived exosomes deliver microRNA-1246 to induce M2 macrophage polarization, which promotes proliferation, migration, and invasion *in vitro* and *in vivo* of glioma cells by targeting telomere binding repeat 2 interacting protein (TERF2IP) through the STAT3 and NF-κB pathways (44). It was also found that M2 macrophages enhance phosphoglycerate kinase 1 (PGK1) threonine 243 phosphorylation, which facilitates glycolysis, proliferation, and tumorigenesis in GBM cells (10). In the present study, we also found that the expression levels of *TREM1* may be accompanied by an increase in macrophage M2 polarization. This result contradicts previous indications that *TREM1* is an M1 marker of macrophages in liver biopsies. The reason for this discrepancy may be due to pathological and tissue differences.

Several other studies support a role for *TREM1* in the development of cancer. *TREM1* has been reported to exert pro-inflammatory immune responses not only in acute pathogen-induced reactions but also in chronic and non-infectious inflammatory disorders, including various types of cancer. *TREM1*-/- mice exhibited reduced tumor number and load in an experimental model of inflammation-driven tumorigenesis of colorectal tumor (45). *TREM1* has also been reported to cooperate with diminished DNA damage response *in vivo* to promote expansion and leukemic progression in Fanca-/- pre-leukemia stem cells (46). Our present study demonstrated that increased expression of *TREM1* in macrophages may promote GBM progression through the release of CSF1. The CSF1 receptor (CSF1R) has been investigated as a possible therapeutic target in the treatment of GBM. Inhibition of CSF1R has been shown to alter the expression of activated M2 markers and to reduce intracranial growth of patient-derived glioma xenografts (47). CSF1R ligand expression was also found to be elevated in GBM xenografts treated with ionizing radiation (48). Both studies indicate that inhibition of CSF1R might be a promising strategy to improve the treatment and prognosis of GBM.

Besides *TREM1*, other genes may also have potential therapeutic roles in GBM treatment. For instance, *GBP2* was found to inhibit mitochondrial fission and cell metastasis in breast cancer cells both *in vitro* and *in vivo* (49). Further, *IFITM2* was significantly up-regulated and induced after activation of beta-catenin signaling in colorectal cancers (50), and it was also reported to promote gastric cancer growth and metastasis through the insulin-like growth factor (IGF1)/IGF1 receptor (IGF1R)/STAT3 signaling pathway (51). *CIITA*, a member of the interferon response factor (IRF) pathway, was found by integrative genomic analysis to be a key oncogenic gene in primary mediastinal large B-cell lymphoma (52). *TYROBP*, a downstream effector of *TREM1*, induced the transformation of microglial cells and regulated inflammatory response (53). Thus, all of these genes will be highly interesting for studies of GBM.

In summary, we analyzed the GBM data in the CGGA database using WGCNA to obtain immune-related genes that may promote the progression of GBM. *TREM1* emerged as a gene of interest due to higher expression in GBMs relative to non-neoplastic tissue and association with a worse prognosis. The expression of *TREM1* increased in macrophages under hypoxia, and supernatants from these cells promoted pathological angiogenesis and migration of GBM cells *in vitro*. A possible factor mediating this response is CSF1 (**Figure 7E**). These results underscore the importance of the TME in GBM development. Thus, targeting the tumor microenvironment, or specifically TAMs, allow a vulnerability in the development of GBM to be exploited and should be considered as a viable therapeutic strategy.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: http://cancergenome.nih.gov/abouttcga, http://www.cgga.org.cn/, http://www.betastasis.com/glioma/rembrandt/.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Shandong University.

AUTHOR CONTRIBUTIONS

YK, Z-CF, NY, and X-GL conceived the study. YK and NY were involved in bioinformatics analysis. YK and Z-CF performed experiments. Y-LZ, YM, Z-MZ, and DZ participated in animal experiments. A-JC and BH performed the statistical analysis. YK and Z-CF drafted the paper. X-FL and JW supplemented manuscript. FT, JW, NY, and X-GL contributed substantially to its revision. NY and X-GL supervised the study. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01281/full#supplementary-material

Supplementary Figure 1 | (A) Network topology for different soft-thresholding powers. Numbers in the plots indicate the corresponding soft thresholding powers. The approximate scale-free topology can be attained at a soft-thresholding power of 5. **(B)** Assessing the scale-free topology when the soft-thresholding power was set to 5 (scale-free $R^2 = 0.84$, slope $= -1.35$). **(C)** Eigengene adjacency heatmap. Different colors indicate the degree of correlation between modules.

Supplementary Figure 2 | (A) Heatmap for gene expression in black modules. The average expression of the eigenvectors of the black module is highly

correlated with the expression of genes within the black module. **(B)** Protein–protein interaction network consisting of all genes in the black module.

Supplementary Figure 3 | The expression of intersection genes in different groups based on the CGGA database. **(A)** Expression of IDH wild-type and mutant. **(B)** Expression of LGG and GBM. **(C)** Expression of the non-mesenchymal subgroup and mesenchymal subgroup.

Supplementary Figure 4 | Quantitative graph of IHC staining in normal tissue and glioma samples. **(A)** TREM1; **(B)** GBP2; **(C)** IFITM2**; (D)** CIITA; and **(E)** TYROBP. Significant difference between the two groups: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Supplementary Figure 5 | (A) *TREM1* mRNA expression levels in both LGG and GBM samples from the CGGA database. **(B)** Scatter plot displaying the correlation between *TREM1* mRNA expression levels and NDRG1pT346 protein levels. **(C)** GSEA highlighting a positive association of increased *TREM1* expression levels with inflammatory response, IL2-STAT5 signaling, and allograft rejection. **(D)** Immunofluorescence staining of GBM tissue sections. Red represents TREM1, blue represents DAPI (scale bar: 100 μm). **(E)** Western blot analysis of TREM1 protein levels in three GBM cell lines under normoxia and hypoxia. **(F)** The correlation between *TREM1* mRNA expression and immune infiltration levels in GBM. These images were generated using TIMER.

Supplementary Figure 6 | (A) Immunofluorescence staining of GBM tissue sections. Red represents TREM1, green represents CD11b and CD68, respectively, and blue represents DAPI (scale bar: 100 μm). **(B)** Immunofluorescence staining of VM for U87MG under hypoxia condition plus control or si-TREM1. Green represents VEGFR2, and blue represents DAPI (scale bar: 100 μm). **(C)** mRNA expressions of *TGF-α*, *IL1β*, *IL6*, *IL10*, *CSF1*, *CSF2*, *CXCL*, and *VEGFA* were detected under normoxia and hypoxia. **(D)** Representative images of Transwell migration for U87MG and LN229 in both control and pexidartinib treatment under hypoxia (scale bar: 200 μm). Significant difference between the two groups: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Supplementary Table 1 | Nomogram for predicting the proportion of glioma patients with OS based on the TCGA database.

REFERENCES

1. Ostrom QT, Cioffi G, Gittleman H, Patil N, Waite K, Kruchko C, et al. CBTRUS statistical report: primary brain and other central nervous system tumors diagnosed in the United States in 2012-2016. *Neuro Oncol.* (2019) 21:1–v100. doi: 10.1093/neuonc/noz150
2. Wesseling P, Capper D. WHO 2016 Classification of gliomas. *Neuropathol Appl Neurobiol.* (2018) 44:139–50. doi: 10.1111/nan.12432
3. Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, Burger PC, Jouvet A, et al. The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathol.* (2007) 114:97–109. doi: 10.1007/s00401-007-0243-4
4. Conniot J, Scomparin A, Peres C, Yeini E, Pozzi S, Matos AI, et al. Immunization with mannosylated nanovaccines and inhibition of the immune-suppressing microenvironment sensitizes melanoma to immune checkpoint modulators. *Nat Nanotechnol.* (2019) 14:891–901. doi: 10.1038/s41565-019-0512-0
5. Nakagawa K, Garon EB, Seto T, Nishio M, Ponce Aix S, Paz-Ares L, et al. Ramucirumab plus erlotinib in patients with untreated, EGFR-mutated, advanced non-small-cell lung cancer (RELAY): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet Oncol.* (2019) 20:1655–69. doi: 10.1016/S1470-2045(19)30634-5
6. Meti N, Esfahani K, Johnson NA. The role of immune checkpoint inhibitors in classical Hodgkin Lymphoma. *Cancers.* (2018) 10:204. doi: 10.3390/cancers10060204
7. Buerki RA, Chheda ZS, Okada H. Immunotherapy of primary brain tumors: facts and hopes. *Clin Cancer Res.* (2018) 24:5198–205. doi: 10.1158/1078-0432.CCR-17-2769
8. Brennan CW, Verhaak RG, McKenna A, Campos B, Noushmehr H, Salama SR, et al. The somatic genomic landscape of glioblastoma. *Cell.* (2013) 155:462–77. doi: 10.1016/j.cell.2013.09.034
9. Woroniecka K, Chongsathidkiet P, Rhodin K, Kemeny H, Dechant C, Farber SH, et al. T-cell exhaustion signatures vary with tumor type and are severe in glioblastoma. *Clin Cancer Res.* (2018) 24:4175–86. doi: 10.1158/1078-0432.CCR-17-1846
10. Zhang Y, Yu G, Chu H, Wang X, Xiong L, Cai G, et al. Macrophage-Associated PGK1 Phosphorylation Promotes Aerobic Glycolysis and Tumorigenesis. *Mol Cell.* (2018) 71:201–15. doi: 10.1016/j.molcel.2018.06.023
11. Shi Y, Ping YF, Zhou W, He ZC, Chen C, Bian, et al. Tumour-associated macrophages secrete pleiotrophin to promote PTPRZ1 signalling in glioblastoma stem cells for tumour growth. *Nat Commun.* (2017) 8:15080. doi: 10.1038/ncomms15080
12. Langfelder P, Horvath S. WGCNA: an R package for weighted correlation network analysis. *BMC Bioinformatics.* (2008) 9:559. doi: 10.1186/1471-2105-9-559
13. The Cancer Genome Atlas Research Network Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature.* (2008) 455:1061–8. doi: 10.1038/nature07385
14. Zhao Z, Meng F, Wang W, Wang Z, Zhang C, Jiang T. Comprehensive RNA-seq transcriptomic profiling in the malignant progression of gliomas. *Sci Data.* (2017) 4:170024. doi: 10.1038/sdata.2017.24
15. Madhavan S, Zenklusen JC, Kotliarov Y, Sahni H, Fine HA, Buetow K. Rembrandt: helping personalized medicine become a reality through integrative translational research. *Mol Cancer Res.* (2009) 7:157–67. doi: 10.1158/1541-7786.MCR-08-0435
16. Huang DW, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res.* (2009) 37:1–13. doi: 10.1093/nar/gkn923
17. Kanehisa M, Sato Y, Kawashima M, Furumichi M, Tanabe M. KEGG as a reference resource for gene and protein annotation. *Nucleic Acids Res.* (2016) 44:457–62. doi: 10.1093/nar/gkv1070
18. von Mering C, Huynen M, Jaeggi D, Schmidt S, Bork P, Snel B. STRING: a database of predicted functional associations between proteins. *Nucleic Acids Res.* (2003) 31:258–61. doi: 10.1093/nar/gkg034
19. Dennis GJ, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, et al. DAVID: database for annotation, visualization, and integrated discovery. *Genome Biol.* (2003) 4:R60. doi: 10.1186/gb-2003-4-9-r60
20. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.* (2003) 13:2498–504. doi: 10.1101/gr.1239303
21. Bader GD, Hogue CW. An automated method for finding molecular complexes in large protein interaction networks. *BMC Bioinformatics.* (2003) 4:2. doi: 10.1186/1471-2105-4-2
22. Uhlen M, Zhang C, Lee S, Sjostedt E, Fagerberg L, Bidkhori G, et al. A pathology atlas of the human cancer transcriptome. *Science.* (2017) 357:eaan2507. doi: 10.1126/science.aan2507
23. Puchalski RB, Shah N, Miller J, Dalley R, Nomura SR, Yoon JG, et al. An anatomic transcriptional atlas of human glioblastoma. *Science.* (2018) 360:660–3. doi: 10.1126/science.aaf2666
24. Liu Y, Li F, Yang YT, Xu XD, Chen JS, Chen TL, et al. IGFBP2 promotes vasculogenic mimicry formation via regulating CD144 and MMP2 expression in glioma. *Oncogene.* (2019) 38:1815–31. doi: 10.1038/s41388-018-0525-4
25. Cai J, Chen Q, Cui Y, Dong J, Chen M, Wu P, et al. Immune heterogeneity and clinicopathologic characterization of IGFBP2 in 2447 glioma samples. *Oncoimmunology.* (2018) 7:e1426516. doi: 10.1080/2162402X.2018.1426516
26. Lin KW, Liao A, Qutub AA. Simulation predicts IGFBP2-HIF1alpha interaction drives glioblastoma growth. *PLoS Comput Biol.* (2015) 11:e1004169. doi: 10.1371/journal.pcbi.1004169
27. Zhou YH, Hess KR, Liu L, Linskey ME, Yung WK. Modeling prognosis for patients with malignant astrocytic gliomas: quantifying the expression of multiple genetic markers and clinical variables. *Neuro Oncol.* (2005) 7:485–94. doi: 10.1215/S1152851704000730

28. Dyer LM, Schooler KP, Ai L, Klop C, Qiu J, Robertson KD, et al. The transglutaminase 2 gene is aberrantly hypermethylated in glioma. *J Neurooncol.* (2011) 101:429–40. doi: 10.1007/s11060-010-0277-7
29. Lei Z, Chai N, Tian M, Zhang Y, Wang G, Liu J, et al. Novel peptide GX1 inhibits angiogenesis by specifically binding to transglutaminase-2 in the tumorous endothelial cells of gastric cancer. *Cell Death Dis.* (2018) 9:579. doi: 10.1038/s41419-018-0594-x
30. Kaffes I, Szulzewsky F, Chen Z, Herting CJ, Gabanic B, Velazquez Vega JE, et al. Human mesenchymal glioblastomas are characterized by an increased immune cell presence compared to proneural and classical tumors. *Oncoimmunology.* (2019) 8:e1655360. doi: 10.1080/2162402X.2019.1655360
31. Kaka N, Hafazalla K, Samawi H, Simpkin A, Perry J, Sahgal A, et al. Progression-free but no overall survival benefit for adult patients with bevacizumab therapy for the treatment of newly diagnosed glioblastoma: a systematic review and meta-analysis. *Cancers.* (2019) 11:1723. doi: 10.3390/cancers11111723
32. Demeure K, Fack F, Duriez E, Tiemann K, Bernard A, Golebiewska A, et al. Targeted proteomics to assess the response to anti-angiogenic treatment in Human Glioblastoma (GBM). *Mol Cell Proteomics.* (2016) 15:481–92. doi: 10.1074/mcp.M115.052423
33. Mahase S, Rattenni RN, Wesseling P, Leenders W, Baldotto C, Jain R, et al. Hypoxia-mediated mechanisms associated with antiangiogenic treatment resistance in glioblastomas. *Am J Pathol.* (2017) 187:940–53. doi: 10.1016/j.ajpath.2017.01.010
34. Subramanian A, Kuehn H, Gould J, Tamayo P, Mesirov JP. GSEA-P: a desktop application for gene set enrichment analysis. *Bioinformatics.* (2007) 23:3251–3. doi: 10.1093/bioinformatics/btm369
35. Bosco MC, Pierobon D, Blengio F, Raggi F, Vanni C, Gattorno M, et al. Hypoxia modulates the gene expression profile of immunoregulatory receptors in human mature dendritic cells: identification of TREM-1 as a novel hypoxic marker *in vitro* and *in vivo*. *Blood.* (2011) 117:2625–39. doi: 10.1182/blood-2010-06-292136
36. Murat A, Migliavacca E, Hussain SF, Heimberger AB, Desbaillets I, Hamou MF, et al. Modulation of angiogenic and inflammatory response in glioblastoma by hypoxia. *PLoS ONE.* (2009) 4:e5947. doi: 10.1371/journal.pone.0005947
37. Li T, Fan J, Wang B, Traugh N, Chen Q, Liu JS, et al. TIMER: a web server for comprehensive analysis of tumor-infiltrating immune cells. *Cancer Res.* (2017) 77:e108–10. doi: 10.1158/0008-5472.CAN-17-0307
38. Newman AM, Liu CL, Green MR, Gentles AJ, Feng W, Xu Y, et al. Robust enumeration of cell subsets from tissue expression profiles. *Nat Methods.* (2015) 12:453–7. doi: 10.1038/nmeth.3337
39. Vergadi E, Chang MS, Lee C, Liang OD, Liu X, Fernandez-Gonzalez A, et al. Early macrophage recruitment and alternative activation are critical for the later development of hypoxia-induced pulmonary hypertension. *Circulation.* (2011) 123:1986–95. doi: 10.1161/CIRCULATIONAHA.110.978627
40. Pickert G, Lim HY, Weigert A, Haussler A, Myrczek T, Waldner M, et al. Inhibition of GTP cyclohydrolase attenuates tumor growth by reducing angiogenesis and M2-like polarization of tumor associated macrophages. *Int J Cancer.* (2013) 132:591–604. doi: 10.1002/ijc.27706
41. Rolny C, Mazzone M, Tugues S, Laoui D, Johansson I, Coulon C, et al. HRG inhibits tumor growth and metastasis by inducing macrophage polarization and vessel normalization through downregulation of PlGF. *Cancer Cell.* (2011) 19:31–44. doi: 10.1016/j.ccr.2010.11.009
42. O'Rourke DM, Nasrallah MP, Desai A, Melenhorst JJ, Mansfield K, Morrissette JJD, et al. A single dose of peripherally infused EGFRvIII-directed CAR T cells mediates antigen loss and induces adaptive resistance in patients with recurrent glioblastoma. *Sci Transl Med.* (2017) 9:aaa0984. doi: 10.1126/scitranslmed.aaa0984
43. Hung AL, Maxwell R, Theodros D, Belcaid Z, Mathios D, Luksik AS, et al. TIGIT and PD-1 dual checkpoint blockade enhances antitumor immunity and survival in GBM. *Oncoimmunology.* (2018) 7:e1466769. doi: 10.1080/2162402X.2018.1466769
44. Qian M, Wang S, Guo X, Wang J, Zhang Z, Qiu W, et al. Hypoxic glioma-derived exosomes deliver microRNA-1246 to induce M2 macrophage polarization by targeting TERF2IP via the STAT3 and NF-kappaB pathways. *Oncogene.* (2019) 39:428–42. doi: 10.1038/s41388-019-0996-y
45. Kozik JH, Trautmann T, Carambia A, Preti M, Lutgehetmann M, Krech T, et al. Attenuated viral hepatitis in Trem1-/- mice is associated with reduced inflammatory activity of neutrophils. *Sci Rep.* (2016) 6:28556. doi: 10.1038/srep28556
46. Du W, Amarachintha S, Wilson A, Pang Q. The immune receptor Trem1 cooperates with diminished DNA damage response to induce preleukemic stem cell expansion. *Leukemia.* (2017) 31:423–33. doi: 10.1038/leu.2016.242
47. Pyonteck SM, Akkari L, Schuhmacher AJ, Bowman RL, Sevenich L, Quail DF, et al. CSF-1R inhibition alters macrophage polarization and blocks glioma progression. *Nat Med.* (2013) 19:1264–72. doi: 10.1038/nm.3337
48. Stafford JH, Hirai T, Deng L, Chernikova SB, Urata K, West BL, et al. Colony stimulating factor 1 receptor inhibition delays recurrence of glioblastoma after radiation by altering myeloid cell recruitment and polarization. *Neuro Oncol.* (2016) 18:797–806. doi: 10.1093/neuonc/nov272
49. Zhang J, Zhang Y, Wu W, Wang F, Li, X, et al. Guanylate-binding protein 2 regulates Drp1-mediated mitochondrial fission to suppress breast cancer cell invasion. *Cell Death Dis.* (2017) 8:e3151. doi: 10.1038/cddis.2017.559
50. Andreu P, Colnot S, Godard C, Laurent-Puig P, Lamarque D, Kahn A, et al. Identification of the IFITM family as a new molecular marker in human colorectal tumors. *Cancer Res.* (2006) 66:1949–1955. doi: 10.1158/0008-5472.CAN-05-2731
51. Xu L, Zhou R, Yuan L, Wang S, Li X, Ma H, et al. IGF1/IGF1R/STAT3 signaling-inducible IFITM2 promotes gastric cancer growth and metastasis. *Cancer Lett.* (2017) 393:76–85. doi: 10.1016/j.canlet.2017.02.014
52. Mottok A, Hung SS, Chavez EA, Woolcock B, Telenius A, Chong LC, et al. Integrative genomic analysis identifies key pathogenic mechanisms in primary mediastinal large B-cell lymphoma. *Blood.* (2019) 134:802–13. doi: 10.1182/blood.2019001126
53. Chen B, Zhou M, Zhang H, Wang C, Hu X, Wang B, et al. TREM1/Dap12-based CAR-T cells show potent antitumor activity. *Immunotherapy.* (2019) 11:1043–55. doi: 10.2217/imt-2019-0017

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REVIEW
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Molecular Heterogeneity and Immunosuppressive Microenvironment in Glioblastoma

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Glioblastoma (GBM) is the most aggressive primary brain tumor in adults, with a poor prognosis, despite surgical resection combined with radio- and chemotherapy. The major clinical obstacles contributing to poor GBM prognosis are late diagnosis, diffuse infiltration, pseudo-palisading necrosis, microvascular proliferation, and resistance to conventional therapy. These challenges are further compounded by extensive inter- and intra-tumor heterogeneity and the dynamic plasticity of GBM cells. The complex heterogeneous nature of GBM cells is facilitated by the local inflammatory tumor microenvironment, which mostly induces tumor aggressiveness and drug resistance. An immunosuppressive tumor microenvironment of GBM provides multiple pathways for tumor immune evasion. Infiltrating immune cells, mostly tumor-associated macrophages, comprise much of the non-neoplastic population in GBM. Further understanding of the immune microenvironment of GBM is essential to make advances in the development of immunotherapeutics. Recently, whole-genome sequencing, epigenomics and transcriptional profiling have significantly helped improve the prognostic and therapeutic outcomes of GBM patients. Here, we discuss recent genomic advances, the role of innate and adaptive immune mechanisms, and the presence of an established immunosuppressive GBM microenvironment that suppresses and/or prevents the anti-tumor host response.

Keywords: glioblastoma, microenvironment, brain tumor, immunity, microglia, astrocytes

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INTRODUCTION

Glioblastoma (GBM) is the most common primary brain tumor with an annual incidence of 3.19 per 100,000 population (1). GBM is a Grade IV astrocytoma, characterized by uncontrolled cellular proliferation, local infiltration, extensive genomic instability, tendency for necrosis, angiogenesis, and resistance to therapy. Histopathologically, GBM is composed of a heterogeneous cell population, consisting of differentiated and undifferentiated tumor cells, along with differences in morphology and capacity for self-renewal and proliferation (2, 3). Despite aggressive treatment including surgical resection and radiotherapy with concomitant

chemotherapy, prognosis remains poor due to GBM recurrence, with a median survival of 14.6 months (4). In molecular terms, this poor prognosis is mostly characterized by dysregulation of many key signaling pathways involving cell survival, growth, proliferation and apoptosis due to genomic mutations (5). GBM is a robust malignant tumor, distinguished by its local invasion pattern (6, 7). Generally, GBM does not metastasize extracranially; however, there have been rare cases in which 0.44% of GBM have spread to other parts of the body usually when patients have undergone craniotomy (8, 9).

GBM is highly invasive, lack clear margins, and therefore, poses a challenge for complete surgical resection and almost inevitably recurs in patients who have been treated. Despite recent advances in genomics, chemotherapy, immunotherapy, and technological approaches to cancer models, the treatment outcome for GBM patients has remained consistently poor. Clinical symptoms vary and depend on size and location of tumor; it may include headache, nausea, dizziness, confusion, speech difficulties, and change in personality, new onset of seizures and focal neurological deficit. The tumor is generally located in the frontal and temporal lobes of the brain and can also rarely occur in the brainstem, cerebellum and spinal cord (10, 11). GBM is most often *de novo* i.e., primary GBM, which account for ∼90% of GBM cases and are predominately found in patients older than 45 years (5). The remaining 10% of GBM cases develop from a lower-grade tumor progressing to a higher-grade malignancy (secondary GBM) over a 5–10 year period, and is primarily present in patients younger than 45 years. These subtypes have distinct genetic aberrations but are histologically indistinguishable (5, 12, 13).

Despite advances in our understanding of cancer biology, managing GBM remains a challenge. It is important to understand why treatment for GBM is largely ineffective; it is mainly due to the heterogeneous nature of the tumor microenvironment. It has not been possible to produce appropriate cancer models for GBM that would help us study the properties by which GBM is promoted and sustained. Therefore, it is vital to study the role of the immune system in the GBM microenvironment. This review aims to analyze the recent genomic advances in dissecting the considerable molecular and cellular heterogeneity in GBM and the innate and adaptive immune mechanisms that are suppressed, which ultimately contribute to tumorigenesis.

GENOMIC LANDSCAPE OF THE GBM MICROENVIRONMENT

GBM shows considerable cellular and molecular heterogeneity, both between patients and within the tumor microenvironment itself. GBM subtyping via histological examinations is a poor prognostic indicator for gliomas. Glioma is an overarching term used for brain tumors of glial cells: astrocytes, glioblastoma, oligodendrocytes, oligodendroglioma, ependymal cells, ependymoma, and was improved by combining histology with molecular genotyping of key markers (e.g., iso-citrate dehydrogenase (IDH), ATP-dependent helicase (ATRX), Lys-27-Met mutations in histone 3 (H3K27M), p53 mutations, and 1p/19q chromosomal deletion (14). However, the era of genomics and next generation sequencing (NGS) has led to a greater understanding of the formation and pathogenesis of these tumors by identifying core molecular pathways affected, facilitating the design of novel treatment regimens. The Cancer Genome Atlas (TCGA) network was among the first to conduct a major genomic study interrogating 33 different types, with particular emphasis on GBM, leading to the whole genome characterization and molecular genotyping of 600 GBM and 516 other low-grade gliomas (15). Novel genomic variations were identified, e.g., deletions of neurofibromin gene (NF1) and parkin RBR E3 ubiquitin protein ligase (PARK2) as well as copy number variations (CNVs) of AKT serine/threonine kinase 3 (AKT3) and other single nucleotide variations (SNVs). Furthermore, patients who had undergone treatment were shown to have higher genetic variability in their recurrent tumors than untreated patients, showing additional layers of complexity in the pathogenesis and progression of GBM. These data allowed the TCGA to group GBM into distinct molecular subtypes (16). Subsequent studies further refined this classification using additional genomic and transcriptomic data to give the following three most clinically relevant molecular subtypes of GBM: proneural (PN), mesenchymal (MSC), and classical (CL) (**Table 1**). This classification was based on platelet-derived growth factor receptor A (PDGFRA) gene/IDH mutation, NF1 mutation, and epidermal growth factor receptor (EGFR) expression, respectively (15, 22). EGFR is also an important marker for proliferation and MSC subtype (23).

These GBM classifications have been key in trying to associate genomic/molecular variation to clinical phenotypes, particularly in recurrent episodes and treatment failures, such as the PN-MSC subtype-switch in the tumor aggressiveness and resistance. In line with this, a recent study (where glioma cells were treated with varying concentrations of cytokines) revealed that cytokine storm in the GBM tumor microenvironment enforces PN-subtype switch to MES-subtype by transcriptional networking and induces radiation-resistance properties (24). Similarly, another study shows that post-translational modification of oncogenic transcription factors (TF) such as OLIG2, switches the proliferative nature of glioma cells into a highly invasive phenotype by controlling the inflammatory cytokine, TGF-β (30). Prognostically, GBM patients with the MSC subtype tend to have a poor survival and resistance to therapy in comparison to other subtypes. Inevitably, NF1 drives mutations and a characteristic NF-κB transcriptome profile, an important inflammatory TF that seems to be very specific to MSC subtype (17). Moreover, NF1 is an RAS-GTPase and an important tumor suppressor gene. Its disruption, through mutation or deletion, is associated with enhanced tumor aggression and invasiveness (31). Deficiency in NF1 is also key in macrophage/microglia recruitment (32–34).

Most of the early TCGA studies have utilized tissue from one single random location in the tumor, but as mentioned above, GBM has high levels of cellular heterogeneity, with several factors affecting the molecular subtype, including anatomical location. Using RNA-Seq, a single GBM sample was shown to contain cells from 3 different subtypes (25). Approximately

TABLE 1 | Adult (WHO Grade IV) Glioblastoma multiforme (GBM) subtypes defined by genomic, transcriptome and epigenomic markers.

GBM phenotype	Methylation status	Genotypic/phenotypic abnormality	
Proneural (PN)	G-CIMP+*	IDH1/IDH2 mutations	Ch10 deletion
	MGMT gene promoter (high)	ARTX mutation	MYC
		TP53 mutation	CDKN2A/CDKN2B deletion
	G-CIMP–*	IDH1 wildtype	RTKI
		TERT promoter mutation PDGRFA amplification Ch7 insertion/chr10 deletion	CDK4 amplification DLL3, OLIG2 and NKX2-2
Classic (CL)	Cluster M3* MGMT gene promoter (moderate)	EGFR amplification/mutation RTKII	CDKN2A/CDKN2B deletion PTEN deletion
		EGFRvIII	TERT promoter mutation
		Ch7 insertion/chr10 deletion	IDH1/IDH2 wildtype
Mesenchymal (MSC)	Cluster M1*	NF1 mutation	VEGRF2
		TP53 mutation	CD40, CD31, CD68
		S100A1, PTPRC TERT promoter mutation	CHI3L1/YKL-40, MET EGFR amplification (MSC subtypes)
		Ch7 insertion/chr10 deletion	↑NF-κB driven inflammation

*Neural "subtype" not used in classification as no gene clustering observed in several studies (15, 17–20). G-CIMP, Glioma CpG island methylator phenotype; MGMT, O^6-methylguanine-DNA methyltransferase; TERT, Telomerase reverse transcriptase; RTKI, RTKII, Receptor tyrosine kinase I and II; EGFR, Epidermal growth factor receptor; VEGRF2, vascular endothelial growth factor receptor 2; PTPRC, Protein Tyrosine Phosphatase Receptor Type C; S100A1, S100 Calcium Binding Protein A1; MET, MET-Proto-Oncogene, Receptor Tyrosine Kinase. *Methylation cluster and G-CIMP phenotype defined by Brennan et al. (21). ↑, enhanced. Ch, Chromosome. Table compiled using data from the following: Cancer Genome Atlas Research Network (16), Verhaak et al. (22), Wang et al. (15), Phillips et al. (23), Bhat et al. (24), Patel et al. (25), Noushmehr et al. (26), de Souza et al. (27), Reifenberger et al. (28), and Waker et al. (29).*

8% of the GBM samples contain more than one subtype. Therefore, there needs to be a refinement of these genomic approaches to characterize genetic and protein changes to both single cell and specific cell populations within the tumor (35). Understanding the nature and consequences of cellular and molecular heterogeneity in GBM is crucial in identifying new biomarkers and therapeutic interventions. To date, there has been little evidence of significant association between molecular subtype and prognosis, although recently poorer prognosis has been observed in the MSC subtype, compared to other subtypes (17). Furthermore, enhanced survival was observed in GBM samples of low heterogeneity in 20% of the total GBM samples analyzed (15).

Further sub-classification and refinement of subtypes has also required an epigenetic approach. In gliomas, the mutational status of IDH is an important marker, and interestingly, gliomas with mutated IDH also have a particular cytosine-phosphate-guanine (CpG) island methylator phenotype (G-CIMP). The G-CIMP of DNA methylation seems to identify a distinct subgroup of glioma, with G-CIMP "high" subgroup of tumors in younger patients at diagnosis that having better overall prognosis. The G-CIMP "high" phenotype is also more commonly observed in lower-grade gliomas than GBM and tends to have the PN molecular subtype (21, 26). Furthermore, in patients treated with temozolomide (TMZ), those that had recurrences and had lost methylation of the O(6)-methylguanine-DNA methyl transferase (MGMT) promoter, had increased genetic mutations compared to untreated patients, indicating that this methylation phenotype could contribute to the chemotherapeutic resistance of the tumor (21, 26). However, MGMT methylation status is also predictive of treatment response in IDH wild-type GBM patients (36) and abnormal methylation of MGMT has increased prognosis in some GBM patients after TMZ treatment (37) (**Figure 1**). Recently, small non-coding RNA molecules (ncRNAs or miRNAs) have been suggested to be involved in a number of cancers. Five miRNAs were found to be involved in MGMT alterations and tumor suppressor functions of TP53 (miR-21, miR-125b, miR-34a, miR-181d, and miR-648) in GBM progression (38). In particular, miR-21 and miR-181d were associated with GBM tumorigenesis (39–42), as have a number of other miRNAs, miR-144 and miR-29a (43–45). These miRNAs may prove to be important biomarkers for GBM, but their specificity needs to be further validated.

IDH mutation has been linked with chromosomal abnormalities and prognosis in low-grade gliomas. Correlations have been observed in 3 subtypes: IDH mutant with 1p/19q co-deletion correlating to increase survival (46, 47), whilst IDH mutant without 1p/19q co-deletion and IDH wild-type was correlated with poor prognosis that is similar to GBM (16). Furthermore, patients with oligodendroglioma (which often contain the 1p/19q deletion) tended to respond better to chemo- and radiotherapy, with an enhanced prognosis overall (14, 48). EGFR-TACC fusion via a chromosomal translocation has been described in a small number of GBM patients, but its clinical significance is unclear (35), but may have strong sensitivity to some tyrosine kinase inhibitors (49).

Further studies have identified known oncogenic pathways in GBM such as RB, p53, RTK/RAS/P13K (16); a putative attempt at linking GBM molecular subtypes to cell types of the central nervous system (CNS) has also been suggested based on

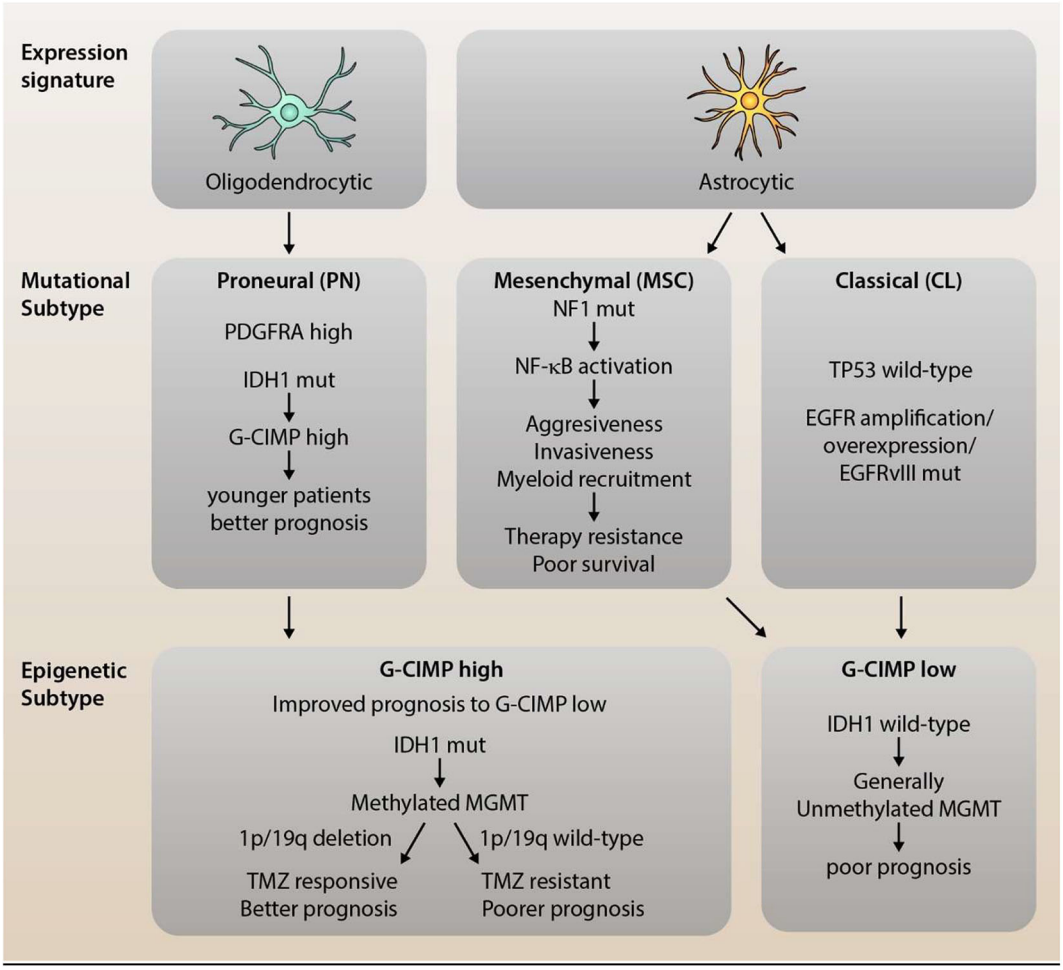

FIGURE 1 | Dissection of Mutational and Epigenetic GBM Subtype Classifications. Glioblastoma (GBM) is a highly heterogeneous disease with distinct, recurring molecular subtypes that differ in their associated expression profile, mutational signature, and epigenetic modifications. GBM can be classified into three main subtypes: the proneural (PN), mesenchymal (MSC), and classical (CL) subtype. PN gliomas tend to display an expression profile resembling oligodendrocytes, high levels of PDGFRα (due to amplifications or mutations) as well as characteristic mutations in IDH1. The latter leads to an epigenetic CpG island methylator phenotype (C-GIMP), which is associated with younger patients and a better prognosis. MSC subtype tumors, on the other hand, show a high rate of NF1 mutations which, in turn, promotes NF-κB activation and, thereby, aggressiveness, invasiveness, and myeloid recruitment. This translates into a therapy resistant phenotype for MSC gliomas with poorer survival compared to the other subtypes. The third subtype is the classical subtype, which preserves wild-type p53 expression, but shows over-expression and/or mutation of EGFR. Both MSC and CL tumor cells resemble (cultured) astrocytic gene expression profiles as well as epigenetically a G-CIMP low phenotype. The distinction between G-CIMP high and low is not only prognostically relevant (as G-CIMP high shows improved prognosis), but also predictively. Methylation of MGMT, which is observed in G-CIMP high tumors, in conjunction with 1p/19q deletion, has been shown to sensitize cells to TMZ treatment, leading to significantly improved survival.

gene expression signature: PN subtype—oligondendrocytic, CL subtype-astrocytic and MSC subtype–astrocytic (cultured cells) (22, 50). This remains to be fully substantiated. However, the MSC subtype generally is the most heterogeneous, showing its complexity compared to other non-MSC tumors (22). A few studies have also reported a switch between molecular subtypes in recurrent tumors that may be driven by the accumulation of new genetic mutations (23, 51, 52). It has been suggested that recurrent tumors may acquire extra mutations and evolve along two distinct molecular pathways governed by p53 mutation (Type 1 GBM) or EGFR amplification (Type 2 GBM) (51). Although the MSC subtype is the most common subtype in GBM, the shift from PN to MSC has not been clearly shown to occur (15).

Comparative studies between initial and recurrent GBM have been conducted using specific known markers and genome-wide analysis to further understand tumorigenesis and progression. Immunohistochemistry has been used to study proteins thought to be involved in DNA repair and tumor growth such as MutL homolog 1 (MLH1), MutS homolog2 (MSH2), and tumor suppressor p53 (53). These were found to be expressed significantly lower in recurrent GBM. Furthermore, reduction of MLH1 and post-meiotic segregation increased 2 (PMS2) proteins conferred TMZ resistance and is associated with

recurrent TMZ (54). Genomic, transcriptomic and epigenetic approaches have been utilized in a number of longitudinal studies using whole epigenome sequencing (WES), targeted genome sequencing (TES), loss of heterozygosity (LOS), quantitative PCR, RNA-Seq, transcriptome profiling and whole genome sequencing (WGS). These studies have identified numerous additional pathways, biomarkers and deciphered the mutational behavior of the tumor with and without treatment. Genetic differences in tumor evolution were observed in primary and recurrent tumors, sharing relatively few initial mutations (55). Subtype switching was also found to be common (66%) in primary GBM and may be a result of accumulation of additional mutations in highly expressed genes (56). A new mutation in latent TGF-β-binding protein 4 (LTBP4) gene was found in 10% of recurrent GBM, whilst the TGF-β pathway was also found to be involved in tumor pathogenesis (56). Primary GBM tumors without p53 and EGFR mutations gain novel EGFR amplification during recurrence and can follow two distinct pathways, depending on the genetic type of the original tumor (51). In another study, using WES, considerable tumor heterogeneity, mediated by EGRF overexpression, was observed in GBM, as well as a deletion on chromosome 10, losing phosphatase and tensin homolog (PTEN) and cyclin-dependant kinase inhibitor 2A (CDKN2A) genes (57). A further study analyzed the evolution of mutations in GBM by using paired samples and found that 67.9% were clonal in nature, whilst 29.8% were sub-clonal (55). Of these, 90% of p53 and PIK3CA/PIK3R1 mutations were also clonal, suggesting that the nature of p53 mutations in GBM has implications for tumorigenesis (55). TMZ treatment also influences the nature and rate of mutations in recurrent GBM tumors (58). Transcriptomic profiling revealed that a macrophage/microglia-rich tumor microenvironment is key for the development of the MSC molecular subtype, which is further facilitated by NF1 depletion (15) (**Figure 1**).

Epigenomic analysis has offered important insights into molecular mechanisms, such as methylation, underpinning clinical phenotypes. Promoter methylation of the DNA-repair gene MGMT results in gene silencing which was associated with significantly better prognosis in patients treated with TMZ, than those that did not have a methylated MGMT promoter (59). In this study, 45% of 206 GBM cases were found to have MGMT promoter methylation (59). In a recent study, a comprehensive DNA methylation analysis of 200 tumors from 77 GBM patients identified biomarkers which, at the time of diagnosis, were found to be predictive of GBM recurrence and prognosis. Patients in the G-CIMP "high" subgroup, with IDH mutation and intact 1p19q were found to have a good clinical outcome upon recurrence compared to patients with altered and lowered methylation (G-CIMP "low"), at the time of diagnosis, with the latter having an increased risk of recurrence and significantly poorer clinical outcome (27). Another important recent study conducted a detailed survey of DNA methylation in GBM tumors using the reduced representation bisulfite sequencing (RRBS) technique and RNA-Seq, and made significantly findings in dissecting out tumor heterogeneity based on DNA methylation profile (60). Transcriptional subtypes of tumor were identified as well as DNA methylation profiles, predictive of immune cell infiltration, necrosis and tumor cell morphology. Furthermore, de-methylation of Wnt signaling promoters upon recurrence and progression was also associated with worse clinical outcome (60).

These promising studies showing genomic variations, transcriptional profiles, molecular abnormalities of G-CIMP and other global DNA methylation profiles, along with the changes in the local tumor microenvironment, will lead to a greater understanding of the complex tumor-immune heterogeneity, and enable interventions to prevent GBM tumorigenesis and progression in the future (**Figure 1**). One such key player is the complement system, the most potent and versatile humoral innate immune system.

COMPLEMENT SYSTEM AND GBM

The complement system is one of the first lines of defense of innate immunity in the brain and is comprised of more than 30 different glycoproteins which are soluble proteins, cell associated regulators or receptors (61). Complement can be activated by pathogens and altered-self cells or indirectly by pathogen-bound antibodies. Activation of complement opsonises target pathogens or altered-self cells for phagocytic uptake, inducing an inflammatory response and enabling cell lysis. Complement is activated through 3 different pathways which are the Alternative, Classical and Lectin pathways (**Figure 2**) (62, 63). The alternative pathway is auto-activated by a process termed 'tick-over', where C3 (the most abundant complement protein) is spontaneously hydrolyzed, designated C3(H_2O). Complement protein Factor B associates with C3(H_2O) and in-turn is cleaved by Factor D generating Ba and Bb. The larger cleaved product Bb remains associated and forms the protease complex C3(H_2O)Bb which cleaves additional C3 to form the cleaved products C3a and C3b. The cleaved anaphylatoxin C3a can elicit inflammation whereas C3b can bind to and opsonize pathogens and also bind to C3 convertase (C3bBb) to form C5 convertase (C3bBbC3b). An amplification loop can also be initiated when C3b generated from the Classical and Lectin pathway bind with Factor B from the alternative pathway allowing Factor D to cleave it similarly to "tick-over" (63, 64). The activation of the Classical pathway is through the binding of C1q directly to pathogens, altered-self cells or to antibody antigen complexes. This triggers the C1r to activate C1s which cleaves C4 and C2 to generate C4a anaphylatoxin, C4b opsonin, C2a and C2b. C4b and C2b bind to form C3 convertase (C4b2b) (65). Similarly, in the Lectin pathway both C4 and C2 are also cleaved producing the same products that generate C3 convertase (C4b2b). The lectin pathway is activated by mannose binding lectin (MBL) binding to oligosaccharides on pathogens. The associated enzyme mannan-binding lectin serine protease (MASP) 2 are responsible for the cleavage of C4 and C2 (66, 67). All 3 pathways converge at C3 convertase enabling the cleavage of the central complement component C3 to form C3a and C3b. The opsonin C3b binds to C3 convertase and generate C5 convertase (C3bBbC3b) (C4b2Bc3b), which enables the cleavage of C5 to form anaphylatoxin C5a, and opsonin C5b. C5b binds to the pathogen

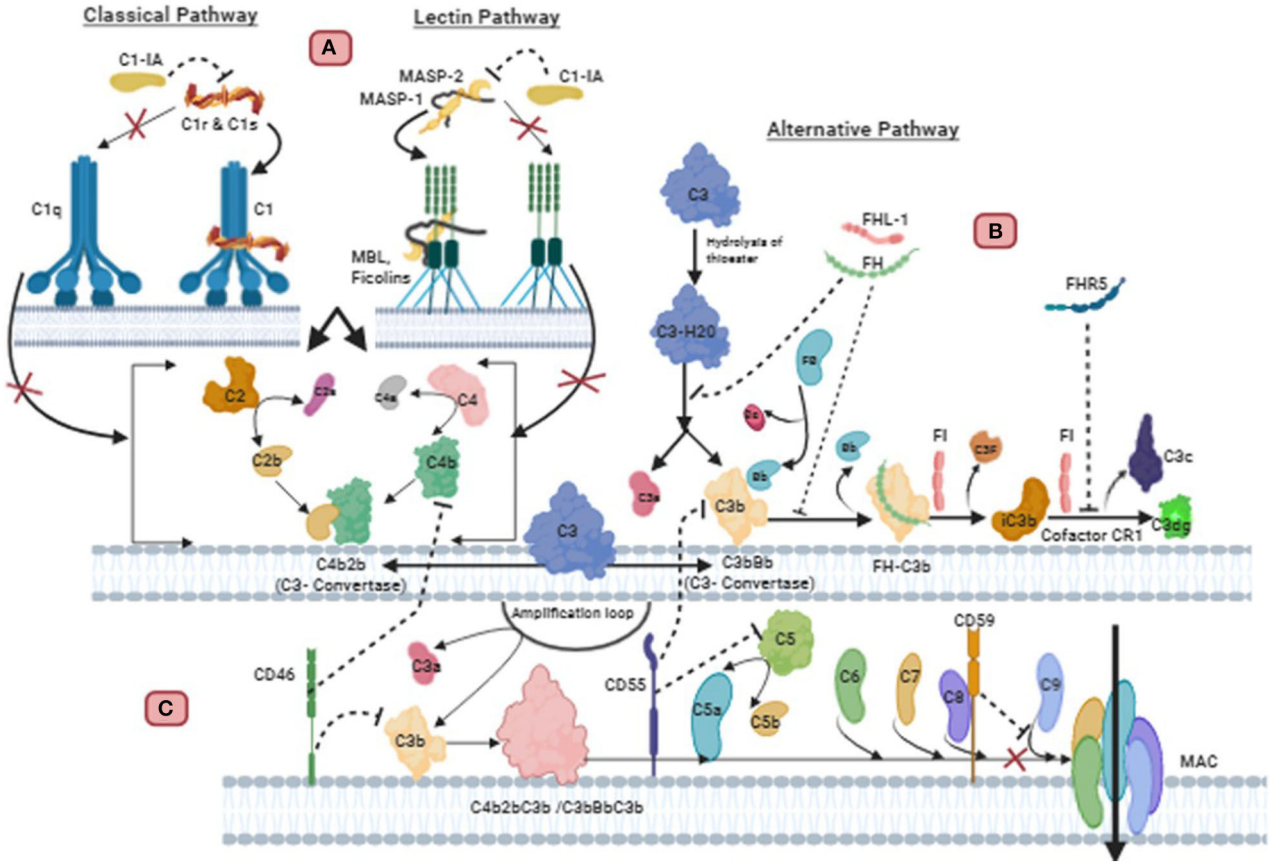

FIGURE 2 | Regulation of complement pathways in Glioblastoma: **(A)** C1 inactivator (C1-IA), also called C1 inhibitor (C1-Inh), binds covalently to the active site of C1r and C1s, blocking their function. It also dissociates $C1r_2C1s_2$ from C1, releasing C1q. This inactivation subsequently prevents the cleavage of C4 and C2 mediated classical pathway. C1-IA can also inhibit the function of MASP-1 and MASP-2 and prevent cleavage of C4 and C2 of the lectin pathway. **(B)** Endogenous or GBM synthesized Factor H (FH) and FH-like protein 1 (FHL-1) can successfully bind to GBM cell membrane. FH is a decay accelerating factor for C3 convertase. This plasma alternative pathway regulator FH binds with C3b in the convertase, displacing Factor Bb to inactivate the convertase. This FH-C3b also acts as a cofactor for cleavage of C3b by Factor I (FI) to yield the inactive product iC3b. CR1 allows FI to perform the second cleavage generating C3c and C3dg. Complement factor H related protein 5 (FHR5) secreted from GBM also exhibits functional activity similar to factor H. FHR5 functions as a co-factor for factor I mediated cleavage of C3b, and decay acceleration of C3 convertase, thus inhibiting complement mediated lysis. **(C)** The membrane bound regulators such as CD59, CD55, and CD46 are also important for resisting complement attack on GBM cells. CD59 binds to C5b-8 complex and blocks the sites for C9 attachment, thus, preventing polymerization of C9 and inhibition of MAC formation. CD55 inhibits the formation and accelerates the decay of C3 and C5 convertase of alternative and classical pathway. CD46 causes inactivation of C3b and C4b deposited on the membrane.

and also to C6, C7, C8, and C9, to produce a membrane attack complex (MAC) which generates pores through the pathogen's cell membrane, leading its destruction by osmotic cell lysis (61).

The complement system plays an important role in defense against pathogens, angiogenesis, neuroinflammation and neurodegeneration, as well as regulation of adaptive immunity. Apart from these functions, complement system also has a key role to play in cancer immunotherapy, cytotoxicity and tumorigenesis (68). Over the years, studies have shown that GBM is resistant to complement-mediated killing and this is facilitated by membrane-bound and soluble complement inhibitors. These regulators include Factor H (FH), FH-like protein 1 (FHL-1), C1 inactivator (C1-IA; also called C1-inhibitor:C1-inh), protectin (CD59), membrane co-factor protein (MCP; CD46) and decay accelerating factor (DAF; CD55) (69–71). FH is an important soluble regulator of the Alternative pathway, as it competes with factor B for C3b binding, to prevent the formation of C3 convertases and thus accelerates the decay of C3 convertase (C3bBb) to disassemble the enzyme (**Figure 2**). FH also acts as a co-factor for factor I to inactivate C3b by cleaving the α-C3b chain into 2 fragments (72, 73). FH is composed of 20 complement control proteins (CCPs) of which CCP 1–4 facilitate the functional activity of FH. FHL-1 represents the truncated form of FH as its 7 CCPs are identical to the N-terminal of FH, and therefore elicit the same inhibitory ability (73, 74). In the presence of glycosaminoglycans and sialic acid, which are present on self-cells, the affinity of FH increases for surface bound C3b via the 3 binding sites at CCPs 1–4, 7–15, and 19–20. The polyanions are only present on self-cells, thus enabling FH to differentiate between self and non-self-cells (72, 75).

TABLE 2 | Immune system components associated with Glioblastoma multiforme (GBM) microenvironment.

Immune system component	Source	Effect on GBM microenvironment	References
Cytokine			
IL-10	TAM	Enhances Immunosuppression, promotes tumorigenesis, decreases expression of MHC class II on monocytes, promotes Tregs, inhibits expression of TNF-α and IFN-γ, suppresses anti-tumor effect of immune cells	(76–78)
TGF-β	TAM and GSC [TGFB2]	Suppresses anti-tumor immune response, promotes tumorigenesis, blocks NK cells activity, Inhibits T-cells, promotes Tregs, downregulates IL-2, Inhibits NKG2D on $CD8^+$ T-cells, upregulates $CD133^+$	(79–83)
IL-6	TAM	Suppresses immune effector cells	(84, 85)
CSF-1	TAM	Enhances immunosuppression	(86–88)
Complement system			
FH	GBM cells	Enhances immunosuppression, inactivates C3b, inhibits activation of the complement alternative pathway	(70)
C1-IA	GBM cells	Enhances immunosuppression, prevents activation of the complement classical pathway	(69)
CD59	GBM cells	Enhances immunosuppression, inhibits the formation of MAC, prevents activation of the complement pathway	(70)
CFHR5	GBM cells	Inhibits complement-mediated lysis and decay acceleration of C3 convertase	(89)
TAM			
TAM	Microglia and macrophage/ monocyte	Polarises toward M2 phenotype, enhances immunosuppression, promotes tumor invasion, secretes anti-tumor cytokines, expresses FasL which act as an immunosuppressant, expresses MMPs which promote tumor invasion, promotes proliferation of growth factors	(86, 90)

IL, interleukin; TGF, transforming growth factor; CSF, colony stimulating factor; FH, factor H; C1-1A, complement 1-inactivator A; CFHR5, complement factor H related protein 5; TAM, tumor-associated macrophage.

Complement Regulators

Complement regulatory proteins are important in protecting healthy self-cells from complement attack by exerting tight regulatory functions. Regulation is required at all major checkpoints of complement activation and amplification to prevent a deleterious effect on self-cells from an over-reactive complement system. Healthy cells express soluble regulators such as FH and membrane bound regulators including CD59, CD55, and CD46 (**Table 2**), which all use different mechanisms to provide protection (91, 92). Soluble regulators inactivate complement as they are attracted to self-structure over foreign surfaces (93, 94). However, soluble and membrane-bound complement regulators can act as double-edged swords by overregulating the complement system to the point it is unable to eliminate tumor cells. Studies suggests that the expression of complement regulators by tumors including GBM allows these cells to proliferate unchecked. This highlights the significance that complement regulators play in the tumor cells' avoidance of complement attack. As knowledge of the relationship between complement regulatory proteins and tumors evolves, it is possible that their therapeutic blockade can have an important role in tumor treatment (70, 71).

Factor H

Factor H is secreted by GBM cell lines such as H2, U138, U118, and U87 (95). In another study by Junnikkala et al., expression of RNA and protein production of FHL-1 in the malignant cells was found to exceed that of FH, in contrast to normal serum where the concentration of FH is greater than FHL-1 (70) (**Table 2**). It appears that endogenously synthesized and fluid phase FH and FHL-1 from plasma can successfully bind to the GBM cell membrane, efficiently regulating complement activation and promoting the cleavage of membrane deposited C3b into its inactive form iC3b. Ultimately, this mechanism prevents activation of the late stages of complement activity, to elicit cell lysis via MAC formation because there is reduced C5b-9 deposition. The inhibitory effect of secreted FH and FHL-1 can be overcome through neutralization of FH and FHL-1 with antibodies that target the C3b binding site and by the removal of sialic acid to sensitize GBM cells to complement lysis. FH and FHL-1 play a crucial role in GBM tumorigenesis by enabling the acquisition of GBM cells' exceptional resistance to complement mediated killing (70). In a more recent study on primary tumor cells derived from 3 GBM patients, secretion of complement Factor H related protein 5 (FHR5) was also reported (89). It was found that the cells secreted FHR5, but not FH, and that FHR5 inhibited complement-mediated lysis and decayed acceleration of C3 convertase (89).

Complement 1 Inactivator A

GBM resistance to complement-mediated lysis can be acquired by the production of Complement 1 inactivator (C1-IA) or C1 inhibitor (C1-inh) (**Table 2**). C1-IA, a serine protease, is able to regulate classical pathway activation by irreversibly binding to C1r and C1s proteases, which along with C1q, form the multiprotein complex C1, which is the first component in the

initiation of the classical pathway (96, 97). The ability of C1-Inh to bind to C1r and C1s protease subsequently prevents C1r autoactivation and C1s activation, which in turn, prevents the cleavage of C4 and C2. This ultimately stops the formation of the Classical pathway's C3 convertase (C4b2a) (98). Gene expression and mRNA analysis in human GBM tissues showed an upregulation of C1-inh (69). Inhibition of C1-inh in rats with GBM, using appropriate antibodies, was found to increase survival but also led to decreased levels of cytokines IL-1β and GM-CSF, which are associated with an immunosuppressive tumor microenvironment (69, 99).

Membrane-Bound Complement Regulators

The ability of GBM cells to avoid complement attack is not only determined by soluble inhibitors but also by membrane bound regulators such as CD59, CD55, and CD46 (70, 71) (**Table 2**). CD59 is a major protective element against complement mediated lysis. It binds to C5b-8 complex and blocks the sites to which C9 can attach, thus, preventing the insertion and polymerization of C9. As a result, the final step of MAC assembly on the cell membrane is prevented (100). CD55 is an anchored membrane regulator that inhibits the formation and accelerates the decay of C3 and C5 convertase of the alternative and classical pathway to prevent complement activation (101). The complement cascade is also regulated by CD46, which serves as a co-factor of factor I inactivation of C3b and C4b, deposited on the membrane (102).

In a study by Maenpaa et al., it was shown that CD59 was expressed in 14 human glioma tissues as well as 7 glioma cell lines (71). In normal astrocytes, the expression of CD59 is weak as the need to protect these cells from complement is reduced due to the blood-brain barrier, which restricts entry of many pathogens into the brain (71). Successful binding of CD59 to C5b-8 complex inhibits the formation of MAC at the point of insertion of C9 into GBM cell membrane, thus protecting the cell from complement mediated killing (70). The inhibition of CD59 by neutralizing antibodies enables the cells to overcome the resistance of GBM to complement mediated cytolysis (70). In the same study, CD55 and CD46 were also shown to be moderately expressed in GBM cell lines, and neutralizing them with respective antibodies showed moderate complement-mediated cytolysis, although CD59 was considered to be the most important complement regulator on GBM cells (70).

Role of Microglia and Macrophages in GBM

The CNS has historically been considered an immune privileged site. This is primarily because it lacks a traditional lymphatic system, containing only a few antigen presenting cells which would mount an extremely weak immune response (103). Considering recent data, the characteristics of immune privilege have been redefined and are no longer considered absolute (103). The concept of immune privilege had stemmed from the ability of antigens within the brain to avoid systemic immunological recognition (104). It is now evident that immune privilege is specific to brain parenchyma which is imperative for damage limitation during inflammation. The brain parenchyma is an extremely sensitive part of the organ with poor regenerative capacity and is protected by the blood brain barrier, a semi-permeable membrane consisting of endothelial cells that separate the blood from the cerebro-spinal fluid (104).

The CNS is able to coordinate a robust immune response involving both the innate and adaptive immune systems (105). During inflammation, immune cells are able to migrate to perivascular spaces following chemotaxis (106). Studies have shown that antigens can enter the cervical lymph nodes by passing through the Virchow Robin Perivascular Space within the walls of the cerebral arteries (107). It is also possible for immunoglobulins to cross the blood-brain barrier via carrier mediated transporters by attaching to FcRn receptor (108). Antigen presentation occurs as dendritic cells (DCs) can travel outside of the brain and present antigens to T-cells located in the cervical lymph nodes (109). However, inflammation and disease in the CNS can compromise the integrity of the blood-brain barrier, thereby enabling circulating immune cells to migrate past it and infiltrate the parenchyma (110).

Microglia are the resident macrophage of the CNS comprising 5–20% of the total glial cell population. In the brain, microglia are involved in immune surveillance and are a crucial component of the first line of defense (111). Originally discovered over a century ago by Pio Del Rio Hortega, it is now clear that resident microglia originate from haematopoietic precursor cells of immature yolk sac during early embryogenesis (112). Microglia are usually found in a "resting" state; microglia having branched extensions or processes actively patrol and perform surveillance of local areas. Following inflammatory stimuli, inflammatory stimuli, circulating microglia change into "amoeboid" shape, and additional recruitment of macrophage from infiltrating circulating monocytes takes place (113, 114). Apart from surveillance, microglia actively contribute to brain development and CNS homeostasis by apoptotic cell removal, maintenance and pruning of synapses, and regulation of neuronal activity (114, 115). In GBM, a second group of macrophages derived from peripheral bone marrow, are present (116). In the brain, macrophages are restricted to the perivascular, choroid and meningeal locations. However, disruption to the blood-brain barrier by disease or inflammation allows macrophage to gain entry to the parenchyma (117). These mononuclear cells are difficult to differentiate from microglia as they intermingle in GBM (118).

Traditional approaches to distinguish macrophage and microglia involved use of CD45 antibody as microglia are defined as $CD45^{low}$, whereas macrophages are defined as $CD45^{high}$ (118, 119). Despite this, it is still unclear as to whether microglia or macrophage make up most of the mononuclear density in GBM. Parney et al. suggested that gliomas contained more recruited macrophages than resident microglia (120). However, Muller et al. challenged this concept as they demonstrated resident microglia were the main source of mononuclear cells in gliomas and that the microglia present had increased their expression of CD45 (121). Together, microglia and macrophages in GBM are generally referred to as tumor-associated macrophages (TAM) (**Figure 3**) (122).

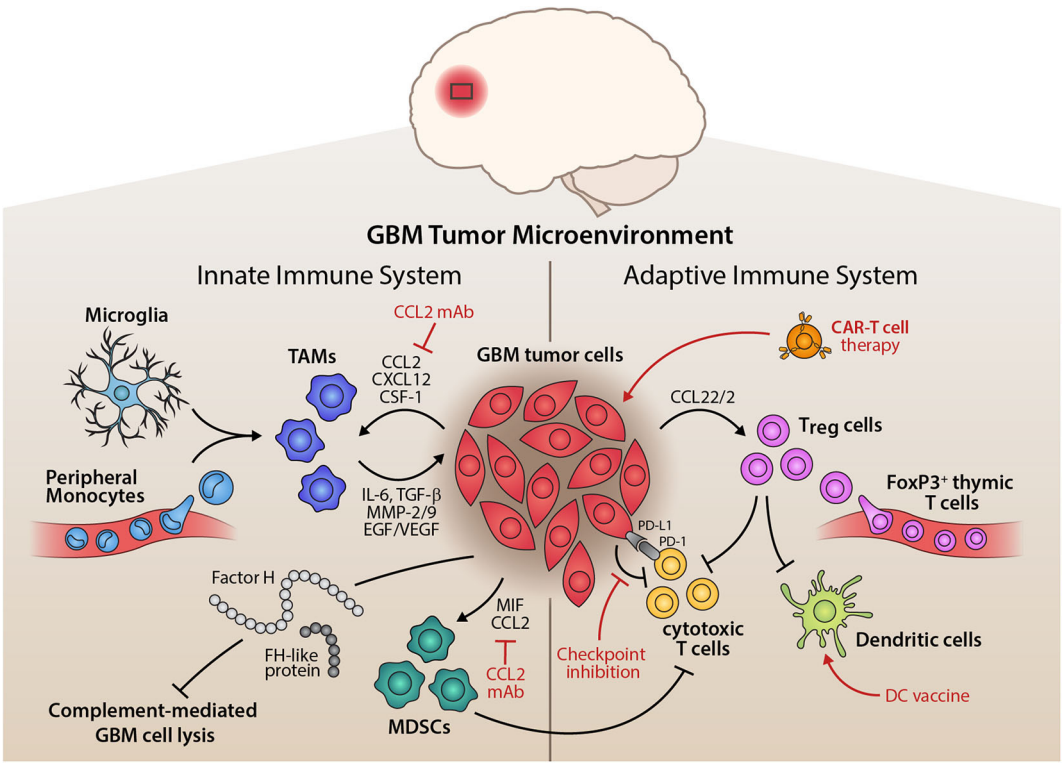

FIGURE 3 | Inflammatory Tumor Microenvironment of GBM and its Therapeutic Implications. Illustration of the interplay of innate and adaptive immune components within the glioma microenvironment. On the side of the innate immune system, tumor-associated macrophages (TAMs), mainly comprised of microglia and peripheral monocytes, are attracted by tumor cells, which release pro-inflammatory cytokines, matrix remodelers, and growth factors to aid tumorigenesis. Myeloid-derived suppressor cells (MDSCs) are also recruited by the tumor and potently suppress anti-tumor immunity. Alternative pathway molecules factor H (FH) and FH-like protein 1 of the complement system enhance immunosuppression and prevent complement-mediated lysis of the tumor cells. The adaptive immune system, on the other hand, is largely suppressed in its function through the recruitment of regulatory T cells (T_{reg}). These inhibit the action of cytotoxic T cells and dendritic cells, disturbing a competent anti-tumor immune response. Tumor cells also exert direct suppression of adaptive immunity through immune checkpoint expression, e.g., PD-L1 or CTLA-4. Therapeutically, this tumor-immune crosstalk can be targeted by inhibiting chemoattractants of pro-tumor immune cells, such as anti-CCL2 monoclonal antibody, by immune checkpoint inhibition, dendritic cell vaccination approaches or adoptive transfer of chimeric antigen receptor (CAR) T cells that target the glioma cells (red indicators).

It has also been reported that in the MES subtype, deficiency of NF1 leads to increased infiltration of TAM (15). This may explain why GBM subtype-specific cell autonomous functions drive tumor aggressiveness and therapy resistance and have poorer prognosis. Furthermore, this study also highlighted that the tumor microenvironment in recurrent GBM showed the presence of more resident microglia/macrophages as compared to peripherally-derived monocytes, indicating that treatment (such as radiotherapy) may have an impact on monocytes, and thus in recurrent GBM; more efforts need to be made to address resident cells in the brain. This elegant study also showed increased $CD8^+$ T cells in TMZ-induced hypermutated recurrent GBM (15).

Microglial cells have been known to enhance infiltration leading to increased invasiveness of the tumor. A murine microglial cell study on mouse glioma cells found that tumor cell migration occurred sooner and was higher when compared to tumor cells without microglia (123). Another study using murine brain slices found that microglia stimulated the extracellular matrix metalloprotease (MMP)-2, which led to increased invasiveness of the tumor (124). Pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α, secreted by microglia, have been shown to increase tumor invasiveness *in vitro* (125). By specifically targeting microglia, using propentofylline which blocks secretion of IL-1β, IL-6 and TNF-α, tumor growth was found to regress (126).

GBM cells secrete a range of chemo-attractants such as CCL2, CXCL12, and SDF-1, which actively recruit microglia and macrophages (127, 128). Various CC and CXC chemokines are secreted including CCL2, CXCL12, and their receptors (129, 130). CCL2 is one of the most important CC chemokines commonly expressed by GBM as it plays a key role in regulating the penetrative migration of TAM to the GBM microenvironment (131). It was the first TAM chemo-attractant identified in GBM; the level of CCL2 expression is associated with glioma grade (132). CCL2 is highly expressed in GBM at mRNA and protein levels, thus contributing to a high influx of TAM (133). Inhibiting CCL2 activity in mice studies (GL261

glioma and xenograft of human U87 models) with relevant antibodies has been shown to reduce infiltration and ultimately prolong survival (134). The receptor for CCL2 is CCR2 which are also present on microglia (135). In addition, microglia from the GBM tumor microenvironment have the capacity to secrete CCL2, thereby stimulating more microglia recruitment to the tumor (130).

CXCL12, also known as stromal derived factor 1 (SDF-1), a chemokine, promotes TAM recruitment in high-grade gliomas. A murine high-grade model, ALTS1C1, demonstrated the chemo-attractant ability of SDF-1 for microglia and macrophages. High expression of SDF-1 promoted the accumulation of TAM to areas of hypoxia in brain and tumor invasion (136). GBM cells also express colony stimulating factor-1 (CSF-1) which functions as TAM chemo-attractant (86, 87). CSF-1 is overexpressed in GBM, thus contributing to the high influx of microglia/macrophages, promoting tumor invasion (86, 87). High glucose has been shown to increase proliferation and inhibit apoptosis in a study on human GBM U87 cell line, by upregulation of vascular endothelial growth factor (VEGF) and is mediated by increased expression of chemotactic receptors including EGFR (137). A recent murine study showed that osteopontin is an important chemokine that attracts TAM to the GBM site, via integrin $\alpha_v\beta_5$ (138). Further, $\alpha_v\beta_5$ deficiency was found to lead to a direct $CD8^+$ T cell cytotoxic effect at the tumor site (138).

Majority of newly recruited TAMs acquire an alternatively activated M2 phenotype under the direct influence of tumor cells to produce a pro-tumor microenvironment. M2 polarized TAMs produce mediators that contribute to the immunosuppressive microenvironment established by the tumor cells (139). TAMs are known to secrete anti-inflammatory cytokines such as IL-6, IL-10 and TGF-β, thereby enhancing immunosuppression in tumor microenvironment, leading to promotion of GBM cell growth and angiogenesis (84). Studies have shown that these anti-inflammatory cytokines supress M1 phenotypes as TGF-β inhibits pro-inflammatory cytokine expression and microglia proliferation whilst IL-10 polarizes microglia to a M2 phenotype (88). TAMs are also known to express Fas ligand (FasL) which acts as an immunosuppressant in GBM, as it contributes to the reduced presence of tumor infiltrating leukocytes (90).

The pro-tumor microenvironment of GBM is supported by the expression of MMPs by TAM, including MMP-2 and MMP-9, which are involved in tumor growth by having an impact on angiogenesis, apoptosis and cell proliferation (140). Subsequent inhibition of MMPs derived from TAM have shown a reduction in tumor growth and angiogenesis (141, 142). A study has shown that membrane type 1 (MT1) MMP is enhanced in TAM, which in turn, activates MMP-2 in GBM, via microglial cells, thus increasing tumor invasion (143). TGF-β1 derived from microglia in GBM plays an important role in TAM-mediated promotion of tumorigenesis (79). It has been shown that TGF-β1, released by TAM, induces Epithelial-to-Mesenchymal Transition (EMT) and enhanced invasion of $CD133^+$ Glioma stem cells (GSCs) which led to a pro-tumorigenic environment (80). Moreover, TAMs also contribute to tumorigenesis in GBM by providing proliferation promoting factors such as EGF and VEGF (86).

IL-10 from TAM in GBM have the ability to promote tumor growth i*n vitro* via JAK2/STAT3 pathway (76). Activation of Signal Transducer and Activator of Transcription 3 (STAT3) co-ordinates the expression of immunosuppressive molecules by decreasing expression of major histocompatibility complex (MHC) class II and co-stimulatory molecule, CD40 (77). An activation loop is formed as the stimulation of STAT3 by IL-10 enables activation of this transcription factor in nearby immune cells (77). These cells include macrophage, natural killer (NK) cells and DCs. As a result, the anti-tumor activity of these immune cells is supressed (78). IL-10 derived by TAM also supresses MHC class II expression on monocytes and down-regulates the production of IFN-γ and TNF-α in GBM, thus preventing anti-tumor activity (144). The overall effect of IL-10 secreted by TAM on GBM is immunosuppression which ultimately promotes a pro-tumor milieu (145).

DCs are antigen-presenting cells, involved in surveillance against pathogens and tumorigenic cells, and present these to T cells, thereby serving as an important link between innate and adaptive immunity. This is utilized in anti-tumor therapies, to help induce a cytotoxic response against the tumor cells. In GBM, DCs are considered to present tumor cell peptides, leading to cytotoxic T cells response, and secretion of pro-inflammatory cytokines. Pre-clinical studies on murine glioma models have found DCs to be effective in inducing an effective tumor-response and increasing survival (146, 147). Phase I clinical trials have shown DC vaccination therapy to be safe and to elicit cytotoxic T cell responses (148, 149). Early results from a subsequent Phase III clinical trial involving an autologous tumor-lysate pulsed DC vaccine was shown to be feasible and safe and may extend survival in GMB (150).

Microglia in GBM are a major source of TGF-β, which plays a key role in contributing to the immunosuppressive GBM microenvironment (135). TGF-β enhances immunosuppression in GBM through a range of mechanisms including blocking T-cell activation and proliferation, inhibiting the activation of NK cells, down regulating IL-2 production, and promoting T_{regs} (81). Blocking T cell activation can be achieved by the ability of TGF-β2 to supress HLA-DR antigen expression which is essential for tumor associated antigen presentation to $CD4^+$ T-cells (82). TGF-β is also capable of facilitating immune escape by inhibiting NKG2D (an activating receptor responsible for host-response to pathogen and tumor cells) on $CD8^+$ T cells and NK cells ultimately rendering the cells less effective at cytotoxic destruction of GBM (83). Strategies which inhibit TGF-β expression can restore anti-tumor immunity in GBM. Transient silencing of TGF-β, using siRNA, has been shown to prevent NKG2D expression and increase GBM susceptibility to destruction by immune cells (151). Murine glioma models also showed that blocking TGF-β1 receptor increased the number of long-term survivors by 33%, as opposed to the 6% observed in the control group. The level of $CD8^+$ T cells were also increased, demonstrating a reversal of the immunosuppressive effect when TGF-β1 is inhibited (152).

NK cells are known for its anti-viral and anti-tumor response, and secrete cytokines such as IFN-γ and TNF-α. Pre-clinical models of GBM have shown NK cells to be effective in HLA

class I-mediated tumor lysis (153); IL-2 activated NK cells' ability to kill GBM cells (154), and NK cells' effectiveness in preventing metastasis in the GBM xenograft mouse model have been reported (155).

ADAPTIVE IMMUNITY AND T_{REG} CELLS

T_{reg} cells play a major role in mediating immune suppression of anti-tumor immune cells. In non-tumorigenic environments, T_{regs} usually are involved in preventing autoimmunity (156). T_{regs} are a sub-population of $CD4^+$ T-cells and can be categorized into two groups based on their developmental origin. Thymus derived T_{regs} develop after antigen presentation by thymic epithelial cells and are characterized by high level expression of the transcription factor Forkhead Fox P3 (FoxP3) (157). By contrast, peripherally induced T_{regs} differentiate in the periphery upon antigen presentation and recognition by naive conventional $CD4^+$ T-cells. IL-10 and TGF-β signaling are key contributors in supporting the induction of peripherally induced T_{regs} which have negligible FoxP3 expression (158). Studies have shown that there is a high influx of T_{regs} predominately of thymic origin, accounting for 25% of tumor infiltrating lymphocytes (159, 160). The abundance of T_{regs} is associated with poor prognosis, as they shift the tumor cytokine milieu toward immunosuppression (161). This enhanced immunosuppression is achieved by T_{regs} ability to restrict the function of infiltrating T cells by preventing production of IL-12 (162). The high influx of T_{regs} in GBM is likely due to CCL22 and CCL2 secreted by GBM, as they bind to CCR4 commonly expressed by T_{regs} (163, 164).

Immune Checkpoint

Immune checkpoints are co-stimulatory and co-inhibitory pathways that restrict the function of the immune system. These regulatory pathways supress T-cell activation and proliferation, ensuring that immune responses are limited to maintaining self-tolerance which prevents the immune system attacking self-cells (165). An immune checkpoint involved in GBM immune evasion is programmed cell death protein 1 ligand (PD-L1), which is a transmembrane glycoprotein of the B7 family co-stimulatory molecules (166). PD-L1 is not usually expressed in the CNS, therefore, its presence in this location is associated with a pathological or tumorigenic environment (167). PD-L1 is activated by binding to the receptor programmed cell death protein 1 (PD-1) to exert its inhibitory effect (168). In GBM, activation of PD-L1 suppresses the proliferation and function of tumor resident cytotoxic T cells, which would otherwise destroy the tumor cells. PD-L1 can also enhance T_{reg} activity which will promote a pro-tumorigenic microenvironment (168) (**Figure 3**).

Various immune cells express PD-L1 in GBM, such as $CD4^+$ and $CD8^+$ T cells (169). TAM express PD-L1 on their surfaces, whilst promoting PD-L1 expression on GBM cells (166). Genetic alterations have also been shown to contribute to PD-L1 expression as the loss of PTEN tumor suppressor gene enhances the expression of PD-L1 on glioma cells (170). The expression pattern of PD-L1 is positively correlated with glioma grade and is also associated with poor survival of GBM patients (169). A study in mouse glioma cell-line has shown that inhibiting PD-L1 with antibodies on glioma cells in combination with radiotherapy has clear survival benefits (171). PD-L1 expression was found to be dependent on IL-6; inhibition of IL-6 signaling diminished expression of PD-L1, leading to increased survival and reduced tumor growth in orthotopic murine glioma model (85).

Cytotoxic T Lymphocyte Antigen 4 (CTLA-4) is another immune checkpoint molecule which plays a role in GBM immune evasion, as it modulates the early stages of T lymphocyte activation. CTLA-4 is expressed on activated T-cell and T_{reg} in a tumor microenvironment (172). Targeting CTLA-4 in glioma models with anti CTLA-4 antibodies proved useful in reversing immune evasion. This study showed an increase in long term survival, increased resistance to T_{reg} mediated suppression and enhanced proliferation of $CD4^+CD25^-$ T-cells (172).

Despite several biological and clinical approaches, including the 2018 Nobel Prize for immune checkpoint blockade in cancer immunotherapy, no specific immune therapy treatment for GBM has been successful in phase III or randomized controlled trials due to either lack of positive response, or due to side-effects (173). Some of the clinical trials that did not show significant survival benefit include nivolumab (anti-PD-1) and ipilimubab (anti-CTLA-4) in recurrent GBM (174); nivolumab vs. TMZ and radiation therapy in newly-diagnosed GBM (175); and nivolumab in combination with TMZ and radiation therapy in newly-diagnosed GBM (176).

Other emerging themes in cancer immunotherapy include inhibition of VEGF to reduce angiogenesis and vascular permeability, and cancer vaccine-based therapy such as use of DCs to activate T cells (173). The overall survival and progression-free survival was found to be increased in newly diagnosed GBM patients who received TMZ, GM-CSF, and targeted cytomegalovirus (CMV) with DCs (177). CMV proteins have been found to be expressed in GBM but not in normal brain tissue, and this has been utilized to generate specific T-cell immune response to lyse GBM tumor cells (178). A follow-on randomized trial in GBM patients showed significant progression-free and overall survival in patients who received CMV-specific DC vaccination (179). Another exciting theme involves use of CART-cell therapy (chimeric-antigen receptor T-cell therapy), in which immune receptors are specifically engineered to generate an immune response when they face tumor proteins (180). A study in recurrent GBM patients, targeting a type of EGF, using CART-cell therapy, was found to kick-start an immune response at the site of the glioma including infiltration by T_{reg} cells (181). This preliminary study is the first in humans and involved 10 patients with recurrent GBM. They were treated with a single peripheral dose of autologous T-cells targeted to EGFR variant III, which is found in about 30% of GBM patients and associated with poorer prognosis (182). This particular CART-cell therapy was found to be safe, the infused product reached tumor site in the brain, and also found to assert anti-tumor activity by decreasing EGFR variant III expression (**Figure 3**).

Glioma Stem-Like Cells (GSCs)

Cancer stem cell hypothesis relates to presence of cells with stem-cell like properties in the tumor microenvironment (i.e., cells

that possess ability to differentiate into various cell lineages or generate new tumor or resistance to treatment) (183). The GBM microenvironment is thought to contain such cells called as GSCs that possess properties of self-renewal, pluripotency or ability to give rise to differentiated cell types, and resistance to multiple drug and radiation therapy. The presence of GSCs in GBM was first discovered by Singh et al., and since then numerous studies on GBM microenvironment have established their role in therapeutic resistance, tumor migration and invasion, capability to metastasise, as well as continued maintenance of stem cell-like state of cells (35, 184).

GSCs are considered to have the ability to escape immune response by down-regulating expression of MHC class I, thereby leading to failure of activation of cytotoxic T cells (185). One of the important mechanisms involves PD-L1 present on extracellular vesicles (lipid membrane-bound vesicles secreted by cells; also called exosomes and microvesicles) secreted by GBM cells, which block T-cell receptor by anti-CD3, thereby reducing activation and proliferation of $CD4^+$ and $CD8^+$ T cells (186). GSCs have also been shown to evade immune response by increasing production and infiltration of T_{reg} cells (83), and by increasing levels of TGF-β produced by TAM, which in turn, increase levels of TGF-β, thus, down regulates MHC II and subsequent antigen processing mechanism, causing T-cell anergy (187). GSCs are known to attract TAM *in vitro* via CCL2 and periostin (188) and by secretion of cytokines TGF-β and CSF, which are known to polarize TAM to immunosuppressive mode (88).

Myeloid-Derived Suppressor Cells (MDSCs) in the GBM Microenvironment

One of the major characteristics of GBM is the abundance of Myeloid-derived suppressor cells (MDSCs) in the tumor microenvironment, which largely determines disease prognosis by immune suppressive functions. MDSCs are the key components of innate immune system which essentially originate from the bone marrow derived cells. Significantly, infiltrations of MDSCs in GBM tumor microenvironment were markedly associated with cytotoxic T cells suppression (189, 190). A recent study showed that MDSCs substantially paralyze $CD4^+$ T cell memory functions in GBM patients (191). Moreover, findings in GBM murine models showed that pharmacological targeting of MDSCs by Sunitinib resulted in significantly increased $CD3^+CD4^+$ T cell count in the tumor microenvironment (189, 190). Moreover, MDSCs depletion led to improved animal survival as well as increased T cell activation in the in GBM patients' PBMCs (189, 190). Within GBM, GSCs constitute the major neoplastic compartment, which substantially modulates immune suppressive functions by recruitment of non-neoplastic components such as MDSCs, TAMs, and T_{regs} in the tumor microenvironment (192–195). Previous studies have reported that GSCs produce intrinsic factors such as IL-10, IL-4Rα, and TGF-β to program M2 macrophages and activation of T_{reg} cells for an effective immunosuppressive function (188, 192, 194–196). In solid tumors, cell-intrinsic factors of the neoplastic compartment play a key role in recruiting TAMs and MDSCs for disease progression. For instance, CC chemokine CCL2 (MCP1) is the most abundant chemokine, which significantly correlated with poor prognosis in GBM patients (130, 197). Genetic depletion of CCL2 in the murine model is associated with reduced infiltrations of MDSCs in the GBM microenvironment (198). CCL2 depletion led to a significant recruitment of cytotoxic T cell in the tumor microenvironment, which resulted in glioma growth suppression (198). The immunosuppressive functions of CCL2 is mediated through its binding to CCR2 and CCR4 receptors, which are mainly expressed on T_{regs} and MDSCs in GBM, respectively. Moreover, high expression of CCL2 in the GBM microenvironment leads to infiltration of T_{reg} cells, MDSCs, and TAMS, which subsequently is associated with poor GBM prognosis (130, 163, 198). GSCs produce macrophage migration inhibitory factor (MIF), which recruits MDSCs for immunosuppressive functions and GSC proliferation (195). In addition, TAMs and MDSCs account for up to 50% in the immune compartment of GBM microenvironment; in particular, MDSCs are the main source of TGF-β and PD-L1 (191, 199, 200). Hence, from a clinical viewpoint, targeting the CCL2-CCR axis, MIF, and PD-L1 could potentially offer effective therapies for GBM patients.

Unfortunately, the outcome of recent clinical trials of immunotherapies in GBM did not show any promising results. Therefore, personalized immunotherapy in combination with chemo-radiotherapy strategies for GBM patients are currently under consideration. In line with this, findings from the most recent preclinical study confirmed that combining immuno-radiation therapy exclusively targeting MDSCs and TAMs, did result in improved survival, compared to the monotherapy cohort (194, 201). Collectively, interfering with both cell-intrinsic factors of neoplastic compartments and immunosuppressive components (e.g., MDSCs) of the tumor microenvironment might offer an effective strategy to block GBM progression and overcome resistance to conventional therapies.

CONCLUSIONS

This review highlights the molecular determinants of the complex heterogeneous tumor-immune environment observed in GBM and the mechanisms and interactions of various genetic pathways, transcriptional programming, immune cells and the role of the immune suppressive microenvironment in Glioblastoma. Each aspect of metabolic pathways, innate and adaptive immune responses (including complement system) have a key role to play in the initiation, progression, infiltration, maintenance and suppression of tumor cells, thereby continuing to provide hope for potential effective therapies in future. The multi-dimensional interactions of glioma cells along with immune cells and other metabolic pathways add to the complexity of finding successful treatment avenues. Further research into this interplay of the immune response in GBM, along with the genomic processes underlying this, together with parallel progress in clinical trials, is required to overcome this lethal disease.

AUTHOR'S NOTE

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AUTHOR CONTRIBUTIONS

SD and AS wrote the first draft. AT added molecular concepts. SS and UK reviewed and edited. HY and LK drew illustrations and added supplementary information at the revision stage. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Ostrom QT, Gittleman H, Farah P, Ondracek A, Chen Y, Wolinsky Y, et al. CBTRUS statistical report: primary brain and central nervous system tumors diagnosed in the United States in 2006-2010. *Neuro Oncol.* (2013) 15(Suppl. 2):ii1–56. doi: 10.1093/neuonc/not151
2. Bonavia R, Inda MM, Cavenee WK, Furnari FB. Heterogeneity maintenance in glioblastoma: a social network. *Cancer Res.* (2011) 71:4055–60. doi: 10.1158/0008-5472.CAN-11-0153
3. Soeda A, Hara A, Kunisada T, Yoshimura S, Iwama T, Park DM. The evidence of glioblastoma heterogeneity. *Sci Rep.* (2015) 5:7979. doi: 10.1038/srep09630
4. Strupp R, Mason W, van den Bent M, Weller M, Fisher B, Taphoorn MBK, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med.* (2005). 352:987–96. doi: 10.1056/NEJMoa043330
5. Furnari FB, Fenton T, Bachoo RM, Mukasa A, Stommel JM, Stegh A, et al. Malignant astrocytic glioma: genetics, biology, and paths to treatment. *Genes Dev.* (2007) 21:2683–710. doi: 10.1101/gad.1596707
6. Gabrusiewicz K, Liu D, Cortes-Santiago N, Hossain MB, Conrad CA, Aldape KD, et al. Anti-vascular endothelial growth factor therapy-induced glioma invasion is associated with accumulation of Tie2-expressing monocytes. *Oncotarget.* (2014) 5:2208–20. doi: 10.18632/oncotarget.1893
7. Hermanson M, Funa K, Hartman M, Claesson-Welsh L, Heldin CH, Westermark B, et al. Platelet-derived growth factor and its receptors in human glioma tissue: expression of messenger RNA and protein suggests the presence of autocrine and paracrine loops. *Cancer Res.* (1992) 52:3213–19.
8. Anzil AP. Glioblastoma multiforme with extracranial metastases in the absence of previous craniotomy. Case report. *J Neurosurg.* (1970) 33:88–94. doi: 10.3171/jns.1970.33.1.0088
9. Robert M Wastie M. Glioblastoma multiforme: a rare manifestation of extensive liver and bone metastases. *Biomed Imag Interv J.* (2008). 4:e3. doi: 10.2349/biij.4.1.e3
10. Kanu OO, Mehta A, Di C, Lin N, Bortoff K, Bigner DD, et al. Glioblastoma multiforme: a review of therapeutic targets. *Expert Opin Ther Targets.* (2009) 13:701–18. doi: 10.1517/14728220902942348
11. Holland EC. Glioblastoma multiforme: the terminator. *Proc Natl Acad Sci USA.* (2000) 97:6242–44. doi: 10.1073/pnas.97.12.6242
12. Fujisawa H, Reis RM, Nakamura M, Colella S, Yonekawa Y, Kleihues P, et al. Loss of heterozygosity on chromosome 10 is more extensive in primary (*de novo*) than in secondary glioblastomas. *Lab Invest.* (2000) 80:65–72. doi: 10.1038/labinvest.3780009
13. Maher EA, Brennan C, Wen PY, Durso L, Ligon KL, Richardson A, et al. Marked genomic differences characterize primary and secondary glioblastoma subtypes and identify two distinct molecular and clinical secondary glioblastoma entities. *Cancer Res.* (2006) 66:11502–13. doi: 10.1158/0008-5472.CAN-06-2072
14. van den Bent MJ. Interobserver variation of the histopathological diagnosis in clinical trials on glioma: a clinician's perspective. *Acta Neuropathol.* (2010) 120:297–304. doi: 10.1007/s00401-010-0725-7
15. Wang Q, Hu B, Hu X, Kim H, Squatrito M, Scarpace L, et al. Tumor evolution of glioma-intrinsic gene expression subtypes associates with immunological changes in the microenvironment. *Cancer Cell.* (2017) 32:42–56.e6. doi: 10.1016/j.ccell.2017.06.003
16. Cancer Genome Atlas Research Network. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature.* (2008) 455:1061–68. doi: 10.1038/nature07385
17. Behnan J, Finocchiaro G, Hanna G. The landscape of the mesenchymal signature in brain tumours. *Brain.* (2019) 142:847–66. doi: 10.1093/brain/awz044
18. Behnan J, Stangeland B, Hosainey SA, Joel M, Olsen TK, Micci F, et al. Differential propagation of stroma and cancer stem cells dictates tumorigenesis and multipotency. *Oncogene.* (2017) 36:570–84. doi: 10.1038/onc.2016.230
19. Sturm D, Witt H, Hovestadt V, Khuong-Quang DA, Jones DT, Konermann C, et al. Hotspot mutations in H3F3A and IDH1 define distinct epigenetic and biological subgroups of glioblastoma. *Cancer Cell.* (2012) 22:425–37. doi: 10.1016/j.ccr.2012.08.024
20. Gill BJ, Pisapia DJ, Malone HR, Goldstein H, Lei L, Sonabend A, et al. MRI-localized biopsies reveal subtype-specific differences in molecular and cellular composition at the margins of glioblastoma. *Proc Natl Acad Sci USA.* (2014) 111:12550–5. doi: 10.1073/pnas.1405839111
21. Brennan CW, Verhaak RG, McKenna A, Campos B, Noushmehr H, Salama SR, et al. The somatic genomic landscape of glioblastoma. *Cell.* (2013) 155:462–77. doi: 10.1016/j.cell.2013.09.034
22. Verhaak RG, Hoadley KA, Purdom E, Wang V, Qi Y, Wilkerson MD, et al. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell.* (2010) 17:98–110. doi: 10.1016/j.ccr.2009.12.020
23. Phillips HS, Kharbanda S, Chen R, Forrest WF, Soriano RH, Wu TD, et al. Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. *Cancer Cell.* (2006) 9:157–73. doi: 10.1016/j.ccr.2006.02.019
24. Bhat KPL, Balasubramaniyan V, Vaillant B, Ezhilarasan R, Hummelink K, Hollingsworth F, et al. Mesenchymal differentiation mediated by NF-kappaB promotes radiation resistance in glioblastoma. *Cancer Cell.* (2013) 24:331–46. doi: 10.1016/j.ccr.2013.08.001
25. Patel AP, Tirosh I, Trombetta JJ, Shalek AK, Gillespie SM, Wakimoto H, et al. Single-cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma. *Science.* (2014) 344:1396–401. doi: 10.1126/science.1254257
26. Noushmehr H, Weisenberger DJ, Diefes K, Phillips HS, Pujara K, Berman BP, et al. Identification of a CpG island methylator phenotype that defines a distinct subgroup of glioma. *Cancer Cell.* (2010) 17:510–22. doi: 10.1016/S1040-1741(10)79529-4
27. de Souza CF, Sabedot TS, Malta TM, Stetson L, Morozova O, Sokolov A, et al. A distinct DNA methylation shift in a subset of glioma CpG island methylator phenotypes during tumor recurrence. *Cell Rep.* (2018) 23:637–51. doi: 10.1016/j.celrep.2018.03.107
28. Reifenberger G, Wirsching HG, Knobbe-Thomsen CB, Weller M. Advances in the molecular genetics of gliomas - implications for classification and therapy. *Nat Rev Clin Oncol.* (2017) 14:434–52. doi: 10.1038/nrclinonc.2016.204
29. Waker CA, Lober RM. Brain tumors of glial origin. *Adv Exp Med Biol.* (2019) 1190:281–97. doi: 10.1007/978-981-32-9636-7_18
30. Singh SK, Fiorelli R, Kupp R, Rajan S, Szeto E, Cascio CL, et al. Post-translational modifications of OLIG2 regulate glioma invasion through TGF-β pathway. *Cell Rep.* (2016) 16:950–66. doi: 10.1016/j.celrep.2016.06.045

31. Fadhlullah SFB, Halim NBA, Yeo JYT, Ho RLY, Um P, Ang BT, et al. Pathogenic mutations in neurofibromin identifies a leucine-rich domain regulating glioma cell invasiveness. *Oncogene.* (2019) 38:5367–80. doi: 10.1038/s41388-019-0809-3
32. Daginakatte GC, Gutmann DH. Neurofibromatosis-1 (Nf1) heterozygous brain microglia elaborate paracrine factors that promote Nf1-deficient astrocyte and glioma growth. *Hum Mol Genet.* (2007) 16:1098–112. doi: 10.1093/hmg/ddm059
33. Rutledge WC, Kong J, Gao J, Gutman DA, Cooper LAD, Appin C, et al. Tumor-infiltrating lymphocytes in glioblastoma are associated with specific genomic alterations and related to transcriptional class. *Clin Cancer Res.* (2013) 19:4951–60. doi: 10.1158/1078-0432.CCR-13-0551
34. Solga AC, Pong WW, Kim KY, Cimino PJ, Toonen JA, Walker J, et al. RNA sequencing of tumor-associated microglia reveals Ccl5 as a stromal chemokine critical for neurofibromatosis-1 glioma growth. *Neoplasia.* (2015) 17:776–88. doi: 10.1016/j.neo.2015.10.002
35. Singh D, Chan JM, Zoppoli P, Niola F, Sullivan R, Castano A, et al. Transforming fusions of FGFR and TACC genes in human glioblastoma. *Science.* (2012) 337:1231–5. doi: 10.1126/science.1220834
36. Radke J, Koch A, Pritsch F, Schumann E, Misch M, Hempt C, et al. Predictive MGMT status in a homogeneous cohort of IDH wildtype glioblastoma patients. *Acta Neuropathol Commun.* (2019) 7:89. doi: 10.1186/s40478-019-0745-z
37. Esteller M, Garcia-Foncillas J, Andion E, Goodman SN, Hidalgo OF, Vanaclocha V, et al. Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. *N Engl J Med.* (2000) 343:1350–4. doi: 10.1056/NEJM200011093431901
38. Jesionek-Kupnicka D, Braun M, Trabska-Kluch B, Czech J, Szybka M, Szymanska B, et al. MiR-21, miR-34a, miR-125b, miR-181d and miR-648 levels inversely correlate with MGMT and TP53 expression in primary glioblastoma patients. *Arch Med Sci.* (2019) 15:504–12. doi: 10.5114/aoms.2017.69374
39. ParvizHamidi M, Haddad G, Ostadrahimi S, Ostadrahimi N, Sadeghi S, Fayaz S, et al. Circulating miR-26a and miR-21 as biomarkers for glioblastoma multiform. *Biotechnol Appl Biochem.* (2019) 66:261–5. doi: 10.1002/bab.1707
40. Seo YE, Suh HW, Bahal R, Josowitz A, Zhang J, Song E, et al. Nanoparticle-mediated intratumoral inhibition of miR-21 for improved survival in glioblastoma. *Biomaterials.* (2019) 201:87–98. doi: 10.1016/j.biomaterials.2019.02.016
41. Sippl C, Ketter R, Bohr L, Kim YJ, List M, Oertel J, et al. MiRNA-181d expression significantly affects treatment responses to carmustine wafer implantation. *Neurosurgery.* (2019) 85:147–55. doi: 10.1093/neuros/nyy214
42. Chen YY, Ho HL, Lin SC, Ho TD, Hsu CY. Upregulation of miR-125b, miR-181d, and miR-221 predicts poor prognosis in MGMT promoter-unmethylated glioblastoma patients. *Am J Clin Pathol.* (2018) 149:412–17. doi: 10.1093/ajcp/aqy008
43. Cardoso AMS, Sousa M, Morais CM, Oancea-Castillo LR, Regnier-Vigouroux A, Rebelo O, et al. MiR-144 overexpression as a promising therapeutic strategy to overcome glioblastoma cell invasiveness and resistance to chemotherapy. *Hum Mol Genet.* (2019) 28:2738–51. doi: 10.1093/hmg/ddz099
44. Yang L, Li N, Yan Z, Li C, Zhao Z. MiR-29a-mediated CD133 expression contributes to cisplatin resistance in CD133+ glioblastoma stem cells. *J. Mol. Neurosci.* (2018) 66:369–77. doi: 10.1007/s12031-018-1177-0
45. Zhao Y, Huang W, Kim TM, Jung Y, Menon LG, Xing H, et al. MicroRNA-29a activates a multi-component growth and invasion program in glioblastoma. *J Exp Clin Cancer Res.* (2019) 38:36. doi: 10.1186/s13046-019-1026-1
46. Parsons DW, Jones S, Zhang X, Lin JC, Leary RJ, Angenendt P, et al. An integrated genomic analysis of human glioblastoma multiforme. *Science.* (2008) 321:1807–12. doi: 10.1126/science.1164382
47. Jiao Y, Killela PJ, Reitman ZJ, Rasheed AB, Heaphy CM, de Wilde RF, et al. Frequent ATRX, CIC, FUBP1 and IDH1 mutations refine the classification of malignant gliomas. *Oncotarget.* (2012) 3:709–22. doi: 10.18632/oncotarget.588
48. Cairncross JG, Wang M, Jenkins RB, Shaw EG, Giannini C, Brachman DG, et al. Benefit from procarbazine, lomustine, and vincristine in oligodendroglial tumors is associated with mutation of IDH. *J Clin Oncol.* (2014) 32:783–90. doi: 10.1200/JCO.2013.49.3726
49. Lasorella A, Sanson M, Iavarone A. FGFR-TACC gene fusions in human glioma. *Neuro Oncol.* (2017) 19:475–83. doi: 10.1093/neuonc/now240
50. Cahoy JD, Emery B, Kaushal A, Foo LC, Zamanian JL, Christopherson KS, et al. A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. *J Neurosci.* (2008) 28:264–78. doi: 10.1523/JNEUROSCI.4178-07.2008
51. Martinez R, Rohde V, Schackert G. Different molecular patterns in glioblastoma multiforme subtypes upon recurrence. *J Neurooncol.* (2010) 96:321–9. doi: 10.1007/s11060-009-9967-4
52. Halliday J, Helmy K, Pattwell SS, Pitter KL, LaPlant Q, Ozawa T, et al. *In vivo* radiation response of proneural glioma characterized by protective p53 transcriptional program and proneural-mesenchymal shift. *Proc Natl Acad Sci USA.* (2014) 111:5248–53. doi: 10.1073/pnas.1321014111
53. Stark AM, Witzel P, Strege RJ, Hugo HH, Mehdorn HM. p53, mdm2, EGFR, and msh2 expression in paired initial and recurrent glioblastoma multiforme. *J Neurol Neurosurg Psychiatry.* (2003) 74:779–83. doi: 10.1136/jnnp.74.6.779
54. Shinsato Y, Furukawa T, Yunoue S, Yonezawa H, Minami K, Nishizawa Y, et al. Reduction of MLH1 and PMS2 confers temozolomide resistance and is associated with recurrence of glioblastoma. *Oncotarget.* (2013) 4:2261–70. doi: 10.18632/oncotarget.1302
55. Kim J, Lee IH, Cho HJ, Park CK, Jung YS, Kim Y, et al. Spatiotemporal evolution of the primary glioblastoma genome. *Cancer Cell.* (2015) 28:318–28. doi: 10.1016/j.ccell.2015.07.013
56. Wang J, Cazzato E, Ladewig E, Frattini V, Rosenbloom DI, Zairis S, et al. Clonal evolution of glioblastoma under therapy. *Nat Genet.* (2016) 48:768–76. doi: 10.1038/ng.3590
57. Sottoriva A, Spiteri I, Piccirillo SG, Touloumis A, Collins VP, Marioni JC, et al. Intratumor heterogeneity in human glioblastoma reflects cancer evolutionary dynamics. *Proc Natl Acad Sci USA.* (2013) 110:4009–14. doi: 10.1073/pnas.1219747110
58. Muscat AM, Wong NC, Drummond KJ, Algar EM, Khasraw M, Verhaak R, et al. The evolutionary pattern of mutations in glioblastoma reveals therapy-mediated selection. *Oncotarget.* (2018) 9:7844–58. doi: 10.18632/oncotarget.23541
59. Hegi ME, Diserens AC, Gorlia T, Hamou MF, de Tribolet N, Weller M, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. *N Engl J Med.* (2005) 352:997–1003. doi: 10.1056/NEJMoa043331
60. Klughammer J, Kiesel B, Roetzer T, Fortelny N, Nemc A, Nenning KH, et al. The DNA methylation landscape of glioblastoma disease progression shows extensive heterogeneity in time and space. *Nat Med.* (2018) 24:1611–24. doi: 10.1038/s41591-018-0156-x
61. Carroll MV, Sim RB. Complement in health and disease. *Adv Drug Deliv Rev.* (2011) 63:965–75. doi: 10.1016/j.addr.2011.06.005
62. Whaley K, Schwaeble W. Complement and complement deficiencies. *Semin Liver Dis.* (1997) 17:297–310. doi: 10.1055/s-2007-1007206
63. Thurman JM, Holers, VM. The central role of the alternative complement pathway in human disease. *J Immunol.* (2006) 176:1305–10. doi: 10.4049/jimmunol.176.3.1305
64. Daha MR, Fearon DT, Austen KF. C3 requirements for formation of alternative pathway C5 convertase. *J Immunol.* (1976) 117:630–4.
65. Gigli I, Fujita T, Nussenzweig V. Modulation of the classical pathway C3 convertase by plasma proteins C4 binding protein and C3b inactivator. *Proc Natl Acad Sci USA.* (1979) 76:6596–600. doi: 10.1073/pnas.76.12.6596
66. Wallis R, Dodds AW, Mitchell DA, Sim RB, Reid KB, Schwaeble WJ. Molecular interactions between MASP-2, C4, and C2 and their activation fragments leading to complement activation via the lectin pathway. *J Biol Chem.* (2007) 282:7844–51. doi: 10.1074/jbc.M606326200
67. Heja D, Kocsis A, Dobo J, Szilagyi K, Szasz R, Zavodszky P, et al. Revised mechanism of complement lectin-pathway activation revealing the role of serine protease MASP-1 as the exclusive activator of MASP-2. *Proc Natl Acad Sci USA.* (2012) 109:10498–503. doi: 10.1073/pnas.1202588109
68. Afshar-Kharghan V. The role of the complement system in cancer. *J Clin Invest.* (2017) 127:780–9. doi: 10.1172/JCI90962

69. Fornvik K, Maddahi A, Persson O, Osther K, Salford LG, Nittby Redebrandt H. C1-inactivator is upregulated in glioblastoma. *PLoS ONE.* (2017) 12:e0183086. doi: 10.1371/journal.pone.0183086
70. Junnikkala S, Jokiranta TS, Friese MA, Jarva H, Zipfel PF, Meri S. Exceptional resistance of human H2 glioblastoma cells to complement-mediated killing by expression and utilization of factor H and factor H-like protein 1. *J Immunol.* (2000) 164:6075–81. doi: 10.4049/jimmunol.164.11.6075
71. Maenpaa A, Junnikkala S, Hakulinen J, Timonen T, Meri S. Expression of complement membrane regulators membrane cofactor protein (CD46), decay accelerating factor (CD55), and protectin (CD59) in human malignant gliomas. *Am J Pathol.* (1996) 148:1139–52.
72. Kopp A, Hebecker M, Svobodova E, Jozsi M. Factor H: a complement regulator in health and disease, and a mediator of cellular interactions. *Biomolecules.* (2012) 2:46–75. doi: 10.3390/biom2010046
73. Rodríguez de Córdoba S, Esparza-Gordillo J, Goicoechea de Jorge E, Lopez-Trascasa M, Sánchez-Corral P. The human complement factor H: functional roles, genetic variations and disease association. *Mol Immunol.* (2004) 41:355–67. doi: 10.1016/j.molimm.2004.02.005
74. Hellwage J, Jokiranta TS, Koistinen V, Vaarala O, Meri S, Zipfel PF. Functional properties of complement factor H-related proteins FHR-3 and FHR-4: binding to the C3d region of C3b and differential regulation by heparin. *FEBS Lett.* (1999) 462:345–52. doi: 10.1016/S0014-5793(99)01554-9
75. Ferreira VP, Pangburn MK, Cortes C. Complement control protein factor H: the good, the bad, and the inadequate. *Mol Immunol.* (2010) 47:2187–97. doi: 10.1016/j.molimm.2010.05.007
76. Qi L, Yu H, Zhang Y, Zhao D, Lv P, Zhong, et al. (2016). IL-10 secreted by M2 macrophage promoted tumorigenesis through interaction with JAK2 in glioma. *Oncotarget.* 7:71673–85. doi: 10.18632/oncotarget.12317
77. Wang T, Niu G, Kortylewski M, Burdelya L, Shain K, Zhang S, et al. Regulation of the innate and adaptive immune responses by Stat-3 signaling in tumor cells. *Nat Med.* (2004) 10:48–54. doi: 10.1038/nm976
78. Matsumura Y, Kobayashi T, Ichiyama K, Yoshida R, Hashimoto M, Takimoto T, et al. Selective expansion of foxp3-positive regulatory T cells and immunosuppression by suppressors of cytokine signaling 3-deficient dendritic cells. *J Immunol.* (2007) 179:2170–9. doi: 10.4049/jimmunol.179.4.2170
79. Wesolowska A, Kwiatkowska A, Slomnicki L, Dembinski M, Master A, Sliwa M, et al. Microglia-derived TGF-beta as an important regulator of glioblastoma invasion–an inhibition of TGF-β-dependent effects by shRNA against human TGF-β type II receptor. *Oncogene.* (2008) 27:918–30. doi: 10.1038/sj.onc.1210683
80. Ye XZ, Xu SL, Xin YH, Yu SC, Ping YF, Chen L, et al. Tumor-associated microglia/macrophages enhance the invasion of glioma stem-like cells via TGF-beta1 signaling pathway. *J Immunol.* (2012) 189:444–53. doi: 10.4049/jimmunol.1103248
81. Fontana A, Bodmer S, Frei K, Malipiero U, Siepl C. Expression of TGF-beta 2 in human glioblastoma: a role in resistance to immune rejection? *Ciba Found Symp.* (1991) 157:232-8. doi: 10.1002/9780470514061.ch15
82. Matsushita K, Takenouchi T, Shimada H, Tomonaga T, Hayashi H, Shioya A, et al. Strong HLA-DR antigen expression on cancer cells relates to better prognosis of colorectal cancer patients: possible involvement of c-myc suppression by interferon-gamma *in situ. Cancer Sci.* (2006) 97:57–63. doi: 10.1111/j.1349-7006.2006.00137.x
83. Crane CA, Ahn BJ, Han SJ, Parsa AT. Soluble factors secreted by glioblastoma cell lines facilitate recruitment, survival, and expansion of regulatory T cells: implications for immunotherapy. *Neuro Oncol.* (2012) 14:584–95. doi: 10.1093/neuonc/nos014
84. Liu Q, Li G, Li R, Shen J, He Q, Deng L, et al. IL-6 promotion of glioblastoma cell invasion and angiogenesis in U251 and T98G cell lines. *J Neurooncol.* (2010) 100:165–76. doi: 10.1007/s11060-010-0158-0
85. Lamano JB, Lamano JB, Li YD, DiDomenico JD, Choy W, Veliceasa D, et al. Glioblastoma-Derived IL6 induces immunosuppressive peripheral myeloid cell PD-L1 and promotes tumor growth. *Clin Cancer Res.* (2019) 25:3643–57. doi: 10.1158/1078-0432.CCR-18-2402
86. Coniglio SJ, Eugenin E, Dobrenis K, Stanley ER, West BL, Symons MH, et al. Microglial stimulation of glioblastoma invasion involves epidermal growth factor receptor (EGFR) and colony stimulating factor 1 receptor (CSF-1R) signaling. *Mol Med.* (2012) 18:519–27. doi: 10.2119/molmed.2011.00217
87. De I, Steffen MD, Clark PA, Patros CJ, Sokn E, Bishop SM, et al. CSF1 overexpression promotes high-grade Glioma formation without impacting the polarization status of glioma-associated microglia and macrophages. *Cancer Res.* (2016) 76:2552–60. doi: 10.1158/0008-5472.CAN-15-2386
88. Wu A, Wei J, Kong LY, Wang Y, Priebe W, Qiao W, et al. Glioma cancer stem cells induce immunosuppressive macrophages/microglia. *Neuro Oncol.* (2010) 12:1113–25. doi: 10.1093/neuonc/noq082
89. DeCordova S, Abdelgany A, Murugaiah V, Pathan AA, Nayak A, Walker T, et al. Secretion of functionally active complement factor H related protein 5 (FHR5) by primary tumour cells derived from Glioblastoma multiforme patients. *Immunobiology.* (2019) 224:625–31. doi: 10.1016/j.imbio.2019.07.006
90. Badie B, Schartner J, Prabakaran S, Paul J, Vorpahl J. Expression of Fas ligand by microglia: possible role in glioma immune evasion. *J Neuroimmunol.* (2001) 120:19–24. doi: 10.1016/S0165-5728(01)00361-7
91. Pangburn MK, Fereira VP, Cortes C. Discrimination between host and pathogens by the complement system. *Vaccine.* (2008) 26:15–21. doi: 10.1016/j.vaccine.2008.11.023
92. Schmidt CQ, Lambris JD, Ricklin D. Protection of host cells by complement regulators. *Immunol Rev.* (2017) 274:152–71. doi: 10.1111/imr.12475
93. Cho H. Complement regulation: physiology and disease relevance. *Korean J Pediatr.* (2015) 58:239–44. doi: 10.3345/kjp.2015.58.7.239
94. Tegla CA, Cudrici C, Patel S, Trippe R, Rus V, Noculescu F, et al. Membrane attack by complement: the assembly and biology of terminal complement complexes. *Immunol Res.* (2013) 52:45–60. doi: 10.1007/s12026-011-8239-5
95. Junnikkala S, Hakulinen J, Jarva H, Manuelian T, Bjorge L, Butzow R, et al. Secretion of soluble complement inhibitors factor H and factor H-like protein (FHL-1) by ovarian tumour cells. *Br J Cancer.* (2002) 87:1119–27. doi: 10.1038/sj.bjc.6600614
96. Ratnoff OD, Lepow IH. Some properties of an esterase derived from preparations of the first component of complement. *J Exp Med.* (1957) 106:327–43. doi: 10.1084/jem.106.2.327
97. Sim RB, Reboul A, Arlaud GJ, Villiers CL, Colomb MG. Interaction of 125I-labelled complement subcomponents C-1r and C-1s with protease inhibitors in plasma. *FEBS Lett.* (1979) 97:111–5. doi: 10.1016/0014-5793(79)80063-0
98. Sim RB, Arlaud GJ, Colomb MG. Kinetics of reaction of human C1-inhibitor with the human complement system proteases C1r and C1s. *Biochim Biophys Acta.* (1980) 612:433–49. doi: 10.1016/0005-2744(80)90126-6
99. Fornvik K, Ahlstedt J, Osther K, Salford LG, Redebrandt HN. Anti-C1-inactivator treatment of glioblastoma. *Oncotarget.* (2018) 9:37421–8. doi: 10.18632/oncotarget.26456
100. Davies A, Simmons DL, Hale G, Harrison RA, Tighe H, Lachmann PJ, et al. CD59, an LY-6-like protein expressed in human lymphoid cells, regulates the action of the complement membrane attack complex on homologous cells. *J Exp Med.* (1989) 170:637–54. doi: 10.1084/jem.170.3.637
101. Fodor WL, Rollins SA, Guilmette ER, Setter E, Squinto SP. A novel bifunctional chimeric complement inhibitor that regulates C3 convertase and formation of the membrane attack complex. *J Immunol.* (1995) 155:4135–8.
102. Liszewski MK, Post TW, Atkinson JP. Membrane cofactor protein (MCP or CD46): newest member of the regulators of complement activation gene cluster. *Annu Rev Immunol.* (1991) 9:431–55. doi: 10.1146/annurev.iy.09.040191.002243
103. Louveau A, Harris TH, Kipnis J. Revisiting the mechanisms of CNS immune privilege. *Trends Immunol.* (2015) 36:569–77. doi: 10.1016/j.it.2015.08.006
104. Galea I, Bechmann I, Perry VH. What is immune privilege (not)? *Trends Immunol.* (2007) 28:12–8. doi: 10.1016/j.it.2006.11.004
105. Bailey SL, Carpentier PA, McMahon EJ, Begolka WS, Miller SD. Innate and adaptive immune responses of the central nervous system. *Crit Rev Immunol.* (2006) 26:149–88. doi: 10.1615/CritRevImmunol.v26.i2.40
106. Russo MV, McGavern DB. Immune surveillance of the CNS following Infection and Injury, *Trends Immunol.* (2015) 36:637–50. doi: 10.1016/j.it.2015.08.002
107. Cserr HF, Harling-Berg CJ, Knopf PM. Drainage of brain extracellular fluid into blood and deep cervical lymph and its immunological significance. *Brain Pathol.* (1992) 2:269–76. doi: 10.1111/j.1750-3639.1992.tb00703.x

108. Roopenian DC, Akilesh S. FcRn: the neonatal Fc receptor comes of age. *Nat Rev Immunol.* 7:715–25. doi: 10.1038/nri2155
109. Mohammad MG, Tsai VW, Ruitenberg MJ, Hassanpour M, Li H, Hart PH, et al. Immune cell trafficking from the brain maintains CNS immune tolerance. *J Clin Invest.* (2014) 124:1228–41. doi: 10.1172/JCI71544
110. Engelhardt B, Ransohoff RM. Capture, crawl, cross: the T cell code to breach the blood-brain barriers. *Trends Immunol.* (2012) 33:579–89. doi: 10.1016/j.it.2012.07.004
111. Davies LC, Jenkins SJ, Allen JE, Taylor PR. Tissue-resident macrophages. *Nat Immunol.* (2013) 14:986–95. doi: 10.1038/ni.2705
112. Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S, et al. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science.* (2010) 330:841–5. doi: 10.1126/science.1194637
113. Nimmerjahn A, Kirchhoff F, Helmchen F. Resting microglial cells are highly dynamic surveillants of brain parenchyma *in vivo*. *Science*. (2005) 308:1314–8. doi: 10.1126/science.1110647
114. Ajami B, Bennett JL, Krieger C, McNagny KM, Rossi FM. Infiltrating monocytes trigger EAE progression, but do not contribute to the resident microglia pool. *Nat Neurosci.* (2011) 14:1142–9. doi: 10.1038/nn.2887
115. Casano AM, Peri F. Microglia: multitasking specialists of the brain. *Dev Cell.* (2015) 32:469–77. doi: 10.1016/j.devcel.2015.01.018
116. Hickey WF, Kimura H. Perivascular microglial cells of the CNS are bone marrow-derived and present antigen *in vivo*. *Science.* (1988) 239:290–2. doi: 10.1126/science.3276004
117. Perng P, Lim M. Immunosuppressive mechanisms of malignant gliomas: parallels at non-CNS sites. *Front Oncol.* (2015) 5:153. doi: 10.3389/fonc.2015.00153
118. Badie B, Schartner JM. Flow cytometric characterization of tumor-associated macrophages in experimental gliomas. *Neurosurgery.* (2000) 46:957–61. doi: 10.1227/00006123-200004000-00035
119. Dick AD, Ford AL, Forrester JV, Sedgwick JD. Flow cytometric identification of a minority population of MHC class II positive cells in the normal rat retina distinct from CD45lowCD11b/c+CD4low parenchymal microglia. *Br J Ophthalmol.* (1995) 79:834–40. doi: 10.1136/bjo.79.9.834
120. Parney IF, Waldron JS, Parsa AT. Flow cytometry and *in vitro* analysis of human glioma-associated macrophages. Laboratory investigation. *J Neurosurg.* (2009) 110:572–82. doi: 10.3171/2008.7.JNS08475
121. Muller A, Brandenburg S, Turkowski K, Muller S, Vajkoczy P. Resident microglia, and not peripheral macrophages, are the main source of brain tumor mononuclear cells. *Int J Cancer.* (2015) 137:278–88. doi: 10.1002/ijc.29379
122. Morantz RA, Wood GW, Foster M, Clark M, Gollahon K. Macrophages in experimental and human brain tumors. Part 2: studies of the macrophage content of human brain tumors. *J Neurosurg.* (1979) 50:305–11. doi: 10.3171/jns.1979.50.3.0305
123. Bettinger I, Thanos S, Paulus W. Microglia promote glioma migration. *Acta Neuropathol.* (2002) 103:351–5. doi: 10.1007/s00401-001-0472-x
124. Markovic DS, Glass R, Synowitz M, Rooijen Nv, Kettenmann H. Microglia stimulate the invasiveness of glioma cells by increasing the activity of metalloprotease-2. *J Neuropathol Exp Neurol.* (2005) 64:754–62. doi: 10.1097/01.jnen.0000178445.33972.a9
125. Yeung YT, Bryce NS, Adams S, Braidy N, Konayagi M, McDonald KL, et al. p38 MAPK inhibitors attenuate pro-inflammatory cytokine production and the invasiveness of human U251 glioblastoma cells. *J Neurooncol.* (2012) 109:35–44. doi: 10.1007/s11060-012-0875-7
126. Jacobs VL, Landry RP, Liu Y, Romero-Sandoval EA, de Leo JA. Propentofylline decreases tumor growth in a rodent model of glioblastoma multiforme by a direct mechanism on microglia. *Neuro Oncol.* (2012) 14:119–31. doi: 10.1093/neuonc/nor194
127. Burns JM, Summers BC, Wang Y, Melikian A, Berahovich R, Miao Z, et al. A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development. *J Exp Med.* (2006) 203:2201–13. doi: 10.1084/jem.20052144
128. Rempel SA, Dudas S, Ge S, Gutiérrez JA. Identification and localization of the cytokine SDF1 and its receptor, CXC chemokine receptor 4, to regions of necrosis and angiogenesis in human glioblastoma. *Clin Cancer Res.* (2000) 6:102–11.
129. Salmaggi A, Gelati M, Pollo B, Frigerio S, Eoli M, Silvani A, et al. CXCL12 in malignant glial tumors: a possible role in angiogenesis and cross-talk between endothelial and tumoral cells. *J Neuro Oncol.* (2004) 67:305–17. doi: 10.1023/B:NEON.0000024241.05346.24
130. Chang AL, Miska J, Wainwright DA, Dey M, Rivetta CV, Yu D, et al. CCL2 produced by the glioma microenvironment is essential for the recruitment of regulatory T cells and myeloid-derived suppressor cells. *Cancer Res.* (2016) 76:5671–82. doi: 10.1158/0008-5472.CAN-16-0144
131. Bowman RL, Joyce JA. Therapeutic targeting of tumor-associated macrophages and microglia in glioblastoma. *Immunotherapy.* (2014) 6:663–6. doi: 10.2217/imt.14.48
132. Hambardzumyan D, Gutmann DH, Kettenmann H. The role of microglia and macrophages in glioma maintenance and progression. *Nat Neurosci.* (2016) 19:20–7. doi: 10.1038/nn.4185
133. Takeshima H, Kuratsu J, Takeya M, Yoshimura T, Ushio Y. Expression and localization of messenger RNA and protein for monocyte chemoattractant protein-1 in human malignant glioma. *J Neurosurg.* (1994) 80:1056–62. doi: 10.3171/jns.1994.80.6.1056
134. Zhu X, Fujita M, Snyder LA, Okada H. Systemic delivery of neutralizing antibody targeting CCL2 for glioma therapy. *J Neuro Oncol.* (2011) 104:83–92. doi: 10.1007/s11060-010-0473-5
135. Platten M, Wick W, Weller M. Malignant glioma biology: role for TGF-beta in growth, motility, angiogenesis, and immune escape. *Microsc Res Tech.* (2001) 52:401–10. doi: 10.1002/1097-0029(20010215)52:4<401::AID-JEMT1025>3.0.CO;2-C
136. Wang SC, Hong JH, Hsueh C, Chiang CS. Tumor-secreted SDF-1 promotes glioma invasiveness and TAM tropism toward hypoxia in a murine astrocytoma model. *Lab Invest.* (2012) 92:151–62. doi: 10.1038/labinvest.2011.128
137. Bao Z, Chen K, Krepel S, Tang P, Gong W, Zhang M, et al. High glucose promotes human glioblastoma cell growth by increasing the expression and function of chemoattractant and growth factor receptors. *Transl Oncol.* (2019) 12:1155–63. doi: 10.1016/j.tranon.2019.04.016
138. Wei J, Marisetty A, Schrand B, Gabrusiewicz K, Hashimoto Y, Ott M, et al. Osteopontin mediates glioblastoma-associated macrophage infiltration and is a potential therapeutic target. *J Clin Invest*. (2019) 129:137–49. doi: 10.1172/JCI121266
139. Charles NA, Holland EC, Gilbertson R, Glass R, Kettenmann H. The brain tumor microenvironment. *Glia.* (2011) 59:1169–80. doi: 10.1002/glia.21136
140. Friedberg MH, Glantz MJ, Klempner MS, Cole BF, Perides G. Specific matrix metalloproteinase profiles in the cerebrospinal fluid correlated with the presence of malignant astrocytomas, brain metastases, and carcinomatous meningitis. *Cancer.* (1998) 82:923–30.
141. Belien AT, Paganetti PA, Schwab ME. Membrane-type 1 matrix metalloprotease (MT1-MMP) enables invasive migration of glioma cells in central nervous system white matter. *J Cell Biol.* (1999) 144:373–84. doi: 10.1083/jcb.144.2.373
142. Du R, Petritsch C, Lu K, Liu P, Haller A, Ganss R, et al. Matrix metalloproteinase-2 regulates vascular patterning and growth affecting tumor cell survival and invasion in GBM. *Neuro Oncol.* (2008) 10:254–64. doi: 10.1215/15228517-2008-001
143. Markovic DS, Vinnakota K, Chirasani S, Synowitz M, Raguet H, Stock K, et al. Gliomas induce and exploit microglial MT1-MMP expression for tumor expansion. *Proc Natl Acad Sci USA.* 106:12530–5. doi: 10.1073/pnas.0804273106
144. van Meir EG. Cytokines and tumors of the central nervous system. *Glia.* (1995) 15:264–88. doi: 10.1002/glia.440150308
145. Nduom EK, Weller M, Heimberger AB. Immunosuppressive mechanisms in glioblastoma. *Neuro Oncol.* (2015) 17(Suppl. 7):vii9–14. doi: 10.1093/neuonc/nov151
146. Liau LM, Black KL, Prins RM, Sykes SN, DiPatre PL, Cloughesy TF, et al. Treatment of intracranial gliomas with bone marrow-derived dendritic cells pulsed with tumor antigens. *J Neurosurg.* (1999) 90:1115–24. doi: 10.3171/jns.1999.90.6.1115
147. Pellegatta S, Poliani PL, Corno D, Grisoli M, Cusimano M, Ubiali F, et al. Dendritic cells pulsed with glioma lysates induce immunity against syngeneic intracranial gliomas and increase survival of tumor-bearing mice. *Neurol Res.* (2006) 28:527–31. doi: 10.1179/016164106X116809

148. Liau LM, Prins RM, Kiertscher SM, Odesa SK, Kremen TJ, Giovannone AJ, et al. Dendritic cell vaccination in glioblastoma patients induces systemic and intracranial T-cell responses modulated by the local central nervous system tumor microenvironment. *Clin Cancer Res.* (2005) 11:5515–25. doi: 10.1158/1078-0432.CCR-05-0464
149. Yu JS, Wheeler CJ, Zeltzer PM, Ying H, Finger DN, Lee PK, et al. Vaccination of malignant glioma patients with peptide-pulsed dendritic cells elicits systemic cytotoxicity and intracranial T-cell infiltration. *Cancer Res.* (2001) 61:842–7.
150. Liau LM, Ashkan K, Tran DD, Campian JL, Trusheim JE, Cobbs CS, et al. First results on survival from a large Phase 3 clinical trial of an autologous dendritic cell vaccine in newly diagnosed glioblastoma. *J Transl Med.* (2018) 16:142. doi: 10.1186/s12967-018-1507-6
151. Friese MA, Wischhusen J, Wick W, et al. RNA interference targeting transforming growth factor-beta enhances NKG2D-mediated antiglioma immune response, inhibits glioma cell migration and invasiveness, and abrogates tumorigenicity *in vivo*. *Cancer Res.* (2004) 64:7596–603. doi: 10.1158/0008-5472.CAN-04-1627
152. Tran TT, Uhl M, Ma JY, Janssen L, Sriram V, Aulwurm S, et al. Inhibiting TGF-beta signaling restores immune surveillance in the SMA-560 glioma model. *Neuro Oncol.* (2007) 9:259–70. doi: 10.1215/15228517-2007-010
153. Sivori S, Parolini S, Marcenaro E, Castriconi R, Pende D, Millo R, et al. Involvement of natural cytotoxicity receptors in human natural killer cell-mediated lysis of neuroblastoma and glioblastoma cell lines. *J Neuroimmunol.* (2000) 107:220–5. doi: 10.1016/S0165-5728(00)00221-6
154. Castriconi R, Daga A, Dondero A, Zona G, Poliani PL, Melotti A, et al. NK cells recognize and kill human glioblastoma cells with stem cell-like properties. *J Immunol.* (2009) 182:3530–9. doi: 10.4049/jimmunol.0802845
155. Lee SJ, Kang WY, Yoon Y, Jin JY, Song HJ, Her JH, et al. Natural killer (NK) cells inhibit systemic metastasis of glioblastoma cells and have therapeutic effects against glioblastomas in the brain. *BMC Cancer.* (2015) 15:1011. doi: 10.1186/s12885-015-2034-y
156. Ooi YC, Tran P, Ung N, Thill K, Trang A, Fong BM, et al. The role of regulatory T-cells in glioma immunology. *Clin Neurol Neurosurg.* (2014) 119:125–32. doi: 10.1016/j.clineuro.2013.12.004
157. Allan SE, Passerini L, Bacchetta R, Crellin N, Dai M, Orban PC, et al. The role of 2 FOXP3 isoforms in the generation of human CD4+ Tregs. *J Clin Invest.* (2005) 115:3276–84. doi: 10.1172/JCI24685
158. Colombo MP, Piconese S. Regulatory-T-cell inhibition versus depletion: the right choice in cancer immunotherapy. *Nat Rev Cancer.* (2007) 7:880–7. doi: 10.1038/nrc2250
159. Jacobs JF, Idema AJ, Bol KF, Grotenhuis JA, de Vries IJ, Wesseling P, et al. Prognostic significance and mechanism of Treg infiltration in human brain tumors. *J Neuroimmunol.* (2010) 225:195–9. doi: 10.1016/j.jneuroim.2010.05.020
160. Heimberger AB, Abou-Ghazal M, Reina-Ortiz C, Yang DS, Sun W, Qiao W, et al. Incidence and prognostic impact of FoxP3+ regulatory T cells in human gliomas. *Clin Cancer Res.* (2008) 14:5166–72. doi: 10.1158/1078-0432.CCR-08-0320
161. Elliott LH, Brooks WH, Roszman TL. Activation of immunoregulatory lymphocytes obtained from patients with malignant gliomas. *J Neurosurg.* (1987) 67:231–6. doi: 10.3171/jns.1987.67.2.0231
162. Humphries W, Wei J, Sampson JH, Heimberger AB. The role of tregs in glioma-mediated immunosuppression: potential target for intervention. *Neurosurg Clin N Am.* (2010) 21:125–37. doi: 10.1016/j.nec.2009.08.012
163. Jordan JT, Sun W, Hussain SF, DeAngulo G, Prabhu SS, Heimberger AB. Preferential migration of regulatory T cells mediated by glioma-secreted chemokines can be blocked with chemotherapy. *Cancer Immunol Immunother.* (2008) 57:123–31. doi: 10.1007/s00262-007-0336-x
164. Desbaillets I, Tada M, de Tribolet N, Diserens AC, Hamou MF, van Meir EG. Human astrocytomas and glioblastomas express monocyte chemoattractant protein-1 (MCP-1) *in vivo* and *in vitro*. *Int J Cancer.* (1994) 58:240–7. doi: 10.1002/ijc.2910580216
165. Topalian SL, Drake CG, Pardoll DM. Immune checkpoint blockade: a common denominator approach to cancer therapy. *Cancer Cell.* (2015) 27:450–61. doi: 10.1016/j.ccell.2015.03.001
166. Bloch O, Crane CA, Kaur R, Safaee M, Rutkowski MJ, Parsa AT. Gliomas promote immunosuppression through induction of B7-H1 expression in tumor-associated macrophages. *Clin Cancer Res.* (2013) 19:3165–75. doi: 10.1158/1078-0432.CCR-12-3314
167. Liang SC, Latchman YE, Buhlmann JE, Tomczak MF, Horwitz BH, Freeman GJ, et al. Regulation of PD-1, PD-L1, and PD-L2 expression during normal and autoimmune responses. *Eur J Immunol.* (2003) 33:2706–16. doi: 10.1002/eji.200324228
168. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer.* (2012) 12:252–64. doi: 10.1038/nrc3239
169. Wei B, Wang L, Zhao X, Du C, Guo Y, Sun Z. The upregulation of programmed death 1 on peripheral blood T cells of glioma is correlated with disease progression. *Tumour Biol.* (2014) 35:2923–9. doi: 10.1007/s13277-013-1376-9
170. Parsa AT, Waldron JS, Panner A, Crane CA, Parney IF, Barry JJ, et al. Loss of tumor suppressor PTEN function increases B7-H1 expression and immunoresistance in glioma. *Nat Med.* (2007) 13:84–8. doi: 10.1038/nm1517
171. Zeng J, See AP, Phallen J, Jackson CM, Belcaid Z, Ruzevick J, et al. Anti-PD-1 blockade and stereotactic radiation produce long-term survival in mice with intracranial gliomas. *Int J Radiat Oncol Biol Phys.* (2013) 86:343–9. doi: 10.1016/j.ijrobp.2012.12.025
172. Fecci PE, Ochiai H, Mitchell DA, Grossi PM, Sweeney AE, Archer GE, et al. Systemic CTLA-4 blockade ameliorates glioma-induced changes to the CD4+ T cell compartment without affecting regulatory T-cell function. *Clin Cancer Res.* (2007) 13:2158–67. doi: 10.1158/1078-0432.CCR-06-2070
173. McGranahan T, Therkelsen KE, Ahmad S, Nagpal S. Current state of immunotherapy for treatment of glioblastoma. *Curr Treat Options Oncol.* (2019) 20:24. doi: 10.1007/s11864-019-0619-4
174. Caccese M, Indraccolo S, Zagonel V, Lombardi G. PD-1/PD-L1 immune-checkpoint inhibitors in glioblastoma: a concise review. *Crit Rev Oncol Hematol.* (2019) 135:128–34. doi: 10.1016/j.critrevonc.2018.12.002
175. Arrieta VA, Iwamoto F, Lukas RV, Sachdev S, Rabadan R, Sonabend AM. Can patient selection and neoadjuvant administration resuscitate PD-1 inhibitors for glioblastoma? *J Neurosurg.* (2019)132:1–6. doi: 10.3171/2019.9.JNS192523
176. Adhikaree J, Moreno-Vicente J, Kaur AP, Jackson AM, Patel PM. Resistance mechanisms and barriers to successful immunotherapy for treating glioblastoma. *Cells.* (2020) 9:263. doi: 10.3390/cells9020263
177. Batich KA, Reap EA, Archer GE, Sanchez-Perez L, Nair SK, Schmittling RJ, et al. Long-term survival in glioblastoma with cytomegalovirus pp65-targeted vaccination. *Clin Cancer Res.* (2017) 23:1898–909. doi: 10.1158/1078-0432.CCR-16-2057
178. Nair SK, de Leon G, Boczkowski D, Schmittling R, Xie W, Staats J, et al. Recognition and killing of autologous, primary glioblastoma tumor cells by human cytomegalovirus pp65-specific cytotoxic T cells. *Clin Cancer Res.* (2014) 20:2684–94. doi: 10.1158/1078-0432.CCR-13-3268
179. Mitchell DA, Batich KA, Gunn MD, Huang M-N, Sanchez-Perez L, Nair SK, et al. Tetanus toxoid and CCL3 improve dendritic cell vaccines in mice and glioblastoma patients. *Nature.* (2015) 519:366–9. doi: 10.1038/nature14320
180. Eisenstein M. Immunotherapy offers a promising bet against brain cancer. *Nature.* (2018) 561:S42–4. doi: 10.1038/d41586-018-06705-6
181. ORourke DM, Nasrallah MP, Desai A, Melenhorst JJ, Mansfield K, Morrissette JJD, et al. A single dose of peripherally infused EGFRvIII-directed CAR T cells mediates antigen loss and induces adaptive resistance in patients with recurrent glioblastoma. *Sci Transl Med.* (2017) 9:eaaa0984. doi: 10.1126/scitranslmed.aaa0984
182. Johnson LA, Scholler J, Ohkuri T, Kosaka A, Patel PR, McGettigan SE, et al. Rational development and characterization of humanized anti-EGFR variant III chimeric antigen receptor T cells for glioblastoma. *Sci Transl Med.* (2015) 7:275ra22. doi: 10.1126/scitranslmed.aaa4963
183. Jackson M, Hassiotou F, Nowak A. Glioblastoma stem-like cells: at the root of tumor recurrence and a therapeutic target. *Carcinogenesis.* (2015) 36:177–85. doi: 10.1093/carcin/bgu243
184. Audia A, Conroy S, Glass R, Bhat KPL. The impact of the tumor microenvironment on the properties of glioma stem-like cells. *Front Oncol.* (2017) 7:143. doi: 10.3389/fonc.2017.00143
185. Yang W, Li Y, Gao R, Xiu Z, Sun T. MHC class I dysfunction of glioma stem cells escapes from CTL-mediated immune response via

activation of Wnt/β-catenin signaling pathway. *Oncogene.* (2020) 39:1098–111. doi: 10.1038/s41388-019-1045-6

186. Ricklefs F, Alayo Q, Krenzlin H, Mahmoud A, Speranza M, Nakashima H, et al. Immune evasion mediated by PD-L1 on glioblastoma-derived extracellular vesicles. *Sci Adv.* (2018) 4:eaar2766. doi: 10.1126/sciadv.aar2766
187. Facoetti A, Nano R, Zelini P, Morbini P, Benericetti E, Ceroni M, et al. Human leukocyte antigen and antigen processing machinery component defects in astrocytic tumors. *Clin Cancer Res.* (2005) 11:8304–11. doi: 10.1158/1078-0432.CCR-04-2588
188. Zhou W, Ke SQ, Huang Z, Flavahan W, Fang X, Paul J, et al. Periostin secreted by glioblastoma stem cells recruits M2 tumour-associated macrophages and promotes malignant growth. *Nat Cell Biol.* (2015) 17:170–82. doi: 10.1038/ncb3090
189. Raychaudhuri B, Rayman P, Ireland J, Ko J, Rini B, Borden EC, et al. Myeloid-derived suppressor cell accumulation and function in patients with newly diagnosed glioblastoma. *Neuro Oncol.* (2011) 13:591–9. doi: 10.1093/neuonc/nor042
190. Raychaudhuri, B, Rayman P, Huang P, Grabowski M, Hambardzumyan D, Finke JH, et al. Myeloid derived suppressor cell infiltration of murine human gliomas is associated with reduction of tumor infiltrating lymphocytes. *J Neurooncol.* (2015) 122:293–301. doi: 10.1007/s11060-015-1720-6
191. Dubinski D, Wolfer J, Hasselblatt M, Schneider-Hohendorf T, Bogdahn U, Stummer W, et al. CD4+ T effector memory cell dysfunction is associated with the accumulation of granulocytic myeloid-derived suppressor cells in glioblastoma patients. *Neuro Oncol.* (2016) 18:807–18. doi: 10.1093/neuonc/nov280
192. Di Tomaso T, Mazzoleni S, Wang E, Sovena G, Clavenna D, Franzin A, et al. Immunobiological characterization of cancer stem cells isolated from glioblastoma patients. *Clin Cancer Res.* (2010) 16:800–13. doi: 10.1158/1078-0432.CCR-09-2730
193. Lathia JD, Mack SC, Mulkearns-Hubert EE, Valentim CLL, Rich JN. Cancer stem cells in glioblastoma. *Genes Dev.* (2015) 29:1203–17. doi: 10.1101/gad.261982.115
194. Kamran N, Kadiyala P, Saxena M, Candolfi M, Li Y, Moreno-Ayala MA, et al. Immunosuppressive myeloid cells' blockade in the glioma microenvironment enhances the efficacy of immune-stimulatory gene therapy. *Mol Ther.* (2017) 25:232–48. doi: 10.1016/j.ymthe.2016.10.003
195. Otvos B, Silver DJ, Mulkearns-Hubert EE, Alvarado AG, Turaga SM, Sorensen MD, et al. Cancer stem cell-secreted macrophage migration inhibitory factor stimulates myeloid derived suppressor cell function and facilitates glioblastoma immune evasion. *Stem Cells.* (2016) 34:2026–39. doi: 10.1002/stem.2393
196. Wei J, Barr J, Kong LY, Wang Y, Wu A, Sharma AK, et al. Glioma-associated cancer-initiating cells induce immunosuppression. *Clin Cancer Res.* (2010) 16:461–73. doi: 10.1158/1078-0432.CCR-09-1983
197. Chen Z, Feng X, Herting CJ, Garcia VA, Nie K, Pong WW, et al. Cellular and molecular identity of tumor-associated macrophages in glioblastoma. *Cancer Res.* (2017) 77:2266–78. doi: 10.1158/0008-5472.CAN-16-2310
198. Fujita M, Kohanbash G, Fellows-Mayle W, Hamilton RL, Komohara Y, Decker SA, et al. COX-2 blockade suppresses gliomagenesis by inhibiting myeloid-derived suppressor cells. *Cancer Res.* (2011) 71:2664–74. doi: 10.1158/0008-5472.CAN-10-3055
199. Alban TJ, Alvarado AG, Sorensen MD, Bayik D, Volovetz J, Serbinowski E, et al. Global immune fingerprinting in glioblastoma patient peripheral blood reveals immune-suppression signatures associated with prognosis. *JCI Insight.* (2018) 3:e122264. doi: 10.1172/jci.insight.122264
200. Umemura N, Saio M, Suwa T, Kitoh Y, Bai J, Nonaka K, et al. Tumor-infiltrating myeloid-derived suppressor cells are pleiotropic-inflamed monocytes/macrophages that bear M1- and M2-type characteristics. *J Leukoc Biol.* (2008) 83:1136–44. doi: 10.1189/jlb.0907611
201. Zhang P, Miska J, Lee-Chang C, Rashidi A, Panek WK, An S, et al. Therapeutic targeting of tumor-associated myeloid cells synergizes with radiation therapy for glioblastoma. *Proc Natl Acad Sci USA.* (2019) 116:23714–23. doi: 10.1073/pnas.1906346116

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CASE REPORT
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Encouraging Clinical Evolution of a Pediatric Patient With Relapsed Diffuse Midline Glioma Who Underwent WT1-Targeting Immunotherapy: A Case Report and Literature Review

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Diffuse midline glioma (DMG) in children is a highly aggressive, malignant brain tumor that is fatal when relapsed. Wilms tumor 1 (WT1) is a high-priority antigen target for cancer immunotherapy. We hereby report on a pediatric patient who had DMG that regrew after chemoradiotherapy and underwent WT1 peptide vaccination. A 13-year-old Japanese boy presented with vertigo, diplopia, and right hemiplegia at the initial visit to another hospital, where he was diagnosed with DMG by magnetic resonance imaging (MRI); DMG was categorized to histological grade IV glioma. The patient underwent radiotherapy and chemotherapy with temozolomide. After three cycles of chemotherapy, MRI revealed tumor regrowth that translated into deteriorated clinical manifestations. Immunohistochemically, the H3.3K27M mutation in the biopsy specimen was confirmed and the specimen was positive for WT1 protein. The patient underwent WT1-targeting immunotherapy with the WT1-specific peptide vaccine because of having HLA-A*24:02. Consequently, his quality of life drastically improved so much as to the extent that the patient became capable of conducting nearly normal daily activities at weeks 8 to 12 of vaccination. MRI at week 8 of vaccination revealed an obvious reduction in the signal intensity of the tumor. Furthermore, betamethasone dose could be reduced successively (4, 1, and 0.5 mg/day at weeks 4, 5, and 7, respectively) without deteriorating clinical manifestations. Best response among responses assessed according to the Response Assessment in Neuro-Oncology criteria was stable disease. Overall survival was 6.5 months after vaccination onset and was 8.3 months after relapse; the latter was markedly longer than the reported median OS of 3.2 months for pediatric patients

with relapsed DMG in the literature. Modified WT1 tetramer staining revealed the WT1 peptide vaccine-induced production of WT1-specific cytotoxic T cells, and the interferon-γ (IFN-γ) ELISpot assay of peripheral blood mononuclear cells disclosed the production of IFN-γ. Delayed-type hypersensitivity test became positive. Any treatment-emergent adverse events did not occur except injection site erythema. Our pediatric patient exhibited an encouraging clinical evolution as manifested by stable disease, improved clinical manifestations, steroid dose reductions, a WT1-specific immune response, and a good safety profile. Therefore, WT1-targeting immunotherapy warrants further investigation in pediatric patients with relapsed DMG.

Keywords: WT1 peptide vaccine, immunotherapy, diffuse midline glioma, relapse, WT1-specific cytotoxic T cells, tetramer assay, ELISpot assay, delayed-type hypersensitivity

BACKGROUND

Diffuse midline glioma (DMG) in children is a highly aggressive, malignant brain tumor, and the median overall survival (OS) after relapse for pediatric patients with DMG is 3.2 months (1). Treatment with temozolomide and bevacizumab is effective in adult patients with malignant glioma but not in pediatric patients with relapsed DMG (2, 3). Any effective therapeutic modality for relapsed DMG has not been developed in the last few decades (4), and their prognosis remains dismal, especially in patients with the H3.3K27M mutation [overall survival (OS): 9 months] as compared with those having the H3.1K27M mutation (OS: 15 months) (5). Therefore, the development of an effective treatment for them is required.

Wilms tumor antigen 1 (WT1) was considered as a high-priority antigen target for cancer immunotherapy by the National Cancer Institute because of its high immunogenicity and oncogenicity, as well as its expression in the majority of hematologic malignancies and solid tumors (6–8). In adults with malignant brain tumors (e.g., malignant glioma), the WT1 peptide vaccine was safe and induced favorable clinical and imaging responses (9, 10). We hereby report on a pediatric patient with relapsed DMG, whose H3.3K27M mutation was demonstrated by immunohistochemistry and who exhibited an encouraging clinical evolution during WT1 peptide vaccination.

CASE PRESENTATION

A 13-year-old Japanese boy was diagnosed with DMG by magnetic resonance imaging (MRI) at another hospital—where other primary brain stem tumors were ruled out based on MRI findings, DMG was categorized to histological grade IV glioma based on the biopsy result (H3.3K27M mutation), and the patient underwent radiotherapy (54 Gy) and chemotherapy with temozolomide (280 mg/m^2 PO), intravenously received interferon β, and was found to have HLA-A*24:02 by reverse transcriptase-polymerase chain reaction. Furthermore, DMG was immunohistochemically positive for WT1 protein. The patient did not have medical or family history of particular note. After three cycles of chemotherapy at another hospital, MRI revealed tumor regrowth. At presentation to our hospital, the patient showed lightheadedness, abducens nerve palsy, the deterioration of vertigo, headache, diplopia, and right hemiplegia. Subsequently, the patient was transferred to our hospital for enrollment in a phase I/II clinical trial of WT1-targeting immunotherapy with the WT1 peptide vaccine in patients with refractory pediatric cancers (UMIN 000013252), approved by the ethics committee at Osaka University Hospital and conducted according to the Declaration of Helsinki. The steering committee of the study monitored the efficacy and safety of the regimen and assessed intervention adherence and patient tolerability. The patient received the intradermal injection of 3.0 mg of the Good Manufacturing Practice (GMP)-grade, HLA-A*2402-restricted, 9 mer-modified WT1 peptide vaccine (mp235–243, CYTWNQMNL; Peptide Institute, Osaka, Japan) once/twice weekly for 23 weeks. Before injection, the vaccine was emulsified with an adjuvant Montanide^{TM} ISA 51 at a weight ratio of 1:1.

At the onset of WT1 peptide vaccination, we verified tumor regrowth on a T2-weighted image. Clinical manifestations commenced to improve at week 4 of vaccination, followed by drastic improvements in his quality of life at weeks 8–12 of vaccination so much as to the extent that the patient became capable of conducting nearly normal daily activities. Along with these improvements, MRI at week 8 of vaccination revealed obvious reductions in the high signal intensity of the lesion on T2-weighted (**Figure 1A**) and contrast-enhanced T1-weighted postcontrast (**Figure 1B**) images. Betamethasone dose was reduced from 4 mg/day at the onset of vaccination to 2 mg/day at week 4, followed by reductions to 1, and 0.5 mg/day at weeks 5 and 7, respectively. Best response among responses assessed according to the Response Assessment in Neuro-Oncology criteria was stable disease (11). The patient underwent WT1-specific immunotherapy at our hospital for 23 weeks. OS was 8.3 months after relapse and was 6.5 months after vaccination onset. The patient died of progressed DMG. Any treatment-emergent adverse events did not occur except injection site erythema.

IMMUNOHISTOCHEMICAL ANALYSIS OF WT1 PROTEIN AND H3.3K27M MUTATION

The paraffin-embedded sections of DMG were analyzed immunohistochemically by using the anti-WT1 protein

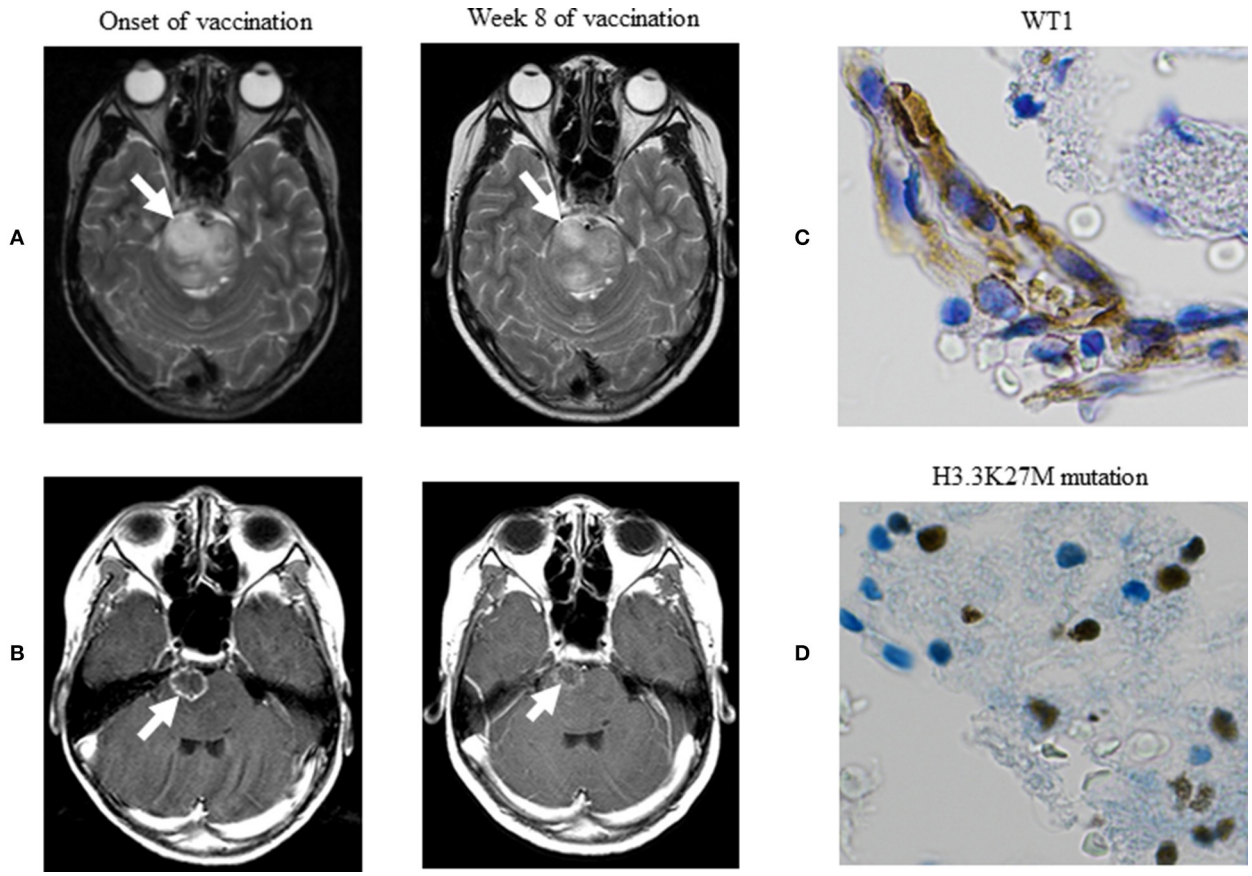

FIGURE 1 | MRI of DMG. **(A)** Axial T2-weighted MR images. **(B)** Axial T1-weighted postcontrast MR images. These images demonstrate a well-circumscribed mass (arrows), and best response among responses assessed with the Response Assessment in Neuro-Oncology criteria was stable disease. **(C)** Immunohistochemical stain demonstrating WT1 protein in the nucleus and cytoplasm of tumor cells, with greater staining intensity in the cytoplasm—a previously known histopathological feature for primary astrocytic tumors (×400). **(D)** Immunohistochemical stain demonstrating the H3.3K27M mutation in the nucleus of tumor cells, displaying highly anaplastic, pleomorphic tumor cells with abnormal nuclear morphology—histopathological evidence supporting the diagnosis of histological grade IV glioma (×400). MRI, magnetic resonance imaging; DMG, diffuse midline glioma; RANO, response assessment in neuro-oncology.

monoclonal antibody (6F-H2 diluted 1:50, Dako) as described previously (9, 10). We newly immunohistochemically stained the preserved biopsy specimen for its presentation in the better staining condition to confirm the expression of WT1 protein and followed the staining procedures as per the manufacturer's instructions. Namely, we used the EnVision™ FLEX immunohistochemical staining system (Agilent Technologies, CA, USA) by which Dako autostainer Link 48 (Agilent Technologies) and EnVision™ FLEX Mini Kit (Agilent Technologies) were applied to automatically stain the specimen by using the primary antibody—monoclonal mouse anti-human WT1 (6F-H2) antibody (Agilent Technologies) at a dilution rate of 400 fold; a whole IgG affinity-pure antibody, AffiniPure Rabbit Anti-Mouse IgG (H+L) (Jackson ImmunoResearch, Pennsylvania, USA), was used at a dilution rate of 200 fold after the completion of the primary antibody response. **Figure 1C** indicates the expression of WT1 protein. In these immunohistochemical staining procedures, kidney podocytes and normal brain tissue were used to conduct control positive and negative staining, respectively.

The biopsy specimen was immunohistochemically stained to verify the occurrence of the H3.3K27M mutation as described previously (12), except Ventara autostainer BenchMark ULTRA (Ventana Medical Systems Roche, Basel-Stadt, Switzerland) and OptiView DAB IHC Detection Kit (Ventana Medical Systems Roche). **Figure 1D** indicates the H3.3K27M mutation in the nucleus of tumor cells.

IMMUNOMONITORING

Modified WT1 Tetramer Staining of WT1-Specific Cytotoxic T Cells (CTLs), (IFN-γ) ELISpot Assay, Determination of Anti-WT1-235 IgG Antibody Titers in Serum, and Delayed-Type Hypersensitivity (DTH) Test

Cellular and/or humoral immune responses were examined by conducting the following four tests as described previously

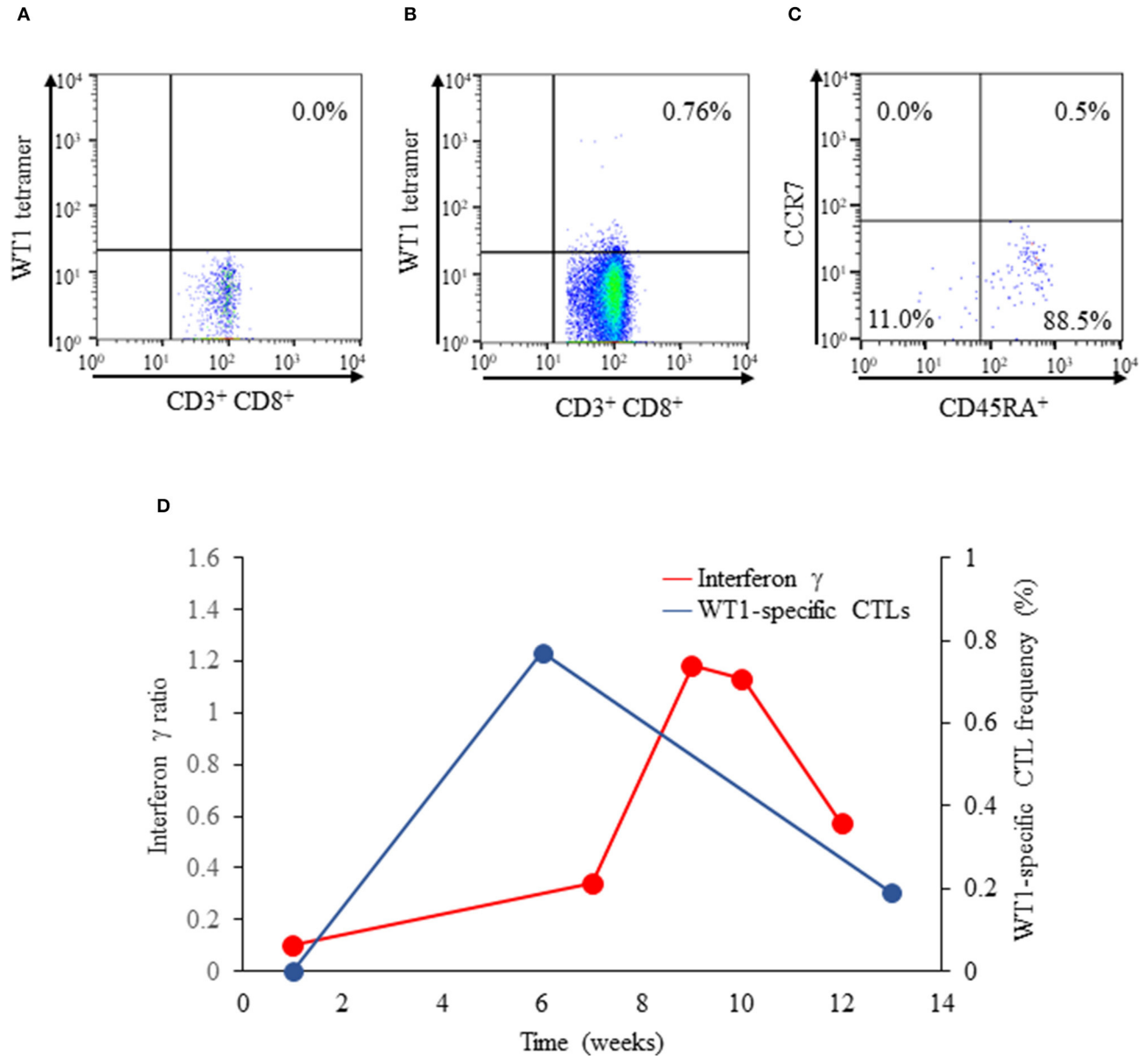

FIGURE 2 | Flow cytograms of WT1-specific CTLs obtained with a flow cytometer (BD FACSCanto^{TM} II). **(A)** WT1 tetramer^{+} CD8^{+} T cells did not emerge at the onset of vaccination (proportion of WT1 tetramer^{+} CD8^{+} T cells: 0.0%). **(B)** WT1-specific CTLs emerged at week 6 of vaccination (proportion of WT1 tetramer^{+} CD8^{+} T cells: 0.76%). **(C)** Diagram showing the phenotypes of WT1-specific CTLs: effector T cells (CD45RA^{+} CCR7^{-}: 88.5%), effector memory T cells (CD45RA^{-} CCR7^{-}: 11.0%), naïve T cells (CD45RA^{+} CCR7^{+}: 0.5%), and central memory T cells (CCR7^{+}, CD45RA^{-}: 0.0%). **(D)** Interferon γ ELISpot assay of PBMCs. Isolated PBMCs were stimulated with WT1-235n (natural peptide: aa 235–243 CMTWNQMNL). The stimulated-to- unstimulated PBMC spot ratio was calculated. Interferon γ production by WT1-specific CTLs was considered positive when the ratio was >1. The frequencies (%) of WT1-specific CTLs at weeks 1 to 13 of vaccination are plotted. WT1, Wilms tumor 1; CTLs, cytotoxic T cells; PBMCs, peripheral blood mononuclear cells.

(13–16): (1) the HLA-A*24:02 modified WT1 tetramer-CYTWNQMNL staining of WT1-specific CTLs—WT1 tetramer^{+} CD8^{+} T cells; (2) IFN-γ ELISpot assay of peripheral blood mononuclear cells (PBMCs); (3) the determination of anti-WT1-235 IgG antibody titers in serum; and (4) DTH test with WT1 peptide.

At the onset and week 6 of vaccination, WT1-specific CTL frequency was determined, and fluorescence-activated cell sorting (FACS) was conducted with a flow cytometer (BD FACSCanto^{TM} II, BD Bioscience, San Jose, CA, USA). Data obtained were analyzed using the FACSDIVA software (BD Bioscience). Consequently, their phenotypes based on CCR7 and CD45RA expressions were determined as follows: naïve T cells (CCR7^{+}, CD45RA^{+}), central memory T cells (CCR7^{+}, CD45RA^{-}), effector memory T cells (CCR7^{-}, CD45RA^{-}), and effector T cells (CCR7^{-}, CD45RA^{+}) (17). WT1 staining of

TABLE 1 | Clinical trials of immunotherapy in pediatric patients with diffuse intrinsic pontine/midline glioma registered at ClinicalTrials.gov as of December 2019.

Identifier no.	Status	Study results (reference)	Phase	Disease	Intervention
NCT01400672	Completed	None available	I	Relapsed or progressive disease	Tumor lysate vaccine, imiquimod
NCT02960230	Recruiting	None available	I	Newly diagnosed	K27M peptide
NCT01058850	Terminated	None available	I	NR	Rindopepimut
NCT02750891	Recruiting	None available	I/II	Relapsed or progressive disease	DSP7888
NCT01130077	Recruiting	Available (19)	NR	Newly diagnosed	Glioma antigen peptide vaccine
NCT02840123	Active; not recruiting	Available (20)	I	Newly diagnosed	Autologous dendritic cell vaccine
NCT02359565	Recruiting	None available	I	Relapsed or progressive disease	Pembrolizumab
NCT03130959	Active	None available	Ib/II	Newly diagnosed	Nivolumab vs. ipilimumab
NCT01952769	Active; not recruiting	Available (21)	I/II	Newly diagnosed	Pidilizumab
NCT02793466	Recruiting	None available	I	Relapsed	Durvalumab
NCT00036569	Completed	Available (22)	II	NR	Pegylated interferon γ-2b
NCT03389802	Active	None available	I	Newly diagnosed, recurrent, or progressive disease	APX005M
NCT03330197	Active	None available	I	NR	Ad-RTS-human interleukin-12
NCT02502708	Recruiting	None available	I	Newly diagnosed	Indoximod in combination with temozolomide, or with radiation followed by indoximod/temozolomide combination

NR, not reported.

TABLE 2 | Results of clinical trials of immunotherapy in pediatric patients with diffuse intrinsic pontine/midline glioma.

Identifier no. (reference)	Phase	Disease	Intervention	No. of patients	Median overall survival after diagnosis	Adverse event
NCT01130077 (17)	NR	Newly diagnosed	Glioma antigen peptide vaccine	26 BSG (14 BSG underwent radiotherapy and 12 BSG/HGG radiochemotherapy)	12.7 months	No grade 3 or 4 adverse events
NCT02840123 (18)	I	Newly diagnosed	Autologous dendritic cell vaccine	9 DIPG	NR[a]	Grade 3 osteomyelitis
NCT01952769 (19)	I/II	Newly diagnosed	Pidilizumab	9 DIPG	15.6 months	Grade 3 neutropenia and BP elevation
NCT00036569 (20)	II	Newly diagnosed	Pegylated interferon γ-2b	32 DIPG	351 days	Grade 3 neutropenia

BP, blood pressure; BSG, brain stem glioma; DIPG, diffuse intrinsic pontine glioma; HGG, high-grade glioma; NR, not reported.
[a]*Vaccination was completed, safety was acceptable, and a WT1-specific immune response was detected.*

immunofuorescent T cells was conducted. Although not detected at the onset of vaccination (**Figure 2A**), the production of WT1-specific CTLs was induced markedly at week 6 of vaccination (0.76%; **Figure 2B**). Effector T cells (88.5%), effector memory T cells (11.0%), and naïve (0.5%) T cells constituted the entirety of WT1-specific T cells at week 8 of vaccination; central memory T cells (0.0%) did not emerge (**Figure 2C**). DTH test with WT1 peptide, which had been negative at the onset of vaccination, turned positive at weeks 8 and 10 of vaccination.

The IFN-γ ELISpot assay of PBMCs was conducted to detect IFN-γ release from CTLs. Briefly, isolated PBMCs were stimulated with WT1 235n (natural peptide: aa 235–243 CMTWNQMNL). PBMCs, with or without peptide stimulation, were smeared onto 96-well-plates precoated with the mouse antihuman IFN-γ antibody; subsequently, the smears were incubated for 18 h. The stimulated-to-unstimulated PBMC spot ratio was calculated. A ratio of >1 indicated that cytokine production from CTLs was positive. In our pediatric patient, IFN-γ production was negative at the onset of vaccination but turned positive at weeks 9 and 10 of vaccination; the positivity lasted up to week 11 of vaccination (**Figure 2D**), and IFN-γ production became undetectable at week 12 of vaccination. Moreover, the proportion of WT1-specific CTLs peaked at week 6 of vaccination and then gradually decreased until week 13 of vaccination (**Figure 2D**), presumably due to the lack of the immunologically relevant production of effector memory T cells. Anti-WT1-235 IgG antibody titers in serum were determined as described previously (15). In brief, WT1 aa 235–245 peptide was used as the capture antigen for the anti-WT1-235 IgG antibody. Anti-WT1-235 IgG antibody titers in serum were measured by enzyme-linked immunosorbent assay at the onset and at weeks 6, 8, 9, 10, 11, 12, 13, 14, and 18 of vaccination. The titers were expressed as absorbance at the wavelength of 450 nm. Consequently, anti-WT1-235

IgG antibody titers were below the detection limit at all measurement points.

DISCUSSION

This is the first case report on a pediatric patient with relapsed DMG, who exhibited an encouraging clinical evolution after WT1-targeting immunotherapy onset. Although best response was stable disease, MRI findings improved, clinical manifestations (e.g., decreased consciousness, vertigo, vomiting, and headache) resolved, and right hemiplegia ameliorated—all transiently at weeks 4–12 of vaccination. Furthermore, betamethasone dose was reduced successfully without deteriorating clinical manifestations. Of note was the fact that DMG remained stable owing to WT1 peptide vaccination alone despite the presence of a large residual mass of the tumor after radiochemotherapy. Namely, the WT1 peptide vaccine showed an obvious antitumor effect and extended the patient's postrelapse OS to 8.3 months as compared with the reported median of 3.2 months for pediatric patients with DMG (1).

We reviewed 14 clinical trials of immunotherapy for pediatric patients with diffuse intrinsic pontine glioma (DIPG)/DMG that were registered at ClinicalTrials.gov website in December 2019–6, 4, and 4 of which used peptide vaccines, immune checkpoint inhibitors, and other treatments, respectively (**Table 1**). Concretely, a peptide vaccine against the point mutation (K27M) of the *histone-3* gene (H3F3A)—a driver gene—seems to be promising and is currently under development. Rindopepimut is a vaccine that targets EGFRvIII protein; its trial in pediatric patients with DMG was discontinued due to the lack of efficacy in the phase III trial for adult glioblastoma (18). DSP7888 contains a peptide that induces WT1-specific CTLs and helper T cells; a phase I/II study of the agent is underway for the treatment of pediatric patients with DMG, grade III glioma, or grade IV glioblastoma in Japan, which used vaccines, a programmed cell death 1 receptor checkpoint inhibitor, and an immunomodulator. Four of these 14 clinical trials, which provided clinical outcomes from pediatric patients with newly diagnosed DIPG/DMG, are summarized in **Table 2**—(1) the glioma-associated antigen-based vaccine (2, 19) the autologous dendritic cell vaccine (3, 20) pidilizumab (21); and (4) pegylated IFN-γ-2b (22). Our pediatric patient, who had relapsed DMG, showed an encouraging clinical evolution presumably due to the following facts: (1) the vaccine is specific to tumor-overexpressed WT1 that is highly immunogenic, tumorigenic, and angiogenic, and that regulates the apoptosis of many malignant brain tumors (23)—the features that drive us to consider that the WT1 peptide vaccine suppresses tumorigenesis in the patient and tumor angiogenesis and enhances immunogenicity of the patient and tumor apoptosis; (2) steroid dose reductions were possible; and (3) the vaccine has a good safety profile. In general, greater refractoriness to treatments is observed in patients with relapsed DMG than in those with newly diagnosed DMG.

A WT1-specific immune response was successfully induced as evidenced by the emergence of WT1-specific CTLs, by IFN-γ production in the IFN-γ ELISpot assay of PBMCs, and by the development of DTH after vaccination onset even during betamethasone administration. The phenotypic analysis of WT1-specific CTLs revealed that the effector T cells (88.5%), effector memory T cells (11.0%), and naïve T cells (0.5%) constituted the entirety of WT1-specific CTLs at week 6 of vaccination. The high frequency (>13%) of effector memory T cells was correlated with longer survival and higher clinical response rates in patients with advanced melanoma (24). In our pediatric patient, as high as 11.0% of WT1 tetramer$^+$ CD8$^+$ T cells emerged as effector memory T cells at week 6 of vaccination, and the IFN-γ ratio surpassed 1.0 at week 9 of vaccination, indicating that WT1-specifc CTLs became functional immunologically. We presume that these facts contributed, at least in part, to 8-week improvements in clinical manifestations and MRI findings.

The development of DTH to the WT1 peptide vaccine, as reported in previous studies (13, 15), indicates a good prognosis of patients with malignant brain tumors. Indeed, our pediatric patient developed DTH but was unable to produce the anti-WT1-235 IgG antibody that requires helper T lymphocytes which are involved in the immunoglobulin class switch. Therefore, co-vaccination with WT1 killer and helper peptides may be required to enhance clinical efficacy and anti-WT1 IgG antibody production.

The central nervous system (CNS), in which an immune response is considered less prone to develop owing to the blood-brain barrier, has immune privilege (25). However, a lymphatic system of the CNS, through which activated T cells can penetrate into the brain parenchyma, was recently discovered (26). In patients with glioblastoma multiforme who underwent peptide-pulsed dendritic cell-based immunotherapy that induces the considerable production of systematically activated T cells (27), the robust infiltration of CTLs and memory T cells into the intracranial tumor was associated with the prolonged survival thereof. Therefore, the increased production of systemically activated T cells and memory T cells may be important for immunotherapy to exert greater efficacy.

Our study has several limitations. First, the long-lasting efficacy of WT1-targeting immunotherapy cannot be expected for pediatric patients with relapsed DMG who have a large residual tumor mass after radiochemotherapy. Second, tumor reduction surgery, which is important for successful immunotherapy, was impossible to conduct for our pediatric patient who had unresectable DMG—the fact that was responsible, at least in part, for his poorer prognosis in comparison with adult patients with glioblastoma who underwent the surgery and for whom the WT1 peptide vaccine induced a clinical response and longer survival than the historical control at our institution (9). Third, the gene expression profiling of our pediatric patient remains to be conducted.

In conclusion, our pediatric patient who had the H3.3K27M mutation and was treated with WT1-targeting immunotherapy with the present peptide vaccine exhibited an encouraging clinical evolution, warranting further clinical research on this therapeutic modality.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Review Committee of Osaka University Faculty of Medicine. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

All authors conceived and designed the study, participated in writing the manuscript, and approved the final version of the manuscript. YH, NK, HSu, YOj, and KO provided study materials or patients. YH, NK, SF, HSa, and NH collected and assembled data. YH, YOk, NK, SF, and NH analyzed and interpreted data.

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REFERENCES

1. Bartels U, Wolff J, Gore L, Dunkel I, Gilheeney S, Allen J, et al. Phase 2 study of safety and efficacy of nimotuzumab in pediatric patients with progressive diffuse intrinsic pontine glioma. *Neuro Oncol.* (2014) 16:1554–9. doi: 10.1093/neuonc/nou091
2. Gururangan S, Chi SN, Young Poussaint T, Onar-Thomas A, Gilbertson RJ, Vajapeyam S, et al. Lack of efficacy of bevacizumab plus irinotecan in children with recurrent malignant glioma and diffuse brainstem glioma: a pediatric Brain Tumor Consortium study. *J Clin Oncol.* (2010) 28:3069–75. doi: 10.1200/JCO.2009.26.8789
3. Cohen KJ, Pollack IF, Zhou T, Buxton A, Holmes EJ, Burger PC, et al. Temozolomide in the treatment of high-grade gliomas in children: a report from the Children's Oncology Group. *Neuro Oncol.* (2011) 13:317–23. doi: 10.1093/neuonc/noq191
4. Wolff JE, Rytting ME, Vats TS, Zage PE, Ater JL, Woo S, et al. Treatment of recurrent diffuse intrinsic pontine glioma: the MD Anderson Cancer Center experience. *J Neurooncol.* (2012) 106:391–7. doi: 10.1007/s11060-011-0677-3
5. Castel D, Philippe C, Calmon R, Le Dret L, Truffaux N, Boddaert N, et al. Histone H3F3A and HIST1H3B K27M mutations define two subgroups of diffuse intrinsic pontine gliomas with different prognosis and phenotypes. *Acta Neuropathol.* (2015) 130:815–27. doi: 10.1007/s00401-015-1478-0
6. Qi XW, Zhang F, Wu H, Liu JL, Zong BG, Xu C, et al. Wilms' tumor 1 (WT1) expression and prognosis in solid cancer patients: a systematic review and meta-analysis. *Sci Rep.* (2015) 5:8924. doi: 10.1038/srep08924
7. Cheever MA, Allison JP, Ferris AS, Finn OJ, Hastings BM, Hecht TT, et al. The prioritization of cancer antigens: a national cancer institute pilot project for the acceleration of translational research. *Clin Cancer Res.* (2009) 15:5323–37. doi: 10.1158/1078-0432.CCR-09-0737
8. Tamaki H, Mishima M, Kawakami M, Tsuboi A, Kim EH, Hosen N, et al. Monitoring minimal residual disease in leukemia using real-time quantitative polymerase chain reaction for Wilms tumor gene (WT1). *Int J Hematol.* (2003) 78:349–56. doi: 10.1007/BF02983561
9. Izumoto S, Tsuboi A, Oka Y, Suzuki T, Hashiba T, Kagawa N, et al. Phase II clinical trial of Wilms tumor 1 peptide vaccination for patients with recurrent glioblastoma multiforme. *J Neurosurg.* (2008) 108:963–71. doi: 10.3171/JNS/2008/108/5/0963
10. Hashimoto N, Tsuboi A, Kagawa N, Chiba Y, Izumoto S, Kinoshita M, et al. Wilms tumor 1 peptide vaccination combined with temozolomide against newly diagnosed glioblastoma: safety and impact on immunological response. *Cancer Immunol Immunother.* (2015) 64:707–16. doi: 10.1007/s00262-015-1674-8
11. Wen PY, Macdonald DR, Reardon DA, Cloughesy TF, Sorensen AG, Galanis E, et al. Updated response assessment criteria for high-grade gliomas: response assessment in neuro-oncology working group. *J Clin Oncol.* (2010) 28:1963–72. doi: 10.1200/JCO.2009.26.3541
12. Bechet D, Gielen GG, Korshunov A, Pfister SM, Rousso C, Faury D, et al. Specific detection of methionine 27 mutation in histone 3 variants (H3K27M) in fixed tissue from high-grade astrocytomas. *Acta Neuropathol.* (2014) 128:733–41. doi: 10.1007/s00401-014-1337-4
13. Nishida S, Ishikawa T, Egawa S, Koido S, Yanagimoto H, Ishii, et al. Combination gemcitabine and WT1 peptide vaccination improves progression-free survival in advanced pancreatic ductal adenocarcinoma: a Phase II randomized study. *Cancer Immunol Res.* (2018) 6:320–31. doi: 10.1158/2326-6066.CIR-17-0386
14. Tsuboi A, Oka Y, Nakajima H, Fukuda Y, Elisseeva OA, Yoshihara S, et al. Wilms tumor gene WT1 peptide-based immunotherapy induced a minimal response in a patient with advanced therapy-resistant multiple myeloma. *Int J Hematol.* (2007) 86:414–7. doi: 10.1007/BF02983998
15. Oji Y, Hashimoto N, Tsuboi A, Murakami Y, Iwai M, Kagawa N, et al. Association of WT1 IgG antibody against WT1 peptide with prolonged survival in glioblastoma multiforme patients vaccinated with WT1 peptide. *Int J Cancer.* (2016) 139:1391–401. doi: 10.1002/ijc.30182
16. Hayashi S, Imanishi R, Adachi M, Ikejima S, Nakata J, Morimoto S, et al. Reader-free ELISPOT assay for immune-monitoring in peptide-based cancer vaccine immunotherapy. *Biomed Rep.* (2020) 12:244–50. doi: 10.3892/br.2020.1289
17. Sallusto F, C R Mackay, Lanzavecchia A. The role of chemokine receptors in primary, effector, and memory immune responses. *Annu Rev Immunol.* (2000) 18:593–620. doi: 10.1146/annurev.immunol.18.1.593
18. Weller M, Butowski N, Tran DD, Recht LD, Lim M, Hirte H, et al. Rindopepimut with temozolomide for patients with newly diagnosed, EGFRvIII-expressing glioblastoma (ACT IV): a randomised, double-blind, international phase 3 trial. *Lancet Oncol.* (2017) 18:1373–85.
19. Pollack IF, Jakacki RI, Butterfield LH, Hamilton RL, Panigrahy A, Potter DM, et al. Antigen-specific immune responses and clinical outcome after vaccination with glioma-associated antigen

peptides and polyinosinic-polycytidylic acid stabilized by lysine and carboxymethylcellulose in children with newly diagnosed malignant brainstem and nonbrainstem gliomas. *J Clin Oncol.* (2014) 32:2050–8. doi: 10.1200/JCO.2013.54.0526

20. Benitez-Ribas D, Cabezón R, Flórez-Grau G, Molero MC, Puerta P, Guillen A, et al. Immune response generated with the administration of autologous dendritic cells pulsed with an allogenic tumoral cell-lines lysate in patients with newly diagnosed diffuse intrinsic pontine glioma. *Front Oncol.* (2018) 8:127. doi: 10.3389/fonc.2018.00127
21. Fried I, Lossos A, Ben Ami T, Dvir R, Toledano H, Ben Arush MW, et al. Preliminary results of immune modulating antibody MDV9300 (pidilizumab) treatment in children with diffuse intrinsic pontine glioma. *J Neurooncol.* (2018) 136:189–95. doi: 10.1007/s11060-017-2643-1
22. Warren K, Bent R, Wolters PL, Prager A, Hanson R, Packer R, et al. A phase 2 study of pegylated interferon alpha-2b (PEG-Intron(R)) in children with diffuse intrinsic pontine glioma. *Cancer.* (2012) 118:3607–13. doi: 10.1002/cncr.26659
23. Kijima N, Hashimoto N, Chiba Y, Fujimoto Y, Sugiyama H, Yoshimine T. Functional roles of Wilms' tumor 1 (WT1) in malignant brain tumors. In: *Wilms Tumor*. Codon Publications. (2016). Available online at: https://www.ncbi.nlm.nih.gov/books/NBK373347 (accessed December 25, 2019).
24. Wistuba-Hamprecht K, Martens A, Heubach F, Romano E, Geukes Foppen M, Yuan J, et al. Peripheral CD8 effector-memory type 1 T-cells correlate with outcome in ipilimumab-treated stage IV melanoma patients. *Eur J Cancer.* (2017) 73:61–70. doi: 10.1016/j.ejca.2016.12.011
25. Barker CF, Billingham RE. Immunologically privileged sites. *Adv Immunol.* (1977) 25:1–54. doi: 10.1016/S0065-2725(08)60012-4
26. Louveau A, Smirnov I, Keyes TJ, Eccles JD, Rouhani SJ, Peske JD, et al. Structural and functional features of central nervous system lymphatic vessels. *Nature.* (2015) 523:337–41. doi: 10.1038/nature14432
27. Yu JS, Wheeler CJ, Zeltzer PM, Ying H, Finger DN, Lee PK, et al. Vaccination of malignant glioma patients with peptide-pulsed dendritic cells elicits systemic cytotoxicity and intracranial T-cell infiltration. *Cancer Res.* (2001) 61:842–7.

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REVIEW
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Microglia-Centered Combinatorial Strategies Against Glioblastoma

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Tumor-associated microglia (MG) and macrophages (MΦ) are important components of the glioblastoma (GBM) immune tumor microenvironment (iTME). From the recent advances in understanding how MG and GBM cells evolve and interact during tumorigenesis, we emphasize the cooperation of MG with other immune cell types of the GBM-iTME, mainly MΦ and T cells. We provide a comprehensive overview of current immunotherapeutic clinical trials and approaches for the treatment of GBM, which in general, underestimate the counteracting contribution of immunosuppressive MG as a main factor for treatment failure. Furthermore, we summarize new developments and strategies in MG reprogramming/re-education in the GBM context, with a focus on ways to boost MG-mediated tumor cell phagocytosis and associated experimental models and methods. This ultimately converges in our proposal of novel combinatorial regimens that locally modulate MG as a central paradigm, and therefore may lead to additional, long-lasting, and effective tumoricidal responses.

Keywords: glioblastoma, immunotherapy, microglia modulation, glioma-associated microglia, glioma- associated macrophages, immune tumor microenvironment

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DEVELOPMENT AND CLASSIFICATION OF GLIOBLASTOMA

Glioblastoma (GBM) is the most aggressive and common primary brain tumor. Despite current treatment modalities, consisting of surgical resection followed by chemo-irradiation, the median overall survival of GBM patients remains only 15 months (1). These tumors arise from astrocytes or their precursors within the central nervous system (CNS) and are genetically and phenotypically heterogeneous (2). World Health Organization (WHO) grade IV glioma that arises *de novo* is designated primary GBM while that developing from the progression of previously diagnosed lower-grade glioma is named secondary GBM (3).

In the course of primary GBM development, chromosome 7 gain and chromosome 10 loss have led to the identification of platelet-derived growth factor subunit A (*PDGFA*) and phosphatase and tensin homolog (*PTEN*) as driver genes (4). Based on genomic, transcriptomic, and proteomic profiles, primary GBM has been further subclassified into classical (CL), proneural (PN), or mesenchymal (MES) subgroups (5–8). While CL-GBM shows frequent epidermal growth factor receptor (*EGFR*) amplification and cyclin-dependent kinase inhibitor 2A (*CDKN2A*) homozygous deletion, PN-GBM is associated with amplification of platelet-derived growth factor receptor alpha (*PDGFRA*) and tumor protein p53 (*TP53*) mutations. Finally, MES-GBM, is associated with additional loss of neurofibromin 1 (*NF1*) gene, and co-mutated *PTEN* and *TP53* tumor suppressor

genes (4, 5). In sum, the genetic alterations that distinguish all 3 GBM subgroups commonly hit the same three major glioma signaling pathways: the RTK/RAS/PI3K (proliferation), TP53 (apoptosis) and RB (cell division) pathways (9). At the clinical level, MES-GBM shows the shortest median survival (11.5 months), compared to CL- and PN-GBM (14.7 and 17 months, respectively) (10) (**Figure 1**). Within these 3 GBM subgroups, limited therapeutic benefit has been observed (5, 6). Additionally, NFKB inhibitor alpha (*NFKBIA*) deletion confers radio-resistance in MES-GBM (20, 21).

Secondary GBM and its precursors harbor isocitrate dehydrogenase [NADP(+)] 1 (*IDH1*) and 2 (*IDH2*) mutations (collectively IDH^{mut}), in addition to either *TP53* mutations in low-grade astrocytoma (LGA) and high-grade astrocytoma (HGA), or co-deletion of chromosome 1p/19q in oligodendroglioma (ODG) (11, 22). In contrast to IDH^{wt}, glioma patients retrospectively identified as IDH^{mut} showed improved survival upon standard of care temozolomide (TMZ) treatment (23). Together with histopathology, *IDH* mutation and 1p/19q co-deletion statuses are now used resulting in the current integrated WHO classification (12). The classification of brain tumors into IDH^{mut} (HGA, LGA, and ODG) or *IDH* wild type (IDH^{wt}; CL-, MES-, and PN-GBM) has been further supported by methylomics (8) (**Figure 1**).

IMPLICATIONS OF GBM SUBTYPE ON IMMUNE CELL INFILTRATES

GBMs frequently contain high proportions of non-neoplastic immune cells that collectively form the immune tumor microenvironment (iTME). The considerable number of immune cells within these tumors may account for the gene expression variability observed between GBM patient biopsies (24). Tumor-associated immune cells primarily enrolled for cytotoxicity against tumor cells, are typically hijacked by the tumor to promote its progression through mutual tumor-immune cell paracrine interactions and genetic reprogramming. Furthermore, the high content of macrophages (MΦ) and microglia (MG) and low frequency of lymphocytes in the GBM-iTME classify GBM as a lymphocyte-depleted tumor (25). Since studies describing the immune cell composition of glioma biopsies *in situ* have used distinct methodologies and calculation modes, interstudy comparison is not quantifiable. Nevertheless, the superimposition of those data shows consistent trends. First, IDH^{wt} primary GBM patients, having shorter overall survival relative to IDH^{mut} secondary GBM patients, show globally higher MG, MΦ, and T lymphocyte composition (13). Then, among IDH^{mut}, from WHO grade II to IV secondary GBM, progressively reduced patient outcome correlates with increased MG, MΦ and T cell contents (14). Finally, primary GBM subgroups show differences in their immune composition,

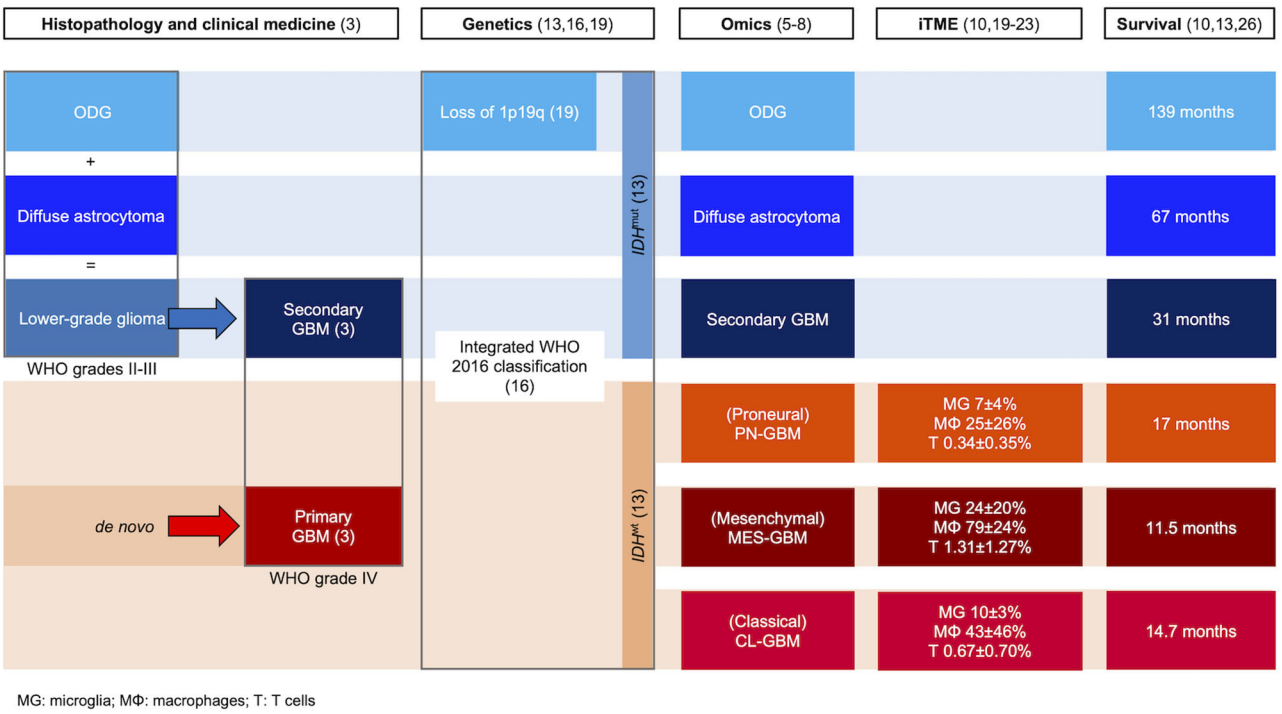

FIGURE 1 | Classification of GBM based on histological, clinical, genetic, -omic, and immune features. IDH^{mut} and IDH^{wt} glioma subtypes are indicated in shades of blue and red, respectively. Arrows indicate tumor progression. The definition of iTME composition is described in the relevant publications. Median survival of ODG and diffuse astrocytoma is based on WHO grade II (3, 5–8, 10–19).

again linking tumor progression and reduced patient survival with higher proportions of immune cells (15–17). Importantly, *NF1* loss (MES subtype) resulted in increased glioma-associated microglia and macrophage (GAM) infiltration, which was even more pronounced in recurrent GBM (10) (**Figure 1**). Thus, there is convincing evidence of increased recruitment of tumor-associated immune cells during brain tumor development, suggesting an oncogenic contribution of the iTME. Hampering this paracrine symbiotic association may lead to greater control of tumor progression. Mechanistically, the accumulation of 2-hydroxyglutarate resulting from *IDH* mutations suppresses the accumulation and activity of infiltrating T cells by impairing the nuclear factor of activated T cells (NFAT) expression in a paracrine manner (26, 27). Further, NF-κB activation of GAMs mediates PN- to MES-GBM transition, while *NF1* inactivation, a hallmark of MES-GBM, results in higher numbers of infiltrating, anti-inflammatory, M2 GAMs and $CD4^+$ memory T cells (10, 21).

In parallel to IDH^{mut} tumors and their hypermethylator phenotype, GBM can acquire a hypermutator phenotype resulting from TMZ-based chemotherapy (18, 28–31). Concurrently, the accumulation of neoantigens stimulates the recruitment of $CD8^+$ T cells into the tumor (10). Thus, the occurrence of spontaneous or TMZ-induced tumor-specific neoantigens represents a potential modulator of iTME composition and T cell-mediated anticancer cytotoxicity in GBM. Altogether, the crosstalk between tumor and infiltrating immune cells suggests possible therapeutic interventions to redirect immune cells against neoplastic cells to further control glioma progression.

CHARACTERIZATION OF THE GBM-iTME

The brain has historically been considered an immune-privileged organ (32, 33). This concept was long supported by three main observations: (a) the existence of a specialized vasculature in the brain, termed the blood-brain barrier (BBB) (34), (b) the lack of a conventional lymphatic system, (c) and a poorly characterized brain-specific immune cell population—MG. This classical dogma has been challenged by several studies that demonstrated that the CNS is in fact actively interacting with the immune system (35). Increasing evidence suggests that inflammation is the prime cause of many neurodegenerative diseases, and it is now generally accepted that the CNS undergoes constant intrinsic and peripheral immune surveillance (36–38). One such mechanism of immunosurveillance has been elucidated by the discovery of a CNS-specific lymphatic system. This study established that antigens and T cells can reach the cervical lymph nodes through cerebrospinal fluid-filled channels (39). In addition, antigens may also enter the cerebral arteries and cervical lymph nodes through the Virchow Robin perivascular spaces, and immunoglobulins are able to cross the BBB via carrier-mediated transport (40, 41). Taken together, these observations point toward the existence of important interactions between the CNS and the immune system, and underscore the role of the immune system in the induction and progression of brain cancers. Moreover, they emphasize the potential for immunotherapeutic approaches in the treatment of brain tumors.

The complex GBM-iTME is dominated by immunosuppressive cytokines such as prostaglandin E_2 (PGE2), transforming growth factor beta 1 (TGFB1), and interleukin (IL)−6 and−10 (42, 43). Important "hubs of immunosuppression" such as high expression of STAT3 or FGL2 by GBM cells might directly act as paracrine mediators on the pleiotropic iTME, and could be universally targeted (44). In parallel, regulatory $CD4^+$ T-helper cells (Tregs) are an important immune population in the GBM-iTME (45). Both natural Tregs (nTregs)—naturally occurring in the thymus—and induced Tregs (iTregs)—induced by activation with antigen or by antigen-presenting cells (APCs)—have been reported to contribute to GBM-mediated immunosuppression, with nTregs reportedly having a dominant role in the GBM-iTME (46). Cytotoxic $CD8^+$ T cells are very rare, accounting for under 20% of all $CD3^+$ lymphocytes, and appear loosely distributed in the GBM parenchyma (47). In an immunohistochemical (IHC) study of tissue microarray cores from 284 gliomas, the number of $CD8^+$ tumor-infiltrating lymphocytes (TILs) correlated negatively with tumor grade whereas the number of $CD4^+$ TILs displayed a positive correlation (48). Another recent study reported that GBM-TILs increased their expression of indoleamine 2,3-dioxygenase (IDO1), an enzyme that catalyzes tryptophan (TRP) degradation, resulting in the depletion of TRP in the local iTME and consequent inhibition of T cell responses (49). Moreover, another study demonstrated that GBM patients and GBM-grafted mice may harbor peripheral blood $CD4^+$ T cell counts as low as acquired immune deficiency syndrome subjects and show T cell-deficient lymphoid organs. Concomitantly, large numbers of T cells were instead found sequestered in the bone marrow (BM), accompanied by tumor-imposed loss of sphingosine 1 phosphate receptor 1 (S1PR1) from the T cell surface (50).

Yet, perhaps the most notable aspect of the GBM-iTME is its population of tumor-associated MΦ and MG—collectively referred to as GAMs. These are the most abundant GBM-infiltrating immune cells and may contribute to up to half of the total tumor mass (51, 52). In addition to the recruitment of brain-resident MG to the tumor site, the high number of GAMs in glioma is a cumulative result of the influx of myeloid-derived MΦ into the brain as a consequence of tumor-induced neoangiogenesis and inflammatory stimuli. This inflammatory iTME acts in an immunosuppressive manner to promote tumor progression [e.g., via reprogramming of GAMs to anti-inflammatory states by paracrine tumor cell-GAM crosstalk (53–56)]. The contribution of GAMs to gliomagenesis continues to unveil the complex interactions of GBM cells with their microenvironment (53, 54, 57). Together, these data suggest that in addition to T cells, GAMs represent an attractive cell population with an intrinsic functional repertoire that may be reprogrammed to target tumor cells. In the **Supplementary Information** and **Supplementary Table 1** of this review, we provide a comprehensive overview of the most recent clinical trials and their strategies in interfering with the innate and adaptive GBM-iTME.

DISTINCTION OF BM-DERIVED MΦ FROM BRAIN-RESIDENT MG

MG are dynamic and specialized CNS-resident immune cells. Their name was first coined by Pío Del Río Hortega, then a student of Santiago Ramón y Cajal, and published in the Bulletin of the Spanish Society of Biology in 1919. MG are constantly monitoring the CNS and become activated in response to pathogens or CNS injury (58, 59).

Various experiments including parabiosis, adoptive transfer and fate mapping studies conducted in mouse models have elucidated our understanding of MG and their distinction from peripheral, BM-derived MΦ (51, 60–65). MG and MΦ are thus distinct and ontogenically different cell populations (54).

Despite the separate origins of MG and MΦ, GAM accumulation within and around GBM has raised interest in dissecting the roles of these cells in tumor progression. Many common chemoattractant factors have been identified for MG and MΦ (57). In the healthy brain, the CX3C motif chemokine receptor 1 (CX3CR1) is mostly expressed by MG and has been established as a reliable marker for MG imaging (57). Notably, a polymorphism in the *CX3CR1* gene has been associated with reduced tumor infiltration by MG which led to increased survival of GBM patients (66). Others reported conflicting findings regarding the importance of CX3CR1 and its ligand—CX3C motif chemokine ligand 1 (CX3CL1)—in tumor-directed MG migration (67, 68). However, infiltrating monocytes, differentiating into MΦ express it as well, implying that CX3CR1 does not represent a MG-specific marker, especially in the context of glioma (67). Notably, a recent study identified perivascular, meningeal, and choroid plexus MΦ as non-parenchymal brain MΦ that mediate immune responses at the brain boundaries and, like MG, express CX3CR1 in the healthy brain (69). One of the first chemoattractant factors identified is CC motif chemokine ligand 2 (CCL2) or MCP1. Ectopic expression of CCL2 in rat glioma cells showed increased tumor growth, with massive infiltration of MG/MΦ, resulting in reduced survival (70). Interestingly, it has been recently described that in mice, MG, in contrast to MΦ, do not express the CCL2 receptor, CC motif chemokine receptor 2 (CCR2), providing a novel model to investigate monocyte subset trafficking within the GBM-iTME (71). In fact, Hutter and colleagues used a *Ccr2* knockout mouse model which limits MΦ infiltration into the tumor site, enabling the specific study of MG within the GBM iTME (72). Colony stimulating factor 1 (CSF1) or M-CSF is another potent GAM-recruiting cytokine. Blocking its receptor, colony stimulating factor 1 receptor (CSF1R) reduced GAM density and attenuated GBM invasion *in vivo* (73, 74). Similar results were reported by a knockdown of its close relative, CSF2, which resulted in reduced MG-dependent invasion in organotypic brain slices as well as diminished growth of intracranial gliomas accompanied by extended survival in animal models (75).

Approaches to distinguish these cell populations have traditionally relied on the expression of the hematopoietic marker CD45, with yolk sac-derived MG being $CD45^{low}$ and infiltrating MΦ of hematopoietic origin $CD45^{high}$ (76). This paradigm has been recently challenged by a study using irradiated chimeras with head protection which impeded the massive unspecific influx of monocytes due to a disrupted BBB. The authors showed that MG are able to upregulate CD45 and represent an inherent part of the $CD45^{high}$ population in the tumor context (77).

Therefore, better targets are needed to accurately distinguish resident MG from infiltrating inflammatory monocytes and non-parenchymal brain MΦ to better understand their contribution in glioma formation, maintenance, and progression.

In a traumatic brain injury model, *in vivo* time-lapse 2-photon imaging of MG revealed their rapid and targeted migration and process extension to the site of injury, establishing a barrier between the healthy and injured tissue. This rapid chemotactic response is mediated by the release of nucleotides following CNS injuries (59). MG express several G protein-coupled receptors, including the G protein-coupled purinergic receptor P2Y 12 (P2RY12), a putative primary site where nucleotides act to induce MG chemotaxis. P2RY12 is also expressed on platelets and required for normal platelet aggregation and blood coagulation (78). In the brain parenchyma, its expression is well-limited to MG, making it a very useful marker in MG identification (79). Another useful marker to distinguish MG from infiltrating MΦ is integrin subunit alpha 4 (ITGA4) or CD49D, which was specifically repressed in the MG of different mouse models of glioma. Its translational relevance has also been shown in human GBM biopsies (53).

Recent advances in RNA sequencing and other cell profiling technologies have enabled the discovery of cell-type-specific signature genes. Among these, a transmembrane protein of unknown function—transmembrane protein 119 (TMEM119)—is exclusively expressed by MG in the human and mouse brain (80). Hence, TMEM119-specific antibodies are now widely used in IHC and flow cytometric (FC) applications. The ongoing large-scale transcriptional profiling of MG further identified novel cell lineage-specific genes like hexosaminidase subunit beta (*HEXB*), which is highly expressed in MG and encodes a subunit of the lysosomal enzyme hexosaminidase, that catalyzes the degradation of gangliosides (81). These novel instruments for cell-specific tracking and genetic modulation will enhance the specificity and sophistication of MG studies as well as our understanding of MG functions in the context of glioma.

MG ACTIVATION AND IMMUNE CELL INTERACTIONS IN THE GBM-iTME

MG accumulated within GBM typically undergo a morphological transformation from a ramified, resting phenotype, to an amoeboid, activated state (51). For MΦ, different types of activation have been defined following *in vitro* stimulation. The pro-inflammatory M1 phenotype is typically acquired after stimulation with IFNγ, alone or in concert with microbial cues such as LPS. Whereas, anti-inflammatory molecules, such as IL-4, -10, and -13, are inhibitors of MΦ activation and induce the alternative M2 phenotype (82, 83). These polarized MΦ subpopulations differ in terms of receptor expression, effector function, and cytokine and chemokine production (83). Given

that these definitions of the different activation states are based on *in vitro* conditions, and the M1 and M2 phenotypes represent the extremes of a broader spectrum of functional states, they are only to some extent translatable to the *in vivo* settings. In the era of single cell sequencing and mass cytometry, and much more detailed functional state analysis, this polarization classification may soon become obsolete in the MG field. Nevertheless, several studies have analyzed the expression of M1 and M2 markers among GBM-associated GAMs and concluded that, similarly to other solid tumor types, they predominantly exhibit an anti-inflammatory M2 polarization and reduced phagocytic activity (54, 84–87). It is believed that glioma-derived molecules such as CSF1 induce the shift of MG and MΦ toward the M2 phenotype and thus create a favorable microenvironment for GBM growth (86). In addition, GAM expression of CD163 and macrophage scavenger receptor 1 (MSR1) or CD204, both of which are considered M2 MΦ markers, was significantly higher in grade IV GBM when compared to low-grade glioma (LGG), indicating that polarization of GBM-associated MG and MΦ toward the M2 phenotype correlates with a more malignant histological grade (55). Accordingly, others identified the expression of CD74, an M1 polarization marker, by human GAMs to be positively correlated with the overall survival of GBM patients (88). However, useful they may have been in establishing and dissecting the functions of MG, the traditional M1 and M2 phenotypes, and the resulting classification of MG responses into a binary system of pro- or anti-inflammatory has so far produced an oversimplified insight to their complex roles in the context of brain diseases (89, 90).

Studies of human and murine neurodegenerative diseases, as well as brain tumors, have identified genes and their encoded proteins previously known to be expressed in the DC compartment of the peripheral immune system. Moreover, transcriptomics data from diverse neurodegenerative disease studies show MG upregulation of genes involved in APC-T cell interactions (91). Interestingly, similar trends have been found in MG isolated from GL261 syngeneic GBM mouse models as well as in tumor biopsies of GBM patients. This upregulated gene set included human and mouse homologs of immunosuppressive modulators (C type lectin domain containing 7A, *CLEC7A*; glycoprotein nmb, *GPNMB*; leukocyte immunoglobulin like receptor B4, *LILRB4*; and *PDCD1*) as well as stimulators (integrin subunit alpha X, *ITGAX* or *CD11C*; and secreted phosphoprotein 1, *SPP1*) of the adaptive immune system. Collectively, these studies show that MG derived from tumor and neurodegenerative states both contribute to immunosuppression and altered T cell responses in the brain (92–94).

In fact, a recent study showed that in the context of Alzheimer's disease (AD), chronically activated MG limit $CD3^+/CD8^+$ T cell recruitment to the brain (95). Another study with GL261 murine glioma models demonstrated that MG are functional APCs and are required for complete antigen-specific $CD8^+$ T cell responses in an MHC class I-dependent manner (96). Given the parallels between the inflammatory states resulting from brain tumors and neurodegenerative diseases, a better understanding of the link between innate and adaptive immune responses in the brain in combination with an improved characterization of MG heterogeneity, remain future directions for targeted immunotherapies against GBM.

Recently, combined high-throughput technologies of regionally annotated MG cells and intratumoral MG have mapped specific functional differences of MG in healthy vs. GBM-burdened brains. In non-neoplastic brains, nine clusters of heterogeneous MG functional states were identified whereas in GBM-associated MG, single-cell RNA sequencing (scRNA-seq) revealed even more heterogeneity–15 clusters—with upregulation of pro-inflammatory and metabolic genes, including *SPP1*, and several type I interferon genes, including apolipoprotein E (*APOE*) and *CD163*. By concurrent mass cytometry, the upregulation of HLA-DR, triggering receptor expressed on myeloid cells 2 (TREM2), APOE, adhesion G protein-coupled receptor G1 (ADGRG1) or GPR56, solute carrier family 2 member 5 (SLC2A5) or GLUT5, and Fc fragment of IgG receptor Ia (FCGR1A) or CD64 was confirmed in GBM-associated MG vs. normal control MG (97). This underscores the diversity and plasticity of MG in the healthy brain and the GBM-iTME, and reiterates the difficulty in targeting these cells for treatment.

MG IN GBM PROGRESSION

Early co-culture studies noted that the motility of murine glioma cells was increased in the presence of MG, and that this glioma-promoting effect could be further enhanced by MG-activating substances like CSF2 (98). GBM cells are known to constitutively release CSF1 and CSF2, which act as chemoattractants for MG and convert GAMs to protumoral phenotypes (74). Consistent with the tumor-promoting effect of CSF1, blockade of CSF1R led to decreased expression of M2 markers in GAMs, resulting in regression of established tumors and increased survival in a mouse GBM model (74). To summarize, once MG and MΦ are recruited to the tumor site and re-educated to a protumorigenic phenotype, mutual paracrine signaling between GAM and GBM cells is established whereby glioma growth and invasion are promoted. Similar effects on glioma cells could be shown by using GAM-conditioned media instead of co-cultures (98). Many of the soluble factors involved in GAM-glioma crosstalk have been identified, such as epidermal growth factor (EGF), which is released by MG and stimulates GBM cell migration and invasion via the commonly upregulated epidermal growth factor receptor (EGFR) on glioma cells (73). Other factors include anti-inflammatory TGFB1 and IL-10, pro-inflammatory molecules like TNF, IL-1B, and IL-6, as well as pro-angiogenic factors like vascular endothelial growth factor A (VEGFA). TGFB1 promotes the migration of glioma cells via processes that likely involve the upregulation of integrin expression and function (99). Furthermore, TGFB1 induces the release of matrix metallopeptidase 2 (MMP2) in its inactive form—pro-MMP2—which becomes activated upon cleavage by the membrane-bound matrix metallopeptidase 14 (MMP14) (99, 100). GBM-associated MG upregulate MMP14 and thereby facilitate the invasion of glioma cells into the brain parenchyma by metalloproteinase-mediated degradation of the extracellular matrix (100). A recent

study by Walentynowicz et al. sought to assess the role of human GBM conditioned media on human MG cell lines on the MG transcriptome. *TGM2* and *GPNMB* were identified across various datasets, but their relevance is awaiting further experimental validation (101).

Along with this paracrine glioma-promoting effect, GAMs also enable GBM engraftment and invasion by failing to efficiently eliminate cancer cells by phagocytosis. Their role as phagocytic innate immune cells is perturbed by glioma cells rendering MG and MΦ to an anti-inflammatory, antiphagocytic M2 phenotype (102). Moreover, upregulation of the so-called "don't eat me" signals on the surface of glioma cells and masking of antigenic sites by overexpressing sialic acid-rich glycoproteins are both effective strategies to inhibit phagocytosis and evade innate immune surveillance (103–105).

MODELING MG-GBM INTERACTIONS

The generation of a mouse strain in which the *Cx3cr1* locus was replaced by a green fluorescent protein (GFP) reporter gene ($Cx3cr1^{+/GFP}$) allowed for the first time the direct study of MG *in vivo* using 2-photon-microscopy (106, 107). This mouse line strongly labels MG and is the best-studied model in MG research (106, 108). To further exploit the *Cx3cr1* promoter activity, the *Cx3cr1* gene was replaced with sequences encoding either Cre recombinase ($Cx3cr1^{Cre}$) or a Cre recombinase fused to a mutant estrogen ligand-binding domain that requires the presence of the estrogen antagonist tamoxifen for activity ($Cx3cr1^{CreERT2}$) (109). These mouse lines enabled a conditional, MG-specific constitutive or inducible gene knockout, which advanced the specificity of MG research significantly (**Table 1**).

Even though CX3CR1 is highly expressed on MG, it is expressed as well on MΦ, monocytes, and DCs (106). P2RY12, on the other hand, was initially investigated for its function as a regulator of platelet adhesion and activation. P2RY12-deficient mice were therefore primarily used to study platelet physiology and blood coagulation (110, 111). Eventually, P2RY12 was identified as a MG-specific marker in the brain parenchyma and $P2ry12^{-/-}$ MG reporter mice were generated, allowing the study of P2RY12-mediated MG chemotaxis to the site of BBB injuries (79, 112) (**Table 1**).

Gene expression profiling not only identified MG specific surface proteins but also MG signature genes such as spalt like transcription factor 1 (*Sall1*), which encodes a transcriptional regulator (113). Accordingly, the introduction of $Sall1^{GFP}$ and $Sall1^{CreERT2}$ knock-in mouse lines represent more distinct models for MG tracking and genetic modulation *in vivo* (114, 115). The ongoing efforts, mainly based on large-scale transcriptional analysis of MG cells, will keep providing novel targets for even more specific *in vivo* imaging and modulation. Very recently demonstrated by the discovery of TMEM119 which was shortly followed by the introduction of a knock-in $Tmem119^{EGFP}$ reporter mouse line and $Tmem119^{CreERT2}$ mice (80, 116) (**Table 1**).

With the increased interest in MΦ-focused immuno-oncology, assays that robustly and reproducibly determine the prophagocytic effect of a therapeutic agent of interest, are constantly evolving as well. While the first reports of the beneficial effect of CD47 disruption in leukemia cells, were mainly based on classical fluorescence microscopy, calculating the phagocytic index by dividing ingested cells by the total number of MΦ, they were soon replaced by FC-based approaches to better identify also smaller effect sizes in other tumor models (103, 117–121). In these experiments, phagocytes were identified by specific markers and co-incubated with cell-dye labeled tumor cells. MΦ that had successfully phagocytosed tumor cells were also positive for the tumor cell stain. However, this method lacks the optical confirmation that the tumor cell has been really engulfed by the phagocytic cell, which is why many studies still included a microscopic assessment or use more elaborately time-lapse live-cell microscopy which offers not only spatial but also temporal information (122). Technological advances enable the better identification of phagocytic events as well, as seen with the introduction of imaging FC, which combines the high throughput analysis of FC with the detailed morphometric information of fluorescence microscopy (123). Besides these *in vitro* phagocytosis assays, many efforts are undertaken to make the complex interplay between tumor cells and phagocytes visible. In many studies, after a specific treatment *in vivo*, the tumor mass is resected and dissociated and within the single-cell suspension, phagocytosis is measured as the ratio of the double-positive MΦ population by FC (118, 120, 122). This approach compared to *in vitro* models allows for a better understanding of the complex interface between innate and adaptive immune systems as they orchestrate the antitumor immune response together (124). More sophisticated and direct approaches employ specific reporter mice that enable *in vivo* imaging using 2-photon microscopy. As shown in their recent publication, Hutter et al. were able to demonstrate real-time phagocytosis of living glioma cells by MG and MΦ upon CD47 disruption using $Ccr2^{+/RFP}$ $Cx3cr1^{+/GFP}$ reporter mice, allowing the direct study of these cells in the TME (72). As new targets in innate immunotherapy are emerging, sophisticated methods will be needed to validate their prophagocytic capacity and clinical potential in cancer therapy, such as 3D cultures and tissue culture bioreactors for improved *ex vivo* tissue preservation (125). Another promising technology to study cell interactions, tissue composition, and spatial distribution of the iTME is high-dimensional multiplexing—CO-Detection by indEXing (CODEX)—that allows *in situ* tissue cytometry with the detection of over 50 parameters (126).

MG TARGETING AND MODULATION

As the largest immune cell population and one that positively correlates with glioma malignancy, invasiveness, and grade, MG represent the primordial target for modulation and antitumor immunotherapy. In this context, most strategies so far aimed at impairing GAM recruitment to the tumor site, thereby preventing their glioma-promoting effects. This included the previously mentioned blockade of CSF1R, disruption of periostin (POSTN), which is secreted by GSCs, and recruits GAMs

through integrin $\alpha_v\beta_3$ signaling, or inhibition of the CXC motif chemokine receptor 4 (CXCR4) chemotactic pathway (53, 74). The latter has been mainly implicated in MΦ mobilization through increased CXC motif chemokine ligand 12 (CXCL12) expression after radiation therapy (127). In combination with radiotherapy, a small molecule inhibitor of CXCL12/CXCR4 interactions prevented GAM infiltration and tumor recurrence (128). Another approach aimed at reversing the MG tumor-promoting effects and re-educating them to an antitumor phenotype. One report showed that activated NK cells combined with an antibody against chondroitin sulfate proteoglycan 4 (CSPG4) on GBM cells, were able to reverse the GAM phenotype (129). Osteopontin (OPN/encoded by *SPP1*) is another promising candidate protein secreted by GBM cells, which has prognostic implications and drives the protumorigenic reprogramming of MG, which can be therapeutically targeted (130, 131).

Recently, the focus has shifted toward the phagocytic role of MG as part of innate immune surveillance, most often targeted through the CD47/signal regulatory protein alpha (SIRPA) and the sialic acid/sialic acid binding immunoglobulin like lectin (SIGLEC) phagocytosis axes. CD47 is a widely expressed transmembrane protein with numerous functions, among which the inhibition of phagocytosis (132). Upon binding and activating its receptor SIRPA on the surface of mononuclear cells, CD47 inhibits the phagocytic activity of MΦ and MG (133). This antiphagocytic signal is transmitted via phosphorylation of the immunoreceptor tyrosine-based inhibitory motif (ITIM) on the cytoplasmic tail of SIRPA. Subsequent binding and activation of the protein-tyrosine phosphatase non-receptor type 6 (PTPN6) and 11 (PTPN11) blocks phagocytosis, putatively by preventing the accumulation of myosin-IIA at the phagocytic synapse (134). However, CD47 expression is best characterized for its role in hematopoietic cell homeostasis, particularly in red blood cells and platelets, where it is required to prevent their elimination by splenic MΦ. CD47 is thus considered a marker of self (133). In pathological processes, inflammation-mobilized hematopoietic stem cells protect themselves from phagocytosis by upregulating CD47 on their surface (117). This CD47 overexpression is co-opted by tumor cells and represents a common feature of hematologic and solid tumors, allowing them to evade innate immune surveillance (103, 117–119).

As a major "don't eat me" signal, CD47 is highly upregulated on the surface of nearly all human tumor cell types, including GBM cells. Transcriptional analysis of glioma patients revealed that high *CD47* mRNA expression levels were associated with decreased progression-free and overall survival, suggesting that *CD47* expression levels may serve as a clinically relevant prognostic factor (103). Willingham et al. were the first to describe the GAM re-educating effect of CD47 blockade in models of GBM. Using targeted monoclonal antibodies against CD47 enabled MΦ-dependent phagocytosis of patient-derived GBM neurospheres *in vitro*. Furthermore, the administration of anti-CD47 antibodies inhibited tumor growth and increased the survival of orthotopic immunodeficient mice transplanted with patient derived GBM cells, providing the first preclinical validation of CD47 as a therapeutic target in GBM (103).

TABLE 1 | Current MG mouse models.

Target gene	Modifications	References
Cx3cr1	$Cx3cr1^{+/GFP}$	(106)
	$Cx3cr1^{GFP/GFP}$	
	$Cx3cr1^{Cre}$	(109)
	$Cx3cr1^{CreERT2}$	
P2ry12	$P2ry12^{-/-}$ $Cx3cr1^{+/GFP}$	(79)
Sall1	$Sall1^{GFP}$	(114)
	$Sall1^{CreERT2}$	(115)
Tmem119	$Tmem119^{EGFP}$	(116)
	$Tmem119^{CreERT2}$	

Additional studies showed that anti-CD47 treatment repolarized GAMs *in vivo* to an M1 phenotype and that both M1- and M2-polarized MΦ alike displayed a higher GBM cell phagocytosis rate under anti-CD47 treatment (120). The therapeutic safety and efficacy of anti-CD47 treatment was also demonstrated in mouse models of murine high-grade glioma as well as five aggressive and etiologically distinct human pediatric brain tumors (medulloblastoma, atypical teratoid/rhabdoid tumor, primitive neuroectodermal tumor, pediatric GBM, and diffuse intrinsic pontine glioma) (121).

More recently, Hutter et al. dissected the response of MG and infiltrating peripheral MΦ upon anti-CD47 treatment in GBM. Using a mouse model with genetically color-coded MΦ ($Ccr2^{RFP}$) and MG ($Cx3cr1^{GFP}$), they showed that even in mice lacking Ccr2-mediated MΦ recruitment to the brain ($Ccr2^{RFP/RFP}$ $Cx3cr1^{GFP/+}$), MG-mediated GBM phagocytosis was sufficient to reduce tumor burden and prolong survival under anti-CD47 treatment. This observation led to the identification of MG as effector cells of GBM cell phagocytosis in response to CD47 blockade (72).

Comparable to CD47 overexpression, the aberrant glycosylation of cancer cells represents a common feature of malignant transformation (135, 136). These glycoproteins and glycolipids are often terminated by negatively charged sialic acids. Sialic acids are derivatives of neuraminic acid, and the predominant sialic acid found in mammalian cells bears at its amino site an acetyl group, therefore termed *N*-acetyl-neuraminic acid. The addition of sialic acids is mediated by sialyltransferases, a family of glycosyltransferases (137). Hypersialylation, meaning the upregulation of sialic acid-containing glycans (sialoglycan) on the cell surface through altered sialyltransferase expression and the increased introduction of non-human sialic acids like *N*-glycolyl-neuraminic acid (xenosialylation) are, together with the altered glycosylation itself, key changes of malignant tissue and important for cancer progression (138, 139).

Sialic acids can modulate the iTME through SIGLEC engagement. To date, 14 human and nine mouse SIGLECs have been identified, differing in their sialic acid ligand specificity and intracellular signaling cascades. SIGLECs are expressed on most cells of the immune system and can transmit immunosuppressive

signals upon binding to sialic acids. Similar to the inhibitory SIRPA receptor—inhibitory SIGLEC receptors contain ITIMs in their intracellular domain that signal negatively via the recruitment of PTPN6 and 11 (140). The physiological role of SIGLECs to recognize sialic acids as self-associated patterns and therefore counter-regulate overshooting immune reactions and limit tissue damage during inflammation can be exploited by cancer cells (141). Hypersialylation of tumor cells can thus contribute to tumor immune evasion (104).

Initially, immunoinhibitory SIGLECs in brain pathologies were primarily associated with CD33 or SIGLEC3 as a genetic risk factor for AD (142–144). Subsequent functional studies showed that CD33 inhibits MG uptake of amyloid-β plaques in diseased brains (145). More recently, CD22 or SIGLEC2 was also identified as a negative regulator of phagocytosis that is upregulated on aged MG. Inhibition of CD22 promoted the clearance of myelin debris, amyloid-β oligomers, and α-synuclein fibrils in an AD model (146). Other studies identified important roles of SIGLECs in neuro-inflammatory diseases, where immunoinhibitory SIGLECs convey neuroprotective functions by alleviating especially MG neurotoxicity (147, 148).

With the paradigm shift in cancer therapy that came with the discovery of immune checkpoint inhibitors, the sialoglycan-SIGLEC pathway attracted recently a great deal of attention as a novel target for cancer immunotherapy. This holds especially true in brain malignancies, since phase II and III clinical trials of classical immunotherapeutic agents like PDCD1 and CD274 inhibitors showed no significant improvement in the median overall survival of GBM patients (149). Correlative single-cell transcriptomic analysis, including The Cancer Genome Atlas (TCGA) data, showed that most members of the SIGLEC family are differentially expressed in glioma. Interestingly, several SIGLEC receptors are predominantly expressed on MΦ and GAMs with higher expression levels observed in high-grade gliomas (150).

In a more translational approach, others investigated the role of immunomodulatory SIGLECs in the treatment with glucocorticosteroids, including dexamethasone, which is frequently used to control tumor-induced edema in brain tumor patients. They found alterations in tumor cell surface sialylation and SIGLEC recognition in response to dexamethasone treatment (151). Specifically, MG showed an upregulation of SIGLEC receptors together with induction of an anti-inflammatory cytokine profile, indicating a crucial role of SIGLECs in dampening the dexamethasone-induced antitumor immunity (152). The first experimental evidence that linked SIGLECs with whole tumor cell phagocytosis in glioma dates back to 2013, when Siglec H, a MG-specific marker, was suggested to be a phagocytic receptor for glioma cells (153–155). Novel insights into the sialic acid-SIGLEC antiphagocytic axis have recently emerged. In particular, SIGLEC10 was identified as the receptor of CD24, an additional "don't eat me" signal. Tumor-expressed CD24 promoted innate immune evasion through its interaction with GAM-expressed SIGLEC10 (122). Another study focused on SIGLEC15 as an immune suppressor and potential target for cancer immunotherapy. Using a genetic mouse model and intracranial injection of murine glioma cells, the authors found significantly slower tumor growth associated with more MΦ and $CD8^+$ T cells in the TME upon genetic ablation of SIGLEC15. Together with *ex vivo* restimulation assays, their data support a role for SIGLEC15 in MΦ-mediated suppression of tumor immunity (156). The mounting evidence of SIGLEC engagement by cancer cells to evade the antitumor immune response, especially innate immune response, make sialic acid-SIGLEC interactions very attractive candidates for potentiating antitumor immunity in GBM.

DISCUSSION: EMERGING LOCAL AND COMBINATORIAL APPROACHES FOR THE TREATMENT OF GBM PLACE MG AT THE CENTER STAGE

Despite advances in surgical techniques, radiation therapy, and chemotherapy, effective treatment of GBM remains an unresolved challenge. Today's unspecific approach of alkylating chemotherapy and radiation therapy causes major toxicities and debilitating side effects. Better ways to control this devastating disease are urgently needed.

We previously showed that modulation of MG within GBM (e.g., by CD47-SIRPA disruption), can control GBM progression by rendering MG tumor-phagocytic. Although disrupting CD47-SIRPA modulates MΦ and MG anti-GBM activity and reprograms the immunosuppressive iTME, GBM represents a heterogeneous tumor entity with a multitude of deregulated cancer pathways. Therefore, a subset of tumor cells will evade the MG-mediated antitumor response and develop resistance. We are thus convinced that reprogramming of MG within the tumor will not suffice by itself to halt GBM entirely, especially in view of emerging insights into MG heterogeneity. On the other hand, pure tumor-targeting approaches, vaccinations with tumor antigens, monoantigenic CAR T cells, or intratumoral cytokine deliveries are all prone to failure because of the overwhelming immunosuppressive contribution of the iTME, and specifically tumor-educated MG. Therefore, more sophisticated combinatorial approaches that target MG, adaptive immunity, and tumor cells at once are mandated.

We believe that MG are at the centerstage for modulation in the iTME, since this will also influence the antitumoral capacity of other components of the iTME such as TILs [e.g., via enhanced antigen presentation (157)]. The capacity to of MG to present antigens (e.g., after tumor cell phagocytosis) needs to be evaluated further and with novel techniques in various experimental contexts since this might offer key insights into potential combinatorial strategies with vaccination studies or T cell checkpoint inhibitor treatments. How MG modulation and reprogramming is best achieved, and which—often redundant—immune evasion mechanisms should be targeted to achieve a durable induction of antitumoral activity is largely unknown. On top, the additional MΦ modulation and recruitment effects caused by the treatments should be considered, since additional recruitment of BM-derived MΦ might cause increased unwanted side effects such as enhanced edema. Prophagocytic pathways

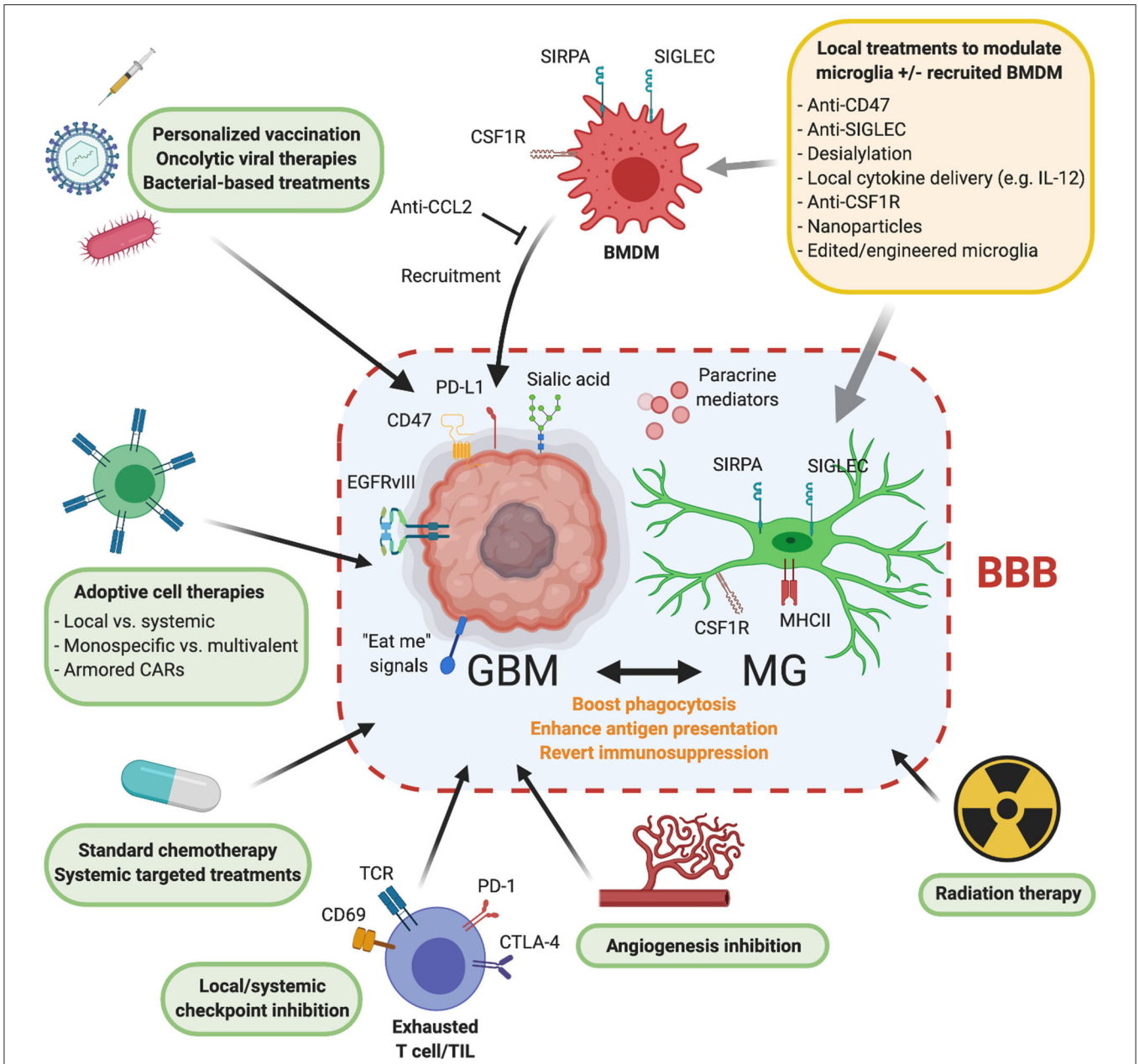

FIGURE 2 | Combinatorial approaches of local MG modulation and other treatment modalities against GBM. Means of local MG modulation (upper right): MG can be targeted locally beyond the BBB by various approaches to influence MG phagocytosis, enhance antigen presentation, and revert generalized immunosuppression. Prominent potential means to locally modulate MG include anti-CD47 or anti-Siglec treatments, intratumoral desialylation, application of pro-inflammatory cytokines, "reprogramming" by blocking CSF1 signaling, or addition of immunomodulatory nanoparticles. Some of these modalities would also interfere with infiltrating BMDMs (e.g., anti-CCL2 blockade). Overall, this would enable other tumor specific or immunotherapeutic regimens to exert their antitumorigenic activity. Combinatorial approaches with MG modulation: *Personalized vaccination, oncolytic viral therapy, or bacterial based approaches*: the effect of tumor-specific vaccinations, oncolytic viruses, or tumor-targeting bacteria might be significantly enhanced when tumor-associated MG is reverted to a less immunosuppressive phenotype. *MG modulation + tumor-targeting CAR T cell therapy:* combining MG modulation with tumor antigen-specific CAR T cell therapy poses another way to circumvent current obstacles in GBM therapy. Local application of MG modulation and tumor-specific CAR T cells might result in better GBM control. Novel CAR T products could combine MG modulation (e.g., by reprogramming MG and targeting the tumor at once). *MG modulation + chemotherapy or targeted treatments:* tumor cells respond to established chemotherapy with increased expression of "eat-me" signals. Combinatorial strategies of already established chemotherapies with inducers of MG phagocytosis could improve treatment responses. *MG modulation + T cell checkpoint therapy:* this dual strategy of targeting the major players of the GBM-iTME—MG—and facilitating an intratumoral T cell response in addition to a putative MG-mediated T cell response might boost tumor regression. Further, the thorough analysis of tumor-phagocytosing MG vs. non-phagocytosing MG, their MHC molecules and linked, presented antigens, could lead to the discovery of novel tumor antigens and result in potential vaccination candidates. *MG modulation + angiogenesis inhibition:* anti-VEGFR treatment serves as a salvage therapy in recurrent GBM. Additional MG activation might prevent development of early resistance. *MG modulation + radiation therapy:* in line with chemotherapy, radiation therapy enhances immune responses and upregulates "eat-me" signals on tumor cells. Additional MG modulation could increase efficacy and long term treatment responses. Created with Biorender.com.

beyond CD47-SIRPA with higher MG specificity might be particularly attractive to tailor the MG response. However, an overshooting MG induction might as well-lead to deleterious effects in the brain (e.g., via hyperphagocytosis), and treatment effects, timing, and delivery need to be carefully validated in future clinical trials. Since most systemic treatments in brain tumors do not effectively reach the tumor because of the BBB, local/continuous application of these treatment regimens might be most effective, and application of these treatments in the early phase of the disease would be preferable over the post-treatment recurrent situation, where the iTME and tumor resistance mechanisms are even more deranged. Besides that, it remains to be studied, whether these treatments should be applied before or after tumor resection, and whether targeting of the peripheral invasion zone of the tumor, where presumably a lot of iTME reprogramming happens, might be advantageous. A multitude of strategies for MG modulation may unleash the inherent antitumoral armamentarium of MG and have translational potential; future translational research and clinical trials should pave the way on how to optimally design these approaches against GBM. In **Figure 2**, we summarize promising combinatorial treatment strategies to overcome these challenges.

AUTHOR CONTRIBUTIONS

GH and TS: conception or design of the work. TAM, PS, and TS: literature collection. TAM, PS, TS, J-LB, M-FR, JvB, SZ, and GH: drafting the article. GH: critical revision of the article. All authors final approval of the version to be published.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.571951/full#supplementary-material

REFERENCES

1. Koshy M, Villano JL, Dolecek TA, Howard A, Mahmood U, Chmura SJ, et al. Improved survival time trends for glioblastoma using the SEER 17 population-based registries. *J Neurooncol.* (2012) 107:207–12. doi: 10.1007/s11060-011-0738-7
2. Urbańska K, Sokołowska J, Szmidt M, Sysa P. Glioblastoma multiforme - an overview. *Contemp Oncol.* (2014) 18:307–12. doi: 10.5114/wo.2014.40559
3. Scherer HJ. A critical review: the pathology of cerebral gliomas. *J Neurol Psychiatry.* (1940) 3:147–77. doi: 10.1136/jnnp.3.2.147
4. Ozawa T, Riester M, Cheng YK, Huse JT, Squatrito M, Helmy K, et al. Most human non-GCIMP glioblastoma subtypes evolve from a common proneural-like precursor glioma. *Cancer Cell.* (2014) 26:288–300. doi: 10.1016/j.ccr.2014.06.005
5. Phillips HS, Kharbanda S, Chen R, Forrest WF, Soriano RH, Wu TD, et al. Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. *Cancer Cell.* (2006) 9:157–73. doi: 10.1016/j.ccr.2006.02.019
6. Verhaak RG, Hoadley KA, Purdom E, Wang V, Qi Y, Wilkerson MD, et al. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell.* (2010) 17:98–110. doi: 10.1016/j.ccr.2009.12.020
7. Brennan C, Momota H, Hambardzumyan D, Ozawa T, Tandon A, Pedraza A, et al. Glioblastoma subclasses can be defined by activity among signal transduction pathways and associated genomic alterations. *PLoS ONE.* (2009) 4:e7752. doi: 10.1371/journal.pone.0007752
8. Capper D, Jones DTW, Sill M, Hovestadt V, Schrimpf D, Sturm D, et al. DNA methylation-based classification of central nervous system tumours. *Nature.* (2018) 555:469–74. doi: 10.1038/nature26000
9. Cancer Genome Atlas Research Network. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature.* (2008) 455:1061–8. doi: 10.1038/nature07385
10. Wang Q, Hu B, Hu X, Kim H, Squatrito M, Scarpace L, et al. Tumor evolution of glioma-intrinsic gene expression subtypes associates with immunological changes in the microenvironment. *Cancer Cell.* (2017) 32:42–56.e6. doi: 10.1016/j.ccell.2017.06.003
11. Yan H, Parsons DW, Jin G, McLendon R, Rasheed BA, Yuan W, et al. IDH1 and IDH2 mutations in gliomas. *N Engl J Med.* (2009) 360:765–73. doi: 10.1056/NEJMoa0808710
12. Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-Branger D, Cavenee WK, et al. The 2016 World Health Organization classification of tumors of the central nervous system: a summary. *Acta Neuropathol.* (2016) 131:803–20. doi: 10.1007/s00401-016-1545-1
13. Amankulor NM, Kim Y, Arora S, Kargl J, Szulzewsky F, Hanke M, et al. Mutant IDH1 regulates the tumor-associated immune system in gliomas. *Genes Dev.* (2017) 31:774–86. doi: 10.1101/gad.294991.116
14. Annovazzi L, Mellai M, Bovio E, Mazzetti S, Pollo B, Schiffer D. Microglia immunophenotyping in gliomas. *Oncol Lett.* (2018) 15:998–1006. doi: 10.3892/ol.2017.7386
15. Martinez-Lage M, Lynch TM, Bi Y, Cocito C, Way GP, Pal S, et al. Immune landscapes associated with different glioblastoma molecular subtypes. *Acta Neuropathol Commun.* (2019) 7:203. doi: 10.1186/s40478-019-0803-6
16. Friebel E, Kapolou K, Unger S, Nunez NG, Utz S, Rushing EJ, et al. Single-cell mapping of human brain cancer reveals tumor-specific instruction of tissue-invading leukocytes. *Cell.* (2020) 181:1626–42.e20. doi: 10.1016/j.cell.2020.04.055
17. Klemm F, Maas RR, Bowman RL, Kornete M, Soukup K, Nassiri S, et al. Interrogation of the microenvironmental landscape in brain tumors reveals disease-specific alterations of immune cells. *Cell.* (2020) 181:1643–60.e17. doi: 10.1016/j.cell.2020.05.007

18. Ohgaki H, Dessen P, Jourde B, Horstmann S, Nishikawa T, Di Patre PL, et al. Genetic pathways to glioblastoma: a population-based study. *Cancer Res.* (2004) 64:6892–9. doi: 10.1158/0008-5472.CAN-04-1337
19. Bigner SH, Matthews MR, Rasheed BK, Wiltshire RN, Friedman HS, Friedman AH, et al. Molecular genetic aspects of oligodendrogliomas including analysis by comparative genomic hybridization. *Am J Pathol.* (1999) 155:375–86. doi: 10.1016/S0002-9440(10)65134-6
20. Bredel M, Scholtens DM, Yadav AK, Alvarez AA, Renfrow JJ, Chandler JP, et al. NFKBIA deletion in glioblastomas. *N Engl J Med.* (2011) 364:627–37. doi: 10.1056/NEJMoa1006312
21. Bhat KPL, Balasubramaniyan V, Vaillant B, Ezhilarasan R, Hummelink K, Hollingsworth F, et al. Mesenchymal differentiation mediated by NF-κB promotes radiation resistance in glioblastoma. *Cancer Cell.* (2013) 24:331–46. doi: 10.1016/j.ccr.2013.08.001
22. Parsons DW, Jones S, Zhang X, Lin JC, Leary RJ, Angenendt P, et al. An integrated genomic analysis of human glioblastoma multiforme. *Science.* (2008) 321:1807–12. doi: 10.1126/science.1164382
23. Hegi ME, Diserens AC, Gorlia T, Hamou MF, de Tribolet N, Weller M, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. *N Engl J Med.* (2005) 352:997–1003. doi: 10.1056/NEJMoa043331
24. Patel AP, Tirosh I, Trombetta JJ, Shalek AK, Gillespie SM, Wakimoto H, et al. Single-cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma. *Science.* (2014) 344:1396–401. doi: 10.1126/science.1254257
25. Thorsson V, Gibbs DL, Brown SD, Wolf D, Bortone DS, Ou Yang TH, et al. The immune landscape of cancer. *Immunity.* (2018) 48:812–30.e14. doi: 10.1016/j.immuni.2018.03.023
26. Kohanbash G, Carrera DA, Shrivastav S, Ahn BJ, Jahan N, Mazor T, et al. Isocitrate dehydrogenase mutations suppress STAT1 and CD8+ T cell accumulation in gliomas. *J Clin Invest.* (2017) 127:1425–37. doi: 10.1172/JCI90644
27. Bunse L, Pusch S, Bunse T, Sahm F, Sanghvi K, Friedrich M, et al. Suppression of antitumor T cell immunity by the oncometabolite (R)-2-hydroxyglutarate. *Nat Med.* (2018) 24:1192–203. doi: 10.1038/s41591-018-0095-6
28. Turcan S, Rohle D, Goenka A, Walsh LA, Fang F, Yilmaz E, et al. IDH1 mutation is sufficient to establish the glioma hypermethylator phenotype. *Nature.* (2012) 483:479–83. doi: 10.1038/nature10866
29. Mathur R, Zhang Y, Grimmer MR, Hong C, Zhang M, Bollam S, et al. MGMT promoter methylation level in newly diagnosed low-grade glioma is a predictor of hypermutation at recurrence. *Neuro Oncol.* (2020) 1–11. doi: 10.1093/neuonc/noaa059
30. Johnson BE, Mazor T, Hong C, Barnes M, Aihara K, McLean CY, et al. Mutational analysis reveals the origin and therapy-driven evolution of recurrent glioma. *Science.* (2014) 343:189–93. doi: 10.1126/science.1239947
31. Choi S, Yu Y, Grimmer MR, Wahl M, Chang SM, Costello JF. Temozolomide-associated hypermutation in gliomas. *Neuro Oncol.* (2018) 20:1300–9. doi: 10.1093/neuonc/noy016
32. Barker CF, Billingham RE. Immunologically privileged sites. *Adv Immunol.* (1977) 25:1–54. doi: 10.1016/S0065-2776(08)60930-X
33. Medawar PB. Immunity to homologous grafted skin; the fate of skin homografts transplanted to the brain, to subcutaneous tissue, and to the anterior chamber of the eye. *Br J Exp Pathol.* (1948) 29:58–69.
34. Graham M, Herndon JN, Casey J, Chaffee S, Ciocci G, Krischer J, et al. High-dose chemotherapy with autologous stem-cell rescue in patients with recurrent and high-risk pediatric brain tumors. *J Clin Oncol.* (1997) 15:1814–23. doi: 10.1200/JCO.1997.15.5.1814
35. Bechmann I. Failed central nervous system regeneration: a downside of immune privilege? *Neuromolecular Med.* (2005) 7:217–28. doi: 10.1385/NMM:7:3:217
36. Grigoriadis N, Grigoriadis S, Polyzoidou E, Milonas I, Karussis D. Neuroinflammation in multiple sclerosis: evidence for autoimmune dysregulation, not simple autoimmune reaction. *Clin Neurol Neurosurg.* (2006) 108:241–4. doi: 10.1016/j.clineuro.2005.11.006
37. Pardo LM, van Duijn CM. In search of genes involved in neurodegenerative disorders. *Mutat Res.* (2005) 592:89–101. doi: 10.1016/j.mrfmmm.2005.06.006
38. Gelders G, Baekelandt V, Van der Perren A. Linking neuroinflammation and neurodegeneration in Parkinson's disease. *J Immunol Res.* (2018) 2018:4784268. doi: 10.1155/2018/4784268
39. Louveau A, Smirnov I, Keyes TJ, Eccles JD, Rouhani SJ, Peske JD, et al. Structural and functional features of central nervous system lymphatic vessels. *Nature.* (2015) 523:337–41. doi: 10.1038/nature 14432
40. Cserr HF, Harling-Berg CJ, Knopf PM. Drainage of brain extracellular fluid into blood and deep cervical lymph and its immunological significance. *Brain Pathol.* (1992) 2:269–76. doi: 10.1111/j.1750-3639.1992.tb0 0703.x
41. Roopenian DC, Akilesh S. FcRn: the neonatal Fc receptor comes of age. *Nat Rev Immunol.* (2007) 7:715–25. doi: 10.1038/nri2155
42. Jackson C, Ruzevick J, Phallen J, Belcaid Z, Lim M. Challenges in immunotherapy presented by the glioblastoma multiforme microenvironment. *Clin Dev Immunol.* (2011) 2011:732413. doi: 10.1155/2011/732413
43. Frei K, Gramatzki D, Tritschler I, Schroeder JJ, Espinoza L, Rushing EJ, et al. Transforming growth factor-beta pathway activity in glioblastoma. *Oncotarget.* (2015) 6:5963–77. doi: 10.18632/oncotarget.3467
44. Nduom E, Weller M, Heimberger A. Immunosuppressive mechanisms in glioblastoma. *Neuro-Oncology.* (2015) 17:9–14. doi: 10.1093/neuonc/nov151
45. Elliott LH, Brooks WH, Roszman TL. Activation of immunoregulatory lymphocytes obtained from patients with malignant gliomas. *J Neurosurg.* (1987) 67:231–6. doi: 10.3171/jns.1987.67.2.0231
46. Wainwright DA, Sengupta S, Han Y, Lesniak MS. Thymus-derived rather than tumor-induced regulatory T cells predominate in brain tumors. *Neuro Oncol.* (2011) 13:1308–23. doi: 10.1093/neuonc/nor134
47. Han S, Ma E, Wang X, Yu C, Dong T, Zhan W, et al. Rescuing defective tumor-infiltrating T-cell proliferation in glioblastoma patients. *Oncol Lett.* (2016) 12:2924–9. doi: 10.3892/ol.2016.4944
48. Han S, Zhang C, Li Q, Dong J, Liu Y, Huang Y, et al. Tumour-infiltrating CD4(+) and CD8(+) lymphocytes as predictors of clinical outcome in glioma. *Br J Cancer.* (2014) 110:2560–8. doi: 10.1038/bjc.2014.162
49. Zhai L, Ladomersky E, Lauing KL, Wu M, Genet M, Gritsina G, et al. Infiltrating T cells increase IDO1 expression in glioblastoma and contribute to decreased patient survival. *Clin Cancer Res.* (2017) 23:6650–60. doi: 10.1158/1078-0432.CCR-17-0120
50. Chongsathidkiet P, Jackson C, Koyama S, Loebel F, Cui X, Farber SH, et al. Sequestration of T cells in bone marrow in the setting of glioblastoma and other intracranial tumors. *Nat Med.* (2018) 24:1459–68. doi: 10.1038/s41591-018-0135-2
51. Charles NA, Holland EC, Gilbertson R, Glass R, Kettenmann H. The brain tumor microenvironment. *Glia.* (2011) 59:1169–80. doi: 10.1002/glia.21136
52. Morantz RA, Wood GW, Foster M, Clark M, Gollahon K. Macrophages in experimental and human brain tumors. Part 2: studies of the macrophage content of human brain tumors. *J Neurosurg.* (1979) 50:305–11. doi: 10.3171/jns.1979.50.3.0305
53. Bowman RL, Klemm F, Akkari L, Pyonteck SM, Sevenich L, Quail DF, et al. Macrophage ontogeny underlies differences in tumor-specific education in brain malignancies. *Cell Rep.* (2016) 17:2445–59. doi: 10.1016/j.celrep.2016.10.052
54. Kennedy BC, Showers CR, Anderson DE, Anderson L, Canoll P, Bruce JN, et al. Tumor-associated macrophages in glioma: friend or foe? *J Oncol.* (2013) 2013:486912. doi: 10.1155/2013/486912
55. Komohara Y, Ohnishi K, Kuratsu J, Takeya M. Possible involvement of the M2 anti-inflammatory macrophage phenotype in growth of human gliomas. *J Pathol.* (2008) 216:15–24. doi: 10.1002/path.2370
56. Engler JR, Robinson AE, Smirnov I, Hodgson JG, Berger MS, Gupta N, et al. Increased microglia/macrophage gene expression in a subset of adult and pediatric astrocytomas. *PLoS ONE.* (2012) 7:e43339. doi: 10.1371/journal.pone.0043339
57. Hambardzumyan D, Gutmann DH, Kettenmann H. The role of microglia and macrophages in glioma maintenance and progression. *Nat Neurosci.* (2016) 19:20–7. doi: 10.1038/nn.4185
58. Hanisch UK, Kettenmann H. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nat Neurosci.* (2007) 10:1387–94. doi: 10.1038/nn1997
59. Davalos D, Grutzendler J, Yang G, Kim JV, Zuo Y, Jung S, et al. ATP mediates rapid microglial response to local brain injury *in vivo*. *Nat Neurosci.* (2005) 8:752–8. doi: 10.1038/nn1472

60. Hickey WF, Kimura H. Perivascular microglial cells of the CNS are bone marrow-derived and present antigen *in vivo*. *Science.* (1988) 239:290–2. doi: 10.1126/science.3276004
61. Massengale M, Wagers AJ, Vogel H, Weissman IL. Hematopoietic cells maintain hematopoietic fates upon entering the brain. *J Exp Med.* (2005) 201:1579–89. doi: 10.1084/jem.20050030
62. Simard AR, Rivest S. Bone marrow stem cells have the ability to populate the entire central nervous system into fully differentiated parenchymal microglia. *FASEB J.* (2004) 18:998–1000. doi: 10.1096/fj.04-1517fje
63. Ajami B, Bennett JL, Krieger C, Tetzlaff W, Rossi FM. Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. *Nat Neurosci.* (2007) 10:1538–43. doi: 10.1038/nn2014
64. Ajami B, Bennett JL, Krieger C, McNagny KM, Rossi FM. Infiltrating monocytes trigger EAE progression, but do not contribute to the resident microglia pool. *Nat Neurosci.* (2011) 14:1142–9. doi: 10.1038/nn.2887
65. Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S, et al. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science.* (2010) 330:841–5. doi: 10.1126/science.1194637
66. Rodero M, Marie Y, Coudert M, Blondet E, Mokhtari K, Rousseau A, et al. Polymorphism in the microglial cell-mobilizing CX3CR1 gene is associated with survival in patients with glioblastoma. *J Clin Oncol.* (2008) 26:5957–64. doi: 10.1200/JCO.2008.17.2833
67. Feng X, Szulzewsky F, Yerevanian A, Chen Z, Heinzmann D, Rasmussen RD, et al. Loss of CX3CR1 increases accumulation of inflammatory monocytes and promotes gliomagenesis. *Oncotarget.* (2015) 6:15077–94. doi: 10.18632/oncotarget.3730
68. Liu C, Luo D, Streit WJ, Harrison JK. CX3CL1 and CX3CR1 in the GL261 murine model of glioma: CX3CR1 deficiency does not impact tumor growth or infiltration of microglia and lymphocytes. *J Neuroimmunol.* (2008) 198:98–105. doi: 10.1016/j.jneuroim.2008.04.016
69. Goldmann T, Wieghofer P, Jordao MJ, Prutek F, Hagemeyer N, Frenzel K, et al. Origin, fate and dynamics of macrophages at central nervous system interfaces. *Nat Immunol.* (2016) 17:797–805. doi: 10.1038/ni.3423
70. Platten M, Kretz A, Naumann U, Aulwurm S, Egashira K, Isenmann S, et al. Monocyte chemoattractant protein-1 increases microglial infiltration and aggressiveness of gliomas. *Ann Neurol.* (2003) 54:388–92. doi: 10.1002/ana.10679
71. Mizutani M, Pino PA, Saederup N, Charo IF, Ransohoff RM, Cardona AE. The fractalkine receptor but not CCR2 is present on microglia from embryonic development throughout adulthood. *J Immunol.* (2012) 188:29–36. doi: 10.4049/jimmunol.1100421
72. Hutter G, Theruvath J, Graef CM, Zhang M, Schoen MK, Manz EM, et al. Microglia are effector cells of CD47-SIRPalpha antiphagocytic axis disruption against glioblastoma. *Proc Natl Acad Sci USA.* (2019) 116:997–1006. doi: 10.1073/pnas.1721434116
73. Coniglio SJ, Eugenin E, Dobrenis K, Stanley ER, West BL, Symons MH, et al. Microglial stimulation of glioblastoma invasion involves epidermal growth factor receptor (EGFR) and colony stimulating factor 1 receptor (CSF-1R) signaling. *Mol Med.* (2012) 18:519–27. doi: 10.2119/molmed.2011.00217
74. Pyonteck SM, Akkari L, Schuhmacher AJ, Bowman RL, Sevenich L, Quail DF, et al. CSF-1R inhibition alters macrophage polarization and blocks glioma progression. *Nat Med.* (2013) 19:1264–72. doi: 10.1038/nm.3337
75. Sielska M, Przanowski P, Wylot B, Gabrusiewicz K, Maleszewska M, Kijewska M, et al. Distinct roles of CSF family cytokines in macrophage infiltration and activation in glioma progression and injury response. *J Pathol.* (2013) 230:310–21. doi: 10.1002/path.4192
76. Ford AL, Goodsall AL, Hickey WF, Sedgwick JD. Normal adult ramified microglia separated from other central nervous system macrophages by flow cytometric sorting. Phenotypic differences defined and direct *ex vivo* antigen presentation to myelin basic protein-reactive CD4+ T cells compared. *J Immunol.* (1995) 154:4309–21.
77. Müller A, Brandenburg S, Turkowski K, Müller S, Vajkoczy P. Resident microglia, and not peripheral macrophages, are the main source of brain tumor mononuclear cells. *Int J Cancer.* (2015) 137:278–88. doi: 10.1002/ijc.29379
78. Srinivasan S, Mir F, Huang JS, Khasawneh FT, Lam SC, Le Breton GC. The P2Y12 antagonists, 2-methylthioadenosine 5'-monophosphate triethylammonium salt and cangrelor (ARC69931MX), can inhibit human platelet aggregation through a Gi-independent increase in cAMP levels. *J Biol Chem.* (2009) 284:16108–17. doi: 10.1074/jbc.M809780200
79. Haynes SE, Hollopeter G, Yang G, Kurpius D, Dailey ME, Gan WB, et al. The P2Y12 receptor regulates microglial activation by extracellular nucleotides. *Nat Neurosci.* (2006) 9:1512–9. doi: 10.1038/nn1805
80. Bennett ML, Bennett FC, Liddelow SA, Ajami B, Zamanian JL, Fernhoff NB, et al. New tools for studying microglia in the mouse and human CNS. *Proc Natl Acad Sci USA.* (2016) 113:E1738–46. doi: 10.1073/pnas.1525528113
81. Butovsky O, Jedrychowski MP, Moore CS, Cialic R, Lanser AJ, Gabriely G, et al. Identification of a unique TGF-beta-dependent molecular and functional signature in microglia. *Nat Neurosci.* (2014) 17:131–43. doi: 10.1038/nn.3599
82. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol.* (2004) 25:677–86. doi: 10.1016/j.it.2004.09.015
83. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol.* (2002) 23:549–55. doi: 10.1016/S1471-4906(02)02302-5
84. Gabrusiewicz K, Ellert-Miklaszewska A, Lipko M, Sielska M, Frankowska M, Kaminska B. Characteristics of the alternative phenotype of microglia/macrophages and its modulation in experimental gliomas. *PLoS ONE.* (2011) 6:e23902. doi: 10.1371/journal.pone.0023902
85. Kees T, Lohr J, Noack J, Mora R, Gdynia G, Todt G, et al. Microglia isolated from patients with glioma gain antitumor activities on poly (I:C) stimulation. *Neuro Oncol.* (2012) 14:64–78. doi: 10.1093/neuonc/nor182
86. Li W, Graeber MB. The molecular profile of microglia under the influence of glioma. *Neuro Oncol.* (2012) 14:958–78. doi: 10.1093/neuonc/nos116
87. Sica A, Schioppa T, Mantovani A, Allavena P. Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy. *Eur J Cancer.* (2006) 42:717–27. doi: 10.1016/j.ejca.2006.01.003
88. Zeiner PS, Preusse C, Blank AE, Zachskorn C, Baumgarten P, Caspary L, et al. MIF receptor CD74 is restricted to microglia/macrophages, associated with a m1-polarized immune milieu and prolonged patient survival in gliomas. *Brain Pathol.* (2015) 25:491–504. doi: 10.1111/bpa.12194
89. Ransohoff RM. A polarizing question: do M1 and M2 microglia exist? *Nat Neurosci.* (2016) 19:987–91. doi: 10.1038/nn.4338
90. Masuda T, Sankowski R, Staszewski O, Bottcher C, Amann L, Sagar, et al. Spatial and temporal heterogeneity of mouse and human microglia at single-cell resolution. *Nature.* (2019) 566:388–92. doi: 10.1038/s41586-019-0924-x
91. Schetters STT, Gomez-Nicola D, Garcia-Vallejo JJ, Van Kooyk Y. neuroinflammation: microglia and t cells get ready to tango. *Front Immunol.* (2017) 8:1905. doi: 10.3389/fimmu.2017.01905
92. Szulzewsky F, Pelz A, Feng X, Synowitz M, Markovic D, Langmann T, et al. Glioma-associated microglia/macrophages display an expression profile different from M1 and M2 polarization and highly express Gpnmb and Spp1. *PLoS ONE.* (2015) 10:e0116644. doi: 10.1371/journal.pone.0116644
93. See AP, Parker JJ, Waziri A. The role of regulatory T cells and microglia in glioblastoma-associated immunosuppression. *J Neurooncol.* (2015) 123:405–12. doi: 10.1007/s11060-015-1849-3
94. Schwartz M, Ziv Y. Immunity to self and self-maintenance: what can tumor immunology teach us about ALS and Alzheimer's disease? *Trends Pharmacol Sci.* (2008) 29:287–93. doi: 10.1016/j.tips.2008.03.006
95. Unger MS, Schernthaner P, Marschallinger J, Mrowetz H, Aigner L. Microglia prevent peripheral immune cell invasion and promote an anti-inflammatory environment in the brain of APP-PS1 transgenic mice. *J Neuroinflammation.* (2018) 15:274. doi: 10.1186/s12974-018-1304-4
96. Malo CS. MHC class I expression by microglia is required for generating a complete antigen-specific CD8 T cell response in the CNS. *J Immunol.* (2018) 200(Suppl.1):99.
97. Sankowski R, Bottcher C, Masuda T, Geirsdottir L, Sagar, Sindram E, et al. Mapping microglia states in the human brain through the integration of high-dimensional techniques. *Nat Neurosci.* (2019) 22:2098–110. doi: 10.1038/s41593-019-0532-y
98. Bettinger I, Thanos S, Paulus W. Microglia promote glioma migration. *Acta Neuropathol.* (2002) 103:351–5. doi: 10.1007/s00401-001-0472-x

99. Wick W, Platten M, Weller M. Glioma cell invasion: regulation of metalloproteinase activity by TGF-beta. *J Neurooncol.* (2001) 53:177–85. doi: 10.1023/A:1012209518843
100. Markovic DS, Vinnakota K, Chirasani S, Synowitz M, Raguet H, Stock K, et al. Gliomas induce and exploit microglial MT1-MMP expression for tumor expansion. *Proc Natl Acad Sci USA.* (2009) 106:12530–5. doi: 10.1073/pnas.0804273106
101. Walentynowicz KA, Ochocka N, Pasierbinska M, Wojnicki K, Stepniak K, Mieczkowski J, et al. In search for reliable markers of glioma-induced polarization of microglia. *Front Immunol.* (2018) 9:1329. doi: 10.3389/fimmu.2018.01329
102. Wu A, Wei J, Kong LY, Wang Y, Priebe W, Qiao W, et al. Glioma cancer stem cells induce immunosuppressive macrophages/microglia. *Neuro Oncol.* (2010) 12:1113–25. doi: 10.1093/neuonc/noq082
103. Willingham SB, Volkmer JP, Gentles AJ, Sahoo D, Dalerba P, Mitra SS, et al. The CD47-signal regulatory protein alpha (SIRPa) interaction is a therapeutic target for human solid tumors. *Proc Natl Acad Sci USA.* (2012) 109:6662–7. doi: 10.1073/pnas.1121623109
104. Bull C, Stoel MA, den Brok MH, Adema GJ. Sialic acids sweeten a tumor's life. *Cancer Res.* (2014) 74:3199–204. doi: 10.1158/0008-5472.CAN-14-0728
105. Veillon L, Fakih C, Abou-El-Hassan H, Kobeissy F, Mechref Y. Glycosylation changes in brain cancer. *ACS Chem Neurosci.* (2018) 9:51–72. doi: 10.1021/acschemneuro.7b00271
106. Jung S, Aliberti J, Graemmel P, Sunshine MJ, Kreutzberg GW, Sher A, et al. Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. *Mol Cell Biol.* (2000) 20:4106–14. doi: 10.1128/MCB.20.11.4106-4114.2000
107. Nimmerjahn A, Kirchhoff F, Helmchen F. Resting microglial cells are highly dynamic surveillants of brain parenchyma *in vivo*. *Science.* (2005) 308:1314–8. doi: 10.1126/science.1110647
108. Wieghofer P, Prinz M. Genetic manipulation of microglia during brain development and disease. *Biochim Biophys Acta.* (2016) 1862:299–309. doi: 10.1016/j.bbadis.2015.09.019
109. Yona S, Kim KW, Wolf Y, Mildner A, Varol D, Breker M, et al. Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity.* (2013) 38:79–91. doi: 10.1016/j.immuni.2012.12.001
110. Foster CJ, Prosser DM, Agans JM, Zhai Y, Smith MD, Lachowicz JE, et al. Molecular identification and characterization of the platelet ADP receptor targeted by thienopyridine antithrombotic drugs. *J Clin Invest.* (2001) 107:1591–8. doi: 10.1172/JCI12242
111. Andre P, Delaney SM, LaRocca T, Vincent D, DeGuzman F, Jurek M, et al. P2Y12 regulates platelet adhesion/activation, thrombus growth, and thrombus stability in injured arteries. *J Clin Invest.* (2003) 112:398–406. doi: 10.1172/JCI17864
112. Lou N, Takano T, Pei Y, Xavier AL, Goldman SA, Nedergaard M. Purinergic receptor P2RY12-dependent microglial closure of the injured blood-brain barrier. *Proc Natl Acad Sci USA.* (2016) 113:1074–9. doi: 10.1073/pnas.1520398113
113. Buttgereit A, Lelios I, Yu X, Vrohlings M, Krakoski NR, Gautier EL, et al. Sall1 is a transcriptional regulator defining microglia identity and function. *Nat Immunol.* (2016) 17:1397–406. doi: 10.1038/ni.3585
114. Takasato M, Osafune K, Matsumoto Y, Kataoka Y, Yoshida N, Meguro H, et al. Identification of kidney mesenchymal genes by a combination of microarray analysis and Sall1-GFP knockin mice. *Mech Dev.* (2004) 121:547–57. doi: 10.1016/j.mod.2004.04.007
115. Inoue S, Inoue M, Fujimura S, Nishinakamura R. A mouse line expressing Sall1-driven inducible Cre recombinase in the kidney mesenchyme. *Genesis.* (2010) 48:207–12. doi: 10.1002/dvg.20603
116. Kaiser T, Feng G. Tmem119-EGFP and Tmem119-CreERT2 transgenic mice for labeling and manipulating microglia. *eNeuro.* (2019) 6:ENEURO.0448-18.2019. doi: 10.1523/ENEURO.0448-18.2019
117. Jaiswal S, Jamieson CH, Pang WW, Park CY, Chao MP, Majeti R, et al. CD47 is upregulated on circulating hematopoietic stem cells and leukemia cells to avoid phagocytosis. *Cell.* (2009) 138:271–85. doi: 10.1016/j.cell.2009.05.046
118. Majeti R, Chao MP, Alizadeh AA, Pang WW, Jaiswal S, Gibbs KD, et al., et al. CD47 is an adverse prognostic factor and therapeutic antibody target on human acute myeloid leukemia stem cells. *Cell.* (2009) 138:286–99. doi: 10.1016/j.cell.2009.05.045
119. Edris B, Weiskopf K, Volkmer AK, Volkmer JP, Willingham SB, Contreras-Trujillo H, et al. Antibody therapy targeting the CD47 protein is effective in a model of aggressive metastatic leiomyosarcoma. *Proc Natl Acad Sci USA.* (2012) 109:6656–61. doi: 10.1073/pnas.1121629109
120. Zhang M, Hutter G, Kahn SA, Azad TD, Gholamin S, Xu CY, et al. Anti-CD47 treatment stimulates phagocytosis of glioblastoma by M1 and M2 polarized macrophages and promotes m1 polarized macrophages *in vivo*. *PLoS ONE.* (2016) 11:e0153550. doi: 10.1371/journal.pone.0153550
121. Gholamin S, Mitra SS, Feroze AH, Liu J, Kahn SA, Zhang M, et al. Disrupting the CD47-SIRPalpha anti-phagocytic axis by a humanized anti-CD47 antibody is an efficacious treatment for malignant pediatric brain tumors. *Sci Transl Med.* (2017) 9:eaaf2968. doi: 10.1126/scitranslmed.aaf2968
122. Barkal AA, Brewer RE, Markovic M, Kowarsky M, Barkal SA, Zaro BW, et al. CD24 signalling through macrophage Siglec-10 is a target for cancer immunotherapy. *Nature.* (2019) 572:392–6. doi: 10.1038/s41586-019-1456-0
123. Klichinsky M, Ruella M, Shestova O, Lu XM, Best A, Zeeman M, et al. Human chimeric antigen receptor macrophages for cancer immunotherapy. *Nat Biotechnol.* (2020) 38:947–53. doi: 10.1038/s41587-020-0462-y
124. von Roemeling CA, Wang Y, Qie Y, Yuan H, Zhao H, Liu X, et al. Therapeutic modulation of phagocytosis in glioblastoma can activate both innate and adaptive antitumour immunity. *Nat Commun.* (2020) 11:1508. doi: 10.1038/s41467-020-15129-8
125. Muraro MG, Muenst S, Mele V, Quagliata L, Iezzi G, Tzankov A, et al. Ex-vivo assessment of drug response on breast cancer primary tissue with preserved microenvironments. *Oncoimmunology.* (2017) 6:e1331798. doi: 10.1080/2162402X.2017.1331798
126. Goltsev Y, Samusik N, Kennedy-Darling J, Bhate S, Hale M, Vazquez G, et al. Deep profiling of mouse splenic architecture with CODEX multiplexed imaging. *Cell.* (2018) 174:968–81.e15. doi: 10.1016/j.cell.2018.07.010
127. Wang SC, Yu CF, Hong JH, Tsai CS, Chiang CS. Radiation therapy-induced tumor invasiveness is associated with SDF-1-regulated macrophage mobilization and vasculogenesis. *PLoS ONE.* (2013) 8:e69182. doi: 10.1371/journal.pone.0069182
128. Kioi M, Vogel H, Schultz G, Hoffman RM, Harsh GR, Brown JM. Inhibition of vasculogenesis, but not angiogenesis, prevents the recurrence of glioblastoma after irradiation in mice. *J Clin Invest.* (2010) 120:694–705. doi: 10.1172/JCI40283
129. Poli A, Wang J, Domingues O, Planaguma J, Yan T, Rygh CB, et al. Targeting glioblastoma with NK cells and mAb against NG2/CSPG4 prolongs animal survival. *Oncotarget.* (2013) 4:1527–46. doi: 10.18632/oncotarget.1291
130. Gieryng A, Pszczolkowska D, Walentynowicz KA, Rajan WD, Kaminska B. Immune microenvironment of gliomas. *Lab Invest.* (2017) 97:498–518. doi: 10.1038/labinvest.2017.19
131. Ellert-Miklaszewska A, Wisniewski P, Kijewska M, Gajdanowicz P, Pszczolkowska D, Przanowski P, et al. Tumour-processed osteopontin and lactadherin drive the protumorigenic reprogramming of microglia and glioma progression. *Oncogene.* (2016) 35:6366–77. doi: 10.1038/onc.2016.55
132. Brown EJ, Frazier WA. Integrin-associated protein (CD47) and its ligands. *Trends Cell Biol.* (2001) 11:130–5. doi: 10.1016/S0962-8924(00)01906-1
133. Barclay AN, Van den Berg TK. The interaction between signal regulatory protein alpha (SIRPα) and CD47: structure, function, and therapeutic target. *Annu Rev Immunol.* (2014) 32:25–50. doi: 10.1146/annurev-immunol-032713-120142
134. Tsai RK, Discher DE. Inhibition of "self" engulfment through deactivation of myosin-II at the phagocytic synapse between human cells. *J Cell Biol.* (2008) 180:989–1003. doi: 10.1083/jcb.200708043
135. Fuster MM, Esko JD. The sweet and sour of cancer: glycans as novel therapeutic targets. *Nat Rev Cancer.* (2005) 5:526–42. doi: 10.1038/nrc1649
136. Pinho SS, Reis CA. Glycosylation in cancer: mechanisms and clinical implications. *Nat Rev Cancer.* (2015) 15:540–55. doi: 10.1038/nrc3982
137. Dall'Olio F, Malagolini N, Trinchera M, Chiricolo M. Sialosignaling: sialyltransferases as engines of self-fueling loops in cancer progression. *Biochim Biophys Acta.* (2014) 1840:2752–64. doi: 10.1016/j.bbagen.2014.06.006
138. Varki A. Loss of N-glycolylneuraminic acid in humans: mechanisms, consequences, and implications for hominid evolution. *Am J Phys Anthropol.* (2001) 33:54–69. doi: 10.1002/ajpa.10018

139. Pearce OM, Laubli H. Sialic acids in cancer biology and immunity. *Glycobiology.* (2016) 26:111–28. doi: 10.1093/glycob/cwv097
140. Schwarz F, Fong JJ, Varki A. Human-specific evolutionary changes in the biology of siglecs. *Adv Exp Med Biol.* (2015) 842:1–16. doi: 10.1007/978-3-319-11280-0_1
141. Varki A. Since there are PAMPs and DAMPs, there must be SAMPs? glycan "self-associated molecular patterns" dampen innate immunity, but pathogens can mimic them. *Glycobiology.* (2011) 21:1121–4. doi: 10.1093/glycob/cwr087
142. Bertram L, Lange C, Mullin K, Parkinson M, Hsiao M, Hogan MF, et al. Genome-wide association analysis reveals putative Alzheimer's disease susceptibility loci in addition to APOE. *Am J Hum Genet.* (2008) 83:623–32. doi: 10.1016/j.ajhg.2008.10.008
143. Hollingworth P, Harold D, Sims R, Gerrish A, Lambert JC, Carrasquillo MM, et al. Common variants at ABCA7, MS4A6A/MS4A4E, EPHA1, CD33 and CD2AP are associated with Alzheimer's disease. *Nat Genet.* (2011) 43:429–35. doi: 10.1038/ng.803
144. Naj AC, Jun G, Beecham GW, Wang LS, Vardarajan BN, Buros J, et al. Common variants at MS4A4/MS4A6E, CD2AP, CD33 and EPHA1 are associated with late-onset Alzheimer's disease. *Nat Genet.* (2011) 43:436–41. doi: 10.1038/ng.801
145. Griciuc A, Serrano-Pozo A, Parrado AR, Lesinski AN, Asselin CN, Mullin K, et al. Alzheimer's disease risk gene CD33 inhibits microglial uptake of amyloid beta. *Neuron.* (2013) 78:631–43. doi: 10.1016/j.neuron.2013.04.014
146. Pluvinage JV, Haney MS, Smith BAH, Sun J, Iram T, Bonanno L, et al. CD22 blockade restores homeostatic microglial phagocytosis in ageing brains. *Nature.* (2019) 568:187c92. doi: 10.1038/s41586-019-1088-4
147. Claude J, Linnartz-Gerlach B, Kudin AP, Kunz WS, Neumann H. Microglial CD33-related Siglec-E inhibits neurotoxicity by preventing the phagocytosis-associated oxidative burst. *J Neurosci.* (2013) 33:18270–6. doi: 10.1523/JNEUROSCI.2211-13.2013
148. Wang Y, Neumann H. Alleviation of neurotoxicity by microglial human Siglec-11. *J Neurosci.* (2010) 30:3482–8. doi: 10.1523/JNEUROSCI.3940-09.2010
149. Reardon DA, Omuro A, Brandes AA, Rieger J, Wick A, Sepulveda J, et al. OS10.3 randomized phase 3 study evaluating the efficacy and safety of nivolumab vs bevacizumab in patients with recurrent glioblastoma: checkmate 143. *Neuro-Oncology.* (2017) 19(Suppl.3):iii21. doi: 10.1093/neuonc/nox036.071
150. Li GZ, Zhang KN, Wang Z, Hu HM, Wang ZL, Huang RY, et al. Siglecs, novel immunotherapy targets, potentially enhance the effectiveness of existing immune checkpoint inhibitors in glioma immunotherapy. *Onco Targets Ther.* (2019) 12:10263–73. doi: 10.2147/OTT.S223406
151. Wielgat P, Trofimiuk E, Czarnomysy R, Braszko JJ, Car H. Sialic acids as cellular markers of immunomodulatory action of dexamethasone on glioma cells of different immunogenicity. *Mol Cell Biochem.* (2019) 455:147–57. doi: 10.1007/s11010-018-3478-6
152. Wielgat P, Czarnomysy R, Trofimiuk E, Car H. The sialoglycan-Siglec-E checkpoint axis in dexamethasone-induced immune subversion in glioma-microglia transwell co-culture system. *Immunol Res.* (2019) 67:348–57. doi: 10.1007/s12026-019-09106-7
153. Gautier EL, Shay T, Miller J, Greter M, Jakubzick C, Ivanov S, et al. Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages. *Nat Immunol.* (2012) 13:1118–28. doi: 10.1038/ni.2419
154. Konishi H, Kobayashi M, Kunisawa T, Imai K, Sayo A, Malissen B, et al. Siglec-H is a microglia-specific marker that discriminates microglia from CNS-associated macrophages and CNS-infiltrating monocytes. *Glia.* (2017) 65:1927–43. doi: 10.1002/glia.23204
155. Kopatz J, Beutner C, Welle K, Bodea LG, Reinhardt J, Claude J, et al. Siglec-h on activated microglia for recognition and engulfment of glioma cells. *Glia.* (2013) 61:1122–33. doi: 10.1002/glia.22501
156. Wang J, Sun J, Liu LN, Flies DB, Nie X, Toki M, et al. Siglec-15 as an immune suppressor and potential target for normalization cancer immunotherapy. *Nat Med.* (2019) 25:656–66. doi: 10.1038/s41591-019-0374-x
157. Watters JJ, Schartner JM, Badie B. Microglia function in brain tumors. *J Neurosci Res.* (2005) 81:447–55. doi: 10.1002/jnr.20485

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Single-Cell RNA-Sequencing Shift in the Interaction Pattern Between Glioma Stem Cells and Immune Cells During Tumorigenesis

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Glioblastoma is one of the most common neoplasms in the central nervous system characterized by limited immune response and unlimited expansion capability. Cancer stem cells (GSCs), a small fraction of the tumor cells, possess a pivotal regulation capability in the tumor microenvironment with a superior proliferation ability. We aimed to reveal the interaction between glioma stem cells (GSCs) and immune cells during tumorigenesis. Single-cell sequencing data from seven surgical specimens of glioblastoma patients and patient-derived GSCs cocultured with peripheral leukocytes were used for the analysis. Cell grouping and trajectory analysis were performed using Seurat and Monocle 3 packages in R software. The gene set of Cancer Genome Anatomy Project was used to define different cell types. Cells with the ability of proliferation and differentiation in glioblastoma tissue were defined as GSCs, which had a similar expression pattern to that in the GSCs *in vitro*. Astrocytes in glioblastoma were mainly derived from differentiated GSCs, while oligodendrocytes were most likely to be derived from different precursor cells. No remarkable evolutionary trajectory was observed among the subgroups of T cells in glioblastoma. The immune checkpoint interaction between GSCs and immune cells was changed from stimulatory to inhibitory during tumorigenesis. The patient-derived GSCs system is an ideal model for GSC research. The above research revealed that the interaction pattern between GSC glioma stem cells and immune cells during tumorigenesis provides a theoretical basis for GSC glioma stem cell-targeted immunotherapy.

Keywords: glioma stem cell, T cell, single cell sequence, immunosuppression, tumorigenesis

INTRODUCTION

Glioblastoma (GBM) is the most lethal type of intracranial malignancy (1). The median survival is about 14.4 months, and the overall survival varies from 3 months to 3 years (2). Among the many factors that contribute to poor outcomes, the existence of glioma stem cells (GSCs) and the immunological "cold tumor" status are considered to be two major pivotal ones (3, 4).

For the past few years, the dysfunction and poor infiltration of T cells in GBM tissue have become a major factor associated with poor prognosis according to a consensus (5). Several strategies for T cell dysfunction in GBM tissue have been described (6). Although T cells are overwhelmed by tumor cells in GBM, not all tumor cells possess the ability of immune regulation. Thus, studying the interaction between tumor cells and T cells may be a new direction in tumorigenesis research.

In recent years, GSCs have become a novel hot spot due to their tumorigenesis and immune regulation capabilities (7). GSCs play a pioneering immunosuppressive role at the time of tumor initiation and gradually lose these capabilities during differentiation to astrocytes and oligodendrocytes. Further, GSCs are considered to be extremely resistant to therapy (8), leading to the failure of multiple treatments, including immunotherapy. Therefore, revealing the interaction between GSCs and T cells may provide novel immunotherapeutic strategies for glioma.

In this article, peripheral T cells and GSC coculture models were built *in vitro* to simulate the initial state of tumor. Taking advantage of the single-cell sequencing data, we were able to identify different subtypes of cells and further analyze the evolutionary relationship between each subtype of tumor cells, as well as immune cells. First, we identified subtypes of GSCs in surgical specimens according to the high proliferation characteristics. Then, we constructed the coculture model of T cells and GSCs. We cross-validated the DNA expression patterns in the GCSs in the established coculture model and surgical specimens. An ideal similarity was detected. Further, we depicted an evolution routine for GSCs in surgical specimens. The astrocytes showed a strong evolutionary relation with GSCs. Since T cells showed various characteristics in those two data sources, we defined the coculture model as the initial stage of tumor progression and the specimens as the advanced stage of tumor. Finally, we simulated the fold change of the immune checkpoint in both T cells and GSCs in those two data sources. The inhibiting checkpoint resulted in an advanced tumor stage. Above all, the *in vitro* model is an ideal tool for unveiling the interaction between peripheral T cells and GSCs, simulating the early microenvironment during tumorigenesis.

MATERIALS AND METHODS

Isolation and Culture of Primary Cells

Tumor tissues obtained during surgery were immediately immersed in the medium and transported to the laboratory on ice for further processing. The tissue was cleaned and shredded mechanically. The tissue was then enzymatically digested into single cells using trypsin. The single cells were filtered using a 200-mesh filter and centrifuged (400 g) for 5 min. After treating the cells with red blood cell lysis, they were centrifuged again. The obtained cells were cultured in a serum-free medium containing DMEM/F12 (Gibco) supplemented with B27 (Gibco), basic fibroblast growth factor (bFGF, 20 ng/mL), epidermal growth factor (EGF, 20 ng/mL), and heparin (2.5 mg/mL). Growth factors (bFGF and EGF) were added twice a week. Primary GSCs were enzymatically dissociated into single cells using Accutase (Sigma Aldrich) and thereafter routinely cultured in the serum-free medium that was replaced every 4–6 days. The stemness of GSCs was verified by multidirectional differentiation immunofluorescence staining (**Figure 2A**).

Normal peripheral blood lymphocytes were obtained from healthy adult male donors. Isolation of peripheral blood T cells was performed following the protocol as previously described (9). In brief, peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation with Lymphoprep (STEMCELL). The PBMCs were resuspended in EasySep™ Buffer (STEMCELL), and T cells were isolated following the manufacturer's instruction (EasySep™ Human T Cell Isolation Kit, STEMCELL). T cells were identified by CD3 staining flow cytometry (**Figure 2A**).

Peripheral blood T cells were cocultured with GSCs for 24 h the day after isolation without CD3/CD28 stimulation. 2×10^6 T cells, together with 1×10^6 GSCs, were directly mixed and resuspended in ImmunoCult™-XF T Cell Expansion Medium (STEMCELL) and were cocultured in a 37°C 5% CO_2 incubator.

Construction of a Single-Cell RNA-Sequencing Library

Single-cell RNA sequencing library construction of the tissue specimens obtained from GBM patients has been described in detail in our previous research (10). The cell preparation for coculture cellular model was done strictly in accordance with the official documentation of 10× Genomics (https://support.10xgenomics.com). Single-cell RNA sequencing was performed using Illumina (HiSeq 2000) according to the manufacturer's instructions by Novogene (Beijing, China).

Cell Clustering Using Seurat

The cell clustering in GBM patients and coculture model of primary normal peripheral blood lymphocytes and GSCs was performed by the R package Seurat (version 3.0, https://satijalab.org/seurat/). Batch effect was removed before the clustering in GBM patients. Subsequently, the cell clustering process in GBM patients and the coculture model were done in the same way. Firstly, cells that have had unique feature counts over 7,500 or <200 and >15% mitochondrial count were removed. Subsequently, after normalizing the data, non-linear dimensional reduction of cells was carried out using UMAP with the default parameters. Finally, the cluster biomarkers were also obtained. In addition, the t-SNE method was also used to verify the reliability of cell grouping of the UMAP method (**Supplementary Figure 7**).

Identification of Cell Clusters

The Cancer Genome Anatomy Project Serial Analysis of Gene Expression (CGAP_SAGE_QUARTILE) was launched to determine the genetic fingerprints of normal, premalignant, and malignant tumor cells based on the transcriptome characteristics of cells (PMID: 10933042). Identification of cell clusters was performed using CGAP_SAGE_QUARTILE analysis in DAVID portal (https://david.ncifcrf.gov/) according to the cluster biomarkers.

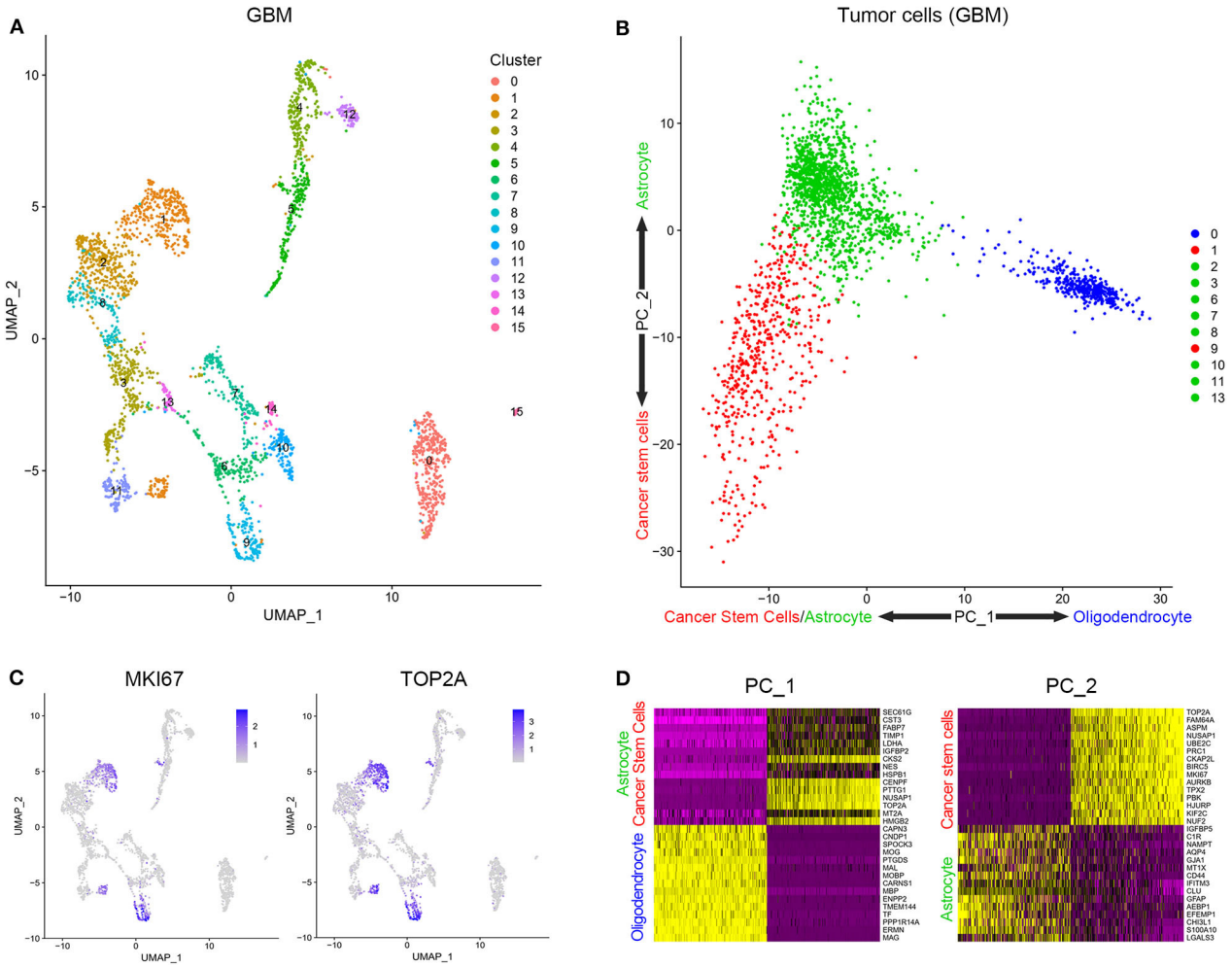

FIGURE 1 | Stratification and identification of cells from surgical specimens. **(A)** The subgroups of cells in surgical specimens. **(B)** The expression level of proliferation-associated biomarkers in different cell subgroups. **(C)** The PCA analysis of tumor cells in surgical samples. **(D)** Genes related to PC1 and PC2.

Functional Enrichment Analysis of Cell Clusters

Gene Ontology (GO) enrichment and KEGG pathway analysis of cell clusters were used to identify the biological significance of each cell type. GO and KEGG pathway analyses were conducted using the cluster biomarkers.

Single-Cell Trajectory Analysis

The R package Monocle 3 was applied to order cells in pseudotime along a trajectory (https://cole-trapnell-lab.github.io/monocle3). After clustering the cells using the above method, the dimensionality was reduced and the results were visualized using the UMAP method. Subsequently, the cells were ordered according to their progress through the developmental program. Monocle measures this progress in pseudotime. In this study, single-cell trajectory analysis of cell subtypes was performed as needed.

Software Availability

Statistical analyses and drawing were performed using the R program (https://www.r-project.org/, version: 4.0), TBtools software (version: 0.67), Java software (version: 12.0.1), and Microsoft office 2016. The Sankey diagram was drawn using online tools (http://sankeymatic.com/build/).

RESULTS

Identification of Glioma Stem Cells in GBM Tissue Samples

Cells from tissue samples of 7 GBM patients were grouped into 16 clusters according to a single-cell sequencing data (**Figure 1A**). Based on CGAP_SAGE_QUARTILE, cell types of 16 clusters were identified according to their gene expression pattern. Clusters 1 and 9 were identified as GSCs. Clusters 4, 5, 12, 14, and 15 were identified as immune cells (**Supplementary Figure 1**). Clusters 0, 2, 3, 6, 7, 8, 10, 11, and 13 were identified as tumor cells (**Supplementary Figure 1**). To further identify

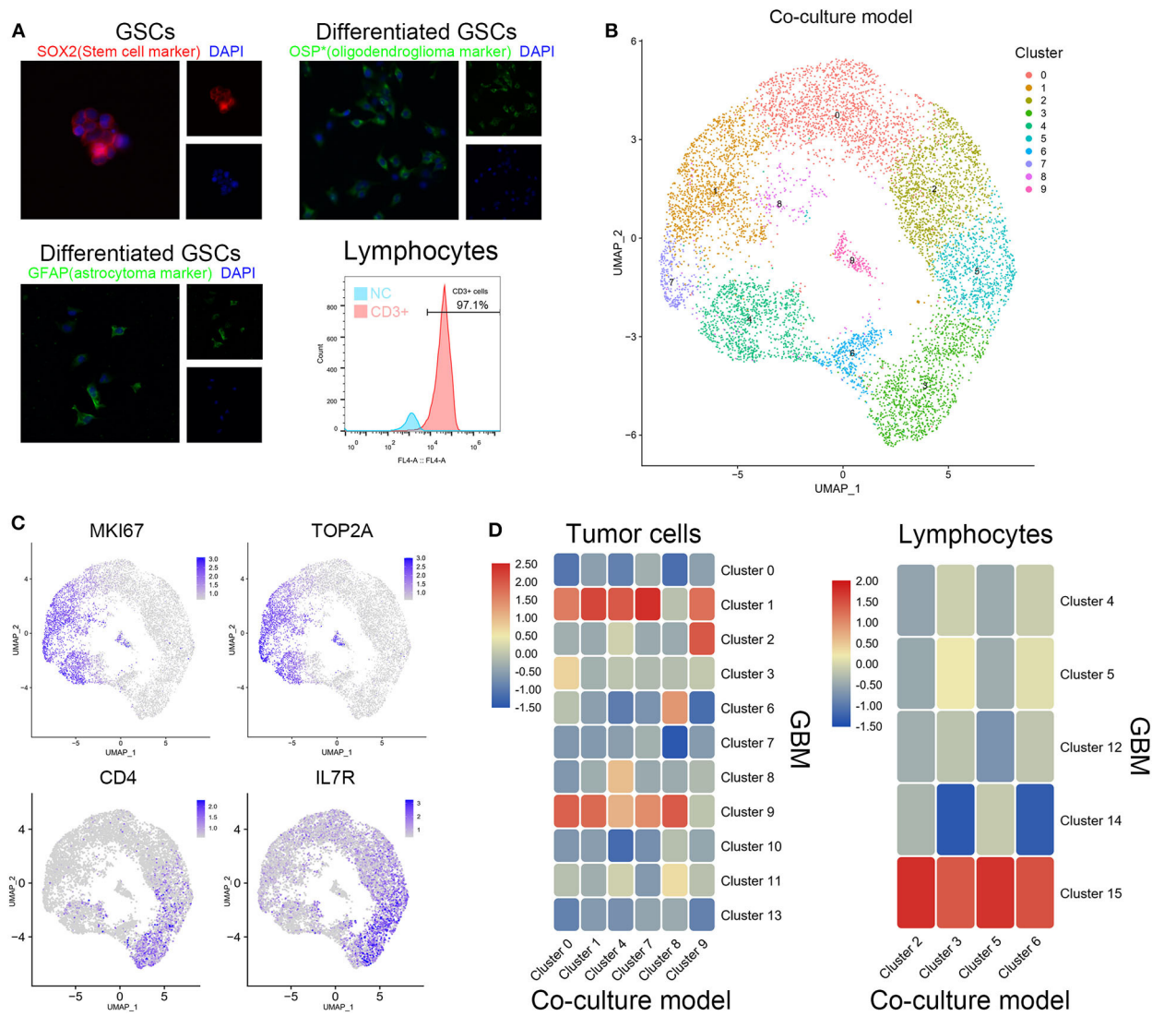

FIGURE 2 | Similarity of cell grouping in the coculture model and surgical specimens. **(A)** The GSCs and T cells were verified by immunofluorescence staining and flow cytometry. OSP: oligodendrocyte specific protein. **(B)** The subgroups of cells in the coculture model. **(C)** Markers of proliferation and immunology in different cell groups. **(D)** The similarity of glioma stem cells and lymphocytes in the coculture model and surgical specimens.

GSCs from these 16 clusters, we examined the proliferation of cells. As a result, clusters 1 and 9 possessed the significantly increased expression of proliferation markers KI67 and TOP2A (**Figure 1B**). In addition, clusters 1 and 9, together with the rest subgroups of tumor cells (Clusters 0, 2, 3, 6, 7, 8, 10, 11, and 13), were further engaged in principal component analysis (PCA). Cells in clusters 1 and 9 possessed low PC-1 and PC-2 values while cells in clusters 2, 3, 6, 7, 8, 10, 11, and 13 possessed low PC-1 and high PC-2 values. On the contrary, cluster 0 possessed high PC-1 and high PC-2 values (**Figure 1C**). The genes that were positively correlated with PC-1 values were mainly oligodendrocyte markers, while those that were negatively correlated with PC-1 values were mainly cancer stem cells and astrocyte markers. On the other hand, the genes that were positively correlated with PC-2 values were mainly astrocyte markers and those that were negatively correlated with PC-2 values were mainly cancer stem cells markers (**Figure 1D** and **Supplementary Table 1**). In short, clusters 1 and 9 containing a group of cells with high proliferation and differentiation abilities had the characteristics of cancer stem cells.

The Coculture Model Was Built to Simulate the Initial State of Tumor Development

Patient-derived GSCs and peripheral blood lymphocytes from healthy adults were cocultured to simulate the initial state of tumor. After identification by cell surface markers, patient-derived GSCs and peripheral blood lymphocytes were mixed (1:2) and cocultured (**Figure 2A**). Single-cell sequencing of the mixed cells was performed after 12 h of coculture. The 10

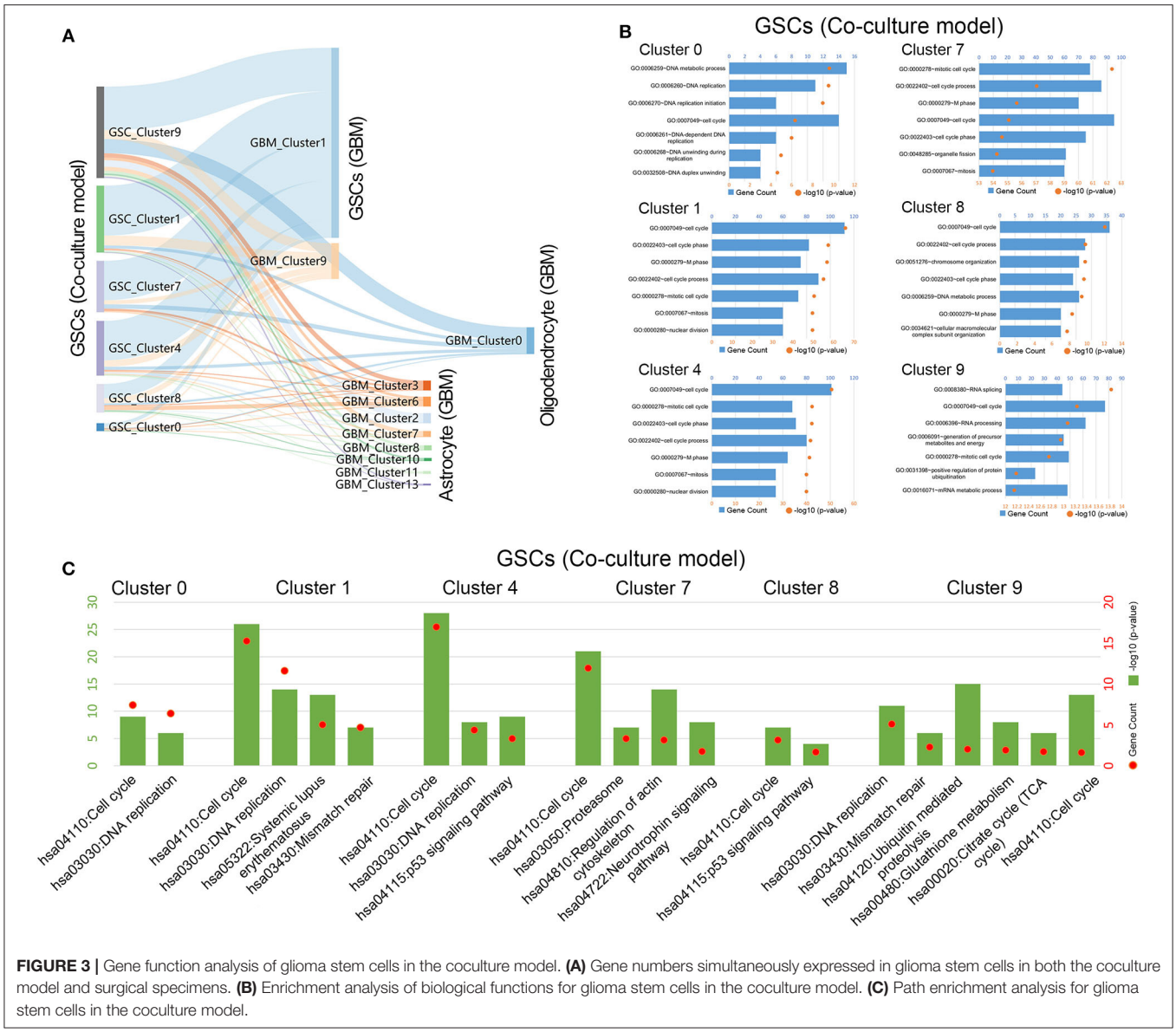

FIGURE 3 | Gene function analysis of glioma stem cells in the coculture model. **(A)** Gene numbers simultaneously expressed in glioma stem cells in both the coculture model and surgical specimens. **(B)** Enrichment analysis of biological functions for glioma stem cells in the coculture model. **(C)** Path enrichment analysis for glioma stem cells in the coculture model.

clusters of the cocultured cells are indicated in **Figure 2B**. As expected, cell clusters could be divided into GSCs and lymphocytes according to their proliferation rate and the expression of immune cell markers. Clusters 0, 1, 4, 7, 8, and 9 with high expression of KI67 and TOP2A were identified as GSCs. Clusters 2, 3, 5, and 6 were considered to be T cells with their extracellular markers, CD4 and IL7R (**Figure 2C**). We further compared the similarity between GSCs and T cells from coculture model and GBM samples. As shown in **Figure 2C**, clusters 0, 1, 4, 7, 8, and 9 in the coculture model possessed the similar gene expression characteristics with clusters 1 and 9 in GBM samples. Clusters 2, 3, 5, and 6 in the coculture model were similar to cluster 15 in GBM samples (**Figure 2D**). The list of cell markers of all clusters is uploaded in **Supplementary Table 2**. Meanwhile, cell types of cocultured cells were also identified based on CGAP_SAGE_QUARTILE. The cell types identified using the CGAP_SAGE_QUARTILE were highly consistent with those defined using the cell markers (**Supplementary Figure 2**).

Stem Cells in the Coculture Model and GBM Samples Showed Highly Similar Expression Characteristics

To further explore the relationship between cells in coculture model and GBM samples, the correlation of the expression characteristics of tumor cells in these two groups was compared. As shown in **Figure 3A**, all clusters of GSCs in the coculture model possessed the majority of the coexpressed genes with clusters 1 and 9 in tumor specimens. Surely, GSCs in the coculture model also had some coexpressed genes with astrocytes as well as oligodendrocytes. Subsequently, functional enrichment

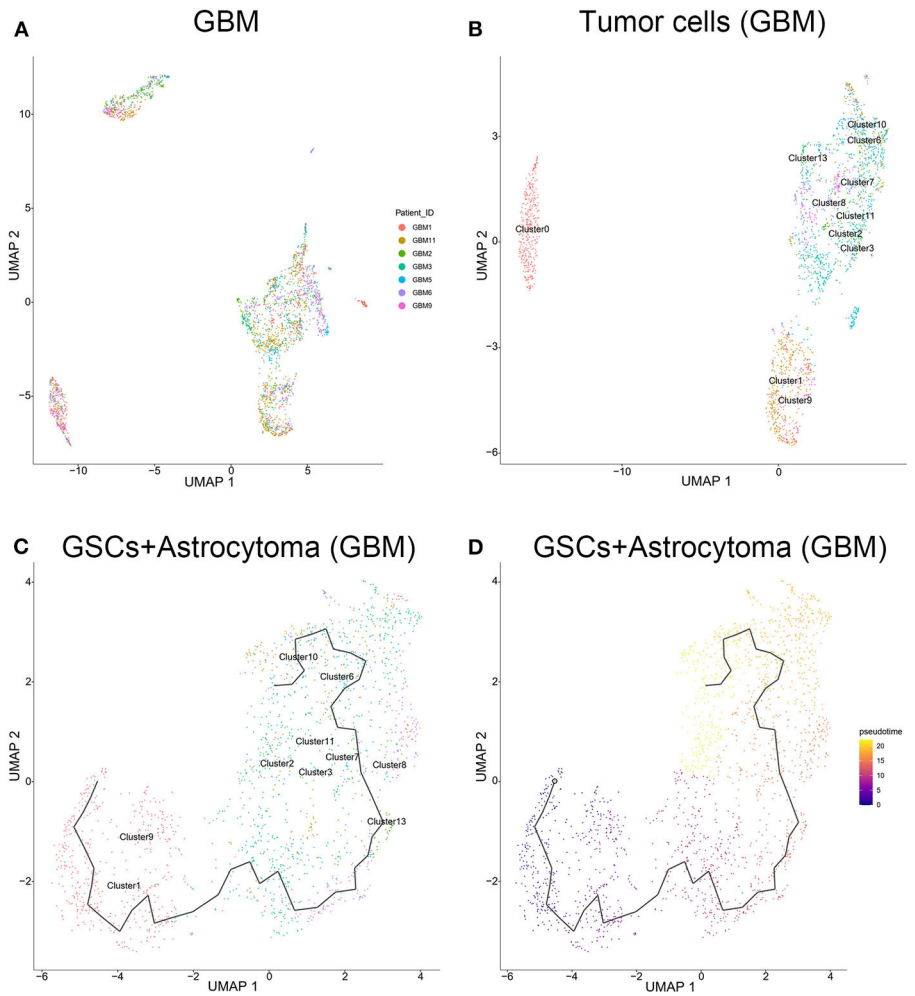

FIGURE 4 | Evolution routine of tumor cells in surgical specimens. **(A)** No significant batch difference in tumor cells from different surgical specimens. **(B)** Tumor cell subgrouping in surgical specimens. **(C)** The evolution direction of tumor cells in surgical specimens. **(D)** The pseudo-time sequence of evolution of tumor cells in surgical specimens.

analysis of tumor cells in the coculture model and GBM samples was performed using GO analysis and KEGG analysis. Stem cells in both groups of cells were characterized by high proliferation capacity (**Figures 3B,C**, **Supplementary Figures 3**, **4**), while other non-stem tumor cells showed significantly different biological characteristics (**Supplementary Figures 3**–**5**). These results suggested that the patient-derived stem cells and the defined GSCs in GBM samples shared a high level of proliferation-related markers, as well as active proliferation pathways, indicating the ultimate proliferation capacity of these cells.

Evolution Routine Can Be Described Between Glioma Stem Cells and Astrocytes in GBM Samples

The tumor cells from the GBM samples were extracted for further study. The results showed that no significant batch effect of tumor cells has been observed among patients (**Figure 4A**). As mentioned above, clusters 0, 1, 2, 3, 6, 7, 8, 9, 10, 11, and 13 of GBM samples were identified as tumor cells, including GSCs (clusters 1 and 9) and astrocytes (clusters 2, 3, 6, 7, 8, 10, 11, and 13) and oligodendrocytes (cluster 0). As shown in **Figure 4B**, among the three groups of cells, the oligodendrocytes were relatively insular compared with the other two cell types. To further unveil the differentiation process from GSC to astrocytes or oligodendrocytes, trajectories of GBM tumor cells were calculated. The results showed GSC's evolution into astrocytes through a certain path in terms of evolution time (**Figures 4C,D**). However, as the subgroups of GSC and oligodendrocytes were far apart on the evolutionary route and there was no fundamental connection between those two cell types, we could conclude that there was no evolutionary relation between GSCs and oligodendrocytes (**Supplementary Figure 6**).

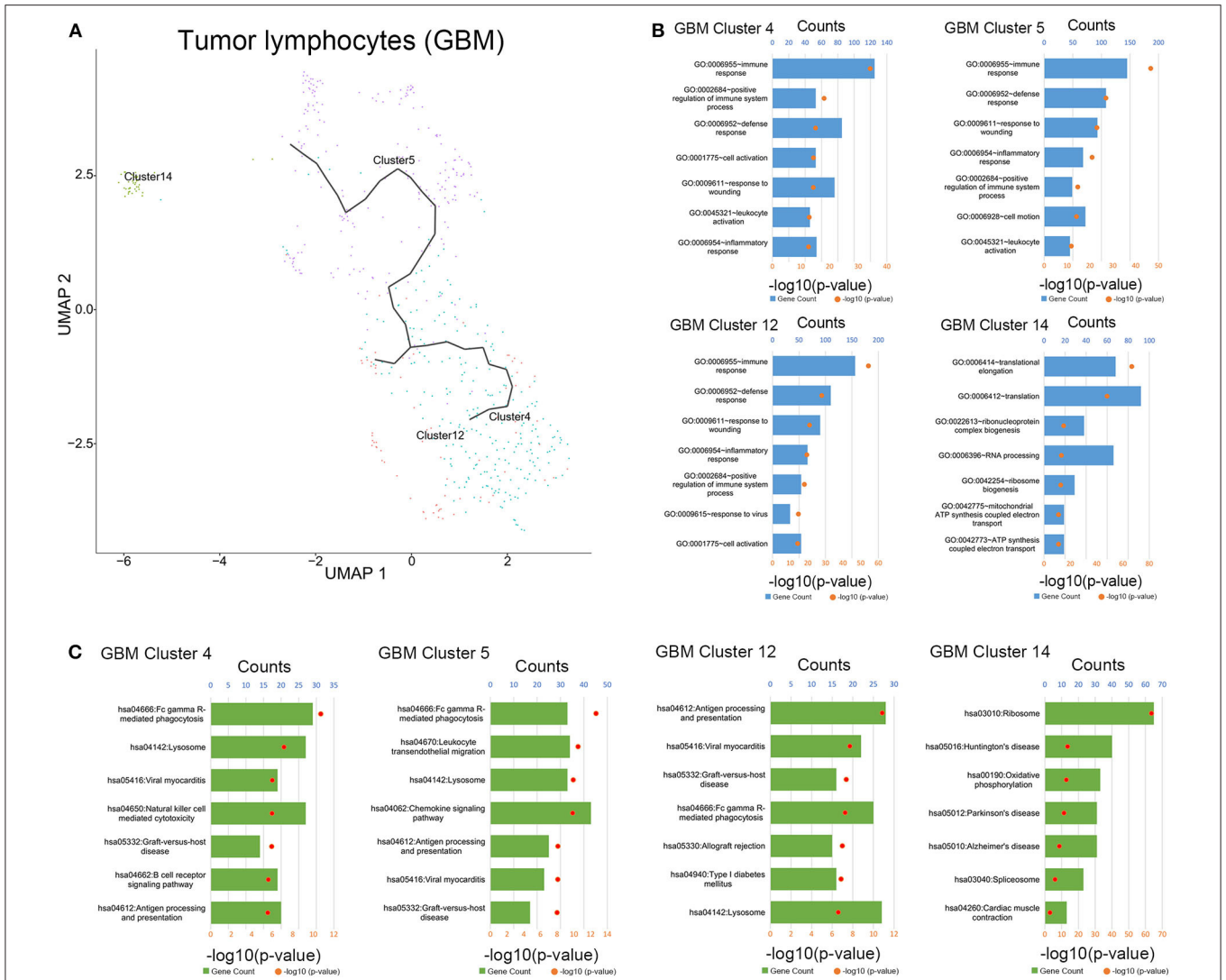

FIGURE 5 | Evolution routine and function enrichment of lymphocytes in surgical specimens. **(A)** The evolution direction of lymphocytes in surgical specimens. **(B)** Enrichment analysis of biological functions for lymphocytes in the surgical specimens. **(C)** Pathway enrichment analysis for lymphocytes in the surgical specimens.

T Cells Showed Differences in Biological Functions and Pathway Activation Between the Coculture Model and GBM Samples

To clarify the evolutionary trajectory of T cells in tumors, trajectories of T cells in GBM were calculated. A clear evolutionary route could be found in T cells (**Figure 5A**). However, the cluster of initial T cells (Cluster 14) was not in the evolution path. The direction of evolution was difficult to determine. Subsequently, GO analysis and KEGG analysis were performed to reveal the biological functions of T cells in the coculture model and GBM samples (**Figures 5B,C**, **Supplementary Figures 3**–**5**). Different groups of T cells showed different biological functions and pathway activations.

Immune Checkpoint Interaction Pattern Changed Significantly Between the Coculture Model and GBM Samples

The interaction of immune checkpoint of T cells and tumor cells were analyzed in the coculture model and GBM samples separately. The stimulatory immune checkpoint genes were expressed mainly in T cells in the coculture model, while inhibitory immune checkpoint genes were enriched in T cells in GBM samples (**Figure 6A**). Similarly, tumor cells mainly expressed ligands of stimulatory immune checkpoints in the coculture model, while tumor cells in GBM samples mainly expressed ligands of inhibitory immune checkpoints (**Figure 6B**). Significant changes in this interaction model may reveal the causes of tumor

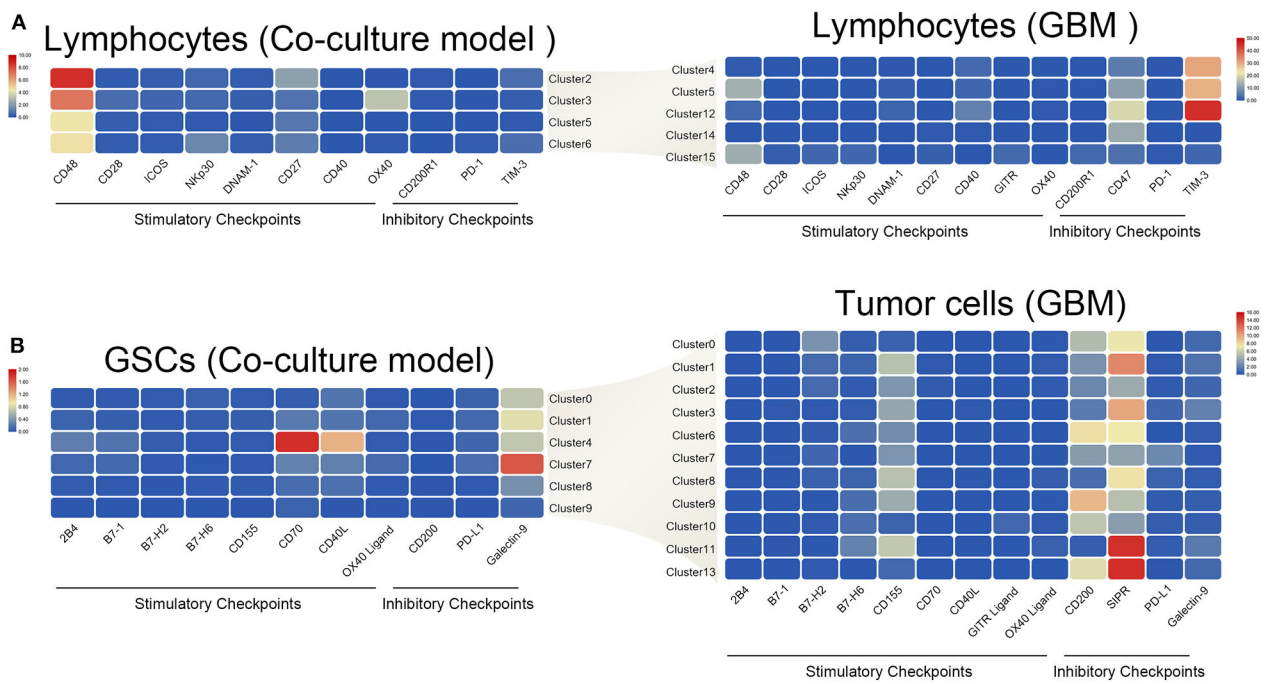

FIGURE 6 | Differences in the interaction pattern between tumor cells and immune cells in early and late stages of glioma. Changes in expression of immune checkpoints and the corresponding receptors in both immune cells **(A)** and glioma stem cells **(B)**, in the early and late stages of glioma.

immunosuppression in the microenvironmental status during tumorigenesis.

DISCUSSION

Numerous studies had confirmed the high level of immunosuppression during GBM processing, which contributes to the dysfunction of the infiltrated immune cells and immunotherapeutic failure (11). Recently, several studies underlined the importance of GSCs for the initiation of immune suppression during tumorigenesis (12, 13). However, the identification of cancer stem cells is challenging. The limited methods for cancer stem cell separation hindered research progress, although beads or flow separation, as well as the introduction of a special culture medium, has been widely used for cancer stem cell sorting. Single-cell sequencing has allowed us to perform multiple analysis of different cell types in a large number of specimens and in *in vitro* culture samples (14).

Thus, by means of single-cell sequencing using the GBM samples, cells with high proliferation and differentiative capacity were defined as GSCs. Similarly, single-cell sequencing data of the established coculture model of the patient-derived GSCs and human peripheral blood T cells were also analyzed. Comparative analysis showed high similarity between GSCs in GBM samples and those in the coculture model. In addition, we have also verified the similarity between these two populations based on the markers, biological function enrichment, and other parameters. It suggests that the coculture model we constructed can simulate the initial status of stem cells in tumors, which could be used in further research. Surprisingly, we found that there were few immune cells (cluster 15) in the surgical specimens that were highly similar to the peripheral blood lymphocytes in the coculture system (cluster 15). We speculated that it was due to the small amount of peripheral blood "contamination" caused by the operation. Since those lymphocytes may not be the original immune cells in the tumor, this phenomenon should be noted when identifying immune cell clusters using single-cell sequencing in the future.

The application of trajectory analysis using single-cell sequencing data in the evolution research has attracted more and more attention. Such technique has been applied to the evolution research of many tumors, e.g., liver cancer (15). Therefore, we used Monocle 3, the most commonly used tool for studying tumor evolution, to analyze the evolution of tumor cells in GBM samples. Our study revealed that GSCs had a differentiation ability. On the other hand, whether astrocytes and oligodendrocytes in tumors were directly originating from GSCs remains controversial. We unveiled that the astrocytes in the tumor were likely derived from GSC. On the contrary, oligodendrocytes showed significantly different characteristics from astrocytes. In addition to possessing significantly different gene expression characteristics, oligodendrocytes were less heterogeneous than astrocytes. Further, oligodendrocytes and astrocytes were proved to have different origins instead of both cell types originating from GSC.

Unfortunately, our study did not find precursor cells of the oligodendrocytes. These research results provide a theoretical basis for the follow-up research and targeted therapy of cancer stem cells.

The immune modulating abilities of GSCs were attributed to inducing cytotoxic T cell (CTL) anergy/apoptosis and expansion of regulatory T cells (Treg) (16). Meanwhile, Tregs were well-known immune suppression cells (17). Nevertheless, an ideal model for simulation of the initial interactions between T cells and GSCs has not yet been reported. Thus, we suppose that the GSC and T cell coculture system might be an ideal model for simulating the early stages of tumorigenesis. Our analysis unveiled that the immune cells in GBM samples had a clear evolutionary trajectory. Clusters 4 and 5 were identified as tumor-associated macrophages. The evolution between clusters 4 and 5 may be the result of the transformation of M1 and M2. This suggests that the evolution of immune cells in the tumor microenvironment may play a role in tumor progression, although the particular mechanism remains unclear. Therefore, we defined the coculture model of GSCs and peripheral blood T cells as the early state of the tumor and the surgical samples of GBM patients as the advanced state of the tumor. By comparing the expression of immune checkpoint-related genes between these two stages, we found that both T cells and tumor cells had a preferential expression of the stimulatory immune checkpoints. However, in the advanced stages of tumors, these two types of cells expressed more suppressive immune checkpoints, which finally evolved into the state of the immune microenvironment consistent with the consensus. A further in-depth study of this transition process may provide new treatment ideas for immunotherapy of gliomas.

In summary, our research confirmed the existence of a group of cells possessing highly proliferative and differentiative capability in the tumor, which are called glioma stem cells. In addition, it also established a reliable *in vitro* model for glioma stem cell research. Our research revealed the evolution of glioma stem cells and the changes in immune status, which can provide new ideas for immunotherapy of gliomas.

DATA AVAILABILITY STATEMENT

The datasets for this study can be found in the CGGA portal (http://www.cgga.org.cn/).

ETHICS STATEMENT

Sample collection and data analyses were approved by Beijing Tiantan Hospital institutional review board (IRB). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

WZ: conception, supervision, and design of this article. YZ and GL: data analysis and editing the manuscript. RL, YC, and YF: data collection and organization of Single-Cell RNA-Sequencing data. YZ, GL, DW, and FW: isolation and culture of primary cells. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.581209/full#supplementary-material

REFERENCES

1. Jiang T, Mao Y, Ma W, Mao Q, You Y, Yang X, et al. CGCG clinical practice guidelines for the management of adult diffuse gliomas. *Cancer Lett.* (2016) 375:263–73. doi: 10.1016/j.canlet.2016.01.024
2. Gerber NK, Goenka A, Turcan S, Reyngold M, Makarov V, Kannan K, et al. Transcriptional diversity of long-term glioblastoma survivors. *Neuro Oncol.* (2014) 16:1186–95. doi: 10.1093/neuonc/nou043
3. Verhaak RG, Hoadley KA, Purdom E, Wang V, Qi Y, Wilkerson MD, et al. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell.* (2010) 17:98–110. doi: 10.1016/j.ccr.2009.12.020
4. Rutledge WC, Kong J, Gao J, Gutman DA, Cooper LA, Appin C, et al. Tumor-infiltrating lymphocytes in glioblastoma are associated with specific genomic alterations and related to transcriptional class. *Clin Cancer Res.* (2013) 19:4951–60. doi: 10.1158/1078-0432.CCR-13-0551
5. Woroniecka K, Chongsathidkiet P, Rhodin K, Kemeny H, Dechant C, Farber SH, et al. T-cell exhaustion signatures vary with tumor type and are severe in glioblastoma. *Clin Cancer Res.* (2018) 24:4175–86. doi: 10.1158/1078-0432.CCR-17-1846
6. Woroniecka KI, Rhodin KE, Chongsathidkiet P, Keith KA, Fecci PE. T-cell dysfunction in glioblastoma: applying a new framework. *Clin Cancer Res.* (2018) 24:3792–802. doi: 10.1158/1078-0432.CCR-18-0047
7. Ma Q, Long W, Xing C, Chu J, Luo M, Wang HY, et al. Cancer stem cells and immunosuppressive microenvironment in glioma. *Front Immunol.* (2018) 9:2924. doi: 10.3389/fimmu.2018.02924
8. Huang Z, Cheng L, Guryanova OA, Wu Q, Bao S. Cancer stem cells in glioblastoma–molecular signaling and therapeutic targeting. *Protein Cell.* (2010) 1:638–55. doi: 10.1007/s13238-010-0078-y

9. Li D, Hu Y, Jin Z, Zhai Y, Tan Y, Sun Y, et al. TanCAR T cells targeting CD19 and CD133 efficiently eliminate MLL leukemic cells. *Leukemia.* (2018) 32:2012–6. doi: 10.1038/s41375-018-0212-z
10. Yu K, Hu Y, Wu F, Guo Q, Qian Z, Hu W, et al. Surveying brain tumor heterogeneity by single-cell RNA sequencing of multi-sector biopsies. *Natl Sci Rev.* (2020) 7:1306–18. doi: 10.1093/nsr/nwaa099
11. Crespo J, Sun H, Welling TH, Tian Z, Zou W. T cell anergy, exhaustion, senescence, and stemness in the tumor microenvironment. *Curr Opin Immunol.* (2013) 25:214–21. doi: 10.1016/j.coi.2012.12.003
12. Wu A, Wei J, Kong LY, Wang Y, Priebe W, Qiao W, et al. Glioma cancer stem cells induce immunosuppressive macrophages/microglia. *Neuro Oncol.* (2010) 12:1113–25. doi: 10.1093/neuonc/noq082
13. Alvarado AG, Thiagarajan PS, Mulkearns-Hubert EE, Silver DJ, Hale JS, Alban TJ, et al. Glioblastoma cancer stem cells evade innate immune suppression of self-renewal through reduced TLR4 expression. *Cell Stem Cell.* (2017) 20:450–61 e454. doi: 10.1016/j.stem.2016.12.001
14. Papalexi E, Satija R. Single-cell RNA sequencing to explore immune cell heterogeneity. *Nat Rev Immunol.* (2018) 18:35–45. doi: 10.1038/nri.2017.76
15. Zheng C, Zheng L, Yoo JK, Guo H, Zhang Y, Guo X, et al. Landscape of infiltrating T cells in liver cancer revealed by single-cell sequencing. *Cell.* (2017) 169:1342–56 e1316. doi: 10.1016/j.cell.2017.05.035
16. Silver DJ, Sinyuk M, Vogelbaum MA, Ahluwalia MS, Lathia JD. The intersection of cancer, cancer stem cells, and the immune system: therapeutic opportunities. *Neuro Oncol.* (2016) 18:153–9. doi: 10.1093/neuonc/nov157
17. Nduom EK, Weller M, Heimberger AB. Immunosuppressive mechanisms in glioblastoma. *Neuro Oncol.* (2015) 17:vii9–14. doi: 10.1093/neuonc/nov151

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Glioblastoma Immune Landscape and the Potential of New Immunotherapies

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Glioblastoma (GBM) are the most common tumors of the central nervous system and among the deadliest cancers in adults. GBM overall survival has not improved over the last decade despite optimization of therapeutic standard-of-care. While immune checkpoint inhibitors (ICI) have revolutionized cancer care, they unfortunately have little therapeutic success in GBM. Here, we elaborate on normal brain and GBM-associated immune landscapes. We describe the role of microglia and tumor-associated macrophages (TAMs) in immune suppression and highlight the impact of energy metabolism in immune evasion. We also describe the challenges and opportunities of immunotherapies in GBM and discuss new avenues based on harnessing the anti-tumor activity of myeloid cells, vaccines, chimeric antigen receptors (CAR)-T and -NK cells, oncolytic viruses, nanocarriers, and combination therapies.

Keywords: glioblastoma, immune response, macrophage, immunotherapy, CART-T cell

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PREFACE

The adult human brain is a tissue of vast complexity, composed of multiple cell types defined by their location, function, or molecular characteristics. Five main classes of cerebral cells have been described: neurons, astrocytes, oligodendrocytes, endothelial cells, and microglia. Interactions among these cell types orchestrate the structure and function of the brain in electrical signaling, axonal ensheathing, regulation of blood flow, metabolic coupling and immune surveillance. For instance, astrocytes which are key effectors of the brain's energy metabolism, convert glucose into lactate, which is delivered to neurons and retro-converted into pyruvate to fuel the Krebs cycle (1). The neurovascular unit (NVU), which encompasses the blood-brain barrier (BBB), is a functional physiological unit that regulates the blood/cerebral parenchyma interface. It is composed of endothelial cells, smooth muscle cells, pericytes, astrocytes, microglia and neurons. The NVU governs brain homeostasis, controlling cerebral perfusion and protecting from potential pathogens or toxins present in the blood. The NVU is significantly altered in CNS malignancy, especially in glioblastoma (GBM), which are grade IV malignant glioma that are highly vascularized with dense tortuous and leaky blood vessels, permitting massive immune cell infiltration in the tumor core. GBMs are mainly derived from neural stem cells, differentiating into astrocytic or neuronal lineages. This cancer is one of the deadliest types in humans, with an average survival time of <15 months upon diagnosis.

Even with the standard-of-care treatment, consisting of surgical resection when possible, followed by radiation and chemotherapy with the drug Temozolomide (TMZ), the estimated recurrence rate is more than 90%. Recurrence is mostly caused by the regrowth of highly invasive cells that spread out of the tumor core, partially due to its hypoxic and acidic environment (2), and are therefore not removed by surgical resection. The long-standing assumption that GBM tumors were clonal masses with identical molecular characteristics have recently been challenged. Indeed, tumor single cell transcriptomics have identified several GBM cellular states with notable plasticity modulated by the tumor microenvironment (3, 4).

IMMUNE MECHANISMS OF THE HEALTHY CENTRAL NERVOUS SYSTEM (CNS)

Prior to delving into the immune landscape and immunosuppressive mechanisms of GBM, we briefly overview the architecture of the CNS immune system under physiological conditions, highlighting its unique lymphatic drainage system, immune cell populations and leukocyte trafficking (**Figure 1A**). Anatomically, the brain parenchyma is surrounded by the meninges, a series of three membranes under the skull, namely the dura mater, the arachnoid membrane and the pia mater (**Figure 1B**). The brain bathes in cerebrospinal fluid (CSF), generated at the blood-CSF barrier, by epithelial cells of the choroid plexus, through diffusion, pinocytosis and active transport from arterial blood in fenestrated capillaries (**Figure 1C**). The CSF flows around the brain four ventricles into the subarachnoid space (SAS) in a unidirectional flux through the action of cilia on the choroid plexus and ependymal cells that line the ventricles. It enters the brain parenchyma through aquaporin 4, water channels on the end-feet of astrocytes surrounding the vasculature, and communicates with the interstitial fluid (ISF) through the glymphatic system, a network of perivascular channels formed by astroglia for waste elimination (5). The CSF is reabsorbed by the venous blood in venous sinuses at arachnoid villi. Such turnover occurs three to twelve times daily suggesting that the CSF is an immunologically active fluid. Indeed, the CSF drains trafficking leukocytes to the deep cervical lymph nodes (DCLNs) via the newly discovered meningeal lymphatic vessels in the dura mater (6, 7), or by channeling along cranial nerves through the cribriform plate to the nasal

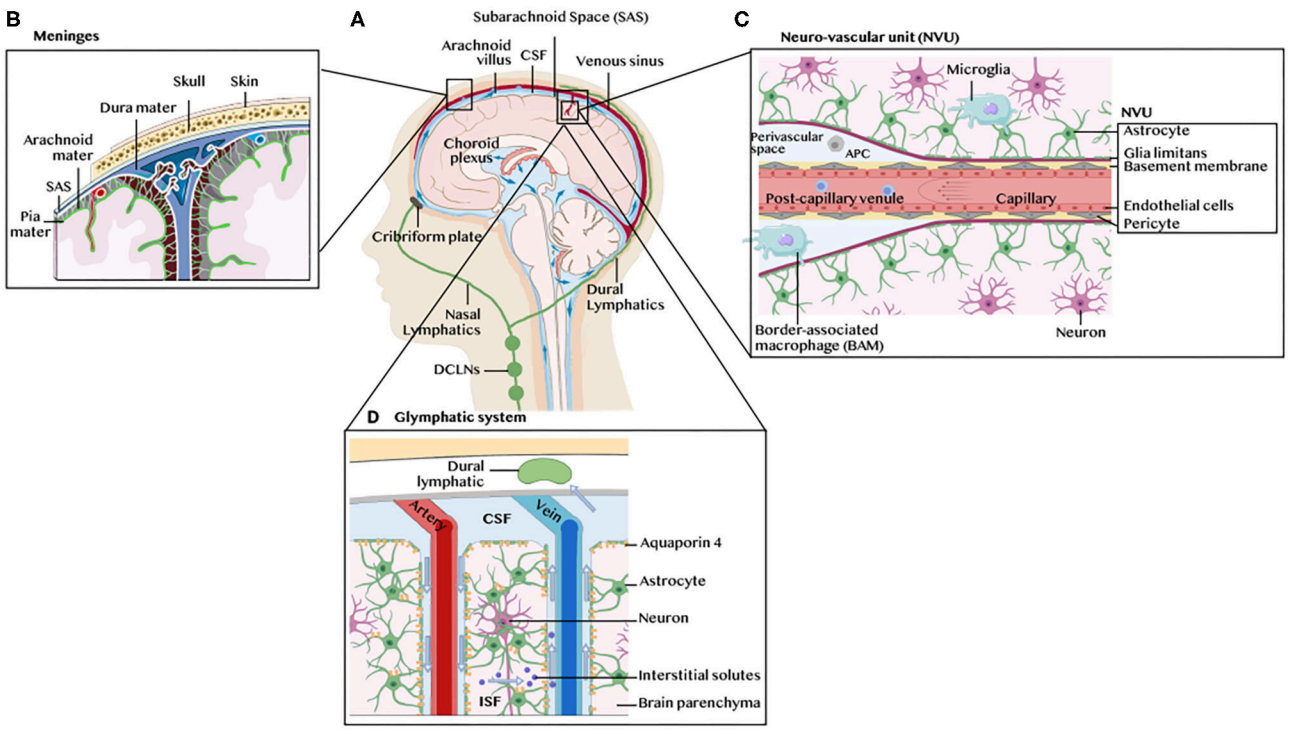

FIGURE 1 | Architecture of the CNS immune system. **(A)** Schematic illustration of the human brain anatomy namely the brain parenchyma, choroid plexus, ventricles, cerebrospinal fluid (CSF), meninges, dural and nasal lymphatics and the deep cervical lymph nodes (DCLNs). **(B)** The meninges. These are three membranes that envelop the brain, namely the dura mater, the arachnoid membrane and the pia mater. **(C)** The neurovascular unit (NVU), blood brain barrier (BBB) and perivascular space. The glia limitans formed by astrocytes end feet ensheath the capillary basement membrane and its pericytes. The perivascular space contains microglia-like perivascular macrophages also dubbed border-associated macrophages (BAMs) and antigen-presenting cells (APC). Microglia are found in the brain parenchyma. **(D)** The glymphatic system. The CSF enters the brain parenchyma through aquaporin 4, water channels on the end-feet of astrocytes surrounding the vasculature, and communicates with the interstitial fluid (ISF) carrying solutes and small antigens through the glymphatic system, a network of perivascular channels formed by astroglia for waste elimination.

mucosa where it accesses its afferent lymphatics. The ISF, which carries parenchymal solutes and small soluble antigens but not parenchymal immune cells, reaches the DCLNs by channeling along the tight space of the basement membrane lining the walls of cerebral capillaries and arteries. The blood supply of the brain enters through capillaries and post-capillary venules, that push the pia mater in the SAS to form perivascular spaces (Virchow-Robin spaces). The brain vasculature is ensheathed by the BBB (**Figure 1D**) formed by endothelial cells connected by complex tight junctions and pericytes in the capillary basement membrane, and surrounded by the pia mater, the subpial space and the glia limitans, a thin membrane barrier at the parenchymal basement membrane formed by astrocyte foot processes.

The CNS has long been considered as a site of immune privilege. This was based on earlier findings that transplanted tissue grafts in the brain parenchyma elicit slow adaptive immune responses and are not readily rejected (8), and on the presumed lack of lymphatic vessels. Further, a paucity of innate immune responses to pathogen- or danger-associated molecular patterns (PAMPs and DAMPs) has been reported (9, 10). However, mounting evidence challenge this notion and demonstrate active immunosurveillance in the healthy CNS (11). Together with the discovery of a dural meningeal lymphatic system (6, 7), several studies have shown that unlike the brain parenchyma, the cerebral ventricles elicit immune responses leading to graft rejection (12, 13). Thus, the CNS exhibits compartment-specific immunity regulated by leukocyte entry across endothelial, epithelial and glial cell layers of the blood-brain and blood-CSF barriers. These barriers segregate the parenchyma from the peripheral immune system at steady state while permitting immune communications in the CSF-filled SAS and ventricular space. Such compartmentalization is also reflected by spatially and functionally diverse resident immune cell subsets.

The recent use of high-dimensional single cell approaches [e.g., mass cytometry and single cell RNA sequencing (scRNAseq)] in mice (14) and humans (15), along with intravascular leukocyte tracking and fate mapping systems in reporter mice, has uncovered diverse resident immune cells in the healthy CNS and mapped their localization to different CNS compartments. Microglia, which are derived from a yolk sac progenitor, are found exclusively in the brain parenchyma. A distinct subset of embryonically-derived microglia-like macrophages line the meninges, the choroid plexus and the perivascular spaces, and are dubbed border-associated macrophages (BAMs). Microglia and BAMs make up the bulk of the healthy CNS immune cells accounting for ∼80% and ∼10% of all CNS steady state leukocytes, respectively. Blood-derived monocytes ($Ly6C^{hi}$ and $Ly6C^{lo}$), monocyte-derived cells (MdCs), dendritic cells (DCs) and neutrophils are also present in the healthy CNS, albeit at lower frequencies (<3%) (14). T and B cells, innate lymphocytes (ILCs), natural killer (NK), NKT, eosinophils and mast cells are rare (<1%) but also found at steady state. While microglia and BAMs share several surface markers ($CD45^{lo}$ $CD11b^{lo}$ $F4/80^{+}$ $CD64^{+}$ $MeTK^{+}$ $Cx3CR1^{+}$), they differ in the expression of SIGLEC-H, which is typically found on microglia but not on BAMs. In contrast, the latter express CD206, CD38 and CD88. Both subsets potentially act as antigen-presenting cells (APCs), as they can upregulate, in a context-dependent fashion, the expression of CD11c, MHCII and co-stimulatory molecules. For instance, microglia of the white matter express higher levels of MHCII, CD68 and HLA-DR compared to gray matter microglia, and upregulate pro-inflammatory cytokines such as SPP1 (osteopontin) with age (15). There is little evidence that microglia and BAMs migrate to the periphery to prime T cells. Instead they are thought to maintain tissue homeostasis and to locally re-stimulate T cells. On the other hand, brain DCs traffic to the DCLNs using one of two routes: a specific route involving the rostral migratory stream (16), olfactory bulb, cribriform plate, and nasal mucosal lymphatics or via the dural lymphatics (**Figure 1A**). At steady state, DC trafficking contributes to CNS immune tolerance by inducing regulatory T cells (T_{reg}). Endothelial cells of the meningeal lymphatic vessels are also presumed to maintain brain antigens-reactive T cells in an anergic state (7). Efferent T cells reach the CNS through the choroid plexus or subarachnoid veins and extravasate into the CSF-filled ventricular space and SAS. In the absence of antigen encounter, T cells are eliminated from the CNS by apoptosis or CSF drainage. Cognate antigen recognition on perivascular or leptomeningeal APCs is required for activated T cells to cross the glia limitans into the parenchyma. T cell activation in the brain is often detrimental leading to neuroinflammation and tissue damage. However, this is not always the case, as T cells can mediate neuroprotective effects in response to CNS injury (17).

GLIOBLASTOMA (GBM) SUBTYPES AND THEIR ASSOCIATED IMMUNE LANDSCAPES

In 2007, the WHO graded CNS tumors based on histological criteria (grade II-IV) (18). In 2010, Verhaak et al. used an unsupervised gene expression analysis of 200 GBM and two normal brain samples to identify four GBM subtypes based on molecular signatures (**Table 1**). These were referred to as neural (NE), proneural (PN), classical (CL) and mesenchymal (MES) (19). The NE subtype, in which the normal brain samples clustered, was characterized by the expression of neuronal gene markers, and was later shown by the same team to be non-tumor specific (20). The PN subtype, associated with the best median patient survival, had two genomic features, *PDGFRA* alterations and point mutations in *IDH1*, and was characterized by elevated expression of oligodendrocytic and pro-neural development genes. The CL subtype had high rates of *EGFR* gene amplification co-occurring with aberrations in the RB pathway. It exhibited high expression of neural precursors and stem cell markers, and elevated expression of effectors of the Notch and sonic hedgehog pathways. The MES subtype, linked to the least favorable outcome, had predominant *NF1* gene aberrations and *PTEN* mutations. As its name implies, it included an epithelial-to-mesenchymal signature indicative of de-differentiated/trans-differentiated tumors. It also had the highest inflammatory signature with a notable upregulation of

TABLE 1 | GBM molecular classification and associated immune phenotypes.

Classifier		Neural	Proneural	Classical	Mesenchymal
Genetics[a]		Expression of neuron markers such as NEFL, GABRA1, SYT1 and SLC12A5 Association with GO categories linked to the neuron projection and axon and synaptic transmission	PDGFRA mutations, especially in the Ig-domain Point mutation in IDH1 associated with higher CpG island methylation Focal amplification of the locus at 4q12 harboring PDGFRA High level of PDGFRA expression TP53 mutation Loss of heterozygosity Chromosome 7 amplification paired to loss of chromosome 10 only in 50% of the cases High expression of oligodendrocytic development genes Expression of proneural development genes	Chromosome 7 amplification paired with chromosome 10 loss High level of EGFR amplification High level of EGFR alterations Lack of TP53 mutations Focal 9p21.3 homozygous deletion, targeting CDKN2A High expression of neural precursors and stem cell markers	Focal hemizygous deletion of a region at 17q11.2 Low expression of NF1 Co-mutations of NF1 and PTEN Expression of mesenchymal markers (CHI3L1, CD44, MERKT, YKL40 and MET) High expression of genes implicated in the NFKB and tumor necrosis factor super family pathways (TRADD, RELB, TNFRSF1A) High expression of microglial markers such as CD68 and PTPRC
Immune cell Infiltrates[b]	Tumor core	Macrophages (CD163)	Macrophages (CD163)	Macrophages (CD163) +	Macrophages (CD163) + + +
	Tumor edge	Microglia (CD68) ++	Microglia (CD68)	Microglia (CD68) +	Microglia (CD68) + + +
	Perivascular area	CD4 T cells ++ CD8 T cells	CD4 T cells CD8 T cells	CD4 T cells + CD8 T cells	CD4 T cells + + + CD8 T cells
Immune markers[c,d]		PD-1	PD-1	IL-12, PD-1	Galectin 3, IL-10, IL-23, TGFβ, PD-L1, CD163, CCR2, CCL-22, CD47, CSF-1, MIC-1, IL-6, CTLA-4, Arginase, CD204, IL1, IL-15, IL-7, CD278, IDO
Re-classification[e]		≪ Healthy brain ≫	Combination of OPC-and NPC-like	AC-like	MES-like
Associated gene mutation with the re-classification [e]			PDGFRA and CDK4 mutations, respectively	EGFR mutation	NF1 mutation

[a]Verhaak RG et al. (19). Cancer Cell 17: 98-110. [b]Martinez-Lage M et al. (28). Acta Neuropathol Commun 7: 203. [c]Doucette T et al. (29). Cancer Immunol Res 1: 112-122. [d]Wang Q et al. (20). Cancer Cell 32: 42-56. [e]Neftel C et al. (4). Cell 178:835-849.

genes in the TNF and NF-κB pathways. Several studies from the Cancer Genome Atlas (TCGA) project subsequently defined a core of recurrent driver genomic alterations in GBM, involving *TP53*, *RB1*, *NF1*, *PDGFRA*, *EGFR*, *PTEN*, and *CTNND2* (21–24). Genetic alterations in *IDH1* or *IDH2*, *TERT*, and co-deletion of chromosome arms 1p and 19q (1p/19q codel) were rather found in low grade gliomas (LGG; grades II-III) (23, 25). In 2016, the WHO reclassified CNS tumors to integrate molecular information to the diagnosis criteria (26). This classification divided adult gliomas into three groups: (1) oligodendrogliomas, which harbor IDH mutations and 1p/19q codel, (2) astrocytomas, which are IDH mutant but without the 1p/19q codel, and (3) GBM, which are mostly IDH wild-type (WT) (**Figure 2**). It also introduced histone 3 K27M mutation as a molecular feature of pediatric diffuse midline glioma (27). More recent integration of results from scRNAseq, *in vivo* single cell lineage tracing and genomic and transcriptomic analyses from TCGA refined the GBM subtypes by identifying four plastic GBM cellular states. These were characterized by six transcriptomic meta-modules and genetic alterations in *EGFR*, *PDGFRA*, *CDK4,* and *NF1* (4). Two meta-modules enriched in mesenchymal genes, including hypoxia and glycolysis genes, were referred to as MES1 and MES2, and corresponded to the TCGA-MES subtype in Verhaak et al. (19). An astrocytes-like (AC) module was consistent with the TCGA-CL, and three additional modules referred to as oligodendrocyte progenitor cells-like (OPC) and neural progenitor cells-like (NPC)1 and NPC2, corresponded to the TCGA-PN sub-type (**Table 1**). Neftel et al. showed, using patient-derived xenografts (PDX) in mice, that tumor cells were able to transit from one cellular state to another, indicative of a plasticity that was modulated by the tumor microenvironment (4).

The immune landscape of the GBM subtypes was initially explored by transcriptomics (19, 20, 29). These studies confirmed that the MES subtype exhibited elevated expression of pro-inflammatory mediators together with immunosuppressive factors and immune checkpoints (**Table 1**). CIBERSORT

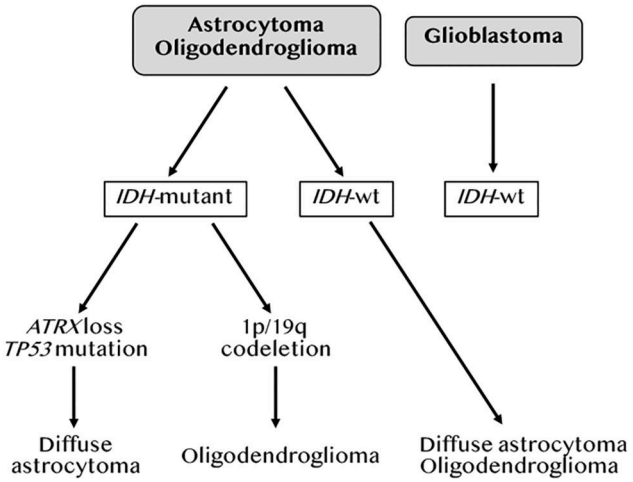

FIGURE 2 | Molecular classification of gliomas. Adapted from the 2016 WHO classification of brain tumors by DeWitt JC et al. (26).

analysis (30) revealed more TAMs, neutrophils and $CD4^+$ T cells expression signatures in MES, whereas an activated DCs signature was found in CL (20). Analysis of a separate glioma classification system based on IDH1 mutation status and DNA methylation (31) similarly revealed elevated TAMs and neutrophils signatures in one subgroup of IDH1 wild-type (WT) tumors, that was of the MES profile (20). To reassess these findings at the protein level, Martinez-Lage et al. used an automated immunohistochemistry-based analysis of tissue microarray (TMA) from a cohort of 98 patients to define the immune cell counts in each GBM subtype. Microglia and blood-derived TAMs were the most prevalent cells in all four GBM subtypes, but were highest (>80% of all leukocytes) in the MES subtype. Whereas, $CD8^+$ T cell frequencies were similar in all groups, the MES subtype had slightly more $CD4^+$ T cells (∼1%) (28).

Alternative stratification of GBM based on consensus immunome clusters (CIC) identified two immunologically active GBM clusters (32). These clusters expressed genes associated with cytotoxic T lymphocyte (CTLs) and NK cell activation, such as granzyme B (*GZMB*) and interferon gamma (*IFNG*), and genes linked to feedback inhibitory mechanisms including *FOXP3*, immune checkpoint inhibitors (*CTLA-4*, *PD-1*, *TIM3*, *VISTA*) and their ligands e.g. *PD-L1* and galectin-9 (32). Nevertheless, these CICs did not discriminate patients with respect to survival outcome, potentially due to the low frequencies of CTLs and NKs and the strong immunosuppressive environment mediated by the myeloid compartment. Indeed, GBM tumor-infiltrating lymphocytes (TILs) display an exhausted phenotype (33), and GBM-infiltrating NK cells express reduced levels of activating receptors e.g., NKp30, NKG2D, and DNAX accessory molecule-1 (DNAM-1) (32).

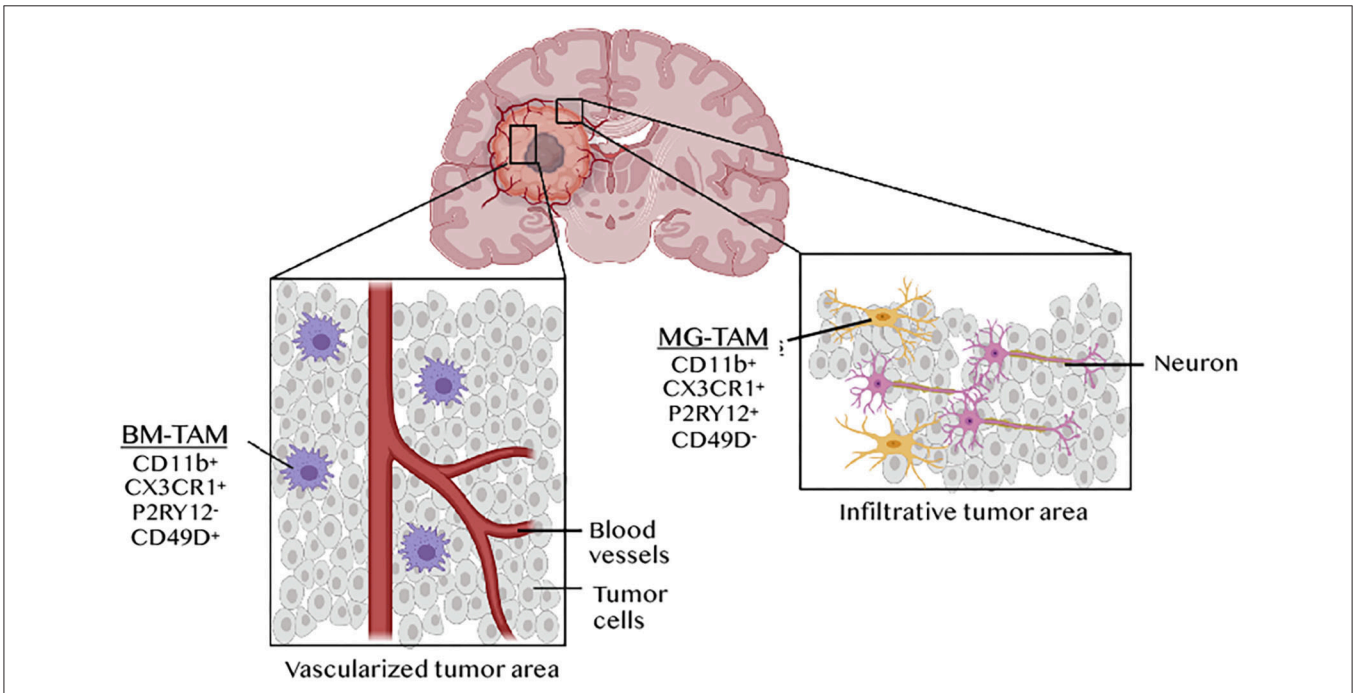

FIGURE 3 | TAM ontogeny and tumor geography in GBM. Tumor-associated macrophages (TAMs) in GBM originates from microglia (MG-TAMs) or from bone marrow-derived monocytes differentiating into macrophages upon recruitment (BM-TAMs). These can be distinguished based on the differential expression of the integrin CD49D on BM-TAMs and of the purinergic receptor P2RY12 on MG-TAMs. BM-TAMs that infiltrate into the tumor core are smaller and less branched than MG-TAMs that are found in the peri-tumoral area.

GBM-ASSOCIATED MYELOID CELLS DIVERSITY, ONTOGENY AND TUMOR GEOGRAPHY

Myeloid cells are key determinants of tumor progression and patient outcome in several cancers (34), and are being actively pursued as targets of new immunotherapies (35, 36). The predominance and diversity of myeloid cells in GBM has warranted extensive analysis of their phenotypes and functions in this cancer. This is critical for discriminate therapy, as general targeting of macrophages with inhibition of colony stimulating factor 1 receptor (CSF1R) failed to enhance overall survival in recurrent GBM (37). The use of lineage tracing systems in glioma mouse models revealed distinct GBM-associated myeloid cell ontogeny, i.e., TAMs derived from microglia (MG-TAMs) or from hematopoietic stem cells in the bone marrow (BM-TAMs) (38). RNAseq analysis of these subsets highlighted the impact of ontogeny-imposed chromatin states and tumor cues on their functions in tumor growth and response to therapy. For instance, differential resistance to the anti-angiogenesis therapy bevacizumab was reported to be mediated by BM-TAMs (39). ATAC-seq and transcription factor (TF) landscape analysis identified TFs linked to microglia identity [e.g., MEF2 (40)] in MG-TAMs, whereas BM-TAMs were enriched in TFs involved in monocyte to macrophage differentiation, i.e. RUNX, CEBP, PU.1, IRF4 and STAT3. Notably, a RUNX-induced gene, integrin subunit alpha 4 (*Itga4*, also known as Cd49d) was identified as a distinguishing cell surface marker between the two TAM subsets in both mice and humans. It is expressed on BM-TAMs but epigenetically suppressed in microglia and MG-TAMs. Further analysis, using three different scRNAseq platforms, uncovered 66 core genes that distinguish the two TAM lineages (41). CX3CR1, which is commonly used to isolate microglia in mice, is not specific to microglia, since monocytes upregulate its expression as they differentiate in tissues. Instead, the purinergic receptor P2RY12 has recently emerged as a new microglia marker. MG-TAMs are therefore $CD11b^+$ $CX3CR1^+$ $P2RY12^+CD49D^-$ whereas BM-TAMs are $CD11b^+$ $CX3CR1^+$ $P2RY12^-$ $CD49D^+$ (41) (**Figure 3**). Both TAM subsets display a "non-canonical" state, expressing both M1 and M2 markers. However, BM-TAMs exhibit higher expression of immunosuppressive cytokines and effectors of oxidative metabolism, characteristic of the M2 phenotype (41). Collectively, while several studies confirm a critical role of BM-TAMs in GBM, MG-TAMs are not mere bystanders. A recent report, exploring the efficacy and targets of the phagocytosis checkpoint inhibitor anti-CD47, demonstrated that MG-TAMs are important effectors of glioma cell phagocytosis contributing to overall survival of glioma-bearing mice (42).

RNAseq analysis of distinct anatomically defined tumor regions (e.g., leading edge, infiltrating region, necrotic zone, blood vessels etc.) and *in situ* hybridization for *BIN1* (an MG-TAM marker) or *TGFBI* (a BM-TAM marker), revealed tumor geographic variation in TAM composition. BM-TAMs were enriched near the blood vessels whereas MG-TAMs were found in infiltrated white matter (41). This was confirmed in a glioma model using the $Cx3cr1^{GFP}$;$Ccr2^{RFP}$ reporter mouse, which showed that BM-TAMs, which constituted 85% of the total TAM population, localized in the perivascular areas of the tumor core, whereas MG-TAMs accounting for 15% of all TAMs, were restricted to the peritumoral area (43) (**Figure 3**). Besides differential gene expression profiles, these two TAM subsets have different morphological and migratory characteristics, as shown by 2-photon microscopy. MG-TAMs are stationary, larger in size and more branched than BMDM-TAMs that are highly mobile and smaller (44). Clinically, BMDM-TAM infiltration correlates with poor patient survival (28, 41).

TAM RECRUITMENT AND IMMUNOSUPPRESSIVE MECHANISMS IN GBM

Interleukin (IL)-6, produced by vascular endothelial cells and TAMs, has been implicated in several pro-tumoral processes in GBM: (1) it contributes to the disruption of the BBB by downregulating intercellular tight junction proteins on endothelial cells (45). Concordantly, endothelial cell-specific deletion of IL-6 prevented glioma growth and improved mouse survival (46); (2) it reinforces GBM metabolic dependence on aerobic glycolysis (47), as discussed below; and (3) it promotes the recruitment of macrophages through the induction of CCL5/CXCL5 and favors their alternative activation through PPARγ/HIF-2α signaling (46). The CCL2-CCR2 pathway is equally important for BM-TAM recruitment. Glioma cells instruct this pathway through indoleamine 2,3-deoxygenase (IDO)-dependent production of kynurenine (KYN), a metabolite that triggers CCR2 upregulation through aryl hydrocarbon receptor (AHR). Myeloid-specific deletion of AHR in mice blunted BM-TAMs glioma infiltration. In humans, the KYN-AHR pathway is upregulated in GBM and is associated with an unfavorable outcome (48). A direct correlate has been established between loss of *PTEN* and BM-TAM recruitment via lysyl oxidase (LOX), a macrophage chemoattractant that signals through the β1 integrin (ITGB1)-PYK2 pathway. Concordantly, YAP1, LOX and β1 integrin are elevated in GBM, and are associated with reduced overall survival. LOX-elicited TAMs infiltrate the tumor microenvironment and support glioma growth via SPP1 (osteopontin), which inhibits glioma cell apoptosis, promotes angiogenesis and sustains the TAM tolerogenic phenotype by signaling through the Integrin αvβ5 (49, 50).

GBM and other brain tumors are notorious for eliciting local and systemic immunosuppression, mediated in great part by TAMs. TAM-derived TGFβ was initially considered as a key inducer of systemic immune tolerance (51). However, targeting this immunosuppressive cytokine alone did not impact the survival of mice bearing brain tumors (52), implicating additional mechanisms. The expression of PD-L1 on circulating monocytes and BM-TAMs might similarly trigger systemic immunosuppression, through a feed forward mechanism involving IL-10 (53). Beyond soluble immunosuppressive cytokines, direct cell-cell contacts, e.g., through PD-L1 (54), tolerogenic HLA molecules (55) and the apoptosis-inducing

TABLE 2 | Clinical trials of immunotherapies for GBM.

Identifier	Study title	Interventions	Number expected to be enrolled	Primary completion
Phase III clinical trials				
NCT04277221	ADCTA for adjuvant immunotherapy in standard treatment of recurrent glioblastoma multiforme (GBM)	Biological: Autologous dendritic cell/tumor antigen, ADCTA	118	December 31, 2022
NCT03548571	Dendritic cell immunotherapy against cancer stem cells in glioblastoma patients receiving standard therapy	Biological: Dendritic cell immunization Drug: Adjuvant temozolomide	60	May 1, 2021
NCT02667587	An investigational immuno-therapy study of temozolomide plus radiation therapy with nivolumab or placebo, for newly diagnosed patients with glioblastoma (GBM, a malignant brain cancer)	Drug: Nivolumab Drug: temozolomide Radiation: Radiotherapy Other: Nivolumab Placebo	693	February 11, 2022
NCT02617589	An investigational immuno-therapy study of nivolumab compared to temozolomide, each given with radiation therapy, for newly-diagnosed patients with glioblastoma (GBM, a malignant brain cancer)	Drug: Nivolumab Drug: Temozolomide Radiation: Radiotherapy	550	January 17, 2019
Phase II clinical trials				
NCT04145115	A study testing the effect of immunotherapy (ipilimumab and nivolumab) in patients with recurrent glioblastoma with elevated mutational burden	Biological: Ipilimumab Biological: Nivolumab	37	May 31, 2023
NCT02649582	Adjuvant dendritic cell-immunotherapy plus temozolomide in glioblastoma patients	Biological: Dendritic cell vaccine plus temozolomide chemotherapy	20	December 2020
NCT03927222	Immunotherapy targeted against cytomegalovirus in patients with newly-diagnosed WHO grade IV unmethylated glioma	Biological: Human CMV pp65-LAMP mRNA-pulsed autologous DCs containing GM CSF Drug: Temozolomide Biological: Tetanus-Diphtheria Toxoid (Td) (and 2 more...)	48	December 2023
NCT03916757	V-Boost immunotherapy in glioblastoma multiforme brain cancer	Biological: V-Boost	20	April 15, 2020
NCT03650257	A large-scale research for immunotherapy of glioblastoma with autologous heat shock protein gp96	Biological: gp96 Drug: Temozolomide radiation: Radiotherapy	150	August 20, 2021
NCT03548571	Dendritic cell immunotherapy against cancer stem cells in glioblastoma patients receiving standard therapy	Biological: Dendritic cell immunization Drug: Adjuvant temozolomide	60	May 1, 2021
NCT04013672	Study of pembrolizumab plus SurVaxM for glioblastoma at first recurrence	Drug: Pembrolizumab Drug: SurVaxM Drug: Sargramostim Drug: Montanide ISA 51	51	December 31, 2020
NCT01567202	Study of DC vaccination against glioblastoma	Procedure: Surgery Drug: Chemotherapy Radiation: Radiotherapy (and 2 more...)	100	December 1, 2019
NCT02799238	Autologous lymphoid effector cells specific against tumor (ALECSAT) as add on to standard of care in patients with glioblastoma	Biological: ALECSAT Radiation: Radiotherapy Drug: Temozolomide	62	June 2020
NCT02799238	Cediranib maleate and olaparib compared to bevacizumab in treating patients with recurrent glioblastoma	Biological: Bevacizumab Drug: Cediranib Drug: Cediranib maleate Drug: Olaparib	70	May 31, 2020
NCT02337686	Pembrolizumab in treating patients with recurrent glioblastoma	Other: Laboratory Biomarker Analysis Biological: Pembrolizumab Other: Pharmacological study Procedure: Therapeutic Conventional Surgery	20	December 31, 2020
NCT01174121	Immunotherapy using tumor infiltrating lymphocytes for patients with metastatic cancer	Biological: Young TIL Drug: Aldesleukin Drug: Cyclophosphamide (and 2 more...)	332	December 29, 2023

(Continued)

TABLE 2 | Continued

Identifier	Study title	Interventions	Number expected to be enrolled	Primary completion
NCT04225039	Anti-GITR/Anti-PD1/Stereotactic radiosurgery, in recurrent glioblastoma	Drug: INCMGA00012 Drug: INCAGN01876 Drug: SRS Procedure: Brain surgery	32	February 2025
NCT04049669	Pediatric trial of indoximod with chemotherapy and radiation for relapsed brain tumors or newly diagnosed DIPG	Drug: Indoximod Radiation: Partial Radiation Radiation: Full-dose Radiation (and 4 more...)	140	October 2, 2024
NCT03491683	INO-5401 and INO-9012 delivered by electroporation (EP) in COMBINATION WITH cemiplimab (REGN2810) in newly-diagnosed glioblastoma (GBM)	Biological: INO-5401 Biological: INO-9012 Biological: Cemiplimab (and 2 more...)	52	January 18, 2021
NCT03047473	Avelumab in patients with newly diagnosed glioblastoma multiforme	Biological: Avelumab	30	September 2022
NCT03174197	Atezolizumab in combination with temozolomide and radiation therapy in treating patients with newly diagnosed glioblastoma	Drug: Atezolizumab Radiation: Radiation therapy Drug: Temozolomide	60	June 30, 2020
NCT03395587	Efficiency of vaccination with lysate-loaded dendritic cells in patients with newly diagnosed glioblastoma	Biological: Autologous, tumor lysate-loaded, mature dendritic cells (DC) Drug: Standard therapy	136	September 6, 2022
NCT03158389	NCT neuro master match–N²M² (NOA-20)	Drug: APG101 Drug: Alectinib Drug: Idasanutlin (and 4 more...)	350	September 30, 2023
NCT03532295	INCMGA00012 and epacadostat in combination with radiation and bevacizumab in patients with recurrent gliomas	Drug: Epacadostat Drug: Bevacizumab Radiation: Radiation therapy Procedure: Peripheral blood draw	55	April 30, 2023
NCT03866109	A phase I/IIa study evaluating temferon in patients with glioblastoma & unmethylated MGMT	Drug: Temferon	21	December 2022
NCT03899857	Pembrolizumab for newly diagnosed glioblastoma	Drug: Pembrolizumab	56	December 2022
NCT01204684	Dendritic cell vaccine for patients with brain tumors	Biological: Autologous tumor lysate-pulsed DC vaccination Biological: Tumor lysate-pulsed DC vaccination+0.2% resiquimod Biological: Tumor-lysate pulsed DC vaccination +adjuvant polyICLC	60	January 31, 2021
NCT02968940	Avelumab with hypofractionated radiation therapy in adults with isocitrate dehydrogenase (IDH) mutant glioblastoma	Biological: Avelumab Radiation: Hypofractionated radiation therapy (HFRT)	43	April 2020
NCT02336165	Phase 2 Study of Durvalumab (MEDI4736) in Patients With Glioblastoma	Drug: Durvalumab Radiation: Standard radiotherapy Biological: Bevacizumab	159	November 2018
NCT04102436	Non-viral TCR gene therapy	Drug: Fludarabine Drug: Cyclophosphamide Drug: aldesleukin Biological: Sleeping Beauty Transposed PBL	210	December 31, 2028
NCT03412877	Administration of autologous T-cells genetically engineered to express T-cell receptors reactive against mutated neoantigens in people with metastatic cancer	Drug: Cyclophosphamide Drug: Fludarabine Drug: Aldesleukin (and 2 more...)	270	March 23, 2027
NCT02794883	Tremelimumab and durvalumab in combination or alone in treating patients with recurrent malignant glioma	Biological: Durvalumab Other: Laboratory Biomarker Analysis Procedure: Surgical Procedure Biological: Tremelimumab	36	December 2019

(Continued)

TABLE 2 | Continued

Identifier	Study title	Interventions	Number expected to be enrolled	Primary completion
NCT03382977	Study to evaluate safety, tolerability, and optimal dose of candidate GBM vaccine VBI-1901 in recurrent GBM subjects	Biological: VBI-1901	38	October 2020
NCT03382977	Study to evaluate safety, tolerability, and optimal dose of candidate GBM vaccine VBI-1901 in recurrent GBM subjects	Biological: DNX-2401 Biological: Pembrolizumab	49	December 2020
Phase I clinical trials				
NCT02649582	Adjuvant dendritic cell-immunotherapy plus temozolomide in glioblastoma patients	Biological: Dendritic cell vaccine plus temozolomide chemotherapy	20	December 2020
NCT04165941	Novel gamma-delta γδ T cell therapy for treatment of patients with newly diagnosed glioblastoma	Biological: DRI cell therapy	12	January 2022
NCT03961971	Trial of anti-tim-3 in combination with anti-PD-1 and SRS in recurrent GBM	Drug: MBG453	15	February 2022
NCT03426891	Pembrolizumab and vorinostat combined with temozolomide for newly diagnosed glioblastoma	Drug: Pembrolizumab Drug: Vorinostat Drug: Temozolomide Radiation: Radiotherapy	32	April 2021
NCT02208362	Genetically modified T-cells in treating patients with recurrent or refractory malignant glioma	Biological: IL13Rα2-specific, hinge-optimized, 41BB-costimulatory CAR/truncated CD19-expressing Autologous T lymphocytes Other: Laboratory biomarker analysis Other: Quality-of-life assessment (and 5 more...)	92	May 2020
NCT04323046	Immunotherapy (nivolumab and ipilimumab) before and after surgery for the treatment of recurrent or progressive high grade glioma in children and young adults	Biological: Ipilimumab Biological: Nivolumab Drug: Placebo Administration (and 2 more...)	45	March 1, 2022
NCT04047706	Nivolumab, BMS-986205, and radiation therapy with or without temozolomide in treating patients with newly diagnosed glioblastoma	Biological: IDO1 inhibitor BMS-986205 Biological: nivolumab Radiation: Radiation Therapy Drug: Temozolomide	30	June 9, 2022
NCT04201873	Pembrolizumab and a vaccine (ATL-DC) for the treatment of surgically accessible recurrent glioblastoma	Biological: Dendritic cell tumor cell lysate vaccine Biological: Pembrolizumab Other: Placebo Administration Drug: Poly ICLC	40	August 1, 2024
NCT04003649	IL13Ralpha2-targeted chimeric antigen receptor (CAR) T cells with or without nivolumab and ipilimumab in treating patients with recurrent or refractory glioblastoma	Biological: IL13Ralpha2-specific Hinge-optimized 4-1BB-co-stimulatory CAR/Truncated CD19-expressing autologous TN/MEM cells Biological: Ipilimumab Biological: Nivolumab (and 2 more...)	60	January 22, 2022
NCT03714334	DNX-2440 oncolytic adenovirus for recurrent glioblastoma	Drug: DNX-2440 injection	24	April 16, 2022
NCT02852655	A pilot surgical trial to evaluate early immunologic pharmacodynamic parameters for The PD-1 checkpoint inhibitor, pembrolizumab (MK-3475), in patients with surgically accessible recurrent/progressive glioblastoma	Drug: MK-3475	35	March 28, 2018
NCT04270461	NKG2D-based CAR T-cells immunotherapy for patient with r/r NKG2DL+ solid tumors	Biological: NKG2D-based CAR T-cells	10	December 1, 2022
NCT03491683	INO-5401 and INO-9012 delivered by electroporation (EP) in combination with cemiplimab (REGN2810) in newly-diagnosed glioblastoma (GBM)	Biological: INO-5401 Biological: INO-9012 Biological: Cemiplimab (and 2 more...)	52	January 18, 2021
NCT03174197	Atezolizumab in Combination with temozolomide and radiation therapy in treating patients with newly diagnosed glioblastoma	Drug: Atezolizumab Radiation: Radiation Therapy Drug: Temozolomide	60	June 30, 2020

(Continued)

TABLE 2 | Continued

Identifier	Study title	Interventions	Number expected to be enrolled	Primary completion
NCT03389230	Memory-enriched T cells in treating patients with recurrent or refractory grade III-IV glioma	Biological: CD19CAR-CD28-CD3zeta-EGFRt-expressing Tcm-enriched T-lymphocytes Biological: CD19CAR-CD28-CD3zeta-EGFRt-expressing Tn/mem-enriched T-lymphocytes Other: Laboratory Biomarker Analysis Procedure: Leukapheresis	42	June 14, 2021
NCT03344250	Phase I EGFR BATs in newly diagnosed glioblastoma	Drug: EGFR BATs with TMZ following SOC RT/TMZ Drug: Weekly EGFR BATs following SOC RT/TMZ	18	October 1, 2020
NCT03158389	NCT neuro master match–N²M² (NOA-20)	Drug: APG101 Drug: Alectinib Drug: Idasanutlin (and 4 more...)	350	September 30, 2023
NCT03866109	A phase I/IIa study evaluating temferon in patients with glioblastoma & unmethylated MGMT	Drug: temFeron	21	December 2022
NCT03392545	Combination of immunization and radiotherapy for malignant gliomas (InSituVac1)	Combination product: Combined immune adjuvants and radiation	30	April 1, 2020
NCT03341806	Avelumab with laser interstitial therapy for recurrent glioblastoma	Drug: Avelumab Combination Product: MRI-guided LITT therapy	30	September 2020
NCT02062827	Genetically engineered HSV-1 phase 1 study for the treatment of recurrent malignant glioma	Biological: M032 (NSC 733972)	36	September 2020
NCT03223103	Safety and immunogenicity of personalized genomic vaccine and tumor treating fields (TTFields) to treat glioblastoma	Drug: Poly-ICLC Device: Tumor Treating Fields Biological: Peptides	20	May 22, 2020
NCT02766699	A study to evaluate the safety, tolerability and immunogenicity of EGFR(V)-EDV-dox in subjects with recurrent glioblastoma multiforme (GBM)	Drug: EGFR(V)-EDV-Dox	20	December 2019
NCT03619239	Dose-escalation study to evaluate the safety and tolerability of GX-I7 in patients with glioblastoma	Drug: GX-I7	15	January 31, 2021
NCT02010606	Phase I study of a dendritic cell vaccine for patients with either newly diagnosed or recurrent glioblastoma	Biological: Dendritic cell vaccination, in addition to standard temozolomide chemotherapy and involved field radiation therapy Biological: Dendritic cell vaccination, with optional bevacizumab treatment for patients previously treated with bevacizumab	39	April 2020
NCT02502708	Study of the IDO Pathway inhibitor, indoximod, and temozolomide for pediatric patients with progressive primary malignant brain tumors	Drug: Indoximod Drug: Temozolomide Radiation: Conformal radiation (and 2 more...)	81	December 12, 2019
NCT03382977	Study to evaluate safety, tolerability, and optimal dose of candidate GBM vaccine VBI-1901 in recurrent GBM subjects	Biological: VBI-1901	38	October 2020
NCT03043391	Phase 1b study PVSRIPO for recurrent malignant glioma in children	Biological: Polio/Rhinovirus Recombinant (PVSRIPO)	12	July 1, 2020
NCT03576612	GMCI, nivolumab, and radiation therapy in treating patients with newly diagnosed high-grade gliomas	Biological: AdV-tk Drug: Valacyclovir Radiation: Radiation (and 3 more...)	36	February 28, 2021
NCT03657576	Trial of C134 in patients with recurrent GBM	Biological: C134	24	September 2022
NCT03152318	A study of the treatment of recurrent malignant glioma with rQNestin34.5v.2	Drug: rQNestin Drug: Cyclophosphamide Procedure: Stereotactic biopsy	108	July 2021

(Continued)

TABLE 2 | Continued

Identifier	Study title	Interventions	Number expected to be enrolled	Primary completion
NCT03911388	HSV G207 in children with recurrent or refractory cerebellar brain tumors	Biological: G207	15	September 1, 2022
NCT02457845	HSV G207 alone or with a single radiation dose in children with progressive or recurrent supratentorial brain tumors	Biological: G207	18	October 2020
NCT00634231	A phase I study of AdV-tk + prodrug therapy in combination with radiation therapy for pediatric brain tumors	Biological: AdV-tk Drug: Valacyclovir Radiation: Radiation	12	December 2015

receptor Fas (56) contribute to immune escape. A recent study reported a role of tumor-associated glycosylation in local and systemic immunosuppression (57). This was mediated through a direct interaction between O-linked glycans on glioma cells with their receptor, Macrophage Galactose-type Lectin (MGL), on TAMs leading to immunosuppression signaling. Of note, the current GBM standard of care often prescribes dexamethasone to alleviate cerebral edema. This immunosuppressive corticosteroid further contributes to the GBM immunosuppressive environment, interfering with anti-tumor immunity and presenting a challenge for the future of immunotherapies in this cancer.

METABOLIC REMODELING OF THE GBM TUMOR MICROENVIRONMENT

Hypoxia and necrosis are well-known features of GBM. HIF-1α, stabilized by the inhibition of prolyl hydroxylase (PHD) activity in hypoxia, is a transcription factor that modifies the expression of thousands of genes, notably effectors of glycolysis and lactic fermentation. The expression of glucose transporters (GLUT1), glycolytic enzymes (PDK1, Hexokinase or PKM2), and lactate dehydrogenase A (LDHA) help in replenishing NAD^+ to support the glycolytic process. Monocarboxylate transporter (MCT)4 expression is also increased following stabilization of HIF-1α, leading to passive release of lactate out of the cells (58). Production of H^+ happens during glycolysis, lactic fermentation, but also during respiration when CO2 is hydrated into $HCO3^-$ and H^+ ions by carbonic anhydrases (CAs). H^+ ions efflux from the cytoplasm via H^+ ATPases and Na^+/H^+ exchangers (NHEs) leads to a decrease in the extracellular pH_e. Tumor acidosis promotes cancer cell invasion through cytoskeletal remodeling, but also by modulating the activity of immune cells in the tumor microenvironment. For instance, LDHA-mediated production of lactic acid was shown to blunt the cytotoxic activity of CTLs and NK cells in melanoma through inhibition of NFAT expression (59). This supports previous findings demonstrating that lactate accumulation in T cells, due to decreased efflux via MCT1 (which controls lactate shuttling in a gradient dependent manner), blunted CTL activity (60). TAMs reinforce GBM metabolic shift to aerobic glycolysis through IL-6 that enhances the activity of phosphoglycerate kinase 1 (PGK1) by promoting its phosphorylation (47).

Glioma cells also display a high dependence on amino acid metabolism accompanied by an elevated uptake of branched chain amino acids (BCAA). Through the overexpression of branched chain amino acid transaminase 1 (BCAT1), glioma tumors excrete elevated levels of branched-chain ketoacids (BCKA) through MCT1. Which influx into TAMs and blunt their phagocytic activity (61). GBM TAMs were also shown to drive T cell dysfunction through elevated expression of the ectonucleosidase CD39 that, together with CD73, induces the production of the immunosuppressive metabolite adenosine (48).

THE FUTURE OF IMMUNOTHERAPIES IN GBM

Immune Checkpoint Inhibitors (ICI)

Immune checkpoint inhibitors (ICI) targeting the PD-1 or CTLA-4 pathways have revolutionized cancer therapy in the last decade. However, they have had little clinical benefit in GBM, at the least in the adjuvant setting. The recently published results of the open-label, randomized, phase 3 trial CheckMate-143, which evaluated nivolumab vs. bevacizumab in patients with recurrent GBM were disappointing, as there was no significant difference in median overall survival (mOS) between the two arms (62). The two ongoing phase 3 trials CheckMate-498 and CheckMate-548 evaluating the use of nivolumab in patients with newly-diagnosed GBM, either methylguanine methyltransferase (MGMT)-unmethylated or MGMT-methylated, also failed to meet their primary endpoints, according to an update by Bristol-Myers Squibb. In the neoadjuvant setting, the results are controversial. The anti-PD-1 nivolumab, administered as a neoadjuvant, did not impact patient survival in resectable GBM in a phase 2 clinical trial (63). In contrast, another study reported a survival benefit of the anti-PD-1 pembrolizumab in 35 patients with recurrent and resectable GBM (64). Collectively, the dismal results of ICI in GBM may be due to the poor immunogenicity of GBM tumors. In 2017, the FDA approved the use of the anti-PD-1 pembrolizumab in solid tumors with microsatellite instability high (MSI-H) or mismatch repair deficiency (dMMR) tumors. This year, it further approved the use of pembrolizumab for the treatment of adult and pediatric patients with non-resectable or metastatic tumor mutation burden-high (TMB-H) solid tumors. dMMR gliomas are rare (65), but earlier results from two case

reports showed a response to pembrolizumab in one pediatric (66) and one adult (67) patients. Despite these promising results, a recent study reported that PD-1 blockade did not impact mOS in hypermutated gliomas, consistent with an observed lack of TILs in these cancers (68). However, another study reported significant clinical and radiological responses of nivolumab in two young siblings with biallelic mismatch repair deficiency (66), suggesting that ICI therapy might benefit pediatric GBM with high mutational burden [e.g., with *MSH6* mutations (69)]. It is plausible that treatments that increase mutational burden might synergize with ICI, as has been shown in other cancers (70). Nanoscale immunoconjugates (NICs), which deliver ICIs, covalently attached on a natural biopolymer scaffold, across the BBB using transferrin receptor (TfR)-mediated transcytosis, or via angiopep-2 (AP-2)- LDLR-related protein 1 (LRP1), were shown to outperform free ICIs in increasing TILs and improving survival in a murine glioma model (71). However, this remains to be tested in patients. Alternative immunotherapies for GBM are being explored. These are primarily focused on vaccines, chimeric antigen receptors (CAR)-T cells, oncolytic viruses and strategies that harness the anti-tumor activity of myeloid cells or the use of adipose stem/stromal cells (ASC) and stromal vascular fraction (SVF) injected in the surgical cavity [reviewed in Bateman et al. (72)].

Vaccines

In the vaccine arena, three phase 3 clinical trials have been completed with different outcomes. ACT IV, a phase III trial evaluating Rindopepimut (also known as CDX-110), a 13-amino acid peptide vaccine targeting EGFRvIII, a constitutively active mutant form of EGFR expressed in ∼30% of GBM patients, in combination with TMZ was terminated for futility, as no significant difference in mOS was observed in patients with newly-diagnosed GBM (73). The failure of this approach might be due to heterogenous expression of EGFRvIII within the tumor or loss of its expression leading to clonal outgrowth of resistant cells. A second phase III trial that evaluated an autologous tumor lysate-pulsed DC vaccine (DCVax®-L) in combination with TMZ showed some clinical benefit, reporting longer progression free survival (PFS) and mOS in patients with recurrent GBM (74). However, this is a logistically complicated approach as it requires personalization, apheresis, and DC expansion prior to administration back into patients. A third phase III trial conducted in Japan using personalized peptide vaccination for HLA-24+ recurrent GBM did not meet the primary nor the secondary endpoints (75). More recently, two phase I/Ib trials reported beneficial effects of personalized peptide vaccines. The first, the Glioma Actively Personalized Vaccine Consortium (GAPVAC), employed two sets of personalized peptide vaccines designed according to patients tumor mutations, transcriptomic and immuno-peptidomic profiles, and showed that these vaccines were able to elicit sustained $CD8^+$ T cell and $CD4^+$ Th1 responses against neoantigens (76). The second, which employed a pool of synthetic long peptides mimicking neoantigens, also reported the generation of poly-functional neoantigen-specific $CD4^+$ T cells and $CD8^+$ T cells in the periphery and enhanced infiltration of TILs (77). Together, these trials indicate that vaccine approaches are feasible as they elicit anti-tumor immune responses but whether this will translate into clinical benefit, as a monotherapy, requires additional testing.

CAR-T and CAR-NK Cells

CAR-T cells are patients-derived T cells engineered to express a CAR, which consists of the antigen-recognition region of an antibody fused in tandem with the cytoplasmic domains of the T cell receptor chain CD3ζ and costimulatory receptors (e.g., CD28 and/or 4-1BB). Currently, approved CAR-T cells target CD19 in B cell malignancies. The challenges of this therapy include the identification of tumor-specific or tumor-associated antigens, especially important in solid tumors, circumventing antigen loss, and countering the exhaustion of transferred CAR-T cells, among others. Several trials and pre-clinical studies have been conducted using CAR-T cells in GBM. The first was a case report that used an IL13Rα2-CAR-T cells in one patient. The CAR-T was delivered through repeated infusions in the resected tumor cavity followed by infusions in the ventricular system. This regimen led to the regression of all cranial and spinal tumors accompanied by a notable immune activity in the CSF (78). A first-in-human study including 10 patients with recurrent GBM followed. This study evaluated EGFRvIII-CAR-T cells injected intravenously. While the CAR-T cells expanded in the blood and trafficked to the tumor, the antigen was lost in 5 out 7 patients and the tumor microenvironment exhibited elevated expression of inhibitory molecules and a high frequency of Treg cells (79). Improvement of CAR-T therapy requires the identification of a tumor-associated antigen expressed stably throughout tumor growth and with limited heterogeneity. Chondroitin sulfate proteoglycan 4 (CSPG4) was found to fit this criterion. It is highly expressed in 67% of GBM cells and is sustained by TNF derived from microglia. Intracranial delivery of CSPG4-CAR-T cells was effective *in vivo* in nude mice transplanted with CSPG4-expressing glioma cells or neurospheres (80). Transgenic expression of cytokines, such as IL-15, was also demonstrated as a mean to improve anti-glioma activity of CAR-T cells, as shown with IL13Rα2-CAR-T cells (81). Since the final CAR-T cell product is a mix of $CD4^+$ CAR-T and $CD8^+$ CAR-T cells, another mean to refine this approach is to characterize the T cell subset that mediates anti-tumor activity. Using orthotopic GBM mouse models and IL13Rα2-CAR-T cells, the $CD4^+$ CAR-T cell subset was found to be more effective than the $CD8^+$ CAR-T cells, which were rapidly exhausted (82). Co-expression of the IL-8 receptor, CXCR1/CXCR2, was found to enhance CAR-T cells trafficking and persistence in the tumor in a glioma mouse model (83). Engineering EGFRvIII-CAR-T cells to co-express a bispecific T-cell engager (BiTE) against wild-type EGFR was demonstrated to ameliorate this therapy by countering the heterogeneity of EGFRvIII expression (84). A CAR-engineered NK cell targeting both WT EGFR and EGFRvIII mutant, NK-92-EGFR-CAR, was similarly efficient in targeting and killing GBM cells in mice engrafted with patients' mesenchymal GBM stem cells (85). Additional CAR target antigens in GBM include B7-H3 (86, 87), HER2 (88–90) and EphA2 (91), as demonstrated in preclinical studies, and in a phase I dose escalation clinical trial using a HER2-CAR (92). Interestingly, generation of a

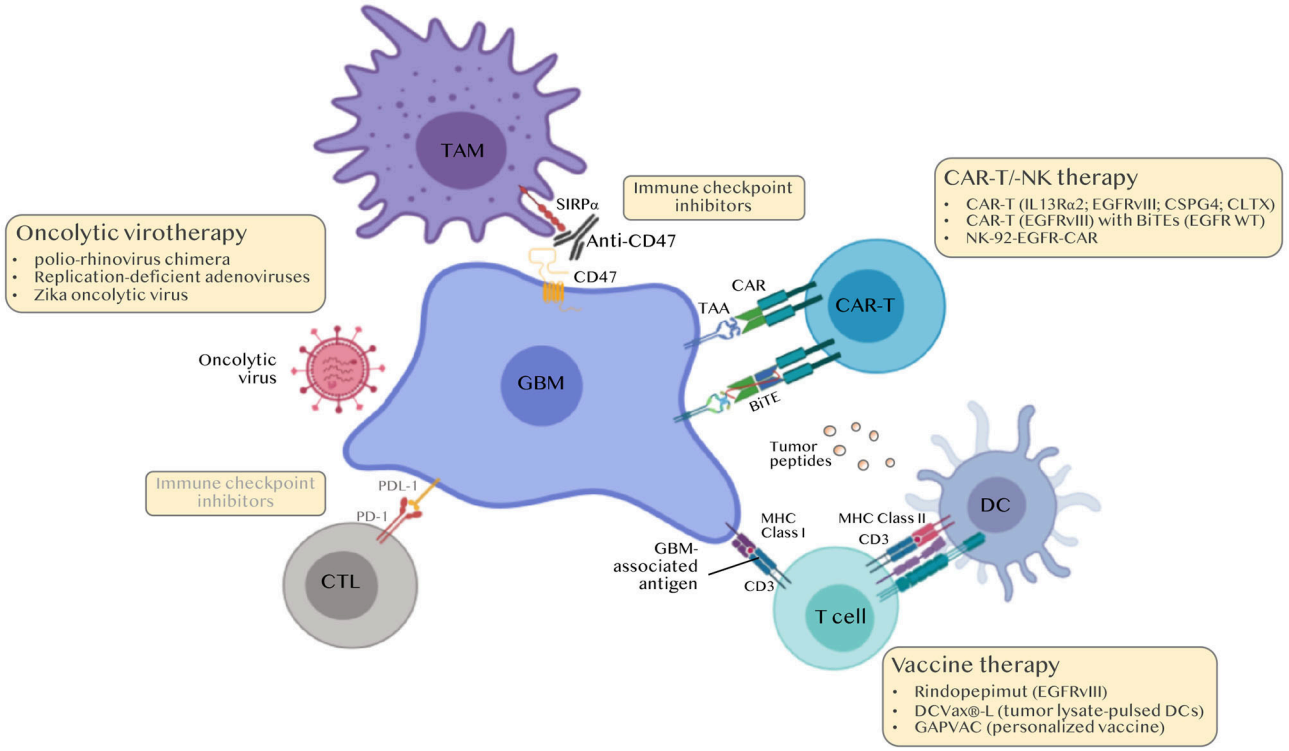

FIGURE 4 | Immunotherapies for the treatment of GBM. Classical immune checkpoint inhibitors (ICI) i.e., anti-PD-1/PDL-1 and anti-CTLA4 were ineffective in GBM. Current approaches include modulating TAMs (anti-CD47 to boost phagocytosis, nano-immunoconjugates to modulate TAM phenotype, aptamers to inhibit TAM recruitment), personalized peptide vaccines, chimeric antigen receptor (CAR)-T and CAR-NK cell approaches and oncolytic viruses. BiTEs, Bi-specific T-cell engagers.

tri-cistronic transgene encoding three CAR molecules against HER2, EphA2 and IL13Rα2, dubbed universal CAR-T (UCAR), was shown to overcome interpatient heterogeneity and target 100% of tumor cells (93). Another approach to overcome problems of tumor heterogeneity and antigen escape, is a new CAR design employing a toxin as the targeting entity was developed and tested in a murine model of glioma. This is based on GBM cells' affinity to bind chlorotoxin (CLTX) by matrix metalloproteinase-2. (CLTX)-CAR-T cells efficiently limited tumor growth in the absence of off-target effects (94).

Oncolytic Viruses

Oncolytic viruses (OV) constitute an interesting therapeutic approach in GBM, as besides their lytic activity, they might overcome GBM immunosuppression by stimulating innate immunity. Several types of OVs have been tested including replication-competent viruses such as polio and measles viruses, Herpes simplex viruses (HSV), adenoviruses and retroviruses. Notably, recombinant non-pathogenic polio-rhinovirus chimera (PVSRIPO), which binds the poliovirus receptor CD155 on cancer cells, was evaluated in 61 GBM patients via intra-tumoral injection and was effective in 21% patients who survived past 36 months (95). Replication-deficient adenoviruses, e.g., aglatimagene besadenovec, have also been used as vectors to deliver tumoricidal genes such as the HSV thymidine kinase that converts ganciclovir into a toxic nucleotide analog that poisons infected dividing cells. Two phase II clinical trials evaluated this Adv-tk viro-immunotherapy in GBM and reported improved PFS and OS (96, 97). An oncolytic HSV expressing E-cadherin, a ligand for the inhibitory NK receptor KLRG1, resulted in a better outcome in a glioma mouse model, by inhibiting NK cells and permitting viral spread (98). More recently, a Zika OV was shown to specifically target GBM stem cells (GSCs) rather than neural precursor cells, through a SOX2-Integrin αvβ5 Axis (99), suggesting a potentially superior anti-tumoral activity for brain tumor therapy. A triple combination of anti-CTLA-4, anti-PD-1 and a recombinant oncolytic HSV expressing mouse IL-12 (G47Δ-mIL12) cured most mice in two glioma models. $CD4^+$ T cells, $CD8^+$ T cells and M1 macrophages mediated this response, highlighting the need for combinatory approaches in future trials (100).

Macrophage-Based Immunotherapies

Additional promising strategies for GBM immunotherapy include harnessing the anti-tumor activity of myeloid and NK cells. Targeting the phagocytosis checkpoint CD47 using a humanized anti-CD47 antibody, Hu5F9-G4, has shown promise in a glioma PDX mouse model of five aggressive pediatric brain cancers (101). Furthermore, anti-CD47 in combination with TMZ was shown to enhance phagocytosis and promote cytotoxic $CD8^+$ T cell priming by stimulating antigen cross-presentation through cGAS-STING activation (102). Members of

the *Let-7* micro-RNA family have also been used as a therapeutic tool in a mouse glioma model; they boosted microglial anti-tumor activity by stimulating TLR7 (103). Alternatively, blocking TAM recruitment or polarization has also shown some efficacy in preclinical models. A 4-1BB–osteopontin (OPN) bi-specific aptamer for instance increased median survival by neutralizing macrophage infiltration while co-stimulating effector T cell activity (50). Di-mannose nanocarriers that bind the mannose receptor CD206 on M2 macrophages, used to deliver *in vitro*-transcribed mRNA encoding M1-polarizing transcription factors, were shown to reprogram TAMs and improve survival in different cancer models (ovarian, lung metastasis) including GBM (104).

Perspectives

There is a significant need to develop novel GBM immunotherapies. To date, more than 70 clinical trials with the terms GBM and immunotherapy are found in the clinicaltrials.gov webpage, of which 7 are phase III, 31 phase II and 37 phase I trials (**Table 2**). These trials explore the various strategies described above notably personalized vaccines, adoptive cell transfer therapy and combinations. It is our hope that this endeavor will soon impact patients' lives (**Figure 4**).

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REFERENCES

1. Machler P, Wyss MT, Elsayed M, Stobart J, Gutierrez R, Von Faber-Castell A, et al. *In vivo* evidence for a lactate gradient from astrocytes to neurons. *Cell Metab*. (2016) 23:94–102. doi: 10.1016/j.cmet.2015.10.010
2. Daubon T, Leon C, Clarke K, Andrique L, Salabert L, Darbo E, et al. Deciphering the complex role of thrombospondin-1 in glioblastoma development. *Nat Commun*. (2019) 10:1146. doi: 10.1038/s41467-019-08480-y
3. Patel AP, Tirosh I, Trombetta JJ, Shalek AK, Gillespie SM, Wakimoto H, et al. Single-cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma. *Science*. (2014) 344:1396–401. doi: 10.1126/science.1254257
4. Neftel C, Laffy J, Filbin MG, Hara T, Shore ME, Rahme GJ, et al. An integrative model of cellular states, plasticity, and genetics for glioblastoma. *Cell*. (2019) 178:835–49 e821. doi: 10.1016/j.cell.2019.06.024
5. Iliff JJ, Wang M, Liao Y, Plogg BA, Peng W, Gundersen GA, et al. A paravascular pathway facilitates CSF flow through the brain parenchyma and the clearance of interstitial solutes, including amyloid beta. *Sci Transl Med*. (2012) 4:147ra111. doi: 10.1126/scitranslmed.3003748
6. Aspelund A, Antila S, Proulx ST, Karlsen TV, Karaman S, Detmar M, et al. A dural lymphatic vascular system that drains brain interstitial fluid and macromolecules. *J Exp Med*. (2015) 212:991–9. doi: 10.1084/jem.20142290
7. Louveau A, Harris TH, Kipnis J. Revisiting the mechanisms of CNS immune privilege. *Trends Immunol*. (2015) 36:569–77. doi: 10.1016/j.it.2015.08.006
8. Medawar PB. Immunity to homologous grafted skin; the fate of skin homografts transplanted to the brain, to subcutaneous tissue, and to the anterior chamber of the eye. *Br J Exp Pathol*. (1948) 29:58–69.
9. Andersson PB, Perry VH, Gordon S. The acute inflammatory response to lipopolysaccharide in CNS parenchyma differs from that in other body tissues. *Neuroscience*. (1992) 48:169–86. doi: 10.1016/0306-4522(92)90347-5
10. Locatelli G, Wortge S, Buch T, Ingold B, Frommer F, Sobottka B, et al. Primary oligodendrocyte death does not elicit anti-CNS immunity. *Nat Neurosci*. (2012) 15:543–50. doi: 10.1038/nn.3062
11. Ransohoff RM, Brown MA. Innate immunity in the central nervous system. *J Clin Invest*. (2012) 122:1164–71. doi: 10.1172/JCI58644
12. Mason DW, Charlton HM, Jones AJ, Lavy CB, Puklavec M, Simmonds SJ. The fate of allogeneic and xenogeneic neuronal tissue transplanted into the third ventricle of rodents. *Neuroscience*. (1986) 19:685–94. doi: 10.1016/0306-4522(86)90292-7
13. Nicholas MK, Antel JP, Stefansson K, Arnason BG. Rejection of fetal neocortical neural transplants by H-2 incompatible mice. *J Immunol*. (1987) 139:2275–83.
14. Mrdjen D, Pavlovic A, Hartmann FJ, Schreiner B, Utz SG, Leung BP, et al. High-dimensional single-cell mapping of central nervous system immune cells reveals distinct myeloid subsets in health, aging, and disease. *Immunity*. (2018) 48:599. doi: 10.1016/j.immuni.2018.01.011
15. Sankowski R, Bottcher C, Masuda T, Geirsdottir L, Sagar Sindram E, Seredenina T, et al. Mapping microglia states in the human brain through the integration of high-dimensional techniques. *Nat Neurosci*. (2019) 22:2098–110. doi: 10.1038/s41593-019-0532-y
16. Mohammad MG, Tsai VW, Ruitenberg MJ, Hassanpour M, Li H, Hart PH, et al. Immune cell trafficking from the brain maintains CNS immune tolerance. *J Clin Invest*. (2014) 124:1228–41. doi: 10.1172/JCI71544
17. Walsh JT, Hendrix S, Boato F, Smirnov I, Zheng J, Lukens JR, et al. MHCII-independent CD4+ T cells protect injured CNS neurons via IL-4. *J Clin Invest*. (2015) 125:2547. doi: 10.1172/JCI76210
18. Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, Burger PC, Jouvet A, et al. The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathol*. (2007) 114:97–109. doi: 10.1007/s00401-007-0243-4
19. Verhaak RG, Hoadley KA, Purdom E, Wang V, Qi Y, Wilkerson MD, et al. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell*. (2010) 17:98–110. doi: 10.1016/j.ccr.2009.12.020
20. Wang Q, Hu B, Hu X, Kim H, Squatrito M, Scarpace L, et al. Tumor evolution of glioma-intrinsic gene expression subtypes associates with immunological changes in the microenvironment. *Cancer Cell*. (2017) 32:42–56 e46. doi: 10.1016/j.ccell.2017.06.003
21. Brennan CW, Verhaak RG, Mckenna A, Campos B, Noushmehr H, Salama SR, et al. The somatic genomic landscape of glioblastoma. *Cell*. (2013) 155:462–77. doi: 10.1016/j.cell.2013.09.034
22. Frattini V, Trifonov V, Chan JM, Castano A, Lia M, Abate F, et al. The integrated landscape of driver genomic alterations in glioblastoma. *Nat Genet*. (2013) 45:1141–9. doi: 10.1038/ng.2734
23. Eckel-Passow JE, Lachance DH, Molinaro AM, Walsh KM, Decker PA, Sicotte H, et al. Glioma groups based on 1p/19q, IDH, and TERT promoter mutations in tumors. *N Engl J Med*. (2015) 372:2499–508. doi: 10.1056/NEJMoa1407279
24. Suzuki H, Aoki K, Chiba K, Sato Y, Shiozawa Y, Shiraishi Y, et al. Mutational landscape and clonal architecture in grade II and III gliomas. *Nat Genet*. (2015) 47:458–68. doi: 10.1038/ng.3273
25. Cancer Genome Atlas Research N, Brat DJ, Verhaak RG, Aldape KD, Yung WK, Salama SR, et al. Comprehensive, integrative genomic analysis of diffuse lower-grade gliomas. *N Engl J Med*. (2015) 372:2481–98. doi: 10.1056/NEJMoa1402121
26. Dewitt JC, Mock A, Louis DN. The 2016 WHO classification of central nervous system tumors: what neurologists need to know. *Curr Opin Neurol*. (2017) 30:643–9. doi: 10.1097/WCO.0000000000000490

27. Khuong-Quang DA, Buczkowicz P, Rakopoulos P, Liu XY, Fontebasso AM, Bouffet E, et al. K27M mutation in histone H3.3 defines clinically and biologically distinct subgroups of pediatric diffuse intrinsic pontine gliomas. *Acta Neuropathol*. (2012) 124:439–47. doi: 10.1007/s00401-012-0998-0
28. Martinez-Lage M, Lynch TM, Bi Y, Cocito C, Way GP, Pal S, et al. Immune landscapes associated with different glioblastoma molecular subtypes. *Acta Neuropathol Commun*. (2019) 7:203. doi: 10.1186/s40478-019-0803-6
29. Doucette T, Rao G, Rao A, Shen L, Aldape K, Wei J, et al. Immune heterogeneity of glioblastoma subtypes: extrapolation from the cancer genome atlas. *Cancer Immunol Res*. (2013) 1:112–22. doi: 10.1158/2326-6066.CIR-13-0028
30. Newman AM, Liu CL, Green MR, Gentles AJ, Feng W, Xu Y, et al. Robust enumeration of cell subsets from tissue expression profiles. *Nat Methods*. (2015) 12:453–7. doi: 10.1038/nmeth.3337
31. Ceccarelli M, Barthel FP, Malta TM, Sabedot TS, Salama SR, Murray BA, et al. Molecular profiling reveals biologically discrete subsets and pathways of progression in diffuse glioma. *Cell*. (2016) 164:550–63. doi: 10.1016/j.cell.2015.12.028
32. Close HJ, Stead LF, Nsengimana J, Reilly KA, Droop A, Wurdak H, et al. Expression profiling of single cells and patient cohorts identifies multiple immunosuppressive pathways and an altered NK cell phenotype in glioblastoma. *Clin Exp Immunol*. (2020) 200:33–44. doi: 10.1111/cei.13403
33. Mohme M, Schliffke S, Maire CL, Runger A, Glau L, Mende KC, et al. Immunophenotyping of newly diagnosed and recurrent glioblastoma defines distinct immune exhaustion profiles in peripheral and tumor-infiltrating lymphocytes. *Clin Cancer Res*. (2018) 24:4187–200. doi: 10.1158/1078-0432.CCR-17-2617
34. Denardo DG, Ruffell B. Macrophages as regulators of tumour immunity and immunotherapy. *Nat Rev Immunol*. (2019) 19:369–82. doi: 10.1038/s41577-019-0127-6
35. Cassetta L, Pollard JW. Targeting macrophages: therapeutic approaches in cancer. *Nat Rev Drug Discov*. (2018) 17:887–904. doi: 10.1038/nrd.2018.169
36. Feng M, Jiang W, Kim BYS, Zhang CC, Fu YX, Weissman IL. Phagocytosis checkpoints as new targets for cancer immunotherapy. *Nat Rev Cancer*. (2019) 19:568–86. doi: 10.1038/s41568-019-0183-z
37. Butowski N, Colman H, De Groot JF, Omuro AM, Nayak L, Wen PY, et al. Orally administered colony stimulating factor 1 receptor inhibitor PLX3397 in recurrent glioblastoma: an Ivy Foundation Early Phase Clinical Trials Consortium phase II study. *Neuro Oncol*. (2016) 18:557–64. doi: 10.1093/neuonc/nov245
38. Bowman RL, Klemm F, Akkari L, Pyonteck SM, Sevenich L, Quail DF, et al. Macrophage ontogeny underlies differences in tumor-specific education in brain malignancies. *Cell Rep*. (2016) 17:2445–59. doi: 10.1016/j.celrep.2016.10.052
39. Castro BA, Flanigan P, Jahangiri A, Hoffman D, Chen W, Kuang R, et al. Macrophage migration inhibitory factor downregulation: a novel mechanism of resistance to anti-angiogenic therapy. *Oncogene*. (2017) 36:3749–59. doi: 10.1038/onc.2017.1
40. Lavin Y, Winter D, Blecher-Gonen R, David E, Keren-Shaul H, Merad M, et al. Tissue-resident macrophage enhancer landscapes are shaped by the local microenvironment. *Cell*. (2014) 159:1312–26. doi: 10.1016/j.cell.2014.11.018
41. Muller S, Kohanbash G, Liu SJ, Alvarado B, Carrera D, Bhaduri A, et al. Single-cell profiling of human gliomas reveals macrophage ontogeny as a basis for regional differences in macrophage activation in the tumor microenvironment. *Genome Biol*. (2017) 18:234. doi: 10.1186/s13059-017-1362-4
42. Hutter G, Theruvath J, Graef CM, Zhang M, Schoen MK, Manz EM, et al. Microglia are effector cells of CD47-SIRPalpha antiphagocytic axis disruption against glioblastoma. *Proc Natl Acad Sci USA*. (2019) 116:997–1006. doi: 10.1073/pnas.1721434116
43. Chen Z, Feng X, Herting CJ, Garcia VA, Nie K, Pong WW, et al. Cellular and molecular identity of tumor-associated macrophages in glioblastoma. *Cancer Res*. (2017) 77:2266–78. doi: 10.1158/0008-5472.CAN-16-2310
44. Chen Z, Ross JL, Hambardzumyan D. Intravital 2-photon imaging reveals distinct morphology and infiltrative properties of glioblastoma-associated macrophages. *Proc Natl Acad Sci USA*. (2019) 116:14254–9. doi: 10.1073/pnas.1902366116
45. Couto M, Coelho-Santos V, Santos L, Fontes-Ribeiro C, Silva AP, Gomes CMF. The interplay between glioblastoma and microglia cells leads to endothelial cell monolayer dysfunction via the interleukin-6-induced JAK2/STAT3 pathway. *J Cell Physiol*. (2019) 234:19750–60. doi: 10.1002/jcp.28575
46. Wang Q, He Z, Huang M, Liu T, Wang Y, Xu H, et al. Vascular niche IL-6 induces alternative macrophage activation in glioblastoma through HIF-2alpha. *Nat Commun*. (2018) 9:559. doi: 10.1038/s41467-018-03050-0
47. Zhang Y, Yu G, Chu H, Wang X, Xiong L, Cai G, et al. Macrophage-associated PGK1 phosphorylation promotes aerobic glycolysis and tumorigenesis. *Mol Cell*. (2018) 71:201–15 e207. doi: 10.1016/j.molcel.2018.06.023
48. Takenaka MC, Gabriely G, Rothhammer V, Mascanfroni ID, Wheeler MA, Chao CC, et al. Control of tumor-associated macrophages and T cells in glioblastoma via AHR and CD39. *Nat Neurosci*. (2019) 22:729–40. doi: 10.1038/s41593-019-0370-y
49. Chen P, Zhao D, Li J, Liang X, Li J, Chang A, et al. Symbiotic macrophage-glioma cell interactions reveal synthetic lethality in PTEN-Null glioma. *Cancer Cell*. (2019) 35:868–84 e866. doi: 10.1016/j.ccell.2019.05.003
50. Wei J, Marisetty A, Schrand B, Gabrusiewicz K, Hashimoto Y, Ott M, et al. Osteopontin mediates glioblastoma-associated macrophage infiltration and is a potential therapeutic target. *J Clin Invest*. (2019) 129:137–49. doi: 10.1172/JCI121266
51. Kuppner MC, Hamou MF, Sawamura Y, Bodmer S, De Tribolet N. Inhibition of lymphocyte function by glioblastoma-derived transforming growth factor beta 2. *J Neurosurg*. (1989) 71:211–7. doi: 10.3171/jns.1989.71.2.0211
52. Jackson CM, Kochel CM, Nirschl CJ, Durham NM, Ruzevick J, Alme A, et al. Systemic tolerance mediated by melanoma brain tumors is reversible by radiotherapy and vaccination. *Clin Cancer Res*. (2016) 22:1161–72. doi: 10.1158/1078-0432.CCR-15-1516
53. Bloch O, Crane CA, Kaur R, Safaee M, Rutkowski MJ, Parsa AT. Gliomas promote immunosuppression through induction of B7-H1 expression in tumor-associated macrophages. *Clin Cancer Res*. (2013) 19:3165–75. doi: 10.1158/1078-0432.CCR-12-3314
54. Parsa AT, Waldron JS, Panner A, Crane CA, Parney IF, Barry JJ, et al. Loss of tumor suppressor PTEN function increases B7-H1 expression and immunoresistance in glioma. *Nat Med*. (2007) 13:84–8. doi: 10.1038/nm1517
55. Wischhusen J, Friese MA, Mittelbronn M, Meyermann R, Weller M. HLA-E protects glioma cells from NKG2D-mediated immune responses *in vitro*: implications for immune escape *in vivo*. *J Neuropathol Exp Neurol*. (2005) 64:523–8. doi: 10.1093/jnen/64.6.523
56. Didenko VV, Ngo HN, Minchew C, Baskin DS. Apoptosis of T lymphocytes invading glioblastomas multiforme: a possible tumor defense mechanism. *J Neurosurg*. (2002) 96:580–4. doi: 10.3171/jns.2002.96.3.0580
57. Dusoswa SA, Verhoeff J, Abels E, Mendez-Huergo SP, Croci DO, Kuijper LH, et al. Glioblastomas exploit truncated O-linked glycans for local and distant immune modulation via the macrophage galactose-type lectin. *Proc Natl Acad Sci USA*. (2020) 117:3693–703. doi: 10.1073/pnas.1907921117
58. Lai SW, Lin HJ, Liu YS, Yang LY, Lu DY. Monocarboxylate transporter 4 regulates glioblastoma motility and monocyte binding ability. *Cancers*. (2020) 12:380. doi: 10.3390/cancers12020380
59. Brand A, Singer K, Koehl GE, Kolitzus M, Schoenhammer G, Thiel A, et al. LDHA-associated lactic acid production blunts tumor immunosurveillance by T and NK cells. *Cell Metab*. (2016) 24:657–71. doi: 10.1016/j.cmet.2016.08.011
60. Fischer K, Hoffmann P, Voelkl S, Meidenbauer N, Ammer J, Edinger M, et al. Inhibitory effect of tumor cell-derived lactic acid on human T cells. *Blood*. (2007) 109:3812–9. doi: 10.1182/blood-2006-07-035972
61. Silva LS, Poschet G, Nonnenmacher Y, Becker HM, Sapcariu S, Gaupel AC, et al. Branched-chain ketoacids secreted by glioblastoma cells via MCT1 modulate macrophage phenotype. *EMBO Rep*. (2017) 18:2172–85. doi: 10.15252/embr.201744154
62. Reardon DA, Brandes AA, Omuro A, Mulholland P, Lim M, Wick A, et al. Effect of nivolumab vs bevacizumab in patients with recurrent glioblastoma: the CheckMate 143 phase 3 randomized clinical trial. *JAMA Oncol*. (2020) 6:1003–10. doi: 10.1001/jamaoncol.2020.1024
63. Schalper KA, Rodriguez-Ruiz ME, Diez-Valle R, Lopez-Janeiro A, Porciuncula A, Idoate MA, et al. Neoadjuvant nivolumab modifies the

tumor immune microenvironment in resectable glioblastoma. *Nat Med*. (2019) 25:470–6. doi: 10.1038/s41591-018-0339-5
64. Cloughesy TF, Mochizuki AY, Orpilla JR, Hugo W, Lee AH, Davidson TB, et al. Neoadjuvant anti-PD-1 immunotherapy promotes a survival benefit with intratumoral and systemic immune responses in recurrent glioblastoma. *Nat Med*. (2019) 25:477–86. doi: 10.1038/s41591-018-0337-7
65. Maxwell JA, Johnson SP, Mclendon RE, Lister DW, Horne KS, Rasheed A, et al. Mismatch repair deficiency does not mediate clinical resistance to temozolomide in malignant glioma. *Clin Cancer Res*. (2008) 14:4859–68. doi: 10.1158/1078-0432.CCR-07-4807
66. Bouffet E, Larouche V, Campbell BB, Merico D, De Borja R, Aronson M, et al. Immune checkpoint inhibition for hypermutant glioblastoma multiforme resulting from germline biallelic mismatch repair deficiency. *J Clin Oncol*. (2016) 34:2206–11. doi: 10.1200/JCO.2016.66.6552
67. Johanns TM, Miller CA, Dorward IG, Tsien C, Chang E, Perry A, et al. Immunogenomics of hypermutated glioblastoma: a patient with germline POLE deficiency treated with checkpoint blockade immunotherapy. *Cancer Discov*. (2016) 6:1230–6. doi: 10.1158/2159-8290.CD-16-0575
68. Touat M, Li YY, Boynton AN, Spurr LF, Iorgulescu JB, Bohrson CL, et al. Mechanisms and therapeutic implications of hypermutation in gliomas. *Nature*. (2020) 580:517–23. doi: 10.1038/s41586-020-2209-9
69. Yang C, Austin F, Richard H, Idowu M, Williamson V, Sabato F, et al. Lynch syndrome-associated ultra-hypermutated pediatric glioblastoma mimicking a constitutional mismatch repair deficiency syndrome. *Cold Spring Harb Mol Case Stud*. (2019) 5:a003863. doi: 10.1101/mcs.a003863
70. Le DT, Durham JN, Smith KN, Wang H, Bartlett BR, Aulakh LK, et al. Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. *Science*. (2017) 357:409–13. doi: 10.1126/science.aan6733
71. Galstyan A, Markman JL, Shatalova ES, Chiechi A, Korman AJ, Patil R, et al. Blood-brain barrier permeable nano immunoconjugates induce local immune responses for glioma therapy. *Nat Commun*. (2019) 10:3850. doi: 10.1038/s41467-019-11719-3
72. Bateman ME, Strong AL, Gimble JM, Bunnell BA. Concise review: using fat to fight disease: a systematic review of nonhomologous adipose-derived stromal/stem cell therapies. *Stem Cells*. (2018) 36:1311–28. doi: 10.1002/stem.2847
73. Weller M, Butowski N, Tran DD, Recht LD, Lim M, Hirte H, et al. Rindopepimut with temozolomide for patients with newly diagnosed, EGFRvIII-expressing glioblastoma (ACT IV): a randomised, double-blind, international phase 3 trial. *Lancet Oncol*. (2017) 18:1373–85. doi: 10.1016/S1470-2045(17)30517-X
74. Liau LM, Ashkan K, Tran DD, Campian JL, Trusheim JE, Cobbs CS, et al. First results on survival from a large Phase 3 clinical trial of an autologous dendritic cell vaccine in newly diagnosed glioblastoma. *J Transl Med*. (2018) 16:142. doi: 10.1186/s12967-018-1552-1
75. Narita Y, Arakawa Y, Yamasaki F, Nishikawa R, Aoki T, Kanamori M, et al. A randomized, double-blind, phase III trial of personalized peptide vaccination for recurrent glioblastoma. *Neuro Oncol*. (2019) 21:348–59. doi: 10.1093/neuonc/noy200
76. Hilf N, Kuttruff-Coqui S, Frenzel K, Bukur V, Stevanovic S, Gouttefangeas C, et al. Actively personalized vaccination trial for newly diagnosed glioblastoma. *Nature*. (2019) 565:240–5. doi: 10.1038/s41586-018-0810-y
77. Keskin DB, Anandappa AJ, Sun J, Tirosh I, Mathewson ND, Li S, et al. Neoantigen vaccine generates intratumoral T cell responses in phase Ib glioblastoma trial. *Nature*. (2019) 565:234–9. doi: 10.1038/s41586-018-0792-9
78. Brown CE, Alizadeh D, Starr R, Weng L, Wagner JR, Naranjo A, et al. Regression of glioblastoma after chimeric antigen receptor T-cell therapy. *N Engl J Med*. (2016) 375:2561–9. doi: 10.1056/NEJMoa1610497
79. O'rourke DM, Nasrallah MP, Desai A, Melenhorst JJ, Mansfield K, Morrissette JJD, et al. A single dose of peripherally infused EGFRvIII-directed CAR T cells mediates antigen loss and induces adaptive resistance in patients with recurrent glioblastoma. *Sci Transl Med*. (2017) 9:984. doi: 10.1126/scitranslmed.aaa0984
80. Pellegatta S, Savoldo B, Di Ianni N, Corbetta C, Chen Y, Patane M, et al. Constitutive and TNFalpha-inducible expression of chondroitin sulfate proteoglycan 4 in glioblastoma and neurospheres: implications for CAR-T cell therapy. *Sci Transl Med*. (2018) 10:eaao2731. doi: 10.1126/scitranslmed.aao2731
81. Krenciute G, Prinzing BL, Yi Z, Wu MF, Liu H, Dotti G, et al. Transgenic expression of IL15 improves antiglioma activity of IL13Ralpha2-CAR T cells but results in antigen loss variants. *Cancer Immunol Res*. (2017) 5:571–81. doi: 10.1158/2326-6066.CIR-16-0376
82. Wang D, Aguilar B, Starr R, Alizadeh D, Brito A, Sarkissian A, et al. Glioblastoma-targeted CD4+ CAR T cells mediate superior antitumor activity. *JCI Insight*. (2018) 3. doi: 10.1172/jci.insight.99048
83. Jin L, Tao H, Karachi A, Long Y, Hou AY, Na M, et al. CXCR1- or CXCR2-modified CAR T cells co-opt IL-8 for maximal antitumor efficacy in solid tumors. *Nat Commun*. (2019) 10:4016. doi: 10.1038/s41467-019-11869-4
84. Choi BD, Yu X, Castano AP, Bouffard AA, Schmidts A, Larson RC, et al. CAR-T cells secreting BiTEs circumvent antigen escape without detectable toxicity. *Nat Biotechnol*. (2019) 37:1049–58. doi: 10.1038/s41587-019-0192-1
85. Han J, Chu J, Keung Chan W, Zhang J, Wang Y, Cohen JB, et al. CAR-engineered NK cells targeting wild-type EGFR and EGFRvIII enhance killing of glioblastoma and patient-derived glioblastoma stem cells. *Sci Rep*. (2015) 5:11483. doi: 10.1038/srep11483
86. Nehama D, Di Ianni N, Musio S, Du H, Patane M, Pollo B, et al. B7-H3-redirected chimeric antigen receptor T cells target glioblastoma and neurospheres. *EBioMedicine*. (2019) 47:33–43. doi: 10.1016/j.ebiom.2019.08.030
87. Tang X, Zhao S, Zhang Y, Wang Y, Zhang Z, Yang M, et al. B7-H3 as a novel CAR-T therapeutic target for glioblastoma. *Mol Ther Oncolytics*. (2019) 14:279–87. doi: 10.1016/j.omto.2019.07.002
88. Burger MC, Zhang C, Harter PN, Romanski A, Strassheimer F, Senft C, et al. CAR-engineered NK cells for the treatment of glioblastoma: turning innate effectors into precision tools for cancer immunotherapy. *Front Immunol*. (2019) 10:2683. doi: 10.3389/fimmu.2019.02683
89. Hegde M, Mukherjee M, Grada Z, Pignata A, Landi D, Navai SA, et al. Tandem CAR T cells targeting HER2 and IL13Ralpha2 mitigate tumor antigen escape. *J Clin Invest*. (2019) 129:3464. doi: 10.1172/JCI131246
90. Shen L, Li H, Bin S, Li P, Chen J, Gu H, et al. The efficacy of third generation antiHER2 chimeric antigen receptor T cells in combination with PD1 blockade against malignant glioblastoma cells. *Oncol Rep*. (2019) 42:1549–57. doi: 10.3892/or.2019.7263
91. Yi Z, Prinzing BL, Cao F, Gottschalk S, Krenciute G. Optimizing EphA2-CAR T cells for the adoptive immunotherapy of glioma. *Mol Ther Methods Clin Dev*. (2018) 9:70–80. doi: 10.1016/j.omtm.2018.01.009
92. Ahmed N, Brawley V, Hegde M, Bielamowicz K, Kalra M, Landi D, et al. HER2-specific chimeric antigen receptor-modified virus-specific T cells for progressive glioblastoma: a phase 1 dose-escalation trial. *JAMA Oncol*. (2017) 3:1094–101. doi: 10.1001/jamaoncol.2017.0184
93. Bielamowicz K, Fousek K, Byrd TT, Samaha H, Mukherjee M, Aware N, et al. Trivalent CAR T cells overcome interpatient antigenic variability in glioblastoma. *Neuro Oncol*. (2018) 20:506–18. doi: 10.1093/neuonc/nox182
94. Wang D, Starr R, Chang WC, Aguilar B, Alizadeh D, Wright SL, et al. Chlorotoxin-directed CAR T cells for specific and effective targeting of glioblastoma. *Sci Transl Med*. (2020) 12:eaaw2672. doi: 10.1126/scitranslmed.aaw2672
95. Desjardins A, Gromeier M, Herndon JE 2nd, Beaubier N, Bolognesi DP, Friedman AH, et al. Recurrent glioblastoma treated with recombinant poliovirus. *N Engl J Med*. (2018) 379:150–61. doi: 10.1056/NEJMoa1716435
96. Ji N, Weng D, Liu C, Gu Z, Chen S, Guo Y, et al. Adenovirus-mediated delivery of herpes simplex virus thymidine kinase administration improves outcome of recurrent high-grade glioma. *Oncotarget*. (2016) 7:4369–78. doi: 10.18632/oncotarget.6737
97. Wheeler LA, Manzanera AG, Bell SD, Cavaliere R, Mcgregor JM, Grecula JC, et al. Phase II multicenter study of gene-mediated cytotoxic immunotherapy as adjuvant to surgical resection for newly diagnosed malignant glioma. *Neuro Oncol*. (2016) 18:1137–45. doi: 10.1093/neuonc/now002
98. Xu B, Ma R, Russell L, Yoo JY, Han J, Cui H, et al. An oncolytic herpesvirus expressing E-cadherin improves survival in mouse models of glioblastoma. *Nat Biotechnol*. (2018) 37:45–54. doi: 10.1038/nbt.4302
99. Zhu Z, Mesci P, Bernatchez JA, Gimple RC, Wang X, Schafer ST, et al. Zika virus targets glioblastoma stem cells through a SOX2-Integrin alphavbeta5 Axis. *Cell Stem Cell*. (2020) 26:187–204 e110. doi: 10.1016/j.stem.2019.11.016

100. Saha D, Martuza RL, Rabkin SD. Curing glioblastoma: oncolytic HSV-IL12 and checkpoint blockade. *Oncoscience*. (2017) 4:67–9. doi: 10.18632/oncoscience.359
101. Gholamin S, Mitra SS, Feroze AH, Liu J, Kahn SA, Zhang M, et al. Disrupting the CD47-SIRPalpha anti-phagocytic axis by a humanized anti-CD47 antibody is an efficacious treatment for malignant pediatric brain tumors. *Sci Transl Med*. (2017) 9:eaaf2968. doi: 10.1126/scitranslmed.aaf2968
102. Von Roemeling CA, Wang Y, Qie Y, Yuan H, Zhao H, Liu X, et al. Therapeutic modulation of phagocytosis in glioblastoma can activate both innate and adaptive antitumour immunity. *Nat Commun*. (2020) 11:1508. doi: 10.1038/s41467-020-15129-8
103. Buonfiglioli A, Efe IE, Guneykaya D, Ivanov A, Huang Y, Orlowski E, et al. let-7 MicroRNAs regulate microglial function and suppress glioma growth through toll-like receptor 7. *Cell Rep*. (2019) 29:3460–71. e3467. doi: 10.1016/j.celrep.2019.11.029
104. Zhang F, Parayath NN, Ene CI, Stephan SB, Koehne AL, Coon ME, et al. Genetic programming of macrophages to perform anti-tumor functions using targeted mRNA nanocarriers. *Nat Commun*. (2019) 10:3974. doi: 10.1038/s41467-019-11911-5

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Immune Escape in Glioblastoma Multiforme and the Adaptation of Immunotherapies for Treatment

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Glioblastoma multiforme (GBM) is the most frequently occurring primary brain tumor and has a very poor prognosis, with only around 5% of patients surviving for a period of 5 years or more after diagnosis. Despite aggressive multimodal therapy, consisting mostly of a combination of surgery, radiotherapy, and temozolomide chemotherapy, tumors nearly always recur close to the site of resection. For the past 15 years, very little progress has been made with regards to improving patient survival. Although immunotherapy represents an attractive therapy modality due to the promising pre-clinical results observed, many of these potential immunotherapeutic approaches fail during clinical trials, and to date no immunotherapeutic treatments for GBM have been approved. As for many other difficult to treat cancers, GBM combines a lack of immunogenicity with few mutations and a highly immunosuppressive tumor microenvironment (TME). Unfortunately, both tumor and immune cells have been shown to contribute towards this immunosuppressive phenotype. In addition, current therapeutics also exacerbate this immunosuppression which might explain the failure of immunotherapy-based clinical trials in the GBM setting. Understanding how these mechanisms interact with one another, as well as how one can increase the anti-tumor immune response by addressing local immunosuppression will lead to better clinical results for immune-based therapeutics. Improving therapeutic delivery across the blood brain barrier also presents a challenge for immunotherapy and future therapies will need to consider this. This review highlights the immunosuppressive mechanisms employed by GBM cancers and examines potential immunotherapeutic treatments that can overcome these significant immunosuppressive hurdles.

Keywords: GBM - Glioblastoma multiforme, immune escape, immunotherapy, combinatorial therapy, treatment, overview

INTRODUCTION

Glioblastoma multiforme (GBM, WHO grade 4) is the most frequently occurring primary brain tumor. Although primarily a disease associated with old age, it can also occur in children. The prognosis for GBM patients is poor and the disease is almost uniformly fatal with only around 5% of patients surviving for a period of 5 years after diagnosis (1). The current course of therapy for GBM patients is surgical resection of the tumor (where possible) followed by concomitant radiotherapy and temozolomide chemotherapy, followed by adjuvant temozolomide. Despite aggressive multimodal therapy, GBM tumors nearly always recur, the majority close to the site of resection (2–4). This recurrence is most likely, and most often, due to the infiltrative nature of GBM making complete resection of tumor cells incredibly difficult. Although progress to improve the surgical removal of tumor cells has been made, such as the use of 5-aminolevulinic acid (5-ALA) which is approved for intraoperative imaging of GBM cells increasing their removal, it is not possible to visualize all individual cancer cells that have migrated further into healthy areas of the brain (5).

GBM tumors are histopathologically characterized by an abundance of poorly differentiated and pleomorphic astrocytes with nuclear atypia and high mitotic activity. GBM tumors are highly vascular and necrosis is often evident within these tumors (6). Metastasis is rarely seen in GBM tumors; however, they are highly invasive, and these tumors employ a plethora of mechanisms to avoid immune detection.

THE BRAIN AS A UNIQUE IMMUNE ENVIRONMENT

In order to understand the complexity of the brain's interaction with the immune system, the presence of the blood-brain barrier (BBB) needs to be considered and understood. The endothelial cells of the brain vasculature are connected by tight junctions that control the permeability of the endothelium. Although these tight junctions under normal physiological conditions are highly regulated, under inflammatory conditions (such as those in GBM) these junctions are not as tightly connected making the endothelium 'leaky' (7). The BBB, a multi-component structure found in the wall of cerebral blood vessels, selectively restricts passage of cells and molecules into the brain from the circulation. The major, but not sole, players in this defense are the endothelial cells of the cerebral vessels, which differ from their peripheral counterparts by the presence of intercellular tight junctions that essentially prevent paracellular transfer of all, but the smallest gases and ions, and the absence of fenestrations and pinocytic mechanisms that restricts bulk transcytosis (8, 9). These features are then reinforced by the presence of numerous efflux transporters that remove xenobiotics and metabolic waste from the brain into the circulation. Beyond the endothelium, the BBB is further composed of a bi-layered basement membrane within which reside pericytes and perivascular macrophages that regulate endothelial function and pose a further barrier to cellular entry, ultimately surrounded by a tight glia limitans formed of astrocyte end-feet that appose and encircle the blood vessel (8, 9).

GBM tumors contain areas of highly metabolic cells that drive local hypoxia, triggering production of vascular endothelial growth factor and angiogenesis (10). This process involves disruption of inter-endothelial tight junctions to permit vascular growth, hence the core of the tumor is associated with a weakened blood-tumor barrier (BTB) with an increased permeability (11, 12). Nevertheless, areas of GBM tumors distal from the hypoxic core, which in diffuse tumors can be a significant proportion, remain behind a BTB that is highly reminiscent of the true BBB, and are thus protected from the entry of chemotherapeutic agents, including most therapeutic antibodies (13). However, these difficulties do not mean that the delivery of effective therapeutics for GBM is futile, and a wide variety of approaches to achieve this are under active exploration.

The brain has traditionally been considered as being an immunoprivileged organ due to a variety of factors, however it is now accepted that there is an active interaction between the brain and the immune system (14, 15). Despite this active immune interaction, the brain is immunologically unique in that immune cells do not freely access the brain parenchyma. Although activated immune cells can cross the BBB, only those specific for antigens within the brain remain there. T cells cross the BBB in a capture, crawl, cross manner with integrins and selectin ligands on T cells binding to selectins and integrin ligands on endothelial cells 'capturing' them (16). Leukocytes are then activated by chemokine secretion resulting in their slowing and eventual transmigration. Once T cells have transmigrated, they downregulate their integrin expression and upregulate expression of matrix metalloproteinases (MMPs) enabling them to break down matrix components allowing cell penetration of the brain parenchyma (7). Inflammation within the brain has been shown to lead to an upregulation of adhesion molecules on the BBB endothelial cells (16, 17). The endothelial cells of the brain vasculature do not just control the immune response by physically excluding immune cells, these cells have also been shown to contribute to immunosuppression in GBM. FasL expression has been seen on GBM associated vascular endothelial cells, and the FasL expressed on these cells is linked to a reduced T cell infiltrate, most likely due to the FasL induced death of T cells (18). There are very few immune cells normally present within the brain, however the microglia can act as antigen presenting cells (APCs). The brain traditionally has low major histocompatibility complex (MHC) class I and class II expression meaning that antigen expression is reduced when compared to other tissues (19). It is important to note, however, that GBM cells themselves have been shown to express MHC class I and II molecules meaning that these cells present antigens to antigen specific $CD8^+$ and $CD4^+$ T cells (20).

Not only does the unique physiology of the brain create an unusual immune environment but it is important to note that the tumors themselves create their own microenvironment. Tumor cells can co-opt stromal cells in order to support their growth and survival (21). The brain extracellular matrix is comprised of

proteoglycans, glycoproteins and glycosaminoglycans. In the GBM setting, significant increases in heparan sulphate proteoglycans (HSPG) have been seen in the tumor microenvironment (TME) (22). The increase of HSPGs in the TME leads to greater retention of growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), thereby supporting tumor nutrition and growth. The increased local concentration of VEGF within the GBM TME results in upregulation of periostin and tenascin C within blood vessels trapping T cells and preventing tumor penetrance (23).

In the case of GBM, as with many cancers T cells are frequently exhausted and dysfunctional and therefore are inadequate at exerting an anti-tumor immune response. Persistent stimulation of T cells by tumor cells results in T cell senescence as indicated by the presence of CD57 on the surface of T cells (24). CD57 positive T cells can secrete cytokines when stimulated by their cognate peptides however they do not proliferate when stimulated (25). Tumor resident senescent T cells have also been shown to down regulate the co-stimulatory molecules CD27 and CD28 contributing to immune dysfunction, causing changes in APC phenotype such as a down regulation of CD80 and CD86 reducing their ability to stimulate T cells further exacerbating the local immune dysfunction (26). When compared to healthy donors GBM patients have a lower number of circulating $CD3^+$ T cells in their peripheral blood mononuclear cells (PBMCs) further indicating a disease related immune dysfunction (27). Glioblastoma multiforme is more frequent in the older population with most cases occurring between the ages of 55 and 60 (28). Increased age is linked to T cell dysfunction; with elderly patients having a higher number of senescent T cells and thymic shrinkage being apparent (24, 29). The chronic stimulation of T cells by tumor cells also leads to the exhaustion of these cells, rendering them ineffective at tumor control. This exhaustion leads to an upregulation of immune checkpoint markers such as PD-1, LAG-3, TIGIT, and CD39 on GBM infiltrating $CD8^+$ T cells (30). TILs isolated from murine GBM tumors show impaired cytokine production compared to peripheral T cells, with reduced levels of interferon gamma, tumor necrosis factor alpha and interleukin 2 being detected via flow cytometry when cells are stimulated *in vitro* (30). Transformed tumor cells also compete with other cells within the TME for glucose, GBM cells have an increased rate of glucose uptake when compared to non-transformed cells. T cells within the TME require glucose in order to perform effector functions and therefore the depletion of glucose by tumor cells results in impaired T cell function and exhaustion (31).

STANDARD OF CARE AND IMMUNOSUPPRESSION

The current standard of care for GBM is maximal surgical resection (where possible) followed by concomitant radiotherapy and temozolomide chemotherapy (32). Patients are also given anti-inflammatory steroids such as dexamethasone to help control peritumoral edema (33). The US Food and Drug Administration (FDA) has also approved the use of tumor treating fields (TTFs) to treat GBMs. This involves using alternating electric fields administered via scalp electrodes to disrupt GBM tumor cell division (34).

Dexamethasone has been shown to lead to the upregulation of the immunosuppressive checkpoint cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) on the surface of T cells, thereby reducing their anti-tumor activity. Dexamethasone has also been shown to lead to a reduction of T cell proliferation (35). Dexamethasone has also been shown to dampen patients' immune responses to immune checkpoint blockade (36).

As previously mentioned, the standard of care involves the use of the chemotherapeutic drug temozolomide (TMZ), which is known to influence the immune system. High dose temozolomide induces lymphopenia, an issue that is exacerbated when TMZ is combined with radiotherapy (37). TMZ has also been shown to result in T and B cell dysfunction in a murine model of GBM (38).

In the GBM setting, radiotherapy can be administered in a variety of ways such as whole brain radiotherapy, stereotactic radiosurgery, image guided radiotherapy and hypofractinated radiotherapy (39). Radiotherapy is known to have a number immune modulating effects (40–42), importantly brain tumor exposure to radiotherapy has been shown to upregulate MHC class I expression by brain tumors, and this improves the antigen presentation capability of these cells. Radiotherapy also increases the repertoire of peptides presented by tumor cells and the phenomenon of antigen spreading can occur – i.e. tumor cells die, and their antigens are taken up by nearby immune cells (43). Research has shown that radiotherapy is less efficient in mice lacking T cells, thereby highlighting the additive effect that radiotherapy has in immune cell-mediated control of cancer (44). Radiotherapy is often thought of as an *in-situ* vaccination that makes tumors susceptible to immune attack (44–46). Although a large amount of evidence points towards radiotherapy stimulating an anti-tumor immune response, radiotherapy can also unfortunately result in the secretion of immunosuppressive cytokines such as IL-6 and IL-10 from treated tumor cells (47, 48).

Combined TMZ, radiotherapy and dexamethasone therapy in GBM patients has been shown to induce a persistent lowering of $CD4^+$ cell counts which is associated with increased rates of infection and poorer survival (49).

IMMUNE INHIBITORY PROTEINS EXPRESSED BY GBM TUMORS

GBM cells secrete many immunosuppressive proteins and express many cell surface and cytoplasmic immune inhibitory proteins (as summarized in **Figure 1**). Intracellular adhesion molecule 1 (ICAM-1), a key regulator of cell-cell interactions, is commonly upregulated within GBM tumors, when compared to immunohistochemically stained normal brain (50). ICAM-1 interacts with lymphocyte function-associated antigen 1 (LFA-1) expressed on myeloid cells to promote migration of these cells into tumors, thereby enhancing intratumoral immune

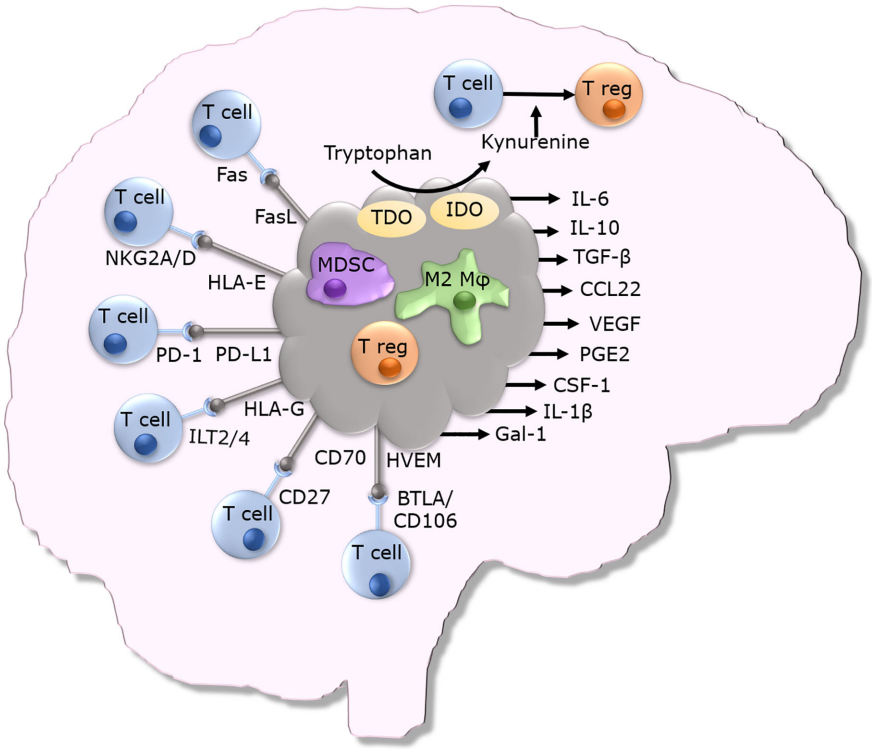

FIGURE 1 | Overview of immunosuppressive mechanisms utilized by GBM tumors.

suppression (51). Myeloid derived suppressor cell (MDSC) accumulation in GBM tumors further contributes to local immune suppression (52). The presence of MDSCs circulating in the blood of GBM patients is also elevated when compared to non-diseased individuals (53). These MDSCs express many immunosuppressive molecules that suppress anti-tumor T cells such as TGF-β and arginase (52). GBM cells have been shown to overexpress galectin-1 (Gal-1), another protein important for the maintenance of cell-cell interactions. Expression of Gal-1 by GBM cells promotes the proliferation and migration of tumor cells (54, 55). Gal-1 expressing GBM cells have also been shown to induce T cell death when the two types of cells are co-cultured (55). Gal-1 interacts with CD45 and CD43 on T cells resulting in their clustering. Gal-1 also binds to CD7 on the T cells and these interactions result in T cell death (56–58).

GBM cells have also been shown to express non-classical MHC class I molecules on their surface which enables them to evade immune cell mediated killing. HLA-G is one such non-classical MHC class I molecule that is involved in immunogenic tolerance of trophoblasts and prevents immune response to the developing semi-allogeneic fetus. In the adult, HLA-G is expressed in thymic epithelial cells, nail matrix and cornea (59). Although HLA-G expression is tightly controlled in the human body, it appears that GBM cells can express HLA-G (59). HLA-G is not just expressed on the cell surface - a soluble isoform that is secreted has been detected in plasma, cerebrospinal fluid and seminal plasma. GBM tumors are frequently infected with cytomegalovirus (hCMV), and hCMV infection has been associated with high levels of HLA-G expression (60). Cytomegalovirus infection is prevalent in the population and infection is lifelong. The immunosuppression linked with GBM results in reduced control of hCMV and this results in reactivation of the virus (60). HLA-G can bind to several receptors, namely the inhibitory receptors Ig-Like Transcript 2 (ILT2) and Ig-Like Transcript 4 (ILT4) (61). HLA-G can also bind the non-inhibitory receptors CD8, CD160, and KIR2DL4. Binding of soluble HLA-G to CD8 on T cells induces a signaling cascade that results in Fas-FasL mediated apoptosis of $CD8^+$ T cells (61). HLA-G binding to ILT2 on natural killer (NK) cells inhibits the polarization of lytic granules and the microtubule-organizing center at the contact zone, ultimately preventing NK cell-mediated lysis (61). HLA-E is another non-classical MHC class I molecule; it is a ligand for both NKG2A and NKG2C expressed on NK cells, $CD8^+$ αβ and γδ T cells. Binding of HLA-E to NKG2C can lead to immune cell activation, and its binding to NKG2A leads to immune cell inhibition. HLA-E, much like HLA-G, is believed to play a role in maternal tolerance of the fetus (62). HLA-E has been shown to be expressed on GBM cells and this HLA-E expression has been shown to prevent NK cell mediated lysis of these tumor cells. Blockade of the NKG2A – HLA-E interaction has been shown to improve NK cell mediated killing of GBM tumor cells (62).

GBM tumors have also been shown to express Fas ligand (CD95L) on their surface, the binding of which to Fas (CD95/

APO-1) on T cells leads to apoptosis of the T cells, thereby enabling GBM cells to evade lysis by Fas-expressing T cells (63). GBMs can also induce T cell death via their expression of CD70. CD70 on GBM cells binds to CD27 on T cells inducing death of activated T cells, and blockade of this interaction has been shown to partially protect T cells from GBM cell induced death (64). GBMs have also been shown to express the immune dampening checkpoint ligand programmed death ligand 1 (PD-L1). PD-L1 binds to its cognate receptor programmed death 1 (PD-1) expressed on activated T cells, and this leads to inhibition of T cell responses to PD-L1 expressing GBM cells. It has been reported that as many as 88% of patient GBM samples express PD-L1 (65). This high level of PD-L1 expression has been shown to be linked with poorer patient survival (66).

Herpes virus entry mediator (HVEM) is an example of another immune checkpoint molecule that has been proven to be expressed in the GBM microenvironment (67). HVEM is usually expressed on T cells, it can have both co-stimulatory and inhibitory effects, depending upon its binding partner (67). HVEM exerts an immune inhibitory effect when bound to B and T lymphocyte attenuator (BTLA) or CD160 expressed by other immune cells (67). High expression of HVEM in GBM tumors has been linked to regulatory T cell differentiation, negatively associated with the regulation of T cell mediated cytotoxicity and with a decreased survival time (67).

Indoleamine 2,3-dioxygenase (IDO) is another protein involved in immunoregulation and prevention of fetal rejection. IDO catabolizes tryptophan into immune-regulatory kynurenines. IDO expression can be induced by a variety of receptors such as the toll like receptors (TLRs), tumor necrosis factor receptor superfamily members (TNFRs), interferon gamma receptors (IFNGRs), transforming growth factor beta receptors (TGFBRs) and aryl hydrocarbon receptors (AhRs) (68). The depletion of tryptophan by IDO activity inhibits immune cell function and prevents dendritic cell (DC) maturation (68). IDO expression is upregulated in recurrent GBMs, with 100% of patients being studied expressing IDO at the time of the second surgery (69). The expression of IDO within GBM tumors is associated with an increased infiltration of $CD4^+$ regulatory T cells, immune escape and a poorer prognosis (70). Increased kynurenine production driven by IDO activity induces the differentiation of naïve $CD4^+$ T cells into immunosuppressive regulatory $CD4^+$ T cells triggered by the binding of kynurenine to the aryl hydrocarbon receptor (AHR) on naïve $CD4^+$ T cells (71). Tryptophan 2,3-dioxygenase (TDO), another enzyme involved in the degradation of tryptophan into kynurenine, can also contribute to an immunosuppressive microenvironment high in kynurenine. TDO is expressed in brain tumors and represents a druggable target for reversing the immunosuppressive microenvironment (72).

GBM tumors also secrete numerous other immunosuppressive factors that shape the immune TME and enable immune evasion. GBM tumors secrete IL-6 (73, 74) and their expression of the IL-6 receptor is upregulated (75). IL-6 mediates signaling via the transcription factor STAT3. Upon activation, STAT3 is phosphorylated and persistent phosphorylation is linked with brain tumor grade; with GBM showing the highest levels of STAT3 phosphorylation. Knockdown of STAT3 in GBM cell lines slows *in vitro* and *in vivo* tumor cell growth (76). Human GBM cells isolated from tumors were shown to secrete the chemokine CCL22 (77) which attracts regulatory $CD4^+$ $CD25^+$ $FoxP3^+$ T cells to the TME (78). GBM tumor cells also secrete the immunosuppressive cytokine TGF-β which reduces ICAM-1 and VCAM-1 expression on GBM endothelial cells and thereby T cell infiltration (79, 80). The active form of TGF-β secreted by GBM cells increases the activity of MMP2 and MMP9 on the surface of GBM cells which in turn increases cell motility and promotes the invasion of GBM cells into the surrounding brain (81). GBM tumor cells also secrete the anti-inflammatory cytokine IL-10 which, in the normal setting prevents excessive inflammation and reduces tissue damage by suppressing the activity of Th1 and $CD8^+$ T cells. Immune cells such as regulatory T cells secrete IL-10 to quell the immune response (82). IL-10 mRNA is highly expressed in GBM tissues (83). More concerning is that IL-10 not only suppresses the immune system, but also increases the proliferation and migration of GBM cells. Intratumoral microglia/macrophages are major contributors to the IL-10 production within GBM tumors (84).

Human cytomegalovirus (hCMV) is a herpes virus that has been shown to persistently infect 50% to 90% of the adult population. Analysis of GBM tumors has revealed that a large proportion of tumors express hCMV proteins indicating the presence of hCMV within these tumors (85, 86). Human cytomegalovirus secretes a homolog of IL-10, known as cmvIL-10 which has the same immunoinhibitory properties as human IL-10 (87). The attenuation of the immune response by cmvIL-10 prevents eradication of the tumor as well as the virus itself. The secretion of cmvIL-10 leads to the differentiation of $CD14^+$ monocytes to macrophages, thereby further supporting hCMV infection (88). *In vitro* studies have revealed that cmvIL-10 affects the maturation and life span of DCs, in that although monocytic DCs exposed to cmvIL-10 reach maturation, their cytokine production is impaired in a non-reversible manner (88). The presence of IL-10 and TGF-β in GBM tumors is believed to downregulate the expression of MHC class I in the TME (89). GBM cells express macrophage migration inhibitory factor (MIF) which renders GBM cells resistant to NK cell mediated killing (90). VEGF secretion by GBM cells stimulates the growth of new blood vessels supplying oxygen and nutrients to rapidly dividing and often hypoxic tumor cells (91, 92). As well as increasing tumor vasculature, VEGF also upregulates expression of the macromolecules tenascin C (TNC) and periostin. TNC blocks the migration of T cells across the blood tumor barrier thereby preventing them from penetrating the tumor parenchyma (23). Periostin also recruits circulating immunoinhibitory M2 macrophages into the tumor parenchyma (93). GBM stem cells secrete the macrophage attracting cytokine periostin. These macrophages support tumor growth and result in a poorer prognosis (93). GBM cells exposed to radiotherapy and chemotherapy have been shown to display increased immunosuppression. This phenomenon has been shown to be due to increased prostaglandin E2 secretion by cells. Blockade of this secreted PGE2 reverses the immunosuppressive capacity of treated cells (47). Colony stimulating factor 1 (CSF-1) is a growth

factor that has been shown to be expressed in GBM tumors and by GBM cell lines (94). CSF-1 can either be secreted by cells or expressed as a transmembrane variant on the cell surface. CSF-1 is secreted by astrocytes within the brain during acute inflammatory responses. CSF-1 can bind to its receptor (CSF-1R) on the surface of macrophages and microglia within the brain promoting their switch to the immunosuppressive M2 phenotype (94, 95). GBM cells also secrete interleukin-1α and -1β (IL-1 α and β) (96). The down regulation of HLA class II expression on the U-105 MG GBM cell line by IL-1β suggests that this could be another mechanism which reduces immune recognition by $CD4^+$ T cells (97).

THE CONTRIBUTION OF IMMUNE CELLS WITHIN GBM TUMORS TO THE IMMUNE INHIBITORY PHENOTYPE

Whilst GBM tumor cells contribute to immunosuppression, the immune cells recruited to the tumor can also exacerbate the immune evasive properties of these tumors. Although immune cells can contribute to tumor control, immunosuppressive populations can also contribute to the immune escape of GBM tumors. Indeed, many of the anti-tumor immune cells recruited to the TME adopt an immunosuppressive phenotype due to the cytokines secreted by the GBM tumors and the unique microenvironment which these tumors create.

Myeloid-derived suppressor cells (MDSCs) can be found within GBM tumors, and these cells contribute to the immunosuppressive phenotype of GBMs (98). MDSCs can be divided into two main types, monocytic and granulocytic. Granulocytic MDSCs are rarely found in GBM tumors, whereas the monocytic subtype are more prevalent (99). Monocytic MDSCs support tumor growth by increasing the recruitment of $CD4^+$ regulatory T cells via chemokine release in the TME (100). $CD4^+$ regulatory T cells are well known immunosuppressive immune cells that dampen the immune response. When compared to healthy controls, the prevalence of regulatory T cells in the peripheral blood is higher in GBM patients. Of even more relevance is that the prevalence of regulatory T cells in lymphocyte populations infiltrating GBM tumors is significantly greater than that in lymphocyte populations from 'normal' brain tissue obtained from seizure patients (101, 102). Although immune cell infiltration is often viewed as a positive prognostic marker, it can also contribute to the pathology of GBM. Lymphocytes entering the tumor have been shown to downregulate costimulatory molecules such as CD28 and CD62L (103). The presence of immunosuppressive regulatory T cells within GBM tumors has been correlated with shorter recurrence-free survival. GBM associated microglia/macrophages, which constitute up to 30% of the GBM tumor bulk are of the immunosuppressive M2 phenotype (103, 104). The expression of PD-L1 by these immunosuppressive M2 cells further contributes to local immunosuppression, as does their secretion of CCL22 which recruits regulatory T cells and MDSCs into the TME (103, 104).

OVERCOMING GBM-DRIVEN IMMUNOSUPPRESSION

Active Immunotherapy via Vaccination

Vaccination presents an attractive method for immunotherapeutically targeting GBMs (ongoing trials are detailed in **Tables 1**–**3**). One issue that can arise with peptide vaccinations is that immune escape variants can develop, and tumors can overcome the immune pressure applied to them. This phenomenon has been seen in the case of Rindopepimut, an EGFRvIII-keyhole limpet hemocyanin peptide conjugate. When Rindopepimut was used to treat GBM patients with EGFRvIII positive tumors, their median overall survival was 26 months compared to the 15 months of matched controls. Although vaccination prolonged the overall survival of patients, tumors recurred in a large proportion of these patients. When the recurrent tumors were analyzed immunohistochemically for EGFRvIII expression, 82% of the tumors examined had lost expression of EGFRvIII and the other 18% only displayed EGFRvIII expression in less than 1% of their tumor cells (114). These data suggest that the targeting of a single antigen can lead to the generation of immune escape variants, as a consequence of which multiple antigens need to be employed in the formulation of such vaccines.

IMA950 is one such multi-peptide vaccine that is being investigated in GBM. IMA950 is made up of 9 CD8 specific peptides derived from BCAN, CSPG4, FABP7, IGF2BP3, NRCAM, NLGN4X, PTPRZ1, and TNC as well as two CD4 specific peptides derived from survivin and c-met (150). This multi-peptide vaccine was given in conjunction with the immune boosting adjuvant poly-ICLC to GBM patients in a phase I/II clinical trial (111). This vaccination was well tolerated by patients and induced antigen specific $CD8^+$ and $CD4^+$ T cell responses (111). The level of response seen in patients varied, and analysis of five tumor samples revealed that no vaccine-specific T cells were present in the TIL population, meaning that there may be issues with the homing of vaccine-induced T cells (111). When samples from the recurrent tumors were tested for expression of the target antigens, no change in the levels of these antigens compared to the pre-vaccination tumor samples was observed, further suggesting that the issues are with T cells not trafficking to the tumor site (111). The ability of tumor cells to present immunogenic epitopes at their surface may also explain the failure of peptide vaccine treatments. Whilst tumors may express the target antigen, they may not present the target epitope on their surface meaning that vaccine generated T cells will not target these tumors. Although vaccination with these peptides generates antigen-specific T cells, there appears to be an issue with the immune TME of these tumors. As a result, the combination of IMA950 vaccination with other modalities, such as immune activating anti-CD27 and anti-PD-1 are being explored in the clinic (111).

A 'personalized' peptide vaccination approach has also been explored in the GBM setting. In a phase I clinical trial, GBM patients were treated with a cocktail of manufactured peptides

TABLE 1 | Peptide vaccine trials for glioblastoma.

Trial name ClinicalTrial.gov identifier	Phase	Immune targets	Associated treatments in active arm	Control arm	Sample size	T cell response(CD4/CD8 response details)	Humoral response	Median PFS(months)	Median OS(months)	Primary endpoint	Results
IMA-950 NCT01222221 (105)	I	BCAN, CSPG4, FABP7, IGF2BP3, NLGN4X, NRCAM, PTPRZ1, TNC, MET, BIRC5, HBcAg	None	None	40	Yes (Up to 1.1% specific CD8)	NA	NA (PFS6 = 74.4%)	15.3	Safety and immunological response	Positive
NOA-16 NCT02454634 (106)	I	IDH1R132H	None	None	32	Results pending	Results pending	Results pending	Results pending	Safety	Safe vaccine
GAPVAC NCT02149225 (107)	I	Personalized vaccine	None	None	15	Yes (Up to 0.02% specific CD8)	NA	14.2	29	Safety and immunological response	Safe vaccine, Trend for immunological response
SurVaxM NCT01250470 (108)	I	Survivin (SVN53-67/M57-KLH peptide)	None	None	9	Yes (CD8 response in 78% patients: at least 1% specific CD8)	Yes (88% patients)	17.6	86.6	Safety	Safe vaccine
NCT01621542 (109)	I	WT2725	None	None	21	Yes (interim results: CD8 response in 10% patients)	Results pending	Results pending	Results pending	Safety and immunological response	Safe vaccine
UMIN000003506 (110)	I	Cocktail of WT1 HLA class I and II peptides	None	None	14	Yes (CD8 response in 64% patients: median specific CD8 = 6% of total CD8)	NA	4 (r- GBM)	6.2 (r-GBM)	Safety	Safe vaccine
PERFORMANCE NCT02864368	I	CMV peptide	Temozolomide	None	70	Results pending	Results pending	Results pending	Results pending	Safety and immunological response	Results pending
NeoVax NCT02287428	Ia/Ib/ Ic	Personalized neoantigen vaccine	Temozolomide plus Pembrolizumab	None	56	Results pending	Results pending	Results pending	Results pending	Feasibility and safety	Results pending
NCT03223103	Ia/Ib	Personalized mutation-derived tumor antigens	TTF	None	20	Ongoing	Ongoing	Ongoing	Ongoing	Safety	Ongoing
IMA-950 NCT01920191 (111)	I/II	BCAN, CSPG4, FABP7, IGF2BP3, NLGN4X, NRCAM, PTPRZ1, TNC, MET, BIRC5, HBcAg	Pembrolizumab	None	13	Results pending (interim results: CD8 response in 63.2% patients)	Results pending	Results pending (interim results: PFS9=63%)	Results pending (interim results: OS=19)	Safety and immunological response	Positive
SL-701 NCT02078648 (112)	I/II	IL-13Ra2, EphrinA2, survivin	Stage 1: imiquimod; Stage 2: Bevacizumab	None	74	Results pending (interim results: CD8 response in stage 2 patients)	Results pending	Results pending	Results pending (interim results: 11.0 for stage 1, 11.7 for stage 2)	Safety, ORR, OS12	Results pending
IMA950-106	I/II	BCAN, CSPG4, FABP7, IGF2BP3, NLGN4X, NRCAM, PTPRZ1, TNC, MET, BIRC5, HBcAg	None	None	24	Ongoing	Ongoing	Ongoing	Ongoing	Safety	Ongoing
UCPVax-Glio NCT04280848	I/II	Telomerase (TERT)	None	None	28	Ongoing	Ongoing	Ongoing	Ongoing	Immunological response	Ongoing
VBI-1901 NCT03382977 (113)	I/II	CMV (pp65 and gB antigens)	None	None	38	Ongoing	Ongoing	Ongoing (Interim results: 3.6 in immunological responders - rGBM)	Ongoing (Interim results: 14.0 in immunological responders - rGBM)	Safety	Ongoing
ROSALIE NCT04116658	I/II	TAAs and microbiome-derived peptides (EO2401)	Nivolumab +/- Bevacizumab	None	32	Ongoing (not yet recruiting)	Ongoing (not yet recruiting)	Ongoing (not yet recruiting)	Ongoing (not yet recruiting)	Safety	Ongoing (not yet recruiting)
ACTIVATe NCT00643097 (114)	II	EGFR-vIII	Temozolomide	None	22	NA	Yes (33% patients)	NA (PFS5.5 = 66%)	26.0	PFS and immunological response	Positive
ACT II NCT00643097 (115)	II	EGFR-vIII	None	None	18	NA	Yes (43% patients)	14.2	26.0	PFS and OS	Positive
ACT III NCT00458601 (116)	II	EGFR-vIII	Temozolomide	None	65	NA	Yes (85% patients)	9.2	21.8	PFS5.5	Positive (PFS5.5 = 66%)
ReACT NCT01498328 (117)	II	EGFR-vIII	Bevacizumab	KLH and GM-CSF plus Bevacizumab	36 (vs. control 37)	NA	Yes (89% patients)	NA	NA	PFS6	Positive (trend)
SurVaxM NCT02455557 (118)	II	Survivin: SVN53-67/M57-KLH peptide	Temozolomide	None	63	Pending results	Pending results	Pending results (interim results: 13.9)	Pending (interim results: OS12=93.4%)	PFS6	Positive

(Continued)

TABLE 1 | Continued

Trial name ClinicalTrial.gov identifier	Phase	Immune targets	Associated treatments in active arm	Control arm	Sample size	T cell response(CD4/CD8 response details)	Humoral response	Median PFS(months)	Median OS(months)	Primary endpoint	Results
WIZARD 201G NCT03149003 (119)	II	WT1	Bevacizumab	Bevacizumab	219	Results pending	Results pending	Results pending	Results pending	Safety and OS	Results pending
SurVaxM NCT04013672 (118)	II	Survivin: SVN53-67/M57-KLH peptide	Pembrolizumab	None	51	Ongoing	Ongoing	Ongoing	Ongoing	PFS6	Ongoing
V-Boost	II	Hydrolyzed GBM antigens and alloantigens	Radiotherapy and Temozolomide	None	20	Ongoing	Ongoing	Ongoing	Ongoing	Antitumor activity	Ongoing
ACT-IV NCT01480479 (120)	III	EGFR-vIII	None	KLH and GM-CSF	369 (vs. control 372)	NA	Yes	NA	20.1	OS	Negative

KLH, keyhole limpet haemocyanin, TTF, Tumor Treating Fields; nd-GBM, newly diagnosed glioblastoma; r-GBM, recurrent glioblastoma; TAA, Tumor Associated Antigen; PFS, progression free survival; OS, overall survival; CMV, Cytomegalovirus; EGFR, Epidermal Growth Factor receptor; WT-1, Wilms' Tumor 1.

derived from known GBM antigens followed by a vaccination that targeted neoepitopes derived from analysis of the patients' tumor immunopeptidome and transcriptome (107). Each patient received a vaccine that was tailored to their tumor antigen expression profile. Vaccines were administered with the adjuvants Poly-ICLC and GM-CSF. The cocktail of 'off the shelf' peptides known as APVAC1 generated $CD8^+$ T cell responses in twelve out of the thirteen patients studied and $CD4^+$ T cell responses were found in nine of the thirteen patients studied (107). The neoepitope vaccine known as APVAC2 generated a predominantly $CD4^+$ T cell response in eight out of the ten patients evaluated. The overall median overall survival of patients receiving this vaccination regime was 29 months (107). Although these findings are promising, these peptide vaccinations are far from curative. Whilst $CD4^+$ and $CD8^+$ responses were detected, these were at a relatively low level, with the frequency of antigen specific T cells being below 4 percent for $CD4^+$ T cells and 1 percent for $CD8^+$ T cells (107). The low frequency of target specific T cells may explain the failure of this therapy to act in a curative manner. Targeting of multiple antigens helps prevent the development of antigen escape variants, however combinatorial methods that enable vaccine-induced T cells to penetrate tumors and overcome the immunosuppressive microenvironment need to be explored.

Targeting Immune Inhibitory Cells and Cytokines

The contribution of macrophages/microglia to the immunosuppressive TME of GBM and their prevalence within the tumor bulk suggest them to be attractive therapeutic targets for the immunotherapeutic targeting of GBM. As mentioned previously, microglia and macrophages in the TME adopt an immunosuppressive M2 phenotype (103, 104). As also previously mentioned, microglia/macrophages express the CSF-1R and GBM cells secrete CSF-1 resulting in the switching of GBM macrophages/microglia to the immune inhibitory M2 phenotype. The blockade of this CSF-1/CSF-1R interaction presents an attractive approach for preventing the switching of tumor resident macrophages/microglia to the immunoinhibitory M2 phenotype. In this regard, blockade of the CSF-1R with the chemical BLZ945 has been shown to improve survival and reduce tumor development in GBM bearing mice without any visible deleterious side-effects. BLZ945 treatment did not alter macrophage numbers within the implanted tumors but reduced the polarization of these macrophages to the M2 phenotype (95). As a result, combining BLZ945 with active immunotherapy represents an exciting therapeutic option for GBM.

As previously discussed GBM cells are known to overexpress MIF, making them resistant to NK cell mediated killing (90). Not only does MIF protect GBM cells from NK cell mediated killing it also exerts effects on macrophages/microglia within the tumors. MIF has been shown to interact with CD74 on microglia resulting in the adoption of the immunosuppressive M2 phenotype. Disruption of the CD74/MIF pathway prevents this M2 phenotype switch and prolongs the survival of GBM tumor bearing mice (151). Immunotoxins have also been used to target

TABLE 2 | Dendritic cell vaccine trials for glioblastoma.

Trial name ClinicalTrial.gov identifier	Phase	Immune targets	Associated treatments in active arm	Control	Sample size	T cell response (CD4/CD8 response details)	Humoral response	Median PFS (months)	Median OS (months)	Primary endpoint	Results
PERCELLVAC NCT02709616 NCT02808364 (121)	I	Personalized TAA	None	None	5	Yes (CD4 and CD8 response in 80% patients: up to 3.5% specific CD8)	NA	NA	19	Safety	Positive
ATTAC NCT00639639 (122, 123)	I	CMV pp65	None	None	11	Yes (up to 4.5% specific CD8 in 55% patients)	NA	25.3	41.1	Safety and feasibility	Safe vaccine
NCT03615404	I	CMV RNA	Td + GM-CSF + DI-TMZ	None	10	Ongoing	Ongoing	Ongoing	Ongoing	Safety and feasibility	Ongoing
NCT00612001 (124)	I	Autologous glioma lysate vs. GAA peptides	None	None	34	NA	NA	9.6	34.4 for lysate-DC, 14.4 for GAA-DC	Safety and feasibility	Safe vaccine
NCT00068510 (125)	I	Autologous glioma lysate	None	None	12	Yes (CTL response in 50% patients)	NA	15.5	23.4	Safety and feasibility	Safe vaccine
Rudnick 2020 (126)	I	Autologous glioma lysate	Gliadel	None	28	Yes (CD8 response in 25% patients, no details in %specific CD8)	NA	3.6	32 for nd-GBM, 16.3 for r-GBM	Safety and clinical outcome	Positive
MC1272 NCT01957956 (127)	I	Autologous glioma lysate	Temozolomide	None	20	Results pending	Results pending	Results pending (interim results: 9.7)	Results pending (interim results: 20.5)	Safety and feasibility	Safe vaccine
NCT02010606 (128)	I	Autologous glioma stem like lysate	Temozolomide for nd-GBM Bevacizumab for r-GBM	None	38	Results pending	Results pending	Results pending (interim results: 8.6 For nd-GBM; 3.14 For r-GBM)	Results pending (interim results: 21.1 for nd-GBM; 12.0 For r-GBM)	Safety	Safe vaccine
ICT-107 (129)	I	AIM-2, MAGE1, TRP-2, gp100, HER2, IL-13Ra2	None	None	16	Yes (specific CD8 increase in 31% patients)	NA	16.9	38.4	Immunological response	Positive (trend)
NCT01808820	I	Autologous glioma lysate	Imiquimod	None	20	Results pending	Results pending	Results pending	Results pending	Safety	Results pending
NCT03360708	I	Autologous glioma lysate	None	None	20	Ongoing	Ongoing	Ongoing	Ongoing	Safety	Ongoing
ATL-DC NCT04201873	I	Autologous glioma lysate	Pembrolizumab	ATL-DC plus poly ICLC plus placebo	40	Ongoing	Ongoing	Ongoing	Ongoing	Safety and immunological response	Ongoing
NCT03360708	I	Autologous glioma lysate	None	None	20	Ongoing	Ongoing	Ongoing	Ongoing	Safety and toxicity	Ongoing
NCT00890032	I	BTSC mRNA	None	None	50	Ongoing	Ongoing	Ongoing	Ongoing	Safety, Feasibility and immune response	Ongoing
NCT03914768	I	Genetically modified tumour cells and neoantigens	Cyclophosphamide + Bevacizumab	None	10	Ongoing	Ongoing	Ongoing	Ongoing	Safety, feasibility and OS12	Ongoing
NCT01171469 (130)	I	Allogenic BTSCs	Imiquimod	None	8	Increase in IL-17 expressing CD4 (Th17) cells in stable patients compared to non-stable patients	None	NA	NA	MTD and immune response	Vaccine well tolerated with not MTD reached
DENDR-STEM NCT02820584	I	Allogenic BTSC	None	None	20	Ongoing	Ongoing	Ongoing	Ongoing	Safety and Immune response	Ongoing
ICT-121 NCT02049489 (131)	I	CD133	None	None	20	Immune response detected to CD133 epitopes)	NA	NA	NA	Safety and Feasibility	Vaccine was safe and well tolerated
NCT00846456 (132)	I/II	Autologous glioma stem cells lysate	None	None	7	Yes (100% patients, defined via proliferation assay)	NA	23.1	25.3	Safety	Safe vaccine
16-184-4412 (133)	I/II	Autologous glioma cells	None	None	32	Yes (CD8 response in 13% patients: up to 5.5% specific CD8 of total CD8 T cells)	NA	10.3 (r-GMB) 18.3 (nd-GBM)	18.0 (r-GMB) 30.5 (nd-GBM)	Safety, feasibility, immunological response	Positive for safety and feasibility
NCT04388033	I/II	Autologous glioma cells	Temozolomide	None	10	Ongoing	Ongoing	Ongoing	Ongoing	Safety and PFS6	Ongoing
DEN-STEM NCT03548571	II/III	Autologous glioma stem cells	Temozolomide	TMZ	60	Ongoing	Ongoing	Ongoing	Ongoing	PFS	Ongoing

(Continued)

TABLE 2 | Continued

Trial name ClinicalTrial.gov identifier	Phase	Immune targets	Associated treatments in active arm	Control	Sample size	T cell response (CD4/CD8 response details)	Humoral response	Median PFS (months)	Median OS (months)	Primary endpoint	Results
ADDIT-GLIO NCT02649582 (134)	I/II	WT1	Temozolomide	None	20	Ongoing (interim results: CD4 response correlated with OS)	Results pending	Results pending	Results pending (interim results: 43.7)	OS	Ongoing
NCT03879512	I/II	Autologous tumor lysate	Metronomic cyclophosphamide	None	25	Ongoing	Ongoing	Ongoing	Ongoing	Safety and Feasibility	Ongoing
ICT-107 NCT01280552 (135)	II	AIM-2, MAGE1, TRP-2, gp100, HER2 and IL-13Ra2	Temozolomide	TMZ	81 (vs. control 43)	Yes (CD8 response in 50% patients)	NA	11.2	17.0	OS	Positive (trend for OS and significant for PFS)
ICT-107 NCT01006044 (136)	II	AIM-2, MAGE1, TRP-2, gp100, HER2 and IL-13Ra2	Radiotherapy-Temozolimide + fluorescence-guided surgery	None	27	Yes (11/27 patients displayed tumor specific responses with increased serum cytokine levels)	NA	12.7	23.4	PFS	Safe vaccine
DENDR1 EUDRACT N° 2008-005035-15 (137)	II	Autologous tumor lysate	Radiotherapy-Temozolimide	None	22	No	NA	10.5	20.1	PFS12	Positive (PFS12 = 41%)
Audencel NCT01213407 (138, 139)	II	Autologous tumor-derived peptides	Temozolomide	TMZ	34 (vs. control 42)	NA	NA	6.8	18.8	PFS12	Negative
NCT00323115 (140)	II	Autologous glioma lysate	None	None	10	Trends (CD8 and CD4)	NA	9.5	28	Immunological response	Positive trend
NCT01567202 (141)	II	Autologous glioma stem-like lysate	None	Placebo	22 (vs. control 21)	NA	NA	7.7	13.7	OS and PFS	Positive (trend for PFS) (Second phase of trial in IDH1wt TERTmt subgroups of GBM patients ongoing)
NCT01204684	II	Autologous glioma lysate	None	None	60	Results pending	Results pending	Results pending	Results pending	Immunological response	Results pending
AV-GBM-1 NCT03400917	II	Autologous glioma cells	TAA-pulsed DC vaccine plus GM-CSF	None	55	Results pending	Results pending	Results pending	Results pending	OS	Results pending
ELEVATE NCT02366728	II	CMV pp65	+-/Basiliximab	None	100	Results pending	Results pending	Results pending	Results pending	OS and DC migration	Results pending
I-ATTAC NCT03927222	II	CMV pp65	Temozolomide	None	48	Ongoing	Ongoing	Ongoing	Ongoing	OS	Ongoing
ATTAC-II NCT02465268	II	CMV pp65	Temozolomide	Unpulsed PBMC and saline	120	Ongoing	Ongoing	Ongoing	Ongoing	OS	Ongoing
DERIVe NCT03688178	II	CMV pp65	Varlilumab plus Temozolomide	Unpulsed DCs	112	Ongoing	Ongoing	Ongoing	Ongoing	OS, Safety and T reg depletion	Ongoing
GlioVax NCT03395587 (142)	II	Autologous glioma lysate	DC vaccine plus TMZ	TMZ	136	Ongoing	Ongoing	Ongoing	Ongoing	OS	Ongoing
ADCV01 NCT04115761	II	Autologous glioma lysate	Temozolomide	TMZ	24	Ongoing	Ongoing	Ongoing	Ongoing	PFS12	Ongoing
NCT00576537	II	Autologous tumor lysate	None	None	50	Ongoing	Ongoing	Ongoing	Ongoing	Safety and feasibility	Ongoing
ADCTA-G NCT02772094 (143)	II	Autologous tumor lysate	TMZ + Radiotherapy	None	42	NA	NA	NA	22.9 (median for this trial and Taiwan DOH/ MA0910072504)	OS and safety	Positive
Combi G-Vax NCT04523688	II	Autologous tumour lysate	TMZ + radiotherapy	None	28	Ongoing	Ongoing	Ongoing	Ongoing	PFS	Ongoing
STING (ICT-107) NCT02546102	III	AIM-2, MAGE1, TRP-2, gp100, HER2 and IL-13Ra2	None	Autologous PBMCs	Estimated 414 but suspended	NA	NA	NA	NA	Overall survival	Suspended
DCVax-L NCT00045968 (144)	III	Autologous tumor lysate	None	Autologous PBMC	331	NA	Results pending	NA	Results pending (interim results: 23.1)	PFS	Results pending
NCT04277221	III	Autologous tumor lysate	Bevacizumab	Bevacizumab	118	Ongoing	Ongoing	Ongoing	Ongoing	OS	Ongoing

KLH, keyhole limpet haemocyanin, TTF, Tumor Treating Fields; nd-GBM, newly diagnosed glioblastoma; r-GBM, recurrent glioblastoma; TAA, Tumor Associated Antigen; PFS, progression free survival; OS, overall survival; Td, Tetanus toxoid; GM-CSF, Granulocyte Macrophage-Colony Stimulating Factor; DI-TMZ, Dose-Intensified Temozolomide; BTSC, Brain Tumor Stem Cells; CMV, Cytomegalovirus; MTD, maximum tolerated dose.

TABLE 3 | Other types of vaccine trials for glioblastoma.

Trial nameClinicalTrial.gov identifier	Phase	Type of vaccine	Immune targets	Associated treatments in active arm	Control	Sample size (evaluable patients)	T cell response(CD4/CD8 response details)	Humoral response	Median PFS (months)	MedianOS (months)	Primary endpoint	Results
NCT04015700	I	DNA	Personalized neoantigen	None	None	6	Ongoing (not yet recruiting)	Ongoing (not yet recruiting)	Ongoing (not yet recruiting)	Ongoing (not yet recruiting)	Safety	Ongoing (not yet recruiting)
VXM01 NCT03750071	I/II	DNA	VEGFR2	Avelumab	None	30	Ongoing	Ongoing	Ongoing	Ongoing	Safety	Ongoing
Glovac – ERC1671 NCT01903330 (145)	II	Tumor cells	Autologous inactivated tumor cells mixed with tumor cell lysates	Cyclophosphamide plus Bevacizumab	placebo plus bevacizumab	84	Ongoing	Ongoing	Ongoing	Ongoing	OS and PFS	Ongoing
HSPPC-96 NCT00293423 (146)	I	HSPPC-96 -peptides	autologous tumor-derived HSPPC-96	None	None	12	Yes (CD8 response in 92% patients - analyzed via re-stimulation essay)	NA	NA	36.0	Safety	Safe vaccine
HSPPC-96 NCT00905060 (147)	II	HSPPC-96 -peptides	autologous tumor-derived HSPPC-96	Temozolomide	None	46	Results pending	Results pending	17.8	23.8 (44.7 in patients with low PD-L1 expression)	Safety, survival	Results pending
NSPPC-96 NCT00293423 (148)	II	HSPPC-96 -peptides	autologous tumor-derived HSPPC-96	Temozolomide	None	41	NA	NA	19.1	42.6	Safety	Safe vaccine
ALLIANCE NCT01814813 (149)	II	HSPPC-96 -peptides	autologous tumor-derived HSPPC-96	Bevacizumab	Bevacizumab	90	Results pending	Results pending	Results pending	Results pending (interim results : 7.5)	OS	Negative
NCT03018288	II	HSPPC-96 -peptides	autologous tumor-derived HSPPC-96	Temozolomide and pembrolizumab	TMZ and Pembrolizumab plus placebo	108	Ongoing	Ongoing	Ongoing	Ongoing	OS12	Ongoing
NCT03650257	II	HSPPC-96 -peptides	HSPPC-96	Temozolomide	Temozolomide	150	Ongoing	Ongoing	Ongoing	Ongoing	OS12	Ongoing

HSPPC-96, heat-shock protein peptide complex 96; KLH, keyhole limpet haemocyanin; PD-L1, Programmed death-ligand 1; PFS, progression free survival; OS, overall surviva.

tumor associated macrophages (TAMs); Nagai et al. (2009) utilized this methodology to selectively target TAMs. Activated TAMs were shown to express folate receptor beta (FRβ), thereby providing a macrophage-specific target. The heavy and light chains of an anti-FRβ antibody were conjugated to the toxin *Pseudomonas exotoxin* (152). The abundance of macrophages within the tumor allows delivery of the toxin to the tumor resulting in the death of tumor cells and the potentially immunosuppressive macrophages. Administration of this immunotoxin intratumorally to a subcutaneous rat C6 glioma tumor reduced tumor growth and the number of TAMs in these tumors (152). It is important to note that this treatment was injected directly into subcutaneous tumors which reduces the potential for any deleterious off-target effects. Although FRβ was not detected in the normal brain, it was detected on macrophages resident in the heart and liver (152). This presents a potential hurdle to the systemic delivery of this immunoconjugate. The ability of this drug to cross the blood brain barrier is also unknown since this study utilized a subcutaneous model. In patients, this immunoconjugate could be administered intratumorally during surgery, or intraventricularly utilizing an Ommaya reservoir (an intracranial catheter device that allows direct delivery of drugs to the ventricles), thereby bypassing the blood brain barrier. However, this method is highly invasive and not without risks (153, 154).

Propentofylline (PPF) is a synthetic methylxanthine drug that is known to reduce the proliferation (155) and expression of inflammatory cytokines (155) by microglia in response to lipopolysaccharide. PPF could therefore be a novel therapeutic for targeting microglia within GBM tumors. In a rat model of GBM utilizing the CNS-1 cell line, a cell line which recapitulates the features of human GBM with minimal immunogenicity, systemic PPF administration reduced the volume of intracranial CNS-1 tumors (156). *In vitro* analysis revealed that PPF did not exert its effects on the CSF-1 cell line, rather its anti-tumor effects were attributed to its effect on microglial migration and the contribution of microglia to tumor cell migration (156). Rather than trying to remove microglia/macrophages from the TME, switching immunosuppressive M2 cells to the immune activating M1 phenotype also represents an attractive therapeutic option.

IL-12 represents an excellent immunotherapeutic candidate due to its ability to activate T-cells and NK cells and provoke antigen-specific immunity (157). As systemic administration of recombinant IL-12 was associated with adverse effects (such as damage to vital organs), gene transfer of IL-12 was achieved by the intracranial administration of an adeno-associated virus (AAV) encoding IL-12 to rats, after which they were challenged by intracranial injection of rat RG2 GBM cells. Treatment improved the survival of tumor challenged mice when compared to PBS injected control mice. Analysis of treated tumors revealed an increase in the microglial activation markers ED1 and TNF-related apoptosis-inducing ligand (TRAIL), and this was accompanied by a downregulation of the proliferation marker Ki67 and an increase in TUNEL staining - an indicator of apoptosis (157).

The blockade of TGF-β presents an attractive adjunct for active immunotherapy, due to its immunoregulating and tumor promoting effects. Trabedersen is an anti-sense RNA for human TGF-β2 mRNA that has been administered via convection-enhanced delivery to patients with recurrent GBM. Although Trabedersen improved the median survival compared to chemotherapy alone, this difference was not of statistical significance (158). In a pre-clinical murine model of metastatic pancreatic cancer, active vaccination was combined with antibody blockade of TGF-β. Soares et al. (2015) treated a murine model of pancreatic cancer using a vaccine comprised of GM-CSF secreting irradiated pancreatic cancer cells known as GVAX. This vaccine was used to treat two models of pancreatic cancer, the Panc02 model and KPC model. When GVAX vaccination was combined with TGF-β blockade, the cure rate of tumor bearing mice was improved in both models when compared to mice given GVAX with an IgG isotype antibody. The anti-tumor effects of GVAX were even further improved when the vaccine was combined with both an anti-TGF-β and anti-PD-1 antibody. This blockade of TGF-β in combination with GVAX reduced the regulatory T cell infiltrate into these tumors, a trend not seen when either therapy was used alone (159).

Immune Checkpoint Blockade

Due to the expression of numerous immunosuppressive checkpoints within the GBM TME, many checkpoint blockade antibodies have been tested in the GBM setting. Immune checkpoint blockade also represents a method for rescuing exhausted T cells. As monotherapies, immune checkpoints have provided lackluster results (160–162). One interesting method for altering the responsiveness to immune checkpoint blockade is to administer these immune checkpoint blocking antibodies in a neoadjuvant setting, as opposed to an adjuvant setting. Neoadjuvant administration of checkpoint blockade involves the dosing of the patient prior to tumor resection and standard therapy as opposed to after surgery and alongside standard therapy. In the GBM setting, neoadjuvant PD-1 blockade has been explored patients with recurrent disease - these patients received neoadjuvant PD-1 and therapy was then continued in the adjuvant setting post-surgery. Neoadjuvant treatment prolonged the overall survival when compared to adjuvant PD-1 blockade, and increased $CD8^+$ T cell infiltration into tumors. An upregulation in the expression of interferon gamma related genes was also seen in the tumors of these patients (163).

Combining checkpoint blockade modalities or combining active immunotherapy with checkpoint blockade are also attractive methods for enhancing protective anti-GBM immunity. In a pre-clinical murine model of GBM, PD-1 blockade was combined with DC vaccination to great effect. Mice bearing intracranial GL261 tumors were vaccinated with DCs loaded with murine GL261 tumor cell lysate. Although this approach increased the infiltration of tumor cells into these intracranial tumors, this did not lead to improved survival in mice with an elevated tumor burden. It was hypothesized that local immune suppression within the TME was preventing tumor-specific lymphocytes from inducing tumor cell death. TILs were shown to have up-regulated their expression of PD-1, as a result of which it was decided to combine anti-PD-1 antibody therapy with DC vaccination. This combination increased the percentage of activated $CD8^+$ T cells within the intracranial tumors and improved the survival of mice when compared to mice given vaccination alone (164).

As CSF-1R inhibition has been shown to reduce polarization of macrophages to the immunosuppressive M2 phenotype (95), combining CSF-1R inhibition with active vaccination and PD-1 blockade has been explored in the GBM setting. Myeloid derived cells recruited to the tumor were shown to express PD-L1 and contribute to the immunosuppressive environment seen in murine GL261 tumors. The presence of vaccine-induced TILs increased the recruitment of these immunosuppressive PD-L1 expressing myeloid cells. As a result, Antonios et al. (2017) combined PD-1 antibody and a CSF-1R inhibitor with active DC vaccination. CSF-1R inhibition increased the presence of TILs within tumors, whereas PD-1 blockade improved the activation of TILs. This triple therapy significantly increased the survival of GL261 tumor bearing mice when compared to non-treated, DC vaccinated and DC vaccinated mice with either CSF-1R or PD-1 blockade alone (165).

As detailed earlier, IDO and TDO expression within GBM tumors contributes to the immunosuppressive nature of these tumors. Targeting IDO alone or as part of a combinatorial strategy therefore also represents an attractive treatment avenue. The anti-viral drug acyclovir has been shown to inhibit both IDO and TDO and preventing the recruitment of regulatory T cells to the TME (166). In a pre-clinical murine model of GBM, the combined blockade of IDO, CTLA-4 and PD-1 reduced regulatory T cell infiltration into tumors and led to 100% long-term survival in mice harboring intracranial GL261 tumors (167).

Engineered CAR T Cells

Chimeric antigen receptor (CAR) T cells provide an avenue for generating tumor targeted T cells that can function in the defective tumor microenvironment. CAR T cells are generated by transfecting autologous T cells taken from patients with a construct combining a single chain variable fragment specific to a tumor cell target with costimulatory domains that enable T cell activation without the need for a secondary co-stimulatory signal (168). Numerous antigens have been targeted utilizing CAR T cells and the design of CAR T cells has been fine-tuned in order to optimize their anti-tumor activity. Traditionally CAR T cells' intracellular signaling domain was derived from the CD3ζ chain of the T cell receptor (first generation), as progress has been made further costimulatory domains have been added to the intracellular region in order to improve the functionality of CAR T cells (second and third generation). These costimulatory domains are often derived from costimulatory CD28, OX-40, ICOS, and 4-1BB (169, 170). Whilst the design of the targeting domain of the CAR T cells has evolved so has the general design of these cells, with the knock in of other genes that enhance anti-

tumor function being explored (see **Table 4**). As mentioned previously, GBM tumors frequently upregulate their expression of FasL (63, 175). CAR T cells generated from patient derived T cells often express Fas, which makes these T cells susceptible to FasL mediated cytotoxicity when entering the TME (176). The development of CAR T cells expressing Fas dominant negative receptors by Yamamoto and colleagues resulted in the persistence of cells without any deleterious side-effects such as autoimmunity or lymphoproliferative disease (177). CAR T cells expressing a dominant negative receptor for TGF-β have also been developed for the treatment of prostate cancer. These CAR T cells target a prostate antigen known as prostate-specific membrane antigen (PSMA) and they also express the dominant negative TGF-βRII that blocks TGF-β signaling. These CAR T cells displayed improved anti-tumor function when compared to CAR T cells that did not have the dominant negative TGF-βRII transfected into them. These CAR T cells appeared to exhibit long-term persistence and resistance to exhaustion (178). CAR T cells have also been engineered to secrete a PD-1 blocking antibody single chain variable fragment (scFv) that binds to PD-1 on the surface of activated T cells (both CAR and bystander T cells), thereby preventing PD-L1 on tumor cells from dampening T cell anti-tumor responses (179). These CAR T cells enhance the survival of PD-L1 expressing tumor bearing mice when compared to CAR T cells that do not secrete the PD-1 scFv combined with an anti-PD-1 antibody. This is believed to be due to the increased amount of PD-1 blockade within the TME when compared to systemic checkpoint blockade. These CAR T cells displayed efficacy against both hematologic and solid tumors (179). CAR T cells have also been modified to express the immune-stimulatory molecule CD40L to improve the anti-tumor function of these cells (180). The interaction of CD40L on these T-cells with CD40 on DCs results in the secretion of the immunostimulatory cytokine IL-12 (180). CD19 directed CAR T cells armed with the CD40 ligand have been shown to lyse CD19 negative cells and prevent their expansion and the development of antigen negative variants that escape an immune response (180). In order to prevent the development of antigen escape variants, CAR T cells have also been developed to produce bi-specific T cell engagers (BiTEs) in the GBM setting. EGFRvIII targeting CAR T cells have been developed to secrete BiTEs that target the wild type epidermal growth factor receptor (EGFR). These BiTEs contain an anti-EGFR domain along with an anti-CD3 domain, homing T cells onto EGFR expressing tumor cells. The secretion of these BiTEs recruits bystander cells that target tumor cells, these CAR T cells can also eradicate tumors that do not express the EGFRvIII antigen, thereby highlighting the importance of the BiTEs produced by these CAR T cells (181). One study looked at utilizing CD123 (IL-3 Receptor α chain) directed CAR T cells to target Hodgkin lymphoma cells. The investigators also hypothesized that as CD123 is expressed on myeloid cells, these CAR T cells could also target these cells and overcome the local immune suppression induced by MDSCs and M2 macrophages. These CAR T cells targeted lymphoma cells *in vitro* and *in vivo*. What was even more interesting was that these CAR T cells were resistant to inhibition by M2 macrophages when compared to classical CD19 targeting CAR T cells (182).

Oncolytic Virotherapy

The design and delivery of immunotherapies must consider the pronounced immunosuppressive environment of the TME in GBM. The use of oncolytic viruses, which can selectively infect and kill tumor cells, is beginning to generate increased interest due to its tumor specificity and the ability of these viruses to turn an immunosuppressive microenvironment into an immune supporting environment (183). Oncolytic viruses are genetically altered to not infect non-transformed cells, and, in some cases, other genes may be knocked down or knocked in to enhance the immune stimulatory properties of these viruses. For example, the oncolytic herpes simplex virus T-VEC has been transfected with the human *granulocyte-macrophage colony-stimulating factor (GM-CSF)* gene. GM-CSF secreted by the virus increases the recruitment of DCs into the TME and thereby enhances antigen presentation and T cell activation (184). Tumor cell lysis by oncolytic viruses also triggers inflammatory immune responses involving the release of antigens, danger associated molecular patterns (DAMPs) and pathogen associated molecular patterns (PAMPs) within the TME (184). Several different types of viruses have been used in the oncolytic virotherapy of GBM, viruses such as the herpes simplex virus (HSV), Newcastle disease virus (NDV), poliovirus, reovirus, adenovirus, measles virus and H1 parvovirus (185) (see **Table 5**). Not only have viruses been used to directly induce the death of tumor cells they have also been used to transfer genes to tumor cells that enable these cells to be targeted. One such example of one of these viruses is Toca 511, a retroviral vector that delivers cytosine deaminase to rapidly dividing malignant cells (193). The transferred cytosine deaminase enzyme then converts the pro-drug 5-fluorocytosine to the active antineoplastic compound 5-fluorouracil resulting in the death of tumor cells (193). The use of this virus pro-drug combination has also been shown to result in an increase of immune cell activity within murine brain tumors, with a decrease in immunosuppressive cells and an increase in interferon gamma positive CD8 T cells within the tumor microenvironment (194). Whilst in treating preclinical models of GBM Toca 511 showed great promise recent however results from a phase II/III clinical trial revealed that Toca 511 in combination with 5-fluorocytosine did not improve overall survival when compared to standard therapy (195). Although oncolytic viral therapy represents an exciting avenue for GBM therapy, it is not without obstacles and as a result combinatorial therapy utilizing oncolytic viruses needs to be considered. Very little virus crosses the blood brain barrier when oncolytic viruses are delivered systemically, yet these therapies are still efficacious in brain tumor models. Oncolytic herpes simplex viruses (HSVs) can be used in combination with various other therapeutics for the treatment of GBM. The virus can also be altered with immunomodulating transgenes to improve anti-tumor efficacy and enable modulation of the TME (196). 'Arming' an oncolytic HSV with the murine IL-4 gene has been shown to increase the survival of mice bearing intracranial GL-261 cells. Conversely, no survival benefit compared to sham treated animals was observed when immunosuppressive IL-10 was transfected into this oncolytic virus (197). Clinical testing of

TABLE 4 | CAR T cell trials for glioblastoma.

Trial name ClinicalTrial.gov identifier	Phase	CAR generation	Targets	Associated treatments in active arm	Sample size (evaluable patients)	Median PFS (months)	MedianOS (months)	Primary endpoint	Results
NCT01109095 (171)	I	Second	HER2 and CMV pp65	None	16	NA	24.8 months for children and 30 months for adults	Safety and feasibility	Positive
NCT02442297	I	Second	HER2	None	28	Ongoing	Ongoing	Safety and feasibility	Ongoing
NCT02208362 (172)	I	Second	IL13Rα2	None	92	Ongoing	Ongoing	Safety and feasibility	Ongoing
NCT02209376 (173)	I	Unknown	EGFRvIII	None	10	Not evaluable	8 months	Safety and feasibility	CAR T cells seen to traffic to tumours, however adaptive changes in TME need to be accounted for
NCT02664363	I	Third	EGFRvIII	TMZ induced lymphodepletion	3	Ongoing	Ongoing	MTD	Ongoing
NCT04003649	I	Second	IL-13Rα2	Ipilimumab and Nivolumab	60	Ongoing	Ongoing	Safety and feasibility	Ongoing
INTERCEPT NCT03283631	I	Unknown	EGFRvIII	None	24	Ongoing	Ongoing	MTD	Ongoing
NCT02844062	I	Unknown	EGFRvIII	None	20	Ongoing	Ongoing	Saftey and feasibility	Ongoing
NCT03726515 (174)	I	Unknown	EGFRvIII	Pembrolizumab	7	Ongoing	Ongoing	Safety, feasibility, OS and PFS	Ongoing
NCT04077866	I	Unknown	B7-H3	TMZ	40	Ongoing	Ongoing	OS	Ongoing
NCT04045847	I	Unknown	CD147	None	31	Ongoing	Ongoing	Safety and feasibility	Ongoing
NCT02937844	I	Second	PD-L1 (PD-1 on CAR T cell linked to co-stimulatory CD28 cytoplasmic domain)	Cyclophosphamide and Fludarabine	20	Ongoing	Ongoing	Safety and feasibility	Ongoing
NCT04270461	I	Third	NKG2D	None	10	Ongoing	Ongoing	Safety and feasibility	Ongoing
NCT04385173	I	Unknown	B7-H3	TMZ	12	Ongoing	Ongoing	Safety, feasibility, OS and PFS	Ongoing
NCT01454596	I/II	Third	EGFRvIII	Chemotherapy induced lumphodepletion and aldesleukin	18	Ongoing	Ongoing	Safety, feasibility and PFS6	Ongoing

PFS, progression free survival; OS, overall survival; MTD, maximum tolerated dose.

TABLE 5 | Viral therapy trials for glioblastoma.

Trial name ClinicalTrial.gov identifier	Phase	Virus used/mode of action	Associated treatments in active arm	Control	Sample size (evaluable patients)	Median PFS(months)	MedianOS(months)	Primary endpoint	Results
NCT00390299	I	Oncolytic carcinoembryonic antigen expressing measles virus (MV-CEA)	None	None	23	Ongoing	Ongoing	Safety, feasibility OS and PFS	Ongoing
NCT02444546	I	Reovirus (REOLYSIN®)	Sargramostim (GM-CSF)	None	6	Ongoing	Ongoing	MTD and safety	Ongoing
NCT00528684	I	Reovirus (REOLYSIN®)	None	None	18	Ongoing	Ongoing	MTD and safety	Ongoing
NCT00031083	I	Adenoviral transfer of IFN-β gene	None	None	35	Suspended	Suspended	Safety and feasibility	Suspended
NCT03043391	I	Poliovirus (PVSRIPO)	None	None	12	Ongoing	Ongoing	Safety, feasibility and OS24	Ongoing
NCT03072134	I	Neural stem cells loaded with adenovirus	None	None	13	Ongoing	Ongoing	Safety and feasibility	Ongoing
NCT03911388	I	HSV G207	+/- Single dose of 5 Gy radiation	None	15	Ongoing	Ongoing	Safety and feasibility	Ongoing
NCT01491893 (186)	I	Poliovirus (PVSRIPO)	None	Historical controls	15	Results pending	Results pending (interim: 12.6)	MTD, safety and feasibility	Positive
NCT02457845 (187)	I	HSV G207	None	None	5	Results pending	Results pending	Safety and feasibility	Positive
D24GBM NCT01956734	I	Adenovirus (DNX-2401)	TMZ	None	31	Pending	Pending	Safety PFS6 and OS12	Pending
NCT02197169 (188)	I	Adenovirus (DNX-2401)	+/- IFNγ	None	27	Results pending	Results pending (interim OS12 = 33 %, interim OS18 = 22 %)	Safety and feasibility	DNX-2401 was well tolerated however the addition of IFNγ made no difference to efficacy
NCT03657576	I	C134-HSV	None	None	24	Ongoing	Ongoing	Safety and efficacy	Ongoing
NCT03152318	I	HSV (RQNestin34.5v.2)	+/- Cyclophosphamide	None	108	Ongoing	Ongoing	MTD	Ongoing
NCT02026271 (189)	I	Ad-RTS-hIL-12	Veledimex	None	31	NA	12.7	Safety and feasibility	Positive
NCT03636477	I	Ad-RTS-hIL-12	Veledimex + Nivolumab	None	21	Ongoing (not recruiting)	Ongoing (not recruiting)	Safety and feasibility	Ongoing (not recruiting)
NCT03896568	I	Allogenic stem cells loaded with adenovirus (DNX-2401)	None	None	36	Ongoing	Ongoing	Safety, feasibility and MTD	Ongoing
NCT03679754	I	Ad-RTS-hIL-12	Veledimex	None	36	Ongoing	Ongoing	Safety and feasibility	Ongoing
NCT01811992	I	Ad-hCMV-TK and Ad-hCMV-Flt3L	None	None	19	Ongoing (not recruiting)	Ongoing (not recruiting)	Safety and feasibility	Ongoing (not recruiting)
NCT03714334	I	DNX-2440	None	None	24	Ongoing	Ongoing	Safety, feasibility and OS	Ongoing
NCT02031965	I	HSV-1716	Dexamethasone + surgery	None	2	Results pending	Results pending	MTD	Results pending
NCT02062827	I	HSV-1	None	None	36	Ongoing	Ongoing	MTD	Ongoing
NCT04327011	I	Toca 511/5-FC	None	None	65	Terminated	Terminated	Safety and OS	Terminated
NCT00028158	I/II	HSV G207	None	None	65	Results pending	Results pending	Safety and feasibility	Results pending
NCT01301430 (190)	I/II	Parovirus H-1 (ParvOryx)	None	None	18	Results pending	Results pending	Safety and feasibility	Results pending

(Continued)

TABLE 5 | Continued

Trial name ClinicalTrial.gov identifier	Phase	Virus used/mode of action	Associated treatments in active arm	Control	Sample size (evaluable patients)	Median PFS(months)	MedianOS(months)	Primary endpoint	Results
ONCOVIRAC NCT03294486	I/II	TG6002/5-FC	None	None	78	Ongoing	Ongoing	Safety and feasibility	Ongoing
NCT01582516	I/II	Adenovirus (Delta-24-rgd)	None	None	20	Results pending	Results pending	Safety, feasibility and OS	Results pending
NCT00589875 (191)	II	AdV-tk	Valacyclovir + standard of care	Matched control cohort	48	12.7	25.1 for patients with maximal resection	Safety, feasibility and OS	Positive
NCT04482933	II	HSV G207	Single dose of 5 Gy radiation	None	30	Ongoing (Not yet recruiting)	Ongoing (Not yet recruiting)	OS	Ongoing (not yet recruiting)
NCT02798406	II	Adenovirus (DNX-2401)	Pembrolizumab	None	49	Ongoing (not recruiting)	Ongoing (not recruiting)	Objective response rate	Ongoing (not recruiting)
NCT00870181 (192)	II	ADV-TK	Ganciclovir + chemotherapy	None	47	8.7	11.4	PFS6	Positive
NCT04406272	II	VB-111	Bevacizumab	Standard of care	45	Ongoing	Ongoing	TIL density and dose limiting toxicity	Ongoing
NCT04006119	II	Ad-RTS-hIL-2	Cemiplimab	None	36	Ongoing (not recruiting)	Ongoing (not recruiting)	Safety, feasibility and OS	Ongoing (not recruiting)
NCT04105374	II/III	Toca 511/Toca FC	TMZ + radiotherapy	Standard of care	Terminated	Terminated	Terminated	PFS and OS	Terminated

PFS, progression free survival; OS, overall survival; MTD, maximum tolerated dose.

oncolytic viruses remains in its relative infancy, with several viral therapies undergoing phase I/II clinical trials (198). The prospect of genetically modifying these viruses provides great hope for the future treatment of GBM. Not only can viruses be genetically manipulated but they can also be combined with other immunotherapeutic modalities to help overcome the immunosuppressive TME. One such example is combination of oncolytic measles virus therapy with anti-PD-1 checkpoint blockade (199). This combination was shown to increase survival in C57BL/6 mice bearing intracranial GL261 tumors when compared to either monotherapy, as well as increasing survival this combinatorial therapy increased T cell infiltrate into these tumors (199). Checkpoint blockade has also been combined with the IL-12 expressing oncolytic HSV in a pre-clinical model of GBM to great effect (200). This IL-12 secreting HSV was combined with both anti-CTLA-4 and anti-PD-1 checkpoint blockade for the treatment of two murine GBM models, this triple therapy reduced the number of regulatory T cells present within tumors and increased the influx of immune supporting M1 macrophages resulting in the complete cure of these mice (200). As previously mentioned GBMs frequently overexpress PGE2 which promotes an immunosuppressive environment and provides an attractive target for therapy. An oncolytic vaccina virus has been developed that expresses 15-(NAD)-hydroxy-prostaglandin-inactivating enzyme (HPGD); an enzyme that inactivates PGE2 (201). This modified vaccinia virus was tested in a variety of mouse solid tumor models and it was found to reduce the number of MDSCs and regulatory T cells within these tumors increasing the response of these tumors to viral therapy and adoptive T cell transfer (201). Whilst viral therapy is in its relative infancy with regards to clinical approval these early findings provide great hope for the future of this treatment modality.

Combining Immunotherapy With Standard Therapy

Adapting current therapies also needs to be considered in the context of immunotherapy for GBM, especially given the likelihood that all new approaches will need to be delivered in the context of current 'standard' therapy. Both TMZ and radiotherapy have immune augmenting effects that can be capitalized upon when considering the immunotherapeutic treatment of GBM. As mentioned previously, TMZ can induce lymphodepletion in patients. This lymphodepletion can be capitalized on to potentially enhance the efficacy of CAR T cell therapy. In a murine model of GBM, EGFRvIII CAR T cells failed to confer a survival advantage for mice bearing intracranial EGFRvIII expressing tumors, despite that fact that these cells were shown to have anti-tumor cell activity *in vitro*. Lymphodepletion with radiotherapy administered prior to CAR T cell therapy was shown to improve the efficacy of CAR T cell therapy by resulting in long-term survival of mice (202). Similarly, TMZ was used to lymphodeplete prior to CAR T cell administration. TMZ was either used in a standard or high dose, with the higher dose inducing more marked lymphodepletion. The lymphodepletion caused by high dose TMZ increased the survival of mice bearing established intracranial tumors when given CAR T cell therapy. This lymphodepletion to led to

persistence of the injected CAR T cells within the blood of treated mice and this correlated with lower tumor burden (202). As well as using high dose TMZ to lymphodeplete, the dosing can also be given as low frequent doses, known as metronomic dosing. Ouyang and colleagues (2016) designed immune activating CpG carbon nanotube conjugates (SWCNT/CpG-2) that prolonged the survival of mice bearing intracranial GL261 tumors. This SWCNT/CpG-2 was used to treat a more invasive GBM model using the KR158B cell line, a model that more faithfully represents the characteristics of human GBM within a murine model. Although this intracranial SWCT/CpG-2 therapy was not curative for this KR158B model, as it was in the case of the GL261 model, when this SWCT/CpG-2 was combined with low dose daily TMZ, it significantly improved survival when compared to SWCT/CpG-2 monotherapy (203). Splenocytes taken from mice that had received metronomic TMZ in combination with SWCT/CpG-2 were more efficient at inducing *in vitro* KR158B tumor cell death than splenocytes from mice given either SWCT/CpG-2 or TMZ alone. This dual therapy did not reduce the number of regulatory T cells in the tumors. However, both SWCT/CpG-2 therapy and dual therapy induced an increased macrophage infiltrate into the tumors. The researchers hypothesized that the metronomic TMZ dosing increased the relative proportions of immune activating M1 macrophages to immune inhibitory M2 macrophages within the tumors (203). Radiotherapy can also be used as an adjunct to immunotherapy in order to boost the anti-tumor immune response. Weiss *et al.* (2018) generated an NKG2D expressing CAR T cell therapy that when systemically administered penetrated brain tumors in a murine GL261 GBM model. These NKG2D CAR T cells were shown to cure 22% of GL261 bearing mice treated. Radiotherapy upregulated the expression of NKG2D ligands on the surface of GBM cells and, as a result, it was decided to combine radiotherapy with NKG2D CAR T cells. Mice were given a single 4 gray (Gy) dose of radiotherapy on day 7 after tumor implant and CAR T cells were given on days 5, 7 and 10. The single radiotherapy dose alone did not alter the survival of tumor bearing mice compared to control mice, however it increased the survival of mice harboring intracranial GL261 cells when combined with the NKG2D CAR T cell therapy. This effect was also shown in mice bearing intracranial SMA-560 tumor cells (204). Another alternative that has been considered is the intratumoral administration of TMZ, as opposed to systemically administered TMZ. This local delivery of TMZ could theoretically prevent the profound lymphodepletion seen in systemic administration due to the therapy being mainly confined to the tumor. This method of administering TMZ was shown to improve the survival of mice bearing GL261 cell-derived tumors when compared to mice given intraperitoneal TMZ. CD4 and CD8 blocking antibodies revealed that T cells are responsible for this improved survival, with mice receiving intracranial TMZ failing to show improved survival if T cell blocking antibodies were used. Survival was improved even further when intracranial TMZ was combined with active immunotherapy using irradiated GL261 cells transfected to express GM-CSF (205). This combined intracranial TMZ and immunotherapy increased $CD8^+$ T cell infiltrate and decreased MDSCs (205).

Overcoming the Blood Brain Barrier (BBB)

The BBB can act as a significant barrier for systemically administered therapeutics, including immune checkpoint blocking antibodies. Several approaches can be used to address this issue. These include direct modification and masking of therapeutic agents, encapsulation of therapeutics within vesicle-based delivery systems, and targeted opening of the BBB/BTB by physical or biochemical disruption. Conceptually, the simplest route to bypass the BTB is direct administration to the brain parenchyma or the cerebrospinal fluid (CSF). Although this is a commonly used approach in pre-clinical, experimental work, it is clinically problematic. Direct intra-parenchymal injection is rarely performed outside of intensive care medicine due to the difficulties associated with infection risk and needle damage. Moreover, although GBM are rarely metastatic (206), direct administration to the tumor site is contra-indicated due to the slow rate of diffusion of therapeutic molecules through compact brain tissue, injected substances rarely travelling more than a few millimeters beyond the injection site (207–209). This route is therefore unlikely to be sufficient for treating GBM, given both the likely tumor size on diagnosis and accessibility issues. Intracerebroventricular or intrathecal injection, delivery to the CSF, has similarly poor distribution issues (210). Passage of drugs from the CSF to the parenchymal tissue is primarily diffusive, which, coupled with rapid removal from the ependymal surface via bulk flow and the glymphatic system, results in minimal transfer of therapeutic agents into the tissue (211, 212). These restrictions are even more relevant to the delivery of large molecules such as therapeutic antibodies (213).

Working on the principle that the simplest way to overcome the BBB/BTB is to remove it, a number of methods of disrupting barrier function have been investigated for their potential use in the treatment of GBM and other neurological disorders. Such techniques were first begun over 50 years ago, with studies employing hypertonic solutions of osmolytes such as mannitol to induce osmotic endothelial shrinkage and tight junction opening (214). Such untargeted disruption, whilst effective in permitting increased therapeutic access to the brain, is also indiscriminate and enables the entry of pro-inflammatory and potentially toxic serum proteins such as albumin and complement factors (215), thereby rendering this non-specific approach unsuitable for clinical use. More targeted methods of inducing increased BBB/BTB permeability have used endogenous bioactive agents such as bradykinin or its synthetic analogues (216). Although such approaches have increased permeability of chemotherapeutic agents to the brain in preclinical models, they have not translated into clinical practice, possibly due to having too brief a duration of action (217).

Rather than chemical or osmotic-mediated disruption, another technique used to circumvent the BBB/BTB is the use of high-power focused ultrasound (218) to generate foci of

increased tissue permeability. Although this approach is effective in opening the barrier to therapeutic antibodies (219), it suffers from producing bystander tissue distortion and damage in experimental animals (220). In an attempt to overcome this issue, the technique has been refined to improve specificity and reduce energy transfer through the use of injected microbubbles (221, 222). In this case, lower frequency ultrasound is used to stimulate microbubble oscillation and cavitation, disrupting the endothelial wall through local shock wave production and permitting access of therapeutic agents to the brain. Although promising, it is not yet clear to what degree brain penetration can be enhanced as efflux transport systems remain active (223), and the long-term consequences of disruption have not yet been studied.

As an alternative to BBB/BTB disruption, numerous attempts have been made to modify the therapeutic agents themselves or their delivery systems to permit greater transfer across an intact BBB/BTB. Building on the rationale that more lipophilic agents are better able to cross the BBB/BTB, initial approaches aimed to improve therapeutic agent lipid solubility. Although such modifications do indeed improve CNS access, this was achieved at the cost of increased non-specific membrane permeability and a consequent rise in off-target effects (224, 225).

To overcome these difficulties, ongoing attempts at achieving effective drug delivery across the BBB/BTB have employed a wide range of different nanocarriers, also termed nanoparticles. These are diverse molecular structures, including lipid micelles, liposome composites of phospholipid and other molecules, and polymer-based particles, with the common property that they form a vesicle that can be loaded with therapeutic agents and which can then cross the BBB to enter the parenchyma (226). Once within the brain, variation in environmental pH at the tumor site, amongst other conditions destabilize the nanocarrier structure and trigger release of the cargo within the tissue (227). Although effective, these nanocarriers are indiscriminate and passively deliver their cargo widely across the brain, a drawback that has spurred the development of more effectively targeted nanocarrier delivery systems.

Targeting can be substantially enhanced by including molecular tags within the vesicle wall using proteins, peptides, nucleic acids or small molecules that specifically recognize tumor-associated receptors, thereby minimizing off-target actions. A wide range of different molecular tags have been exploited for this purpose *in vivo*, including for example, the interaction of nanocarrier borne transferrin with the transferrin receptor TfR-1 on GBM cells (228), the EGFP-EGF1 fusion protein on nanoparticles with tissue factor in tumor cells (229), and cholera toxin with tumor-expressed chloride channels and matrix metalloproteinase-2 (230). Such strategies hold significant promise as they allow for both the concentration of therapeutic agents at the tumor site and, by virtue of the encapsulation, protect therapeutic agents from hepatic metabolism (230). Although a number of nanocarrier-encapsulated small molecule approaches are currently undergoing clinical trial in GBM, as yet none have been approved for use (231). Questions about the efficiency of large molecule, i.e. therapeutic antibody, encapsulation efficiency remain.

As direct lipophilic modification of therapeutic agents and encapsulation strategies have relatively broad specificity, even with improved targeting strategies, interest has grown in the use of direct molecular tagging of the therapies themselves to permit recognition by specific endothelial transporters, e.g. the transferrin receptor, insulin receptor or low density lipoprotein receptor (232, 233), a process sometimes termed receptor mediated transcytosis.

This approach has proven to hold significant promise for the experimental delivery of protein agents, including therapeutic antibodies. Exposure of 'normal' CNS to circulating biologic agents is restricted to less than 0.5% of the concentrations that are present in serum (234, 235), a level at which target engagement is unlikely to occur (236). However, molecular engineering of therapeutic antibodies has enabled significant enhancements in uptake across the BBB. These approaches include the development of bispecific antibodies in which one F(ab) binds the target of interest and the other binds and is transported by an endothelial transporter (237), therapeutic antibodies in which a transporter recognition domain is linked to the immunoglobulin heavy or light chain (238, 239), or, more recently, molecules in which the Fc domain itself is directly recognized by an endothelial transporter (240, 241). Such an approach has not yet been tested directly for the clinical delivery of immunotherapies targeting GBM but does hold significant promise. BBB penetrating Nano immunoconjugates have been developed with the aim of crossing the BBB and penetrating intracranial tumors. Galstyan and colleagues (2019) developed anti-CTLA-4 and anti-PD-1 IgG antibodies conjugated to poly (β-L-malic acid), a natural biopolymer scaffold. These Nano immunoconjugates cross the BBB more efficiently than the anti-PD-1 and CTLA-4 IgG antibodies without polymer conjugation, increased the $CD4^+$ and $CD8^+$ T cell infiltrate into tumors and improved the survival of mice bearing intracranial GL261 tumors compared to those treated with the non-conjugated antibodies (242). Antibody delivery to intracranial tumors can also be improved by disrupting the BBB using focused ultrasound and microbubbles to physically disrupt the tight junctions enabling penetrance of the brain parenchyma. The combination of focused ultrasound (FUS) with microbubbles improves entry of the anti-HER2 antibody Herceptin into brains (243). Similar results have also shown with FUS in combination with microbubbles increases the penetrance of anti-amyloid beta antibodies into the brains of mice in two separate models of Alzheimer's disease (244). FUS is an attractive option since the opening of BBB is transient (245), thereby minimizing the potential for damage to the brain. More interestingly, FUS itself can be used therapeutically to target intracranial tumors due to its immunomodulation action. Ultrasound waves can expand and contract air bubbles present within cells to generate heat and physically damage cells, inducing cell death, leading to the release of antigenic material and an up-regulation of immune activating molecules such as heat shock proteins (246).

CONCLUDING REMARKS

Glioblastoma multiforme (GBM) is the most frequently occurring primary brain tumor. It is uniformly fatal due to its highly invasive nature and resistance to standard therapies. GBM tumors employ several mechanisms to avoid being detected and killed by immune cells. These include the downregulation of important immune activating molecules such as MHC molecules, as well as upregulating expression of molecules that induce the death of immune cells such as Fas ligand, non-classical MHC molecules such as HLA-E and -G, and PD-L1. GBM cells also secrete numerous immunoinhibitory cytokines such as IL-10, TGF-β, Gal-1, IL-6 and PGE2, to name a few. These cytokines result in the inactivation/death of immune cells as well as the recruitment of inhibitory cells such as regulatory T cells and MDSCs to the TME. These cytokines also lead to a conversion of tumor resident macrophages from the immune activating M1 phenotype to the immunosuppressive M2 phenotype further dampening the anti-tumor immune response.

The plethora of immunosuppressive mechanisms that GBM tumors utilize, as well as their physiological location, make treating them with immunotherapy a daunting task. Although these tumors are immunosuppressive, this immunosuppression can be leveraged to try and boost the anti-tumor immune response. The concept of combining immune checkpoint blockade with active vaccination is one such method that can be used, or the use of genetically modified oncolytic viruses and CAR T cells that actively attack tumors whilst overcoming the local immunosuppression, either via the secretion of immune activating cytokines or immune blocking scFvs. Combinatorial immunotherapy along with improvement of BBB penetration represents an encouraging avenue for GBM therapy in the future. The only caveat to these combined therapies is the possibility of an overactive immune response and potential autoimmunity, this will have to be monitored when moving combinatorial immunotherapy forward.

AUTHOR CONTRIBUTIONS

JP wrote the initial draft with SC and SM making significant contributions. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Ostrom QT, Gittleman H, Liao P, Vecchione-Koval T, Wolinsky Y, Kruchko C, et al. CBTRUS Statistical Report: Primary brain and other central nervous system tumors diagnosed in the United States in 2010-2014. *Neuro Oncol* (2017) 19(suppl_5):v1–v88. doi: 10.1093/neuonc/nox158
2. Petrecca K, Guiot MC, Panet-Raymond V, Souhami L. Failure pattern following complete resection plus radiotherapy and temozolomide is at the resection margin in patients with glioblastoma. *J Neurooncol* (2013) 111 (1):19–23. doi: 10.1007/s11060-012-0983-4
3. Brandes AA, Tosoni A, Franceschi E, Sotti G, Frezza G, Amista P, et al. Recurrence pattern after temozolomide concomitant with and adjuvant to radiotherapy in newly diagnosed patients with glioblastoma: correlation With MGMT promoter methylation status. *J Clin Oncol* (2009) 27(8):1275–9. doi: 10.1200/JCO.2008.19.4969
4. Sherriff J, Tamangani J, Senthil L, Cruickshank G, Spooner D, Jones B, et al. Patterns of relapse in glioblastoma multiforme following concomitant chemoradiotherapy with temozolomide. *Br J Radiol* (2013) 86 (1022):20120414. doi: 10.1259/bjr.20120414
5. Hadjipanayis CG, Stummer W. 5-ALA and FDA approval for glioma surgery. *J Neurooncol* (2019) 141(3):479–86. doi: 10.1007/s11060-019-03098-y
6. Young RM, Jamshidi A, Davis G, Sherman JH. Current trends in the surgical management and treatment of adult glioblastoma. *Ann Transl Med* (2015) 3 (9):121–5839. doi: 10.3978/j.issn.2305-5839.2015.05.10
7. Pachter JS, de Vries HE, Fabry Z. The blood-brain barrier and its role in immune privilege in the central nervous system. *J Neuropathol Exp Neurol* (2003) 62(6):593–604. doi: 10.1093/jnen/62.6.593
8. Abbott NJ, Patabendige AA, Dolman DE, Yusof SR, Begley DJ. Structure and function of the blood-brain barrier. *Neurobiol Dis* (2010) 37(1):13–25. doi: 10.1016/j.nbd.2009.07.030
9. McArthur S, Loiola RA, Maggioli E, Errede M, Virgintino D, Solito E. The restorative role of annexin A1 at the blood-brain barrier. *Fluids Barriers CNS* (2016) 13(1):17–016-0043-0. doi: 10.1186/s12987-016-0043-0
10. Jain RK, di Tomaso E, Duda DG, Loeffler JS, Sorensen AG, Batchelor TT. Angiogenesis in brain tumours. *Nat Rev Neurosci* (2007) 8(8):610–22. doi: 10.1038/nrn2175
11. Anderson JC, McFarland BC, Gladson CL. New molecular targets in angiogenic vessels of glioblastoma tumours. *Expert Rev Mol Med* (2008) 10:e23. doi: 10.1017/S1462399408000768
12. Dhermain FG, Hau P, Lanfermann H, Jacobs AH, van den Bent MJ. Advanced MRI and PET imaging for assessment of treatment response in patients with gliomas. *Lancet Neurol* (2010) 9(9):906–20. doi: 10.1016/S1474-4422(10)70181-2
13. Henderson JT, Piquette-Miller M. Blood-brain barrier: an impediment to neuropharmaceuticals. *Clin Pharmacol Ther* (2015) 97(4):308–13. doi: 10.1002/cpt.77
14. Dantzer R. Neuroimmune Interactions: From the Brain to the Immune System and Vice Versa. *Physiol Rev* (2018) 98(1):477–504. doi: 10.1152/physrev.00039.2016
15. Heimberger AB, Sampson JH. Immunotherapy coming of age: what will it take to make it standard of care for glioblastoma? *Neuro Oncol* (2011) 13 (1):3–13. doi: 10.1093/neuonc/noq169
16. Engelhardt B, Ransohoff RM. Capture, crawl, cross: the T cell code to breach the blood-brain barriers. *Trends Immunol* (2012) 33(12):579–89. doi: 10.1016/j.it.2012.07.004
17. Wilson EH, Weninger W, Hunter CA. Trafficking of immune cells in the central nervous system. *J Clin Invest* (2010) 120(5):1368–79. doi: 10.1172/JCI41911
18. Ichinose M, Masuoka J, Shiraishi T, Mineta T, Tabuchi K. Fas ligand expression and depletion of T-cell infiltration in astrocytic tumors. *Brain Tumor Pathol* (2001) 18(1):37–42. doi: 10.1007/BF02478923
19. Vega EA, Graner MW, Sampson JH. Combating immunosuppression in glioma. *Future Oncol* (2008) 4(3):433–42. doi: 10.2217/14796694.4.3.433
20. Mullins C, Walter A, Schmitt M, Classen C, Linnebacher M. Tumor antigen and MHC expression in glioma cells for immunotherapeutic interventions. *World J Immunol* (2013) 11/27:3. doi: 10.5411/wji.v3.i3.62
21. Moserle L, Casanovas O. Anti-angiogenesis and metastasis: a tumour and stromal cell alliance. *J Intern Med* (2013) 273(2):128–37. doi: 10.1111/joim.12018

22. Steck PA, Moser RP, Bruner JM, Liang L, Freidman AN, Hwang TL, et al. Altered expression and distribution of heparan sulfate proteoglycans in human gliomas. *Cancer Res* (1989) 49(8):2096–103.
23. Quail DF, Joyce JA. The Microenvironmental Landscape of Brain Tumors. *Cancer Cell* (2017) 31(3):326–41. doi: 10.1016/j.ccell.2017.02.009
24. Woroniecka KI, Rhodin KE, Chongsathidkiet P, Keith KA, Fecci PE. T-cell Dysfunction in Glioblastoma: Applying a New Framework. *Clin Cancer Res* (2018) 24(16):3792–802. doi: 10.1158/1078-0432.CCR-18-0047
25. Brenchley JM, Karandikar NJ, Betts MR, Ambrozak DR, Hill BJ, Crotty LE, et al. Expression of CD57 defines replicative senescence and antigen-induced apoptotic death of CD8+ T cells. *Blood* (2003) 101(7):2711–20. doi: 10.1182/blood-2002-07-2103
26. Huff WX, Kwon JH, Henriquez M, Fetcko K, Dey M. The Evolving Role of CD8(+)CD28(-) Immunosenescent T Cells in Cancer Immunology. *Int J Mol Sci* (2019) 20(11):2810. doi: 10.3390/ijms20112810
27. Chen PY, Wu CY, Fang JH, Chen HC, Feng LY, Huang CY, et al. Functional Change of Effector Tumor-Infiltrating CCR5(+)CD38(+)HLA-DR(+)CD8(+) T Cells in Glioma Microenvironment. *Front Immunol* (2019) Oct 910:2395. doi: 10.3389/fimmu.2019.02395
28. Hanif F, Muzaffar K, Perveen K, Malhi SM, Simjee S. Glioblastoma Multiforme: A Review of its Epidemiology and Pathogenesis through Clinical Presentation and Treatment. *Asian Pac J Cancer Prev* (2017) 18(1):3–9. doi: 10.22034/APJCP.2017.18.1.3
29. Pangrazzi L, Weinberger B. T cells, aging and senescence. *Exp Gerontol* (2020) 134:110887. doi: 10.1016/j.exger.2020.110887
30. Woroniecka K, Chongsathidkiet P, Rhodin K, Kemeny H, Dechant C, Farber SH, et al. T-Cell Exhaustion Signatures Vary with Tumor Type and Are Severe in Glioblastoma. *Clin Cancer Res* (2018) 24(17):4175–86. doi: 10.1158/1078-0432.CCR-17-1846
31. Mirzaei R, Sarkar S, Yong VW. T Cell Exhaustion in Glioblastoma: Intricacies of Immune Checkpoints. *Trends Immunol* (2017) 38(2):104–15. doi: 10.1016/j.it.2016.11.005
32. Ramirez YP, Weatherbee JL, Wheelhouse RT, Ross AH. Glioblastoma multiforme therapy and mechanisms of resistance. *Pharmaceuticals (Basel)* (2013) 6(12):1475–506. doi: 10.3390/ph6121475
33. Lee EQ, Wen PY. Corticosteroids for peritumoral edema: time to overcome our addiction? *Neuro Oncol* (2016) 18(9):1191–2. doi: 10.1093/neuonc/now167
34. Branter J, Basu S, Smith S. Tumour treating fields in a combinational therapeutic approach. *Oncotarget* (2018) 9(93):36631–44. doi: 10.18632/oncotarget.26344
35. Giles AJ, Hutchinson MND, Sonnemann HM, Jung J, Fecci PE, Ratnam NM, et al. Dexamethasone-induced immunosuppression: mechanisms and implications for immunotherapy. *J Immunother Cancer* (2018) 6(1):51–018-0371-5. doi: 10.1186/s40425-018-0371-5
36. McGranahan T, Therkelsen KE, Ahmad S, Nagpal S. Current State of Immunotherapy for Treatment of Glioblastoma. *Curr Treat Options Oncol* (2019) 20(3):24–019-0619-4. doi: 10.1007/s11864-019-0619-4
37. Sengupta S, Marrinan J, Frishman C, Sampath P. Impact of temozolomide on immune response during malignant glioma chemotherapy. *Clin Dev Immunol* (2012) 2012:831090. doi: 10.1155/2012/831090
38. Karachi A, Dastmalchi F, Mitchell DA, Rahman M. Temozolomide for immunomodulation in the treatment of glioblastoma. *Neuro Oncol* (2018) 20(12):1566–72. doi: 10.1093/neuonc/noy072
39. Gzell C, Back M, Wheeler H, Bailey D, Foote M. Radiotherapy in Glioblastoma: the Past, the Present and the Future. *Clin Oncol (R Coll Radiol)* (2017) 29(1):15–25. doi: 10.1016/j.clon.2016.09.015
40. Foulds GA, Radons J, Kreuzer M, Multhoff G, Pockley AG. Influence of tumors on protective anti-tumor immunity and the effects of irradiation. *Front Oncol* (2013) 3:14. doi: 10.3389/fonc.2013.00014
41. Multhoff G, Rödel F, Pockley AG, Gaipl US. Frontiers research topic: radiation-induced effects and the immune system. *Front Oncol* (2013) 3:55. doi: 10.3389/fonc.2013.00055
42. Gaipl US, Multhoff G, Pockley AG, Rödel F. Editorial: Radioimmunotherapy-Translational Opportunities and Challenges. *Front Oncol* (2020) 10:190. doi: 10.3389/fonc.2020.00190
43. Chow KK, Hara W, Lim M, Li G. Combining immunotherapy with radiation for the treatment of glioblastoma. *J Neurooncol* (2015) 123(3):459–64. doi: 10.1007/s11060-015-1762-9
44. Demaria S, Formenti SC. Role of T lymphocytes in tumor response to radiotherapy. *Front Oncol* (2012) 2:95. doi: 10.3389/fonc.2012.00095
45. Formenti SC, Demaria S. Radiation therapy to convert the tumor into an in situ vaccine. *Int J Radiat Oncol Biol Phys* (2012) 84(4):879–80. doi: 10.1016/j.ijrobp.2012.06.020
46. Vanpouille-Box C, Pilones KA, Wennerberg E, Formenti SC, Demaria S. In situ vaccination by radiotherapy to improve responses to anti-CTLA-4 treatment. *Vaccine* (2015) 33(51):7415–22. doi: 10.1016/j.vaccine.2015.05.105
47. Authier A, Farrand KJ, Broadley KW, Ancelet LR, Hunn MK, Stone S, et al. Enhanced immunosuppression by therapy-exposed glioblastoma multiforme tumor cells. *Int J Cancer* (2015) 136(11):2566–78. doi: 10.1002/ijc.29309
48. Tabatabaei P, Visse E, Bergstrom P, Brannstrom T, Siesjo P, Bergenheim AT. Radiotherapy induces an immediate inflammatory reaction in malignant glioma: a clinical microdialysis study. *J Neurooncol* (2017) 131(1):83–92. doi: 10.1007/s11060-016-2271-1
49. Grossman SA, Ye X, Lesser G, Sloan A, Carraway H, Desideri S, et al. Immunosuppression in patients with high-grade gliomas treated with radiation and temozolomide. *Clin Cancer Res* (2011) 17(16):5473–80. doi: 10.1158/1078-0432.CCR-11-0774
50. Gingras MC, Roussel E, Bruner JM, Branch CD, Moser RP. Comparison of cell adhesion molecule expression between glioblastoma multiforme and autologous normal brain tissue. *J Neuroimmunol* (1995) 57(1-2):143–53. doi: 10.1016/0165-5728(94)00178-Q
51. Piao Y, Henry V, Tiao N, Park SY, Martinez-Ledesma J, Dong JW, et al. Targeting intercellular adhesion molecule-1 prolongs survival in mice bearing bevacizumab-resistant glioblastoma. *Oncotarget* (2017) 8(57):96970–83. doi: 10.18632/oncotarget.18859
52. Kamran N, Kadiyala P, Saxena M, Candolfi M, Li Y, Moreno-Ayala MA, et al. Immunosuppressive Myeloid Cells' Blockade in the Glioma Microenvironment Enhances the Efficacy of Immune-Stimulatory Gene Therapy. *Mol Ther* (2017) 25(1):232–48. doi: 10.1016/j.ymthe.2016.10.003
53. Raychaudhuri B, Rayman P, Ireland J, Ko J, Rini B, Borden EC, et al. Myeloid-derived suppressor cell accumulation and function in patients with newly diagnosed glioblastoma. *Neuro Oncol* (2011) 13(6):591–9. doi: 10.1093/neuonc/nor042
54. Jung TY, Jung S, Ryu HH, Jeong YI, Jin YH, Jin SG, et al. Role of galectin-1 in migration and invasion of human glioblastoma multiforme cell lines. *J Neurosurg* (2008) 109(2):273–84. doi: 10.3171/JNS/2008/109/8/0273
55. Kovacs-Solyom F, Blasko A, Fajka-Boja R, Katona RL, Vegh L, Novak J, et al. Mechanism of tumor cell-induced T-cell apoptosis mediated by galectin-1. *Immunol Lett* (2010) 127(2):108–18. doi: 10.1016/j.imlet.2009.10.003
56. Stillman BN, Hsu DK, Pang M, Brewer CF, Johnson P, Liu FT, et al. Galectin-3 and galectin-1 bind distinct cell surface glycoprotein receptors to induce T cell death. *J Immunol* (2006) 176(2):778–89. doi: 10.4049/jimmunol.176.2.778
57. Pace KE, Hahn HP, Pang M, Nguyen JT, Baum LG. CD7 delivers a pro-apoptotic signal during galectin-1-induced T cell death. *J Immunol* (2000) 165(5):2331–4. doi: 10.4049/jimmunol.165.5.2331
58. Hernandez JD, Nguyen JT, He J, Wang W, Ardman B, Green JM, et al. Galectin-1 binds different CD43 glycoforms to cluster CD43 and regulate T cell death. *J Immunol* (2006) 177(8):5328–36. doi: 10.4049/jimmunol.177.8.5328
59. Wastowski IJ, Simoes RT, Yaghi L, Donadi EA, Pancoto JT, Poras I, et al. Human leukocyte antigen-G is frequently expressed in glioblastoma and may be induced in vitro by combined 5-aza-2'-deoxycytidine and interferon-gamma treatments: results from a multicentric study. *Am J Pathol* (2013) 182(2):540–52. doi: 10.1016/j.ajpath.2012.10.021
60. Rahman M, Dastmalchi F, Karachi A, Mitchell D. The role of CMV in glioblastoma and implications for immunotherapeutic strategies. *Oncoimmunology* (2018) 8(1):e1514921. doi: 10.1080/2162402X.2018.1514921
61. Carosella ED, Rouas-Freiss N, Tronik-Le Roux D, Moreau P, LeMaoult J. HLA-G: An Immune Checkpoint Molecule. *Adv Immunol* (2015) 127:33–144. doi: 10.1016/bs.ai.2015.04.001
62. Wischhusen J, Friese MA, Mittelbronn M, Meyermann R, Weller M. HLA-E protects glioma cells from NKG2D-mediated immune responses in vitro: implications for immune escape in vivo. *J Neuropathol Exp Neurol* (2005) 64(6):523–8. doi: 10.1093/jnen/64.6.523

63. Didenko VV, Ngo HN, Minchew C, Baskin DS. Apoptosis of T lymphocytes invading glioblastomas multiforme: a possible tumor defense mechanism. *J Neurosurg* (2002) 96(3):580–4. doi: 10.3171/jns.2002.96.3.0580
64. Chahlavi A, Rayman P, Richmond AL, Biswas K, Zhang R, Vogelbaum M, et al. Glioblastomas induce T-lymphocyte death by two distinct pathways involving gangliosides and CD70. *Cancer Res* (2005) 65(12):5428–38. doi: 10.1158/0008-5472.CAN-04-4395
65. Berghoff AS, Kiesel B, Widhalm G, Rajky O, Ricken G, Wohrer A, et al. Programmed death ligand 1 expression and tumor-infiltrating lymphocytes in glioblastoma. *Neuro Oncol* (2015) 17(8):1064–75. doi: 10.1093/neuonc/nou307
66. Xue S, Song G, Yu J. The prognostic significance of PD-L1 expression in patients with glioma: A meta-analysis. *Sci Rep* (2017) 7(1):4231–017-04023-x. doi: 10.1038/s41598-017-04023-x
67. Han MZ, Wang S, Zhao WB, Ni SL, Yang N, Kong Y, et al. Immune checkpoint molecule herpes virus entry mediator is overexpressed and associated with poor prognosis in human glioblastoma. *EBioMedicine* (2019) 43:159–70. doi: 10.1016/j.ebiom.2019.04.002
68. Mbongue JC, Nicholas DA, Torrez TW, Kim NS, Firek AF, Langridge WH. The Role of Indoleamine 2, 3-Dioxygenase in Immune Suppression and Autoimmunity. *Vaccines (Basel)* (2015) 3(3):703–29. doi: 10.3390/vaccines3030703
69. Mitsuka K, Kawataki T, Satoh E, Asahara T, Horikoshi T, Kinouchi H. Expression of indoleamine 2,3-dioxygenase and correlation with pathological malignancy in gliomas. *Neurosurgery* (2013) 72(6):1031–8; discussion 1038-9. doi: 10.1227/NEU.0b013e31828cf945
70. Wainwright DA, Balyasnikova IV, Chang AL, Ahmed AU, Moon KS, Auffinger B, et al. IDO expression in brain tumors increases the recruitment of regulatory T cells and negatively impacts survival. *Clin Cancer Res* (2012) 18(22):6110–21. doi: 10.1158/1078-0432.CCR-12-2130
71. Mezrich JD, Fechner JH, Zhang X, Johnson BP, Burlingham WJ, Bradfield CA. An interaction between kynurenine and the aryl hydrocarbon receptor can generate regulatory T cells. *J Immunol* (2010) 185(6):3190–8. doi: 10.4049/jimmunol.0903670
72. Pilotte L, Larrieu P, Stroobant V, Colau D, Dolusic E, Frederick R, et al. Reversal of tumoral immune resistance by inhibition of tryptophan 2,3-dioxygenase. *Proc Natl Acad Sci USA* (2012) 109(7):2497–502. doi: 10.1073/pnas.1113873109
73. Van Meir E, Sawamura Y, Diserens AC, Hamou MF, de Tribolet N. Human glioblastoma cells release interleukin 6 in vivo and in vitro. *Cancer Res* (1990) 50(20):6683–8.
74. Tchirkov A, Rolhion C, Bertrand S, Doré JF, Dubost JJ, Verrelle P. IL-6 gene amplification and expression in human glioblastomas. *Br J Cancer* (2001) 85 (4):518–22. doi: 10.1054/bjoc.2001.1942
75. Kudo M, Jono H, Shinriki S, Yano S, Nakamura H, Makino K, et al. Antitumor effect of humanized anti-interleukin-6 receptor antibody (tocilizumab) on glioma cell proliferation. *Lab Investigation J Neurosurg* (2009) 111(2):219–25. doi: 10.3171/2008.12.JNS081284
76. Luwor RB, Stylli SS, Kaye AH. The role of Stat3 in glioblastoma multiforme. *J Clin Neurosci* (2013) 20(7):907–11. doi: 10.1016/j.jocn.2013.03.006
77. Jacobs JF, Idema AJ, Bol KF, Grotenhuis JA, de Vries IJ, Wesseling P, et al. Prognostic significance and mechanism of Treg infiltration in human brain tumors. *J Neuroimmunol* (2010) 225(1-2):195–9. doi: 10.1016/j.jneuroim.2010.05.020
78. Crane CA, Ahn BJ, Han SJ, Parsa AT. Soluble factors secreted by glioblastoma cell lines facilitate recruitment, survival, and expansion of regulatory T cells: implications for immunotherapy. *Neuro Oncol* (2012) 14 (5):584–95. doi: 10.1093/neuonc/nos014
79. Lohr J, Ratliff T, Huppertz A, Ge Y, Dictus C, Ahmadi R, et al. Effector T-cell infiltration positively impacts survival of glioblastoma patients and is impaired by tumor-derived TGF-beta. *Clin Cancer Res* (2011) 17 (13):4296–308. doi: 10.1158/1078-0432.CCR-10-2557
80. Bodmer S, Strommer K, Frei K, Siepl C, de Tribolet N, Heid I, et al. Immunosuppression and transforming growth factor-beta in glioblastoma. Preferential production of transforming growth factor-beta 2. *J Immunol* (1989) 143(10):3222–9.
81. Platten M, Wick W, Weller M. Malignant glioma biology: role for TGF-beta in growth, motility, angiogenesis, and immune escape. *Microsc Res Tech* (2001) 52(4):401–10. doi: 10.1002/1097-0029(20010215)52:4<401::AID-JEMT1025>3.0.CO;2-C
82. Couper KN, Blount DG, Riley EM. IL-10: the master regulator of immunity to infection. *J Immunol* (2008) 180(9):5771–7. doi: 10.4049/jimmunol.180.9.5771
83. Huettner C, Paulus W, Roggendorf W. Messenger RNA expression of the immunosuppressive cytokine IL-10 in human gliomas. *Am J Pathol* (1995) 146(2):317–22.
84. Wagner S, Czub S, Greif M, Vince GH, Suss N, Kerkau S, et al. Microglial/macrophage expression of interleukin 10 in human glioblastomas. *Int J Cancer* (1999) 82(1):12–6. doi: 10.1002/(SICI)1097-0215(19990702)82:1<12::AID-IJC3>3.0.CO;2-O
85. Cobbs CS, Harkins L, Samanta M, Gillespie GY, Bharara S, King PH, et al. Human cytomegalovirus infection and expression in human malignant glioma. *Cancer Res* (2002) 62(12):3347–50.
86. Lucas KG, Bao L, Bruggeman R, Dunham K, Specht C. The detection of CMV pp65 and IE1 in glioblastoma multiforme. *J Neurooncol* (2011) 103 (2):231–8. doi: 10.1007/s11060-010-0383-6
87. Kotenko SV, Saccani S, Izotova LS, Mirochnitchenko OV, Pestka S. Human cytomegalovirus harbors its own unique IL-10 homolog (cmvIL-10). *Proc Natl Acad Sci USA* (2000) 97(4):1695–700. doi: 10.1073/pnas.97.4.1695
88. Chang WL, Barry PA. Attenuation of innate immunity by cytomegalovirus IL-10 establishes a long-term deficit of adaptive antiviral immunity. *Proc Natl Acad Sci USA* (2010) 107(52):22647–52. doi: 10.1073/pnas.1013794108
89. Brown NF, Carter TJ, Ottaviani D, Mulholland P. Harnessing the immune system in glioblastoma. *Br J Cancer* (2018) 119(10):1171–81. doi: 10.1038/s41416-018-0258-8
90. Mittelbronn M, Platten M, Zeiner P, Dombrowski Y, Frank B, Zachskorn C, et al. Macrophage migration inhibitory factor (MIF) expression in human malignant gliomas contributes to immune escape and tumour progression. *Acta Neuropathol* (2011) 122(3):353–65. doi: 10.1007/s00401-011-0858-3
91. Neurath KM, Keough MP, Mikkelsen T, Claffey KP. AMP-dependent protein kinase alpha 2 isoform promotes hypoxia-induced VEGF expression in human glioblastoma. *Glia* (2006) 53(7):733–43. doi: 10.1002/glia.20326
92. Oka N, Soeda A, Inagaki A, Onodera M, Maruyama H, Hara A, et al. VEGF promotes tumorigenesis and angiogenesis of human glioblastoma stem cells. *Biochem Biophys Res Commun* (2007) 360(3):553–9. doi: 10.1016/j.bbrc.2007.06.094
93. Wu T, Luo Q, Ouyang G. Periostin: a potent chemotactic factor for recruiting tumor-associated macrophage. *Protein Cell* (2015) 6(4):235–7. doi: 10.1007/s13238-015-0141-9
94. Alterman RL, Stanley ER. Colony stimulating factor-1 expression in human glioma. *Mol Chem Neuropathol* (1994) 21(2-3):177–88. doi: 10.1007/BF02815350
95. Pyonteck SM, Akkari L, Schuhmacher AJ, Bowman RL, Sevenich L, Quail DF, et al. CSF-1R inhibition alters macrophage polarization and blocks glioma progression. *Nat Med* (2013) 19(10):1264–72. doi: 10.1038/nm.3337
96. Lu T, Tian L, Han Y, Vogelbaum M, Stark GR. Dose-dependent cross-talk between the transforming growth factor-beta and interleukin-1 signaling pathways. *Proc Natl Acad Sci USA* (2007) 104(11):4365–70. doi: 10.1073/pnas.0700118104
97. Cowan EP, Pierce ML, Dhib-Jalbut S. Interleukin-1 beta decreases HLA class II expression on a glioblastoma multiforme cell line. *J Neuroimmunol* (1991) 33(1):17–28. doi: 10.1016/0165-5728(91)90030-B
98. Kohanbash G, Okada H. Myeloid-derived suppressor cells (MDSCs) in gliomas and glioma-development. *Immunol Invest* (2012) 41(6-7):658–79. doi: 10.3109/08820139.2012.689591
99. Chen Z, Hambardzumyan D. Immune Microenvironment in Glioblastoma Subtypes. *Front Immunol* (2018) 9:1004. doi: 10.3389/fimmu.2018.01004
100. Schlecker E, Stojanovic A, Eisen C, Quack C, Falk CS, Umansky V, et al. Tumor-infiltrating monocytic myeloid-derived suppressor cells mediate CCR5-dependent recruitment of regulatory T cells favoring tumor growth. *J Immunol* (2012) 189(12):5602–11. doi: 10.4049/jimmunol.1201018
101. El Andaloussi A, Lesniak MS. An increase in CD4+CD25+FOXP3+ regulatory T cells in tumor-infiltrating lymphocytes of human

glioblastoma multiforme. *Neuro Oncol* (2006) 8(3):234–43. doi: 10.1215/15228517-2006-006

102. Heimberger AB, Abou-Ghazal M, Reina-Ortiz C, Yang DS, Sun W, Qiao W, et al. Incidence and prognostic impact of FoxP3+ regulatory T cells in human gliomas. *Clin Cancer Res* (2008) 14(16):5166–72. doi: 10.1158/1078-0432.CCR-08-0320
103. Caponegro MD, Tetsuo Miyauchi J, E. Tsirka S. Contributions of immune cell populations in the maintenance, progression, and therapeutic modalities of glioma. *AIMS Allergy Immunol* (2018) 2(1):24–44. doi: 10.3934/Allergy.2018.1.24
104. Kennedy BC, Showers CR, Anderson DE, Anderson L, Canoll P, Bruce JN, et al. Tumor-associated macrophages in glioma: friend or foe? *J Oncol* (2013) 2013:486912. doi: 10.1155/2013/486912
105. Rampling R, Peoples S, Mulholland PJ, James A, Al-Salihi O, Twelves CJ, et al. A Cancer Research UK First Time in Human Phase I Trial of IMA950 (Novel Multipeptide Therapeutic Vaccine) in Patients with Newly Diagnosed Glioblastoma. *Clin Cancer Res* (2016) 22(19):4776–85. doi: 10.1158/1078-0432.CCR-16-0506
106. Platten M, Schilling D, Bunse L, Wick A, Bunse T, Riehl D, et al. ATIM-33. NOA-16: A FIRST-IN-MAN MULTICENTER PHASE I CLINICAL TRIAL OF THE GERMAN NEUROONCOLOGY WORKING GROUP EVALUATING A MUTATION-SPECIFIC PEPTIDE VACCINE TARGETING IDH1R132H IN PATIENTS WITH NEWLY DIAGNOSED MALIGNANT ASTROCYTOMAS. *Neuro-oncology* (2018) 20(suppl_6):8–9. doi: 10.1093/neuonc/noy148.028
107. Hilf N, Kuttruff-Coqui S, Frenzel K, Bukur V, Stevanovic S, Gouttefangeas C, et al. Actively personalized vaccination trial for newly diagnosed glioblastoma. *Nature* (2019) 565(7738):240–5. doi: 10.1038/s41586-018-0810-y
108. Fenstermaker RA, Ciesielski MJ, Qiu J, Yang N, Frank CL, Lee KP, et al. Clinical study of a survivin long peptide vaccine (SurVaxM) in patients with recurrent malignant glioma. *Cancer Immunol Immunother* (2016) 65 (11):1339–52. doi: 10.1007/s00262-016-1890-x
109. Fu S, Piccioni DE, Liu H, Lukas RV, Aregawi D, Yamaguchi K, et al. Initial phase 1 study of WT2725 dosing emulsion in patients with advanced malignancies. *JCO* (2017) 35(15):2066–6. doi: 10.1200/JCO.2017.35.15_suppl.2066
110. Tsuboi A, Hashimoto N, Fujiki F, Morimoto S, Kagawa N, Nakajima H, et al. A phase I clinical study of a cocktail vaccine of Wilms' tumor 1 (WT1) HLA class I and II peptides for recurrent malignant glioma. *Cancer Immunol Immunother* (2019) 68(2):331–40. doi: 10.1007/s00262-018-2274-1
111. Migliorini D, Dutoit V, Allard M, Grandjean Hallez N, Marinari E, Widmer V, et al. Phase I/II trial testing safety and immunogenicity of the multipeptide IMA950/poly-ICLC vaccine in newly diagnosed adult malignant astrocytoma patients. *Neuro Oncol* (2019) 21(7):923–33. doi: 10.1093/neuonc/noz040
112. Peereboom D, Nabors LB, Kumthekar P, Badruddoja M, Fink K, Lieberman F, et al. Results of phase II trial of SL-701, a novel immunotherapy targeting IL-13Ra2, EphA2, and survivin, in adults with second-line recurrent glioblastoma (GBM). *Ann Oncol Off J Eur Soc Med Oncol* (2018) 29: viii122–viii123. doi: 10.1093/annonc/mdy273.361
113. Lassman AB, Reardon DA, Lee EQ, Iwamoto FM, Diaz-Mitoma F, Anderson DE, et al. Interim results of a phase I/IIa trial of a therapeutic CMV vaccine against recurrent glioblastoma (GBM). *JCO* (2019) 37(15):2048–8. doi: 10.1200/JCO.2019.37.15_suppl.2048
114. Sampson JH, Heimberger AB, Archer GE, Aldape KD, Friedman AH, Friedman HS, et al. Immunologic escape after prolonged progression-free survival with epidermal growth factor receptor variant III peptide vaccination in patients with newly diagnosed glioblastoma. *J Clin Oncol* (2010) 28(31):4722–9. doi: 10.1200/JCO.2010.28.6963
115. Sampson JH, Aldape KD, Archer GE, Coan A, Desjardins A, Friedman AH, et al. Greater chemotherapy-induced lymphopenia enhances tumor-specific immune responses that eliminate EGFRvIII-expressing tumor cells in patients with glioblastoma. *Neuro Oncol* (2011) 13(3):324–33. doi: 10.1093/neuonc/noq157
116. Schuster J, Lai RK, Recht LD, Reardon DA, Paleologos NA, Groves MD, et al. multicenter trial of rindopepimut (CDX-110) in newly diagnosed glioblastoma: the ACT III study. *Neuro Oncol* (2015) 17(6):854–61. doi: 10.1093/neuonc/nou348
117. Reardon DA, Desjardins A, Vredenburgh JJ, O'Rourke DM, Tran DD, Fink KL, et al. Rindopepimut with Bevacizumab for Patients with Relapsed EGFRvIII-Expressing Glioblastoma (ReACT): Results of a Double-Blind Randomized Phase II Trial. *Clin Cancer Res* (2020) 26(7):1586–94. doi: 10.1158/1078-0432.CCR-18-1140
118. Ahluwalia MS, Peereboom DM, Ciolfi M, Schilero C, Hobbs B, Ciesielski MJ, et al. Phase II study of pembrolizumab plus SurVaxM for glioblastoma at first recurrence. *JCO* (2020) 38(15):TPS2581–TPS2581. doi: 10.1200/JCO.2020.38.15_suppl.TPS2581
119. De Groot JF, Cloughesy TF, Pitz MW, Narita Y, Nonomura T. A randomized, multicenter phase 2 study of DSP-7888 dosing emulsion in combination with bevacizumab (Bev) versus Bev alone in patients with recurrent or progressive glioblastoma. *JCO* (2018) 36(15):TPS2071–TPS2071. doi: 10.1200/JCO.2018.36.15_suppl.TPS2071
120. Weller M, Butowski N, Tran DD, Recht LD, Lim M, Hirte H, et al. Rindopepimut with temozolomide for patients with newly diagnosed, EGFRvIII-expressing glioblastoma (ACT IV): a randomised, double-blind, international phase 3 trial. *Lancet Oncol* (2017) 18(10):1373–85. doi: 10.1016/S1470-2045(17)30517-X
121. Wang QT, Nie Y, Sun SN, Lin T, Han RJ, Jiang J, et al. Tumor-associated antigen-based personalized dendritic cell vaccine in solid tumor patients. *Cancer Immunol Immunother* (2020) 69(7):1375–87. doi: 10.1007/s00262-020-02496-w
122. Mitchell DA, Batich KA, Gunn MD, Huang MN, Sanchez-Perez L, Nair SK, et al. Tetanus toxoid and CCL3 improve dendritic cell vaccines in mice and glioblastoma patients. *Nature* (2015) 519(7543):366–9. doi: 10.1038/nature14320
123. Batich KA, Reap EA, Archer GE, Sanchez-Perez L, Nair SK, Schmittling RJ, et al. Long-term Survival in Glioblastoma with Cytomegalovirus pp65-Targeted Vaccination. *Clin Cancer Res* (2017) 23(8):1898–909. doi: 10.1158/1078-0432.CCR-16-2057
124. Prins RM, Wang X, Soto H, Young E, Lisiero DN, Fong B, et al. Comparison of glioma-associated antigen peptide-loaded versus autologous tumor lysate-loaded dendritic cell vaccination in malignant glioma patients. *J Immunother* (2013) 36(2):152–7. doi: 10.1097/CJI.0b013e3182811ae4
125. Liau LM, Prins RM, Kiertscher SM, Odesa SK, Kremen TJ, Giovannone AJ, et al. Dendritic cell vaccination in glioblastoma patients induces systemic and intracranial T-cell responses modulated by the local central nervous system tumor microenvironment. *Clin Cancer Res* (2005) 11(15):5515–25. doi: 10.1158/1078-0432.CCR-05-0464
126. Rudnick JD, Sarmiento JM, Uy B, Nuno M, Wheeler CJ, Mazer MJ, et al. A phase I trial of surgical resection with Gliadel Wafer placement followed by vaccination with dendritic cells pulsed with tumor lysate for patients with malignant glioma. *J Clin Neurosci* (2020) 74:187–93. doi: 10.1016/j.jocn.2020.03.006
127. Parney IF, Gustafson MP, Peterson T, Steinmetz SM, Dietz AB. ATIM-13. ALLOGENEIC TUMOR LYSATE/AUTOLOGOUS DENDRITIC CELL VACCINES IN NEWLY DIAGNOSED GLIOBLASTOMA: RESULTS OF CLINICAL TRIAL MC1272. *Neuro-oncology* (2017) 19(Suppl 6):28–9. doi: 10.1093/neuonc/nox168.109
128. Hu J, Rudnick J, Wheeler C, Mazer M, Wang H, Black K, et al. ATIM-18. A PHASE I TRIAL OF VACCINATION WITH AUTOLOGOUS DENDRITIC CELLS PULSED WITH LYSATE DERIVED FROM AN ALLOGENEIC GLIOBLASTOMA STEMLIKE CELL LINE FOR PATIENTS WITH NEWLY DIAGNOSED OR RECURRENT GLIOBLASTOMA. *Neuro-oncology* (2018) 20(suppl_6):4–4. doi: 10.1093/neuonc/noy148.013
129. Phuphanich S, Wheeler CJ, Rudnick JD, Mazer M, Wang H, Nuño MA, et al. Phase I trial of a multi-epitope-pulsed dendritic cell vaccine for patients with newly diagnosed glioblastoma. *Cancer Immunol Immunother* (2013) 62 (1):125–35. doi: 10.1007/s00262-012-1319-0
130. Olin MR, Low W, McKenna DH, Haines SJ, Dahlheimer T, Nascene D, et al. Vaccination with dendritic cells loaded with allogeneic brain tumor cells for recurrent malignant brain tumors induces a CD4(+)IL17(+) response. *J Immunother Cancer* (2014) 2:4–1426-2-4. doi: 10.1186/2051-1426-2-4

131. Rudnick JD, Fink KL, Landolfi JC, Markert J, Piccioni DE, Glantz MJ, et al. Immunological targeting of CD133 in recurrent glioblastoma: A multi-center phase I translational and clinical study of autologous CD133 dendritic cell immunotherapy. *JCO* (2017) 35(15):2059–2059. doi: 10.1200/JCO.2017.35.15_suppl.2059
132. Vik-Mo EO, Nyakas M, Mikkelsen BV, Moe MC, Due-Tønnesen P, Suso EM, et al. Therapeutic vaccination against autologous cancer stem cells with mRNA-transfected dendritic cells in patients with glioblastoma. *Cancer Immunol Immunother* (2013) 62(9):1499–509. doi: 10.1007/s00262-013-1453-3
133. Akasaki Y, Kikuchi T, Homma S, Koido S, Ohkusa T, Tasaki T, et al. Phase I/II trial of combination of temozolomide chemotherapy and immunotherapy with fusions of dendritic and glioma cells in patients with glioblastoma. *Cancer Immunol Immunother* (2016) 65(12):1499–509. doi: 10.1007/s00262-016-1905-7
134. Berneman ZN, Anguille S, Willemen Y, de Velde AV, Germonpre P, Huizing M, et al. Vaccination of cancer patients with dendritic cells electroporated with mRNA encoding the wilms' tumor 1 protein (WT1): correlation of clinical effect and overall survival with T-cell response. *Cytotherapy* (2019) 21(5, Supplement):S10. doi: 10.1016/j.jcyt.2019.03.565
135. Wen PY, Reardon DA, Armstrong TS, Phuphanich S, Aiken RD, Landolfi JC, et al. A Randomized Double-Blind Placebo-Controlled Phase II Trial of Dendritic Cell Vaccine ICT-107 in Newly Diagnosed Patients with Glioblastoma. *Clin Cancer Res* (2019) 25(19):5799–807. doi: 10.1158/1078-0432.CCR-19-0261
136. Inogés S, Tejada S, de Cerio AL, Gállego Pérez-Larraya J, Espinós J, Idoate MA, et al. A phase II trial of autologous dendritic cell vaccination and radiochemotherapy following fluorescence-guided surgery in newly diagnosed glioblastoma patients. *J Transl Med* (2017) 15(1):104–017-1202-z. doi: 10.1186/s12967-017-1202-z
137. Pellegatta S, Eoli M, Cuccarini V, Anghileri E, Pollo B, Pessina S, et al. Survival gain in glioblastoma patients treated with dendritic cell immunotherapy is associated with increased NK but not CD8(+) T cell activation in the presence of adjuvant temozolomide. *Oncoimmunology* (2018) 7(4):e1412901. doi: 10.1080/2162402X.2017.1412901
138. Buchroithner J, Erhart F, Pichler J, Widhalm G, Preusser M, Stockhammer G, et al. Audencel Immunotherapy Based on Dendritic Cells Has No Effect on Overall and Progression-Free Survival in Newly Diagnosed Glioblastoma: A Phase II Randomized Trial. *Cancers (Basel)* (2018) 10(10):372. doi: 10.3390/cancers10100372
139. Erhart F, Buchroithner J, Reitermaier R, Fischhuber K, Klingenbrunner S, Sloma I, et al. Immunological analysis of phase II glioblastoma dendritic cell vaccine (Audencel) trial: immune system characteristics influence outcome and Audencel up-regulates Th1-related immunovariables. *Acta Neuropathol Commun* (2018) 6(1):135–018-0621-2. doi: 10.1186/s40478-018-0621-2
140. Fadul CE, Fisher JL, Hampton TH, Lallana EC, Li Z, Gui J, et al. Immune response in patients with newly diagnosed glioblastoma multiforme treated with intranodal autologous tumor lysate-dendritic cell vaccination after radiation chemotherapy. *J Immunother* (2011) 34(4):382–9. doi: 10.1097/CJI.0b013e318215e300
141. Yao Y, Luo F, Tang C, Chen D, Qin Z, Hua W, et al. Molecular subgroups and B7-H4 expression levels predict responses to dendritic cell vaccines in glioblastoma: an exploratory randomized phase II clinical trial. *Cancer Immunol Immunother* (2018) 67(11):1777–88. doi: 10.1007/s00262-018-2232-y
142. Rapp M, Grauer OM, Kamp M, Sevens N, Zotz N, Sabel M, et al. A randomized controlled phase II trial of vaccination with lysate-loaded, mature dendritic cells integrated into standard radiochemotherapy of newly diagnosed glioblastoma (GlioVax): study protocol for a randomized controlled trial. *Trials* (2018) 19(1):293–018-2659-7. doi: 10.1186/s13063-018-2659-7
143. Huang YC, Chang CN, Wei KC, Lee HC, Chen CC, Cho DY, et al. Atim-42. Safety and Efficacy of Autologous Dendritic Cells/tumor Cell Antigen Adjuvant Therapy of Glioblastoma Multiforme: Results of 59 Cases. *Neuro Oncol* (2018) 20(Suppl 6):vi11. doi: 10.1093/neuonc/noy148.037
144. Liau LM, Ashkan K, Tran DD, Campian JL, Trusheim JE, Cobbs CS, et al. First results on survival from a large Phase 3 clinical trial of an autologous dendritic cell vaccine in newly diagnosed glioblastoma. *J Transl Med* (2018) 16(1):142–018-1507-6. doi: 10.1186/s12967-018-1507-6
145. Bota DA, Chung J, Dandekar M, Carrillo JA, Kong XT, Fu BD, et al. Phase II study of ERC1671 plus bevacizumab versus bevacizumab plus placebo in recurrent glioblastoma: interim results and correlations with CD4(+) T-lymphocyte counts. *CNS Oncol* (2018) 7(3):CNS22–2018-0009. doi: 10.2217/cns-2018-0009
146. Crane CA, Han SJ, Ahn B, Oehlke J, Kivett V, Fedoroff A, et al. Individual patient-specific immunity against high-grade glioma after vaccination with autologous tumor derived peptides bound to the 96 KD chaperone protein. *Clin Cancer Res* (2013) 19(1):205–14. doi: 10.1158/1078-0432.CCR-11-3358
147. Bloch O, Raizer JJ, Lim M, Sughrue M, Komotar R, Abrahams J, et al. Newly diagnosed glioblastoma patients treated with an autologous heat shock protein peptide vaccine: PD-L1 expression and response to therapy. *J Clin Oncol* (2015) 33(15_suppl):2011–1. doi: 10.1200/jco.2015.33.15_suppl.2011
148. Bloch O, Crane CA, Fuks Y, Kaur R, Aghi MK, Berger MS, et al. Heat-shock protein peptide complex-96 vaccination for recurrent glioblastoma: a phase II, single-arm trial. *Neuro Oncol* (2014) 16(2):274–9. doi: 10.1093/neuonc/not203
149. Bloch O, Lim M, Sughrue ME, Komotar RJ, Abrahams JM, O'Rourke DM, et al. Autologous Heat Shock Protein Peptide Vaccination for Newly Diagnosed Glioblastoma: Impact of Peripheral PD-L1 Expression on Response to Therapy. *Clin Cancer Res* (2017) 23(14):3575–84. doi: 10.1158/1078-0432.CCR-16-1369
150. Dutoit V, Herold-Mende C, Hilf N, Schoor O, Beckhove P, Bucher J, et al. Exploiting the glioblastoma peptidome to discover novel tumour-associated antigens for immunotherapy. *Brain* (2012) 135(Pt 4):1042–54. doi: 10.1093/brain/aws042
151. Ghoochani A, Schwarz MA, Yakubov E, Engelhorn T, Doerfler A, Buchfelder M, et al. MIF-CD74 signaling impedes microglial M1 polarization and facilitates brain tumorigenesis. *Oncogene* (2016) 35(48):6246–61. doi: 10.1038/onc.2016.160
152. Nagai T, Tanaka M, Tsuneyoshi Y, Xu B, Michie SA, Hasui K, et al. Targeting tumor-associated macrophages in an experimental glioma model with a recombinant immunotoxin to folate receptor beta. *Cancer Immunol Immunother* (2009) 58(10):1577–86. doi: 10.1007/s00262-009-0667-x
153. Lishner M, Perrin RG, Feld R, Messner HA, Tuffnell PG, Elhakim T, et al. Complications associated with Ommaya reservoirs in patients with cancer. The Princess Margaret Hospital experience and a review of the literature. *Arch Intern Med* (1990) 150(1):173–6. doi: 10.1001/archinte.1990.00390130145023
154. Kennedy BC, Brown LT, Komotar RJ, McKhann G. Stereotactic catheter placement for Ommaya reservoirs. *J Clin Neurosci* (2016) 27:44–7. doi: 10.1016/j.jocn.2015.11.005
155. Si QS, Nakamura Y, Schubert P, Rudolphi K, Kataoka K. Adenosine and propentofylline inhibit the proliferation of cultured microglial cells. *Exp Neurol* (1996) 137(2):345–9. doi: 10.1006/exnr.1996.0035
156. Jacobs VL, Landry RP, Liu Y, Romero-Sandoval EA, De Leo JA. Propentofylline decreases tumor growth in a rodent model of glioblastoma multiforme by a direct mechanism on microglia. *Neuro Oncol* (2012) 14 (2):119–31. doi: 10.1093/neuonc/nor194
157. Chiu TL, Wang MJ, Su CC. The treatment of glioblastoma multiforme through activation of microglia and TRAIL induced by rAAV2-mediated IL-12 in a syngeneic rat model. *J BioMed Sci* (2012) 19:45–0127-19-45. doi: 10.1186/1423-0127-19-45
158. Bogdahn U, Hau P, Stockhammer G, Venkataramana NK, Mahapatra AK, Suri A, et al. Targeted therapy for high-grade glioma with the TGF-β2 inhibitor trabedersen: results of a randomized and controlled phase IIb study. *Neuro Oncol* (2011) 13(1):132–42. doi: 10.1093/neuonc/noq142
159. Soares KC, Rucki AA, Kim V, Foley K, Solt S, Wolfgang CL, et al. TGF-beta blockade depletes T regulatory cells from metastatic pancreatic tumors in a vaccine dependent manner. *Oncotarget* (2015) 6(40):43005–15. doi: 10.18632/oncotarget.5656
160. Blumenthal DT, Yalon M, Vainer GW, Lossos A, Yust S, Tzach L, et al. Pembrolizumab: first experience with recurrent primary central nervous system (CNS) tumors. *J Neurooncol* (2016) 129(3):453–60. doi: 10.1007/s11060-016-2190-1

161. Chamberlain MC, Kim BT. Nivolumab for patients with recurrent glioblastoma progressing on bevacizumab: a retrospective case series. *J Neurooncol* (2017) 133(3):561–9. doi: 10.1007/s11060-017-2466-0
162. Filley AC, Henriquez M, Dey M. Recurrent glioma clinical trial, CheckMate-143: the game is not over yet. *Oncotarget* (2017) 8(53):91779–94. doi: 10.18632/oncotarget.21586
163. Cloughesy TF, Mochizuki AY, Orpilla JR, Hugo W, Lee AH, Davidson TB, et al. Neoadjuvant anti-PD-1 immunotherapy promotes a survival benefit with intratumoral and systemic immune responses in recurrent glioblastoma. *Nat Med* (2019) 25(3):477–86. doi: 10.1038/s41591-018-0337-7
164. Antonios JP, Soto H, Everson RG, Orpilla J, Moughon D, Shin N, et al. PD-1 blockade enhances the vaccination-induced immune response in glioma. *JCI Insight* (2016) 1(10):e87059. doi: 10.1172/jci.insight.87059
165. Antonios JP, Soto H, Everson RG, Moughon D, Orpilla JR, Shin NP, et al. Immunosuppressive tumor-infiltrating myeloid cells mediate adaptive immune resistance via a PD-1/PD-L1 mechanism in glioblastoma. *Neuro Oncol* (2017) 19(6):796–807. doi: 10.1093/neuonc/now287
166. Soderlund J, Erhardt S, Kast RE. Acyclovir inhibition of IDO to decrease Tregs as a glioblastoma treatment adjunct. *J Neuroinflammation* (2010) 7:44–2094-7-44. doi: 10.1186/1742-2094-7-44
167. Wainwright DA, Chang AL, Dey M, Balyasnikova IV, Kim CK, Tobias A, et al. Durable therapeutic efficacy utilizing combinatorial blockade against IDO, CTLA-4, and PD-L1 in mice with brain tumors. *Clin Cancer Res* (2014) 20(20):5290–301. doi: 10.1158/1078-0432.CCR-14-0514
168. Migliorini D, Dietrich PY, Stupp R, Linette GP, Posey AD, June CH. CAR T-Cell Therapies in Glioblastoma: A First Look. *Clin Cancer Res* (2018) 24 (3):535–40. doi: 10.1158/1078-0432.CCR-17-2871
169. Filley AC, Henriquez M, Dey M. CART Immunotherapy: Development, Success, and Translation to Malignant Gliomas and Other Solid Tumors. *Front Oncol* (2018) 8:453. doi: 10.3389/fonc.2018.00453
170. Hyrenius-Wittsten A, Roybal KT. Paving New Roads for CARs. *Trends Cancer* (2019) 5(10):583–92. doi: 10.1016/j.trecan.2019.09.005
171. Ahmed N, Brawley V, Hegde M, Bielamowicz K, Wakefield A, Ghazi A, et al. Autologous HER2 CMV bispecific CAR T cells are safe and demonstrate clinical benefit for glioblastoma in a Phase I trial. *J Immunother Cancer* (2015) 3(Suppl 2):O11–1426-3-S2-O11. doi: 10.1186/2051-1426-3-S2-O11
172. Brown CE, Alizadeh D, Starr R, Weng L, Wagner JR, Naranjo A, et al. Regression of Glioblastoma after Chimeric Antigen Receptor T-Cell Therapy. *N Engl J Med* (2016) 375(26):2561–9. doi: 10.1056/NEJMoa1610497
173. O'Rourke DM, Nasrallah MP, Desai A, Melenhorst JJ, Mansfield K, Morrissette JJD, et al. A single dose of peripherally infused EGFRvIII-directed CAR T cells mediates antigen loss and induces adaptive resistance in patients with recurrent glioblastoma. *Sci Transl Med* (2017) 9(399): eaaa0984. doi: 10.1126/scitranslmed.aaa0984
174. Bagley S, Desai A, Binder Z, Nasrallah M, Hwang W, Maloney-Wilensky E, et al. RBTT-12. A PHASE I STUDY OF EGFRVIII-DIRECTED CAR T CELLS COMBINED WITH PD-1 INHIBITION IN PATIENTS WITH NEWLY, DIAGNOSED, MGMT-UNMETHYLATED GLIOBLASTOMA: TRIAL IN PROGRESS. *Neuro-oncology* (2019) 21:vi221–1. doi: 10.1093/neuonc/noz175.923
175. Jansen T, Tyler B, Mankowski JL, Recinos VR, Pradilla G, Legnani F, et al. FasL gene knock-down therapy enhances the antiglioma immune response. *Neuro Oncol* (2010) 12(5):482–9. doi: 10.1093/neuonc/nop052
176. Tschumi BO, Dumauthioz N, Marti B, Zhang L, Lanitis E, Irving M, et al. CART cells are prone to Fas- and DR5-mediated cell death. *J Immunother Cancer* (2018) 6(1):71–018-0385-z. doi: 10.1186/s40425-018-0410-2
177. Yamamoto TN, Lee PH, Vodnala SK, Gurusamy D, Kishton RJ, Yu Z, et al. T cells genetically engineered to overcome death signaling enhance adoptive cancer immunotherapy. *J Clin Invest* (2019) 129(4):1551–65. doi: 10.1172/JCI121491
178. Kloss CC, Lee J, Zhang A, Chen F, Melenhorst JJ, Lacey SF, et al. Dominant-Negative TGF-beta Receptor Enhances PSMA-Targeted Human CAR T Cell Proliferation And Augments Prostate Cancer Eradication. *Mol Ther* (2018) 26(7):1855–66. doi: 10.1016/j.ymthe.2018.05.003
179. Rafiq S, Yeku OO, Jackson HJ, Purdon TJ, van Leeuwen DG, Drakes DJ, et al. Targeted delivery of a PD-1-blocking scFv by CAR-T cells enhances anti-tumor efficacy in vivo. *Nat Biotechnol* (2018) 36(9):847–56. doi: 10.1038/nbt.4195
180. Kuhn NF, Purdon TJ, van Leeuwen DG, Lopez AV, Curran KJ, Daniyan AF, et al. CD40 Ligand-Modified Chimeric Antigen Receptor T Cells Enhance Antitumor Function by Eliciting an Endogenous Antitumor Response. *Cancer Cell* (2019) 35(3):473–488.e6. doi: 10.1016/j.ccell.2019.02.006
181. Choi BD, Yu X, Castano AP, Bouffard AA, Schmidts A, Larson RC, et al. CAR-T cells secreting BiTEs circumvent antigen escape without detectable toxicity. *Nat Biotechnol* (2019) 37(9):1049–58. doi: 10.1038/s41587-019-0192-1
182. Ruella M, Klichinsky M, Kenderian SS, Shestova O, Ziober A, Kraft DO, et al. Overcoming the Immunosuppressive Tumor Microenvironment of Hodgkin Lymphoma Using Chimeric Antigen Receptor T Cells. *Cancer Discovery* (2017) 7(10):1154–67. doi: 10.1158/2159-8290.CD-16-0850
183. Pearl TM, Markert JM, Cassady KA, Ghonime MG. Oncolytic Virus-Based Cytokine Expression to Improve Immune Activity in Brain and Solid Tumors. *Mol Ther Oncolytics* (2019) 13:14–21. doi: 10.1016/j.omto.2019.03.001
184. Marchini A, Daeffler L, Pozdeev VI, Angelova A, Rommelaere J. Immune Conversion of Tumor Microenvironment by Oncolytic Viruses: The Protoparvovirus H-1PV Case Study. *Front Immunol* (2019) 10:1848. doi: 10.3389/fimmu.2019.01848
185. Wollmann G, Ozduman K, van den Pol AN. Oncolytic virus therapy for glioblastoma multiforme: concepts and candidates. *Cancer J* (2012) 18 (1):69–81. doi: 10.1097/PPO.0b013e31824671c9
186. Desjardins A, Sampson JH, Peters KB, Vlahovic G, Randazzo D, Threatt S, et al. Patient survival on the dose escalation phase of the Oncolytic Polio/Rhinovirus Recombinant (PVSRIPO) against WHO grade IV malignant glioma (MG) clinical trial compared to historical controls. *JCO* (2016) 34 (15):2061–1. doi: 10.1200/JCO.2016.34.15_suppl.2061
187. Friedman G, Bag A, Madan-Swain A, Li R, Kachurak K, Osorio D, et al. IMMU-08. PHASE I TRIAL (NCT02457845) SAFETY, TOLERABILITY AND PRELIMINARY EFFICACY OF IMMUNOVIROTHERAPY WITH HSV G207 IN CHILDREN WITH PROGRESSIVE MALIGNANT SUPRATENTORIAL BRAIN TUMORS. *Neuro Oncol* (2018) 20:i100–0. doi: 10.1093/neuonc/noy059.324
188. Lang FF, Tran ND, Puduvalli VK, Elder JB, Fink KL, Conrad CA, et al. Phase 1b open-label randomized study of the oncolytic adenovirus DNX-2401 administered with or without interferon gamma for recurrent glioblastoma. *JCO* (2017) 35(15):2002–2. doi: 10.1200/JCO.2017.35.15_suppl.2002
189. Chiocca EA, Yu JS, Lukas RV, Solomon IH, Ligon KL, Nakashima H, et al. Regulatable interleukin-12 gene therapy in patients with recurrent high-grade glioma: Results of a phase 1 trial. *Sci Transl Med* (2019) 11(505): eaaw5680. doi: 10.1126/scitranslmed.aaw5680
190. Geletneky K, Huesing J, Rommelaere J, Schlehofer JR, Leuchs B, Dahm M, et al. Phase I/IIa study of intratumoral/intracerebral or intravenous/intracerebral administration of Parvovirus H-1 (ParvOryx) in patients with progressive primary or recurrent glioblastoma multiforme: ParvOryx01 protocol. *BMC Cancer* (2012) 12:99–2407-12-99. doi: 10.1186/1471-2407-12-99
191. Wheeler LA, Manzanera AG, Bell SD, Cavaliere R, McGregor JM, Grecula JC, et al. Phase II multicenter study of gene-mediated cytotoxic immunotherapy as adjuvant to surgical resection for newly diagnosed malignant glioma. *Neuro Oncol* (2016) 18(8):1137–45. doi: 10.1093/neuonc/now002
192. Ji N, Weng D, Liu C, Gu Z, Chen S, Guo Y, et al. Adenovirus-mediated delivery of herpes simplex virus thymidine kinase administration improves outcome of recurrent high-grade glioma. *Oncotarget* (2016) 7(4):4369–78. doi: 10.18632/oncotarget.6737
193. Ostertag D, Amundson KK, Lopez Espinoza F, Martin B, Buckley T, Galvão da Silva AP, et al. Brain tumor eradication and prolonged survival from intratumoral conversion of 5-fluorocytosine to 5-fluorouracil using a nonlytic retroviral replicating vector. *Neuro Oncol* (2012) 14(2):145–59. doi: 10.1093/neuonc/nor199
194. Mitchell LA, Lopez Espinoza F, Mendoza D, Kato Y, Inagaki A, Hiraoka K, et al. Toca 511 gene transfer and treatment with the prodrug, 5-fluorocytosine, promotes durable antitumor immunity in a mouse glioma model. *Neuro Oncol* (2017) 19(7):930–9. doi: 10.1093/neuonc/nox037

195. Cloughesy T, Petrecca K, Walbert T, Butowski N, Salacz M, Perry J, et al. LTBK-08. TOCA 511 & TOCA FC VERSUS STANDARD OF CARE IN PATIENTS WITH RECURRENT HIGH GRADE GLIOMA. *Neuro Oncol* (2019) 21:vi284–4. doi: 10.1093/neuonc/noz219.1199
196. Kanai R, Rabkin SD. Combinatorial strategies for oncolytic herpes simplex virus therapy of brain tumors. *CNS Oncol* (2013) 2(2):129–42. doi: 10.2217/cns.12.42
197. Andreansky S, He B, van Cott J, McGhee J, Markert JM, Gillespie GY, et al. Treatment of intracranial gliomas in immunocompetent mice using herpes simplex viruses that express murine interleukins. *Gene Ther* (1998) 5(1):121–30. doi: 10.1038/sj.gt.3300550
198. Martikainen M, Essand M. Virus-Based Immunotherapy of Glioblastoma. *Cancers (Basel)* (2019) 11(2):186. doi: 10.3390/cancers11020186
199. Hardcastle J, Mills L, Malo CS, Jin F, Kurokawa C, Geekiyanage H, et al. Immunovirotherapy with measles virus strains in combination with anti-PD-1 antibody blockade enhances antitumor activity in glioblastoma treatment. *Neuro Oncol* (2017) 19(4):493–502. doi: 10.1093/neuonc/now179
200. Saha D, Martuza RL, Rabkin SD. Macrophage Polarization Contributes to Glioblastoma Eradication by Combination Immunovirotherapy and Immune Checkpoint Blockade. *Cancer Cell* (2017) 32(2):253–267.e5. doi: 10.1016/j.ccell.2017.07.006
201. Hou W, Sampath P, Rojas JJ, Thorne SH. Oncolytic Virus-Mediated Targeting of PGE2 in the Tumor Alters the Immune Status and Sensitizes Established and Resistant Tumors to Immunotherapy. *Cancer Cell* (2016) 30 (1):108–19. doi: 10.1016/j.ccell.2016.05.012
202. Suryadevara CM, Desai R, Abel ML, Riccione KA, Batich KA, Shen SH, et al. Temozolomide lymphodepletion enhances CAR abundance and correlates with antitumor efficacy against established glioblastoma. *Oncoimmunology* (2018) 7(6):e1434464. doi: 10.1080/2162402X.2018.1434464
203. Ouyang M, White EE, Ren H, Guo Q, Zhang I, Gao H, et al. Metronomic Doses of Temozolomide Enhance the Efficacy of Carbon Nanotube CpG Immunotherapy in an Invasive Glioma Model. *PloS One* (2016) 11(2): e0148139. doi: 10.1371/journal.pone.0148139
204. Weiss T, Weller M, Guckenberger M, Sentman CL, Roth P. NKG2D-Based CAR T Cells and Radiotherapy Exert Synergistic Efficacy in Glioblastoma. *Cancer Res* (2018) 78(4):1031–43. doi: 10.1158/0008-5472.CAN-17-1788
205. Fritzell S, Sanden E, Eberstal S, Visse E, Darabi A, Siesjo P. Intratumoral temozolomide synergizes with immunotherapy in a T cell-dependent fashion. *Cancer Immunol Immunother* (2013) 62(9):1463–74. doi: 10.1007/s00262-013-1449-z
206. Carvalho JADV, Barbosa CCL, Feher O, Maldaun MVC, Camargo VP, Moraes FY, et al. Systemic dissemination of glioblastoma: literature review. *Rev Assoc Med Bras (1992)* (2019) 65(3):460–8. doi: 10.1590/1806-9282.65.3.460
207. Dykstra KH, Arya A, Arriola DM, Bungay PM, Morrison PF, Dedrick RL. Microdialysis study of zidovudine (AZT) transport in rat brain. *J Pharmacol Exp Ther* (1993) 267(3):1227–36.
208. Morrison PF, Laske DW, Bobo H, Oldfield EH, Dedrick RL. High-flow microinfusion: tissue penetration and pharmacodynamics. *Am J Physiol* (1994) 266(1 Pt 2):R292–305. doi: 10.1152/ajpregu.1994.266.1.R292
209. Krewson CE, Klarman ML, Saltzman WM. Distribution of nerve growth factor following direct delivery to brain interstitium. *Brain Res* (1995) 680(1-2):196–206. doi: 10.1016/0006-8993(95)00261-N
210. Pardridge WM. CSF, blood-brain barrier, and brain drug delivery. *Expert Opin Drug Delivery* (2016) 13(7):963–75. doi: 10.1517/17425247.2016.1171315
211. Rizk ML, Zou L, Savic RM, Dooley KE. Importance of Drug Pharmacokinetics at the Site of Action. *Clin Transl Sci* (2017) 10(3):133–42. doi: 10.1111/cts.12448
212. Louveau A, Smirnov I, Keyes TJ, Eccles JD, Rouhani SJ, Peske JD, et al. Structural and functional features of central nervous system lymphatic vessels. *Nature* (2015) 523(7560):337–41. doi: 10.1038/nature14432
213. Larson SM, Carrasquillo JA, Cheung NK, Press OW. Radioimmunotherapy of human tumours. *Nat Rev Cancer* (2015) 15(6):347–60. doi: 10.1038/nrc3925
214. Rapoport SI. Effect of concentrated solutions on blood-brain barrier. *Am J Physiol* (1970) 219(1):270–4. doi: 10.1152/ajplegacy.1970.219.1.270
215. Siegal T, Rubinstein R, Bokstein F, Schwartz A, Lossos A, Shalom E, et al. In vivo assessment of the window of barrier opening after osmotic blood-brain barrier disruption in humans. *J Neurosurg* (2000) 92(4):599–605. doi: 10.3171/jns.2000.92.4.0599
216. Raymond JJ, Robertson DM, Dinsdale HB. Pharmacological modification of bradykinin induced breakdown of the blood-brain barrier. *Can J Neurol Sci* (1986) 13(3):214–20. doi: 10.1017/S0317167100036301
217. Warren K, Jakacki R, Widemann B, Aikin A, Libucha M, Packer R, et al. Phase II trial of intravenous lobradimil and carboplatin in childhood brain tumors: a report from the Children's Oncology Group. *Cancer Chemother Pharmacol* (2006) 58(3):343–7. doi: 10.1007/s00280-005-0172-7
218. Hynynen K, McDannold N, Sheikov NA, Jolesz FA, Vykhodtseva N. Local and reversible blood-brain barrier disruption by noninvasive focused ultrasound at frequencies suitable for trans-skull sonications. *Neuroimage* (2005) 24(1):12–20. doi: 10.1016/j.neuroimage.2004.06.046
219. Janowicz PW, Leinenga G, Götz J, Nisbet RM. Ultrasound-mediated blood-brain barrier opening enhances delivery of therapeutically relevant formats of a tau-specific antibody. *Sci Rep* (2019) 9(1):9255–019-45577-2. doi: 10.1038/s41598-019-45577-2
220. Vykhodtseva NI, Hynynen K, Damianou C. Histologic effects of high intensity pulsed ultrasound exposure with subharmonic emission in rabbit brain in vivo. *Ultrasound Med Biol* (1995) 21(7):969–79. doi: 10.1016/0301-5629(95)00038-S
221. Hynynen K, McDannold N, Vykhodtseva N, Jolesz FA. Noninvasive MR imaging-guided focal opening of the blood-brain barrier in rabbits. *Radiology* (2001) 220(3):640–6. doi: 10.1148/radiol.2202001804
222. Kung Y, Huang HY, Liao WH, Huang AP, Hsiao MY, Wu CH, et al. A Single High-Intensity Shock Wave Pulse With Microbubbles Opens the Blood-Brain Barrier in Rats. *Front Bioeng Biotechnol* (2020) 8:402. doi: 10.3389/fbioe.2020.00402
223. Goutal S, Gerstenmayer M, Auvity S, Caillé F, Mériaux S, Buvat I, et al. Physical blood-brain barrier disruption induced by focused ultrasound does not overcome the transporter-mediated efflux of erlotinib. *J Control Release* (2018) 292:210–20. doi: 10.1016/j.jconrel.2018.11.009
224. Manallack DT. The pK(a) Distribution of Drugs: Application to Drug Discovery. *Perspect Medicin Chem* (2007) 1:25–38. doi: 10.1177/1177391X0700100003
225. Nagpal K, Singh SK, Mishra DN. Drug targeting to brain: a systematic approach to study the factors, parameters and approaches for prediction of permeability of drugs across BBB. *Expert Opin Drug Delivery* (2013) 10 (7):927–55. doi: 10.1517/17425247.2013.762354
226. Meng J, Agrahari V, Youm I. Advances in Targeted Drug Delivery Approaches for the Central Nervous System Tumors: The Inspiration of Nanobiotechnology. *J Neuroimmune Pharmacol* (2017) 12(1):84–98. doi: 10.1007/s11481-016-9698-1
227. White BD, Duan C, Townley HE. Nanoparticle Activation Methods in Cancer Treatment. *Biomolecules* (2019) 9(5):202. doi: 10.3390/biom9050202
228. Luo M, Lewik G, Ratcliffe JC, Choi CHJ, Mäkilä E, Tong WY, et al. Systematic Evaluation of Transferrin-Modified Porous Silicon Nanoparticles for Targeted Delivery of Doxorubicin to Glioblastoma. *ACS Appl Mater Interfaces* (2019) 11(37):33637–49. doi: 10.1021/acsami.9b10787
229. Zhang B, Wang H, Liao Z, Wang Y, Hu Y, Yang J, et al. EGFP-EGF1-conjugated nanoparticles for targeting both neovascular and glioma cells in therapy of brain glioma. *Biomaterials* (2014) 35(13):4133–45. doi: 10.1016/j.biomaterials.2014.01.071
230. Fang C, Wang K, Stephen ZR, Mu Q, Kievit FM, Chiu DT, et al. Temozolomide nanoparticles for targeted glioblastoma therapy. *ACS Appl Mater Interfaces* (2015) 7(12):6674–82. doi: 10.1021/am5092165
231. Jena L, McErlean E, McCarthy H. Delivery across the blood-brain barrier: nanomedicine for glioblastoma multiforme. *Drug Delivery Transl Res* (2020) 10(2):304–18. doi: 10.1007/s13346-019-00679-2
232. Guo L, Ren J, Jiang X. Perspectives on brain-targeting drug delivery systems. *Curr Pharm Biotechnol* (2012) 13(12):2310–8. doi: 10.2174/138920112803341770
233. Pardridge WM. Delivery of Biologics Across the Blood-Brain Barrier with Molecular Trojan Horse Technology. *BioDrugs* (2017) 31(6):503–19. doi: 10.1007/s40259-017-0248-z
234. Poduslo JF, Curran GL, Berg CT. Macromolecular permeability across the blood-nerve and blood-brain barriers. *Proc Natl Acad Sci USA* (1994) 91 (12):5705–9. doi: 10.1073/pnas.91.12.5705

235. St-Amour I, Paré I, Alata W, Coulombe K, Ringuette-Goulet C, Drouin-Ouellet J, et al. Brain bioavailability of human intravenous immunoglobulin and its transport through the murine blood-brain barrier. *J Cereb Blood Flow Metab* (2013) 33(12):1983–92. doi: 10.1038/jcbfm.2013.160
236. Yu YJ, Watts RJ. Developing therapeutic antibodies for neurodegenerative disease. *Neurotherapeutics* (2013) 10(3):459–72. doi: 10.1007/s13311-013-0187-4
237. Yu YJ, Atwal JK, Zhang Y, Tong RK, Wildsmith KR, Tan C, et al. Therapeutic bispecific antibodies cross the blood-brain barrier in nonhuman primates. *Sci Transl Med* (2014) 6(261):261ra154. doi: 10.1126/scitranslmed.3009835
238. Boado RJ, Zhang Y, Zhang Y, Pardridge WM. Humanization of anti-human insulin receptor antibody for drug targeting across the human blood-brain barrier. *Biotechnol Bioeng* (2007) 96(2):381–91. doi: 10.1002/bit.21120
239. Niewoehner J, Bohrmann B, Collin L, Urich E, Sade H, Maier P, et al. Increased brain penetration and potency of a therapeutic antibody using a monovalent molecular shuttle. *Neuron* (2014) 81(1):49–60. doi: 10.1016/j.neuron.2013.10.061
240. Ullman JC, Arguello A, Getz JA, Bhalla A, Mahon CS, Wang J, et al. Brain delivery and activity of a lysosomal enzyme using a blood-brain barrier transport vehicle in mice. *Sci Transl Med* (2020) 12(545):eaay1163. doi: 10.1126/scitranslmed.aay1163
241. Kariolis MS, Wells RC, Getz JA, Kwan W, Mahon CS, Tong R, et al. Brain delivery of therapeutic proteins using an Fc fragment blood-brain barrier transport vehicle in mice and monkeys. *Sci Transl Med* (2020) 12(545):eaay1359. doi: 10.1126/scitranslmed.aay1359
242. Galstyan A, Markman JL, Shatalova ES, Chiechi A, Korman AJ, Patil R, et al. Blood-brain barrier permeable nano immunoconjugates induce local immune responses for glioma therapy. *Nat Commun* (2019) 10(1):3850–019-11719-3. doi: 10.1038/s41467-019-11719-3
243. Kinoshita M, McDannold N, Jolesz FA, Hynynen K. Noninvasive localized delivery of Herceptin to the mouse brain by MRI-guided focused ultrasound-induced blood-brain barrier disruption. *Proc Natl Acad Sci USA* (2006) 103(31):11719–23. doi: 10.1073/pnas.0604318103
244. Raymond SB, Treat LH, Dewey JD, McDannold NJ, Hynynen K, Bacskai BJ. Ultrasound enhanced delivery of molecular imaging and therapeutic agents in Alzheimer's disease mouse models. *PloS One* (2008) 3(5):e2175. doi: 10.1371/journal.pone.0002175
245. Burgess A, Shah K, Hough O, Hynynen K. Focused ultrasound-mediated drug delivery through the blood-brain barrier. *Expert Rev Neurother* (2015) 15(5):477–91. doi: 10.1586/14737175.2015.1028369
246. Cohen-Inbar O, Xu Z, Sheehan JP. Focused ultrasound-aided immunomodulation in glioblastoma multiforme: a therapeutic concept. *J Ther Ultrasound* (2016) 4:2–016-0046-y. doi: 10.1186/s40349-016-0046-y

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Identification of Immune Cell Infiltration and Immune-Related Genes in the Tumor Microenvironment of Glioblastomas

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Glioblastoma (GBM) is one of the most prevalent malignant brain tumors with poor prognosis. Increasing evidence has revealed that infiltrating immune cells and other stromal components in the tumor microenvironment (TME) are associated with prognosis of GBM. The aim of the present study was to identify immune cells and immune-related genes extracted from TME in GBM. RNA-sequencing and clinical data of GBM were downloaded from The Cancer Genome Atlas (TCGA). Four survival-related immune cells were identified *via* Kaplan-Meier survival analysis and immune-related differentially expressed genes (DEGs) screened. Functional enrichment and protein-protein interaction (PPI) networks for the genes were constructed. In addition, we identified 24 hub genes and the expressions of 6 of the genes were significantly associated with prognosis of GBM. Finally, the genes were validated in single-cell sequencing studies of GBM, and the immune cells validated in an independent GBM cohort from the Chinese Glioma Genome Atlas (CGGA). Overall, 24 immune-related genes infiltrating the tumor microenvironment were identified in the present study, which could serve as novel biomarkers and immune therapeutic targets.

Keywords: glioblastoma, tumor microenvironment, immune infiltration, immune therapy, TCGA

INTRODUCTION

Glioblastoma (GBM) is the most common primary malignant brain tumor accounting for approximately 80% of all primary malignant brain tumors, and has a dismal prognosis and poor quality of life, with a median overall survival (OS) often < 1 year. Hereditary syndromes and ionizing radiation are the most common risk factors for GBM (1). The standard care of GBM is surgical resection followed by concomitant radiation therapy and chemotherapy with temozolomide (TMZ). Although multiple treatments have improved due to the development of gene therapy, immunotherapy, vaccine therapy, and others (2), therapeutic options for managing

recurrence in GBM are limited. Immune checkpoint inhibitors (ICIs) such as anti-programmed cell death protein-1 (PD-1)/programmed death ligand-1 (PD-L1) and anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) have been extensively studied for both primary and recurrent glioblastomas in medical research. However, most of the clinical studies for GBM based on ICIs and trials with vaccine therapies have been unsuccessful. The cause of the failure in clinical trials of GBM *via* immunotherapy is attributed to several factors, including a highly immunosuppressive environment and multiple mechanisms of therapeutic resistance. GBM induces local immune dysfunction and systemic immunosuppression, which causes more complex coupling relationships between GBM and the surrounding tumor microenvironment (TME). Studying the mechanisms of GBM immunosuppression enhances our understanding on development of immunotherapy strategies (3).

TME is one of the crucial factors of local immune dysfunction, which establishes a niche for cancer cells, multiple stromal cells (endothelial cells, immune cells, etc.) and extracellular components (extracellular matrix, cytokines, growth factors, etc.). TME plays a critical role in the establishment of specific conditions, thereby interfering with angiogenesis, cell death, oxidative stress, and immune escape (4). Increasing studies have revealed that TME is not only pivotal in tumor initiation, progression, and migration, but it also affects generation of therapeutic resistance and malignancy. Cellular composition of TME and accessibility of immune cells exhibit large variations among GBM subtypes and patients. Such factors contribute to immunosuppression of GBM, which in turn lead to immunotherapeutic treatment failure (5). Identification of actively involved types of immune genes and immune cells associated with the TME facilitates elucidation of the general mechanisms of GBM immunosuppression.

Therefore, the present study investigated survival-related immune cells in GBM and identified hub genes associated with immune cell infiltration. We acquired RNA-sequencing (RNA-seq) expression data and corresponding clinical data of 166 patients with GBM from The Cancer Genome Atlas (TCGA) database. A total of 22 types of infiltrating immune cells in the 166 patients were estimated using the method of estimating relative subsets of RNA transcripts (CIBERSORT) (6). Subsequently, four survival-related immune cells were identified from the survival analyses of 22 types of immune cells. Immune-related genes were ranked through differential gene expression analyses and 24 hub genes selected from the protein-protein interaction (PPI) network established using Cytoscape (7). Six hub genes associated with overall survival were identified. Finally, immune cells were validated in an independent GBM cohort from the Chinese Glioma Genome Atlas (CGGA), and hub genes verified in single-cell sequencing studies of GBM. All analyses were conducted using R software. The findings of the present study provide valuable information that will guide patient-specific clinical immunotherapeutic strategies, and further construction of prediction models for prognosis of GBM. Moreover, immune cells infiltrating in the tumor microenvironment could act as therapeutic targets for the clinical treatment of GBM.

MATERIALS AND METHODS

Raw Data Collection

RNA-Seq expression profiles of immune cells and corresponding clinical data of 166 patients with GBM were downloaded from TCGA database. The file format of RNA-seq expression was FPKM. The expression profile of each sample included age, gender, expression subclass, and MGMT promoter status. RNA-Seq expression information of immune cells from CGGA were also downloaded for the validation. Data acquisition and analyses were performed using R software (8).The entire research data analysis process is presented in **Figure 1**.

Identification of Survival-Related Tumor-Infiltrating Immune Cells

CIBERSORT is an analytical algorithm, which can characterize cell composition of complex tissues based on normalized gene

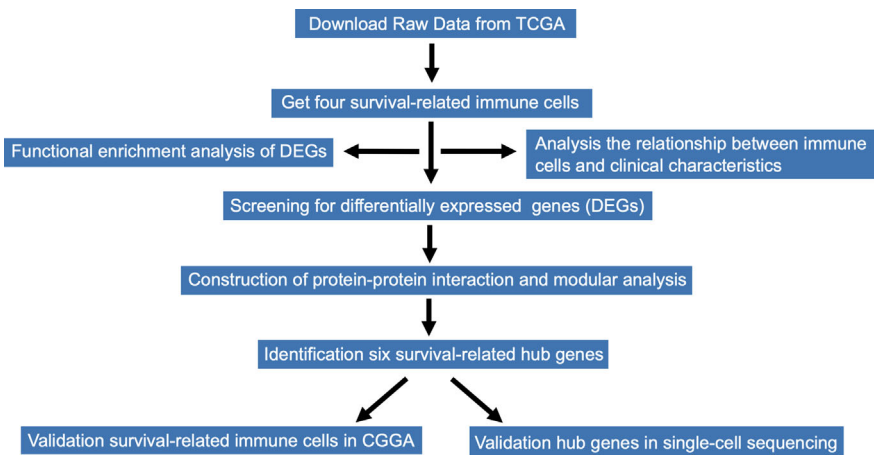

FIGURE 1 | Flow chart of the whole analysis process.

expression profiles (9). We used CIBERSORT to estimate the ratio of 22 infiltrating immune cell types based on each GBM sample. Afterward, 57 samples with $P \leq 0.05$ were selected and correlation analyses conducted to analyze contents of the 22 immune cells (10). Survival analyses of the filtered immune cells in the tumor microenvironment were performed by the Kaplan-Meier survival analysis, with a cut-off level set at the median value. The results were tested by log-rank test. All the analyses were conducted using R software.

Relationship Between Clinical Characteristics and Survival-Related Immune Cells

To determine the relationship between survival-related immune cells and clinical features such as age, gender, expression subclass, and MGMT promoter status, 57 samples were analyzed. An independent sample t-test was used to compare means of two groups, while one-way analysis of variance (ANOVA) test was used to compare the means of four groups.

Identification and Functional Enrichment Analysis of Immune-Related Genes

Immune related-genes were analyzed using survival-related cells that had been obtained previously. Data analysis was performed using the edgeR R package, and $|\log FC| \geq 1.0$ and $P < 0.05$ were set as the cut-offs to screen for immune-related genes. Subsequently, a Venn diagram was used to visualize genes displayed by the online tool (http://bioinformatics.psb.ugent.be/webtools/Venn/) (11). DAVID software (https://david.ncifcrf.gov/) was used to analyze immune-related genes in the Gene Ontology(GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (12). Results of GO analysis revealed the functions of immune-related genes in biology process, cell component, and molecular function (13). KEGG pathway analyses results revealed the role of development-related signaling pathways.

Construction of PPI Network, Selection and Analysis of Hub Genes

PPI networks of immune-related genes were predicted using the Search Tool for the Retrieval of Interacting Genes (STRING, https://string-db.org/) (14). An interaction combined score of >0.4 was considered statistically significant. Cytoscape is an open-access software platform designed to analyze and visualize complex interaction networks (7). Molecular Complex Detection(MCODE) plugin of Cytoscape was used to cluster the networks based on topology to identify densely connected regions with MCODE score > 5, degree cut-off = 2, node score cut-off = 0.2, max depth = 100, and k-score = 2 (15). Hub genes were defined based on module connectivity (16).

Identification and Immune Infiltration of Survival-Related Hub Genes

Kaplan-Meier plots were used to identify immune-related genes in relation to the overall survival of patients. These results were analyzed by long-rank test. The correlation between 24 hub genes and 22 immune cells was determined using Person's correlation analysis and CIBERSORT to reveal the relationship between hub genes and immune cells (17). Afterward, comprehensive correlation analysis between six selected survival-related hub genes and tumor-infiltrating immune cell signatures for GBM were performed using Tumor Immune Estimation Resource (TIMER 1.0, https://cistrome.shinyapps.io/timer/) (18).

Distribution of Immune-Related Hub Genes in TME of GBM From Single-Cell Data

Data for the single cell GBM analysis was derived from the paper "An Integrative Model of Cellular States, Plasticity, and Genetics for Glioblastoma", and the Seurat R package was used to reprocess the count matrix in which the dimensional reduction plot and cell type annotation were both retrieved from published meta data (19). The distribution of expressions of the hub genes was created using the Feature Plot function.

RESULTS

Data Source and Identification of Survival-Related Immune Cells

The workflow of the study is presented in **Figure 1**. Publicly available data for the 166 cases of GBM, including RNA-Seq (FPKM and counts format) and clinical data were downloaded from TCGA database. The abundance ratios of 22 immune cells in the 57 samples are presented in the **Figure 2A**, and the relationship between abundance ratios of the immune cells is presented in **Figure 2B**. Consequently, the correlations between abundance ratios of immune cells were analyzed using Kaplan-Meier survival analysis to elucidate the potential role of the abundance ratios of immune cells in overall survival. The four immune cells that were associated with survival are presented in **Figures 2C–F**. The results of survival analyses indicated that there was a significant negative correlation between M0 Macrophages, while monocytes, activated NK cells, and eosinophils predicted positive overall survival.

Clinical Data Correlated With Survival-Related Immune Cells

To determine the effect of immune cells on the clinical characteristics of GBM, relevant GBM clinical data were downloaded to investigate correlation with the abundance ratios of survival-related immune cells. The clinical characteristics included age, gender, expression subclass, and MGMT promoter status. The odds ratio of monocytes and eosinophils increased in neural and proneural types and was higher in males than in females (**Figure 3**).

Screening of Immune-Related Genes

The immune-related genes were categorized into high- and low-expression groups in GBM to identify genes that associated with the four survival-related immune cells. Unique genes expression profiles of the four survival-related immune cells are presented

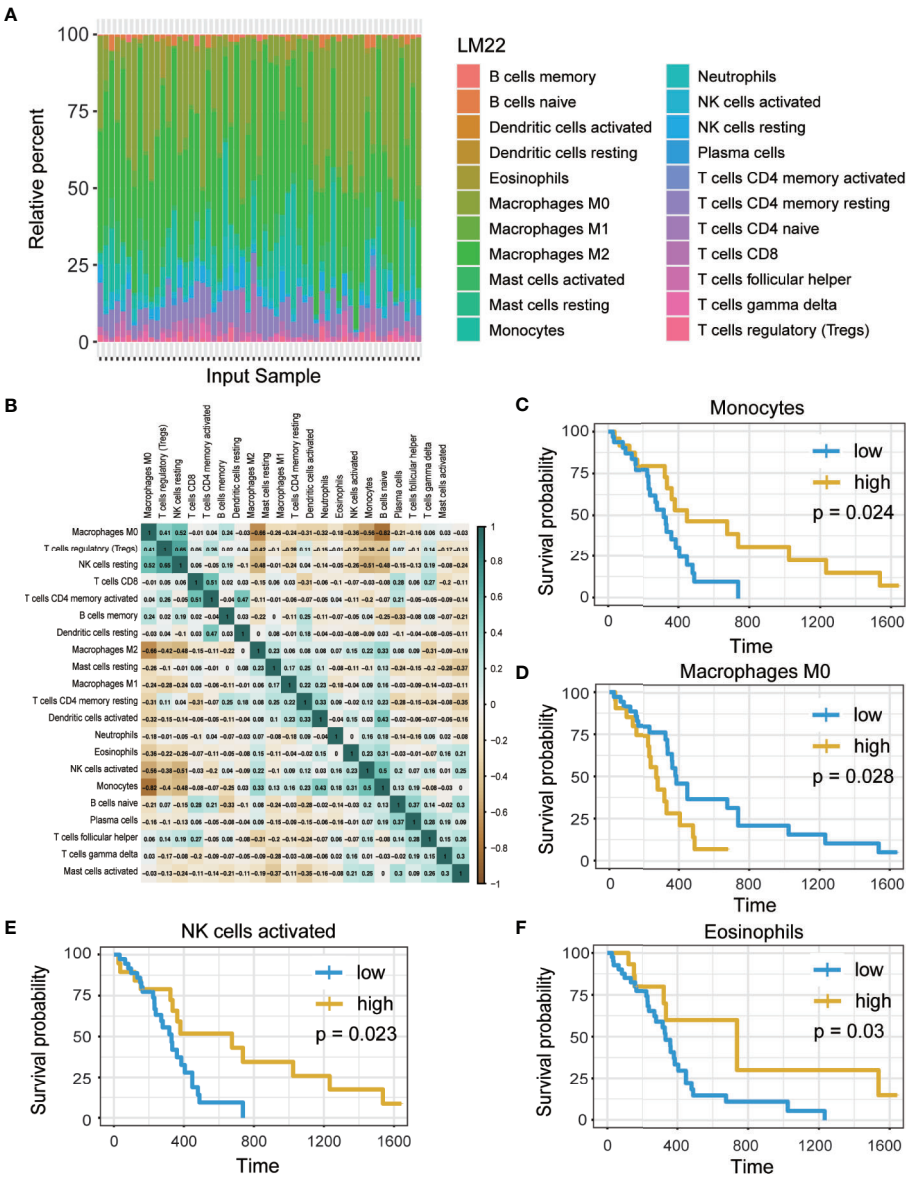

FIGURE 2 | The abundance ratios of 22 immune cells and overall survival analysis. **(A)** The abundance ratios of immune cells in the 57 samples. The specific 22 immune cells corresponded to to one sample by different colors as shown in barplot. **(B)** The abundance ratios matrix of 22 immune cells. The value represents the correlation value, green represents the positive correlation while brown represents negative correlation. **(C–F)** Overall survival analysis of four immune cells based on Kaplan Meier-plotter from the comparison of groups of high (yellow line) and low (blue line) genes expression. ($p<0.05$).

by volcano plots in **Figure 4**. A total of 1,107 genes were identified in monocytes, 1,137 genes in macrophages M0, 1,742 genes in activated NK cells, and 1,336 genes in eosinophils (**Figures 4A–D**). In addition, 38 identical genes expressed in infiltration of the four immune cells are presented by Venn diagrams in **Figure 4E**.

Functional Enrichment Analysis of Immune-Related Genes

Functional enrichment analysis of immune-related genes was performed *via* DAVID website to reveal the potential functions of immune-related genes (**Figure 5**). GO term analysis revealed that immune-related genes were significantly enriched in the biological processes (BP) of nervous system development, cell adhesion, extracellular matrix organization, and chemical synaptic transmission (**Figure 5A**). Genes in the cellular components (CC) groups (**Figure 5B**) were primarily enriched in the plasma membrane, extracellular exosome, extracellular space, and extracellular region; the molecular functions (MF) were enriched in protein binding, calcium binding, structural constituent of cytoskeleton, and microtubule binding (**Figure 5C**). Moreover, the KEGG analysis revealed that immune-related

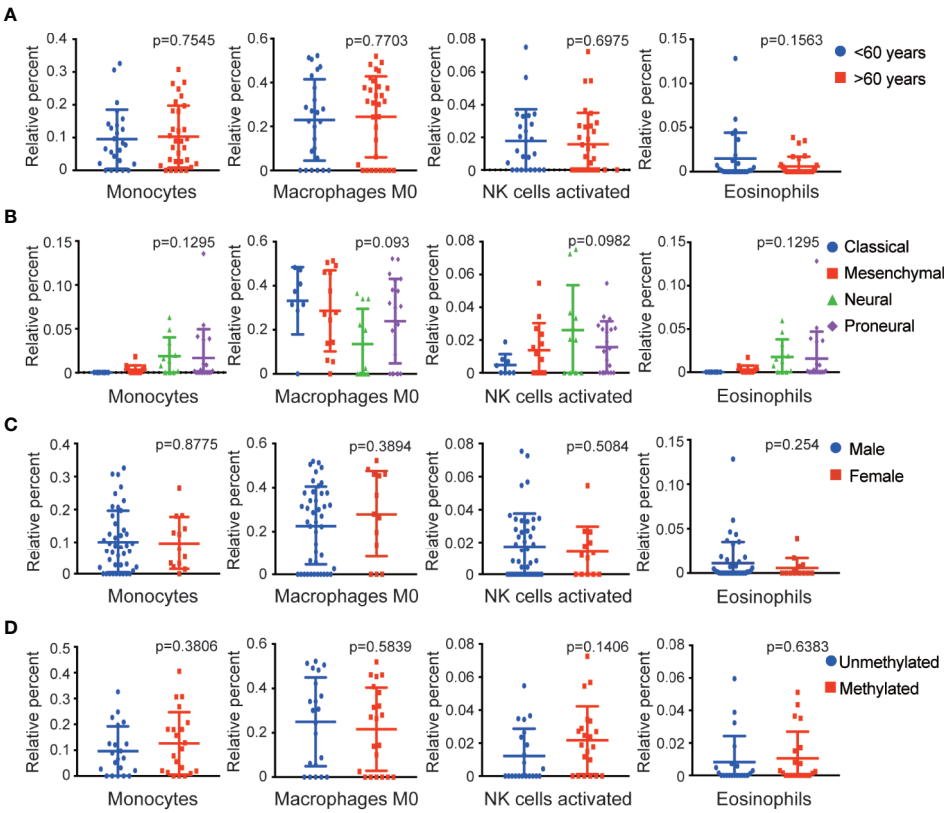

FIGURE 3 | Relationship between four survival-related immune cells and clinical features. **(A–D)** The relationship between four survival-related immune cells and age, gender, expression subclass, and MGMT status.

genes were linked to cell adhesion molecules, cAMP signaling pathway, leukocyte transendothelial migration, protein digestion and absorption, and Toll-like receptor signaling pathway (**Figure 5D**). These results demonstrated that the genes were associated with the extracellular matrix of tumor microenvironment and cellular interaction.

Modular Analysis Based on PPI Network

Considering the limitation of the PPI networks regarding the number of genes, we screened all the differentially expressed genes but selected the genes only co-expressed in at least two immune cells. Overall, we identified 920 genes from 4,122 genes. These genes were imported into the online STRING tool to elucidate the interaction of immune-related genes. Finally, we got the PPI network with 357 genes which the combined-score was set to ≥ 0.4 (**Figure 6A**). We selected the most significant module for further functional enrichment analysis (**Figure 6B**).

Identification of Survival-Related Hub Genes

A total of 24 hub genes with high connectivity in the modules were identified from the PPI network based on the cut-off criteria. We subsequently elevated the biological enrichment analysis of the 24 hub genes using the online tool (http://www.ncbi.nlm.nih.gov/gene) (**Table 1**). Six of the hub genes were significantly correlated with survival (**Figure 7**). GRIA1, BST2, B2M, and TRIM21 were positively correlated with the overall survival. GRIA2 and MAP2 were correlated with poor prognosis. The relationship between 24 hub genes and 22 immune cells analyzed using Person's correlation analysis is performed in **Figure 8A**. The remarkable relationship between infiltration levels of immune cell types and survival-related hub genes was validated in TIMER. The results indicated that infiltration levels of CD8+ T cells, neutrophils, and dendritic cells were significantly associated with GRIA1, GRIA2, and MAP2 (**Figure 8B**). Furthermore, BST2 and B2M were correlated with B cells, macrophages, and dendritic cells, and TRIM21 was associated with B cells and neutrophils.

Validation of the Correlation Between Immune Cell Infiltration and Survival-Related Hub Genes

The correlation between survival-related hub genes and immune cell infiltration in GBM was analyzed after determining the prognostic value of hub genes (**Figure 9**).

In addition, gene expression data of immune cells for 134 GBM samples were downloaded from CGGA database to investigate the significance of immune cells identified from TCGA database. The results we obtained from CCGA revealed that activated NK cells (**Figure S1**, p = 0.019) and monocytes (**Figure S1**, p = 0.023) were

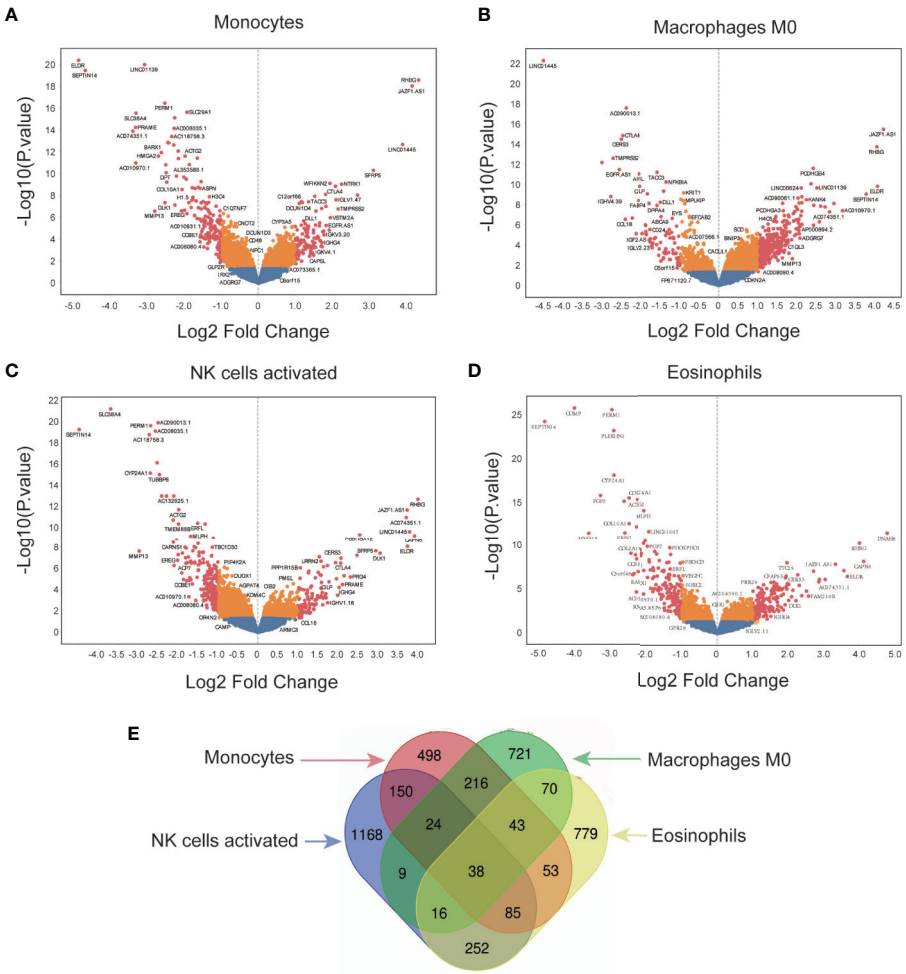

FIGURE 4 | Screening for immune-related genes. **(A–D)** The volcano plot of all quantified genes in the analysis of monocytes, macrophages M0, NK cells activated, and eosinophils. **(E)** Venn diagram indicates the overlap of differentially expressed genes across the four different immune cells.

associated with positive prognosis, which are consistent with the data we have gotten previously from the TCGA database (**Figure S1**).

Validation of the Expression of Immune-Related Hub Genes by Single-Cell Sequencing

The cells were classified as malignant and non-malignant cell types by combining three approaches; high expression of markers classified as non-malignant cells such as macrophages, T cells, and oligodendrocytes. The distribution of hub genes expressions in the four cell clusters is displayed in **Figure 9**. *A1F1*, *C3AR1*, *FCGR1A*, *MNDA*, *HMOX1*, and *TLR2* were only expressed in macrophages. *B2M*, *CCT3*, *HSPA8*, and *TUBA1A* were significantly expressed in all the four cell clusters. With reference to survival-related genes, *BST2* was detected in macrophages, T-cells, and malignant cells. *GRIA1* and *GRIA2* were expressed in oligodendrocytes and malignant cells. *MAP2* was only detected in malignant cells. However, *TRIM21* was not detected in any of the cells types. Notably, microglia are the vital macrophages of the brain, and they act as the primary form of immune defense in the central nervous system. A specific microglial marker in humans, TMEM 119, was used to distinguish microglia from macrophages in the brain (**Figure S2**). We subsequently identified the expression of hub genes in microglia and found *AIF1*, *B2M*, *BST2*, *C3AR1*, *CCND1*, *CCT3*, *FCGR1A*, *GNG7*, *HMOX1*, *HSPA8*, *MNDA*, *TLR2*, and *TUBA1A* were significantly expressed (**Figure S2**).

DISCUSSION

The present study analyzed immune cells and immune-related genes in TME of GBM to establish a potential strategy for GBM immunotherapy. The study identified immune-related genes in TME, which significantly contributed to the survival of patients with GBM from TCGA database. Four survival-related immune cells were initially identified from GBM samples and the genes

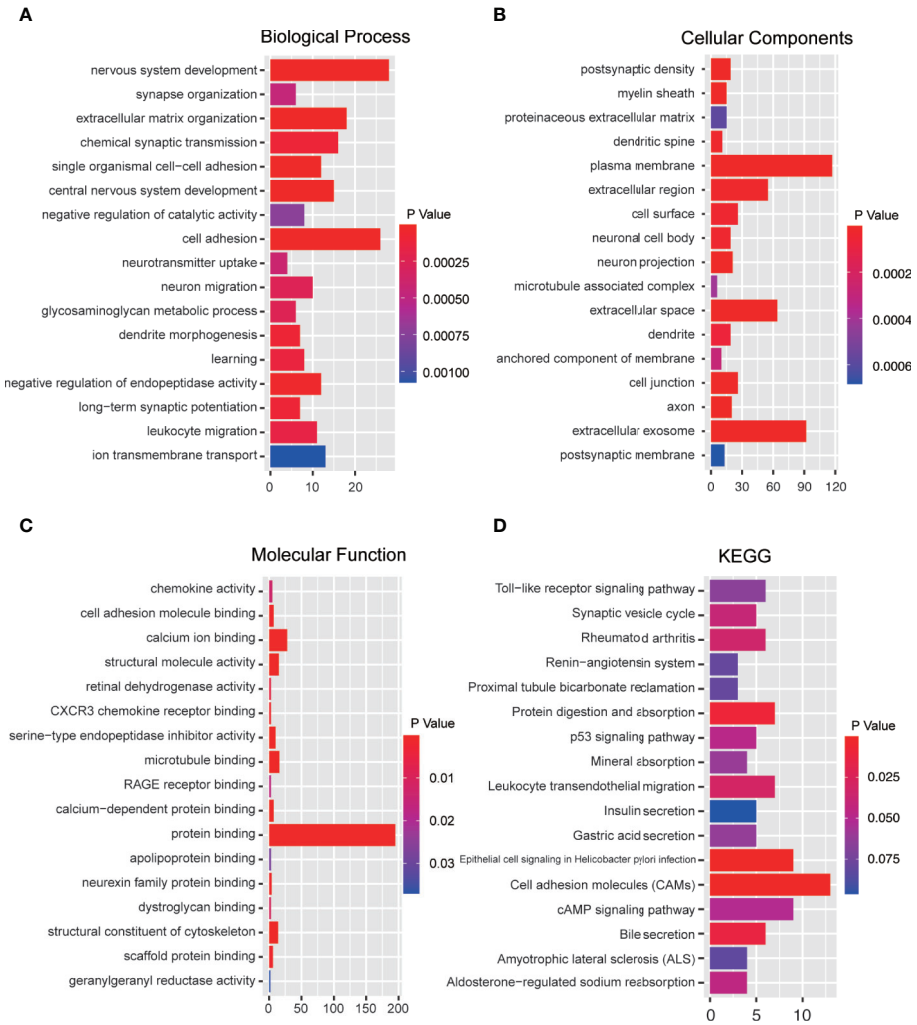

FIGURE 5 | Functional enrichment analysis of immune-related genes. **(A)** Biological process analysis. **(B)** Cellular components analysis. **(C)** Molecular function. **(D)** KEGG pathway analysis ($p < 0.05$).

correlating to the levels of four immune cells analyzed. Furthermore, GO and KEGG enrichment analysis were conducted to investigate the biological functions of immune-related genes. Subsequently, all the immune-related genes were imported to construct a PPI network, and 24 hub genes obtained. Finally, the immune cell types in patients with GBM were validated using CGGA database, and hub genes validated in single-cell sequencing.

Four types of survival-related immune cells associated with GBM were identified from TCGA database, including M0 macrophage, monocytes, NK cells and eosinophils. Previous research has indicated that immune cells, especially tumor-associated macrophages (TAMs) in TME interact with tumor cells through direct contact or different signaling pathways. TAMs are crucial components of infiltrating immune cells, accounting for 30–40% of the cellular components in GBM (20). Immune cell populations in GBM are classified into two categories: microglia and bone marrow-derived monocytes. The BBB is damaged during tumor progression (21). With the accumulation of a family of monocyte chemoattractant family of proteins (MCPs), monocytes from the periphery infiltrate into the tumor across the BBB, and then differentiate into macrophages. Tumor-associated macrophages are often regarded as the facilitators of tumor proliferation due to their proangiogenic and immunosuppressive effects (21). M0 macrophages, which are referred to as 'alternatively activated macrophages,' can be polarized into M1 or M2 phenotypes by environmental signals (22). M1 macrophages can produce pro-inflammatory cytokines that are essential for host defense and exert tumoricidal effects in GBM (21). However, M2 macrophage phenotype is considerably involved in tumor cell proliferation and prediction of poor clinical prognosis in patients with GBM patients (23). M1 and M2 macrophages are plastic and heterogeneous immune cells, and the TME facilitates the regulation of functional polarization of TAMs (24). Currently, researchers have been working on promoting the reversal of

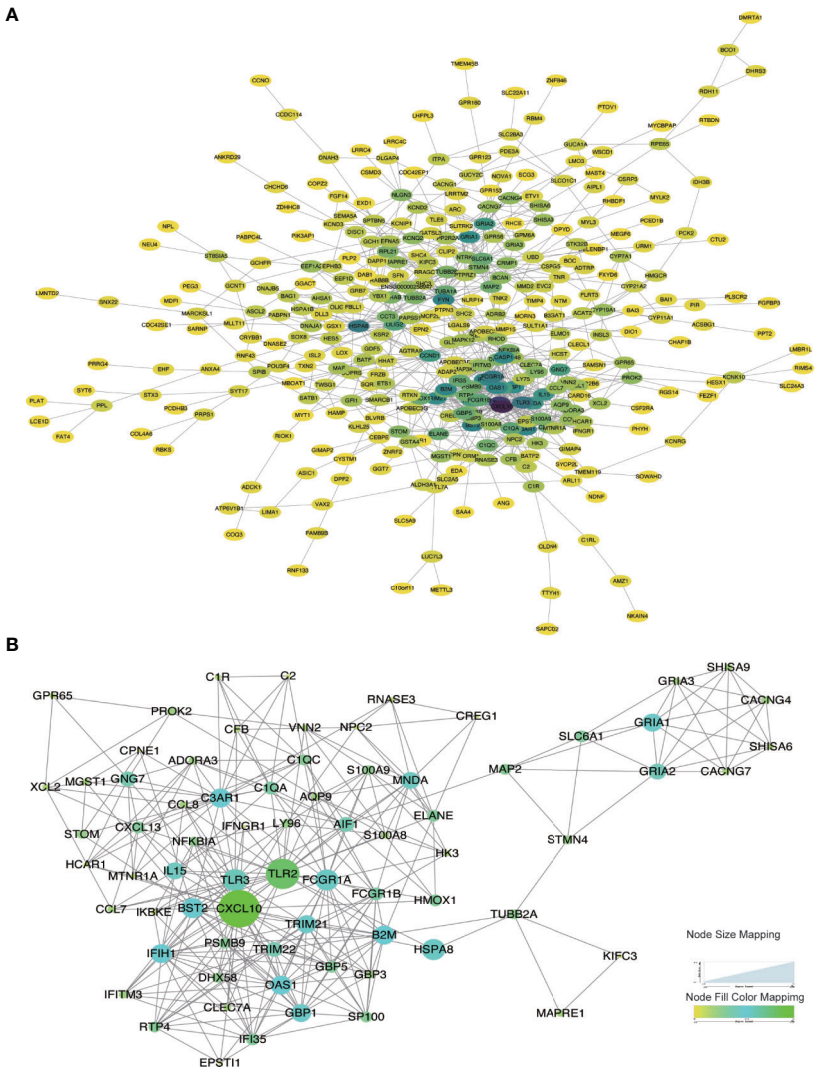

FIGURE 6 | Protein-Protein interaction network construction and modular analysis. **(A)** PPI network was constructed using a total of DEGs. **(B)** The most significant module was marked. The color of a node reflects the log(Fc) value of the gene expression, the size of a node suggests the numbers of interacting genes with others.

TAMs from M2 to M1 based on their polarization (25, 26). Therefore, the results may indicate that the macrophages in TME of GBM could be used as potential therapeutic targets for GBM immunotherapy.

NK cells accounts for 2.11% of the total cellular components in GBM, which constitutes the lowest proportion of all immune cells infiltrating in GBM (27). NK cells have been reported to recognize target cells that are deficient in the surface expression of major histocompatibility complex (MHC) molecules, and can directly lyse tumor cells without prior activation (28). However, TME influences the immune function of NK cells and causes immune evasion. The upregulation of growth factor signaling pathways or the loss of cell cycle regulators promotes evasion of GBM from surveillance through resistance to NK-derived cytotoxicity (29). Moreover, GBM cells express high levels of MHC class I molecules and human leukocyte antigens (HLA)-A, HLA-B, and HLA-C ligands, which inhibit functions of NK cells *via* killer immunoglobulin-like receptors (KIRs) (30). Therefore, blocking KIRs could disrupt the tumor microenvironment and attenuate the activity of NK cells to kill GBM cells. Increasing the number of NK cells infiltrating the GMB microenvironment and modification of NK cells could be a potential treatment intervention for GBM (31, 32). Emerging evidence has demonstrated that the activation of eosinophils induces initiation, promotion and progression of GBM (33).

Previous advances have indicated the eosinophil-derived neurotoxin (EDN) and eosinophil cationic protein (ECP) play a critical role in preventing GBM initiation (34). During GBM

TABLE 1 | The function of hub genes.

Number	Name	Full name	Function
1	FYN	FYN proto-oncogene, Src family tyrosine kinase	G-protein signaling_RhoA regulation pathway and Lipoprotein metabolism
2	HSPA8	Heat shock protein family A (Hsp70) member 8	ubiquitin protein ligase binding
3	CCND1	Cyclin D1	protein kinase activity and enzyme binding
4	GRIA1	Glutamate ionotropic receptor AMPA type subunit 1	PDZ domain binding and extracellularly glutamate-gated ion channel activity
5	TLR2	Toll like receptor 2	protein heterodimerization activity and transmembrane signaling receptor activity
6	B2M	Beta-2-microglobulin	identical protein binding
7	AIF1	Allograft inflammatory factor 1	calcium ion binding and actin filament binding
8	MAP2	Microtubule associated protein 2	structural molecule activity and calmodulin binding
9	OLIG2	Oligodendrocyte transcription factor 2	homodimerization activity and transcription factor activity, RNA polymerase II distal enhancer sequence-specific binding.
10	CXCL10	C-X-C motif chemokine ligand 10	signaling receptor binding and chemokine activity
11	GCH1	GTP cyclohydrolase 1	calcium ion binding and GTP binding
12	FCGR1A	Fc fragment of IgG receptor Ia	obsolete signal transducer activity, downstream of receptor and IgG binding
13	C3AR1	Complement C3a receptor 1	G protein-coupled receptor activity and complement component C3a receptor activity
14	TUBA1A	Tubulin alpha 1a	structural molecule activity
15	CCT3	Chaperonin containing TCP1 subunit 3	unfolded protein binding
16	HMOX1	Heme oxygenase 1	protein homodimerization activity and oxidoreductase activity
17	GNG7	G protein subunit gamma 7	obsolete signal transducer activity
18	C1R	Complement C1r	calcium ion binding and serine-type peptidase activity
19	BST2	Bone marrow stromal cell antigen 2	obsolete signal transducer activity
20	CYP19A1	Cytochrome P450 family 19 subfamily A member 1	iron ion binding and electron transfer activity
21	GRIA2	Glutamate ionotropic receptor AMPA type subunit 2	ionotropic glutamate receptor activity and AMPA glutamate receptor activity
22	MNDA	Myeloid cell nuclear differentiation antigen	Innate Immune System and Apoptosis and Autophagy
23	MAF	MAF bZIP transcription factor	DNA-binding transcription factor activity and DNA-binding transcription activator activity, RNA polymerase II-specific
24	TRIM21	Tripartite motif containing 21	identical protein binding and ligase activity

promotion, eosinophils are activated by GBM mediators, which in turn lead to the production of tumor promoting growth factors (35). Nevertheless, the mechanisms of immune response in GBM remain indeterminate; therefore, further studies are required to investigate the mechanism involved.

More importantly, KEGG enrichment analysis indicated that these differential immune-related genes were enriched in the classical pathway, such as cell adhesion molecules (CAMs) and cAMP signaling pathway. CAMs are glycol-proteins expressed on the cell surface and play a critical role in multiple biologic processes during tumor development (36). It has been reported that CAMs mediate the process of immune responses in the tumor microenvironment, such as immune cell recruitment, immune cell activation, and formation of immunological synapse between immune cells and tumor cells (37). The cAMP signaling pathway, which acts as universal second messengers regulates pivotal physiological processes. The increases of intracellular cAMP inhibits innate immune functions (38). At the BP level, these differential immune-related genes were significantly enriched in cell adhesion, and extracellular matrix organization. In the CC groups, the differential immune-related genes were related to extracellular exosome, extracellular space, and extracellular region; the MF groups were enriched in protein binding, the structural constituent of cytoskeleton, and microtubule binding. Theses pathways are all related to the extracellular matrix components and cell's cytoskeleton in the microenvironment. These above results further indicate the reliability of the immune differential genes and their relevance to the GBM tumor microenvironment.

Furthermore, we identified 24 hub genes, and 6 of these genes (GRIA2, GRIA1, BST2, MAP2, B2M, and TRIM21) have significant correlation with prognosis and were considered as predictive biomarkers that could provide valuable insights into new immunotherapy strategies. Previous studies have demonstrated that glioma cells can secrete excitotoxicity glutamate that mediates neuronal death in glioma microenvironment. Moreover, glutamate secretion promotes tumor expansion by inducing inflammatory response within the surrounding areas (39). Researchers have established that the expression of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) protects GBM cells from the glutamate-rich tumor microenvironment (40). AMPARs are complexes consisting of four subunits (GluR1, GluR2, GluR3, and GluR4). GRIA1 and GRIA2 are also referred to as GluR1and GluR2, respectively. Glutamate receptors (GluRs) are receptors that bind to glutamate, and they function as ligand-gated ion channels in the central nervous system and mediate transmission in excitatory synapses (41). The subunit composition of AMPARs depends on the conductance properties of Ca^{2+}. Absence of GluR2

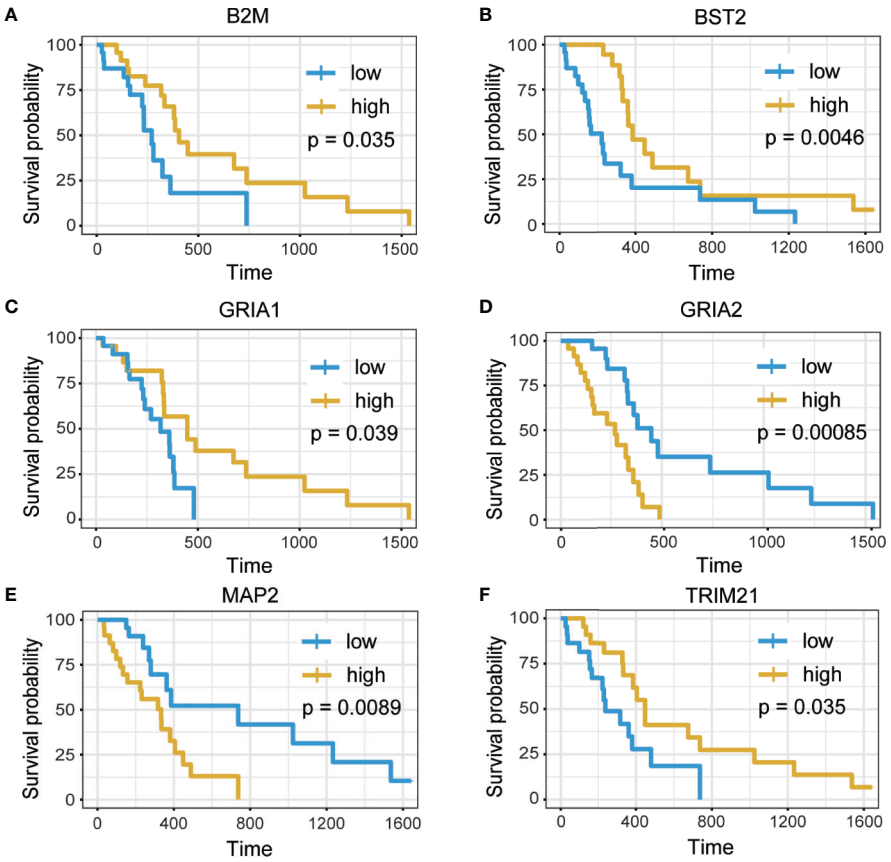

FIGURE 7 | Overall survival analysis of six hub genes. **(A)** B2M. **(B)** BST2. **(C)** GRIA1. **(D)** GRIA2. **(E)** MAP2. **(F)** TRIM21 ($p < 0.05$).

subunit promotes permeability to Ca^{2+}, whereas presence of GluR2 inhibits permeability to Ca^{2+} (42). However, GluR1 and GluR4 subunits also function as Ca^{2+}- permeable AMPARs. Ishiuchi et, al found GluR1 proteins were substantially expressed in most tumor cells, whereas GluR2 was mainly expressed in normal tissues in human glioblastoma samples (43). Furthermore, it has been suggested that blockage of Ca^{2+} influx through GluR2 expression suppresses migration and induces apoptosis in human glioblastoma cells (44). In addition, knocking down GluR1 inhibits glioma growth (45). Therefore, the conversion of Ca^{2+}- permeable AMPARs to Ca^{2+}-impermeable could be a potential therapeutic target for brain tumors (43). TRIM21 expression is correlated with prognosis, which acts as a tumor suppressor in patients with GBM (46). TRIM21 depletion in GBM enhanced cell proliferation and tumor growth. Lee et, al found that phosphofructokinase 1 (PFK1) expression promotes human glioblastoma progression, while TRIM21 exert anti-tumor effect by mediating poly ubiquitination and degradation of PFK1 (46). Therefore, TRIM21 is a novel target for glioblastoma treatment.

The expression of 24 hub genes in human glioblastomas was validated using single-cell sequencing. Conventional RNA-seq is regularly performed on a bulk level and only measures the average gene expression based on mixed cell populations in samples. Genes that contribute to cell-by-cell variations cannot be detected using conventional RNA-seq data of GBM downloaded from TCGA database (47). However, single-cell RNA-seq (scRNA-seq) profiles for intracellular transcriptome at individual cell level can reveal potential heterogeneous tumors and the composition of glioblastoma tumor microenvironment (48). ScRNA-seq can easily identify highly variable genes in all cell types in the TME of GBM, including the two primary cell types: microglia/macrophages and oligodendrocytes, which are limited in conventional RNA-seq (49). For example, as we have mentioned above, the results of ScRNA-seq revealed that GluR1 and GluR2 were expressed in oligodendrocytes and malignant cells. The expression of Ca2+-permeable GluR confers protection against excitotoxicity and promotes progression of tumor (50). BST2 expression increases in the malignant cells of glioma during tumor progression (51). TLR2 expressed in microglia can promote glioblastoma progression by up-regulating the expression of MT1-MMP in microglia (52). The expression of CCND1 in microglia cells contributes to the differential diagnosis of oligodendrogliomas (53). The use of scRNA-seq to detect the expression of hub genes could significantly help us to accurately understand the function of hub genes in each cell (54). In addition, scRNA-seq demonstrates transcriptional heterogeneity associated with spatial specificity in distinct TME

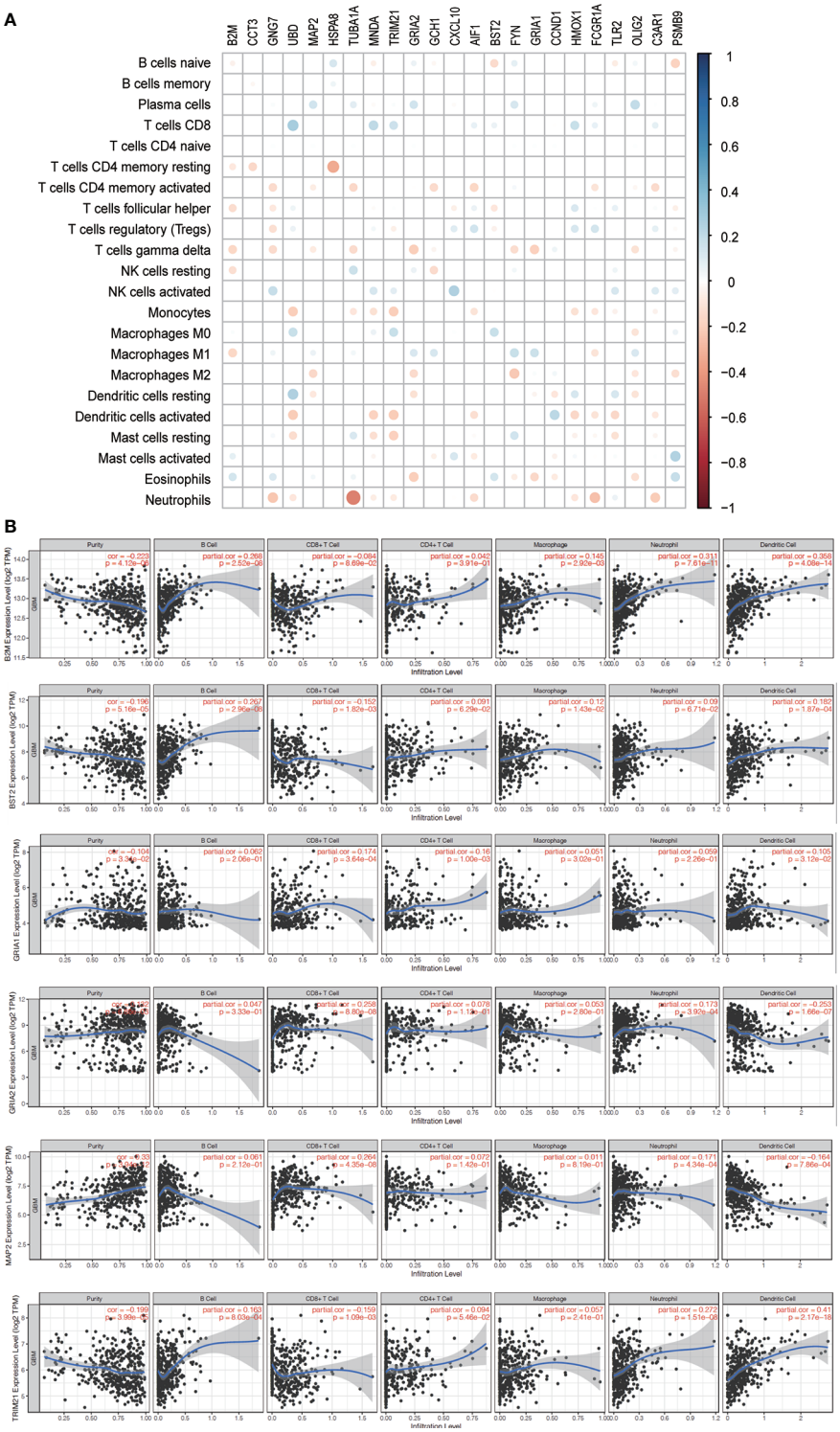

FIGURE 8 | Immune infiltration of survival-related genes. **(A)** The correlation between expression proportion of hub genes and immune cells. Red suggests the positive correlation while the blue represents negative correlation. The size of point indicates P-value, and the color reflects the correlation. **(B)** The correlation analysis between survival-related genes and tumor infiltrating immune cells was performed. Scatter plots were generated with partial Spearman's correlation and statistical significance.

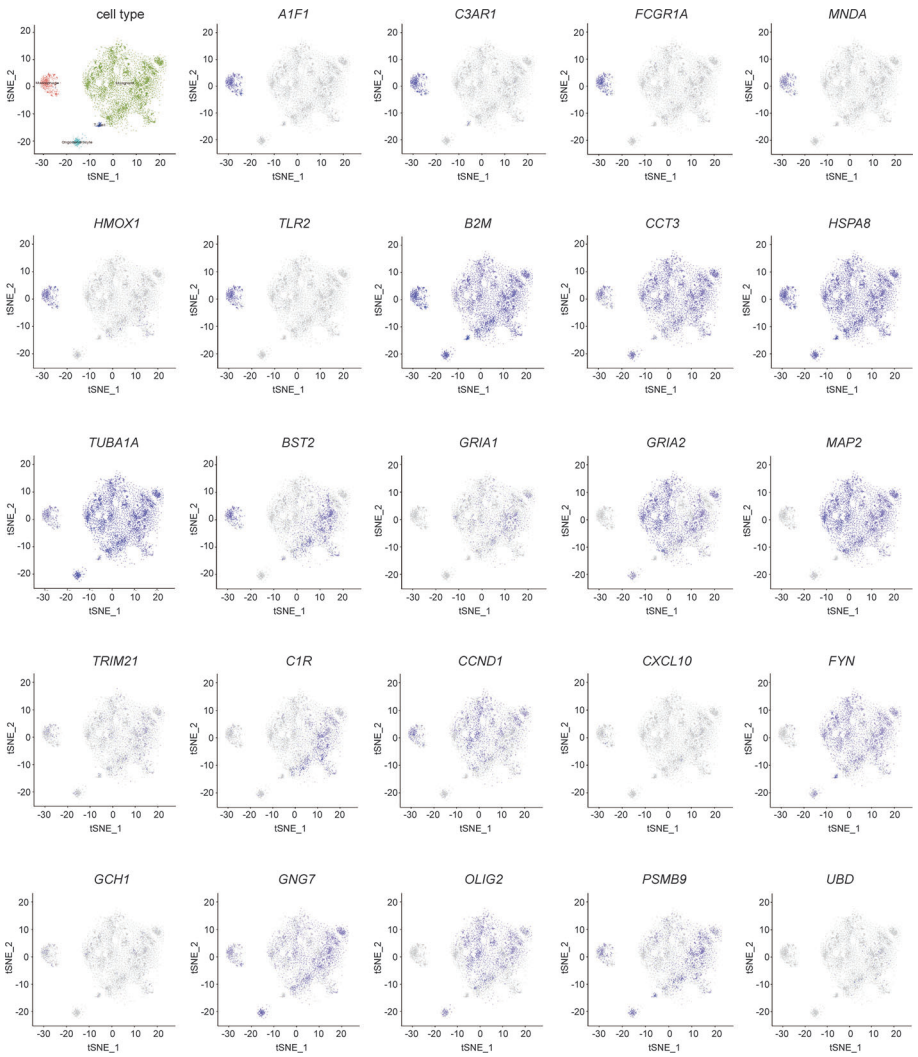

FIGURE 9 | Validation of hub genes in single- cell sequencing in GBM. t-distributed neighbor embedding(tSNE) plot of all single cells. The color represents the expression of markers for Malignant cells (green), marcophages(magenta), oligodendrocytes (cyan), and T-cells (blue).

patterns (55). ScRNA-seq has emerged as a revolutionary tool to enhance our understanding of the profiles of hub genes in GBM, and offers insights with implications for both targeted and immune therapies for GBM (49).

In summary, the study identified four types of survival-related immune cells from TCGA database and 24 TME-related hub genes in glioblastoma. The correlation between immune cells and hub genes in patients with GBM was validated using single-cell sequencing data. The results revealed that the hub genes are involved in the development and progression of GBM. Therefore, the candidate genes identified in the study can be used as potential prognostic biomarkers for GBM. However, further studies on the immune cells and hub genes in GBM tumor microenvironment should be conducted to investigate the underlying mechanisms. The present study provides novel insights into the potential association between immune cell TME and GBM prognosis.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

SH, ZS, JS, and JP designed the study. SH, ZS, XH, TZ, and KH collected and analyzed data. SH, ZS, XH, QZ, and JP wrote the

manuscript. All the authors approved the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.585034/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | Overall survival analysis of 22 immune cells based on Kaplan Meier-plotter from the data of CGGA.

SUPPLEMENTARY FIGURE 2 | Hub genes expression in microglia. Microglia classified from macrophages are marked in dark red, while hub genes are in orange.

REFERENCES

1. Touat M, Idbaih A, Sanson M, Ligon KL. Glioblastoma targeted therapy: updated approaches from recent biological insights. *Ann Oncol* (2017) 28 (7):1457–72. doi: 10.1093/annonc/mdx106
2. Omuro A, DeAngelis LM. Glioblastoma and other malignant gliomas: a clinical review. *Jama* (2013) 310(17):1842–50. doi: 10.1001/jama.2013.280319
3. Medikonda R, Dunn G, Rahman M, Fecci P, Lim M. A review of glioblastoma immunotherapy. *J Neurooncol* (2020). doi: 10.1007/s11060-020-03448-1
4. Da Ros M, De Gregorio V, Iorio AL, Giunti L, Guidi M, de Martino M, et al. Glioblastoma Chemoresistance: The Double Play by Microenvironment and Blood-Brain Barrier. *Int J Mol Sci* (2018) 19(10):2879. doi: 10.3390/ijms19102879
5. Tomaszewski W, Sanchez-Perez L, Gajewski TF, Sampson JH. Brain Tumor Microenvironment and Host State: Implications for Immunotherapy. *Clin Cancer Res* (2019) 25(14):4202–10. doi: 10.1158/1078-0432.ccr-18-1627
6. Newman AM, Liu CL, Green MR, Gentles AJ, Feng W, Xu Y, et al. Robust enumeration of cell subsets from tissue expression profiles. *Nat Methods* (2015) 12(5):453–7. doi: 10.1038/nmeth.3337
7. Doncheva NT, Morris JH, Gorodkin J, Jensen LJ. Cytoscape StringApp: Network Analysis and Visualization of Proteomics Data. *J Proteome Res* (2019) 18(2):623–32. doi: 10.1021/acs.jproteome.8b00702
8. Jia D, Li S, Li D, Xue H, Yang D, Liu Y. Mining TCGA database for genes of prognostic value in glioblastoma microenvironment. *Aging (Albany NY)* (2018) 10(4):592–605. doi: 10.18632/aging.101415
9. Gentles AJ, Newman AM, Liu CL, Bratman SV, Feng W, Kim D, et al. The prognostic landscape of genes and infiltrating immune cells across human cancers. *Nat Med* (2015) 21(8):938–45. doi: 10.1038/nm.3909
10. Cao J, Yang X, Li J, Wu H, Li P, Yao Z, et al. Screening and Identifying Immune-Related Cells and Genes in the Tumor Microenvironment of Bladder Urothelial Carcinoma: Based on TCGA Database and Bioinformatics. *Front Oncol* (2019) 9:1533:1533. doi: 10.3389/fonc.2019.01533
11. Heberle H, Meirelles GV, da Silva FR, Telles GP, Minghim R. InteractiVenn: a web-based tool for the analysis of sets through Venn diagrams. *BMC Bioinf* (2015) 16(1):169. doi: 10.1186/s12859-015-0611-3
12. Huang DW, Sherman BT, Tan Q, Kir J, Liu D, Bryant D, et al. DAVID Bioinformatics Resources: expanded annotation database and novel algorithms to better extract biology from large gene lists. *Nucleic Acids Res* (2007) 35(Web Server issue):W169–75. doi: 10.1093/nar/gkm415
13. Szklarczyk D, Morris JH, Cook H, Kuhn M, Wyder S, Simonovic M, et al. The STRING database in 2017: quality-controlled protein-protein association networks, made broadly accessible. *Nucleic Acids Res* (2017) 45(D1):D362–d368. doi: 10.1093/nar/gkw937
14. Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, et al. STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res* (2019) 47(D1):D607–d613. doi: 10.1093/nar/gky1131
15. Xu WH, Xu Y, Wang J, Wan FN, Wang HK, Cao DL, et al. Prognostic value and immune infiltration of novel signatures in clear cell renal cell carcinoma microenvironment. *Aging (Albany NY)* (2019) 11(17):6999–7020. doi: 10.18632/aging.102233
16. Chen Q, Yu D, Zhao Y, Qiu J, Xie Y, Tao M. Screening and identification of hub genes in pancreatic cancer by integrated bioinformatics analysis. *J Cell Biochem* (2019) 120(12):19496–508. doi: 10.1002/jcb.29253
17. Wu Y, Zhang S, Yan J. IRF1 association with tumor immune microenvironment and use as a diagnostic biomarker for colorectal cancer recurrence. *Oncol Lett* (2020) 19(3):1759–70. doi: 10.3892/ol.2020.11289
18. Li T, Fan J, Wang B, Traugh N, Chen Q, Liu JS, et al. TIMER: A Web Server for Comprehensive Analysis of Tumor-Infiltrating Immune Cells. *Cancer Res* (2017) 77(21):e108–10. doi: 10.1158/0008-5472.can-17-0307
19. Neftel C, Laffy J, Filbin MG, Hara T, Shore ME, Rahme GJ, et al. An Integrative Model of Cellular States, Plasticity, and Genetics for Glioblastoma. *Cell* (2019) 178(4):835–849.e21. doi: 10.1016/j.cell.2019.06.024
20. Charles NA, Holland EC, Gilbertson R, Glass R, Kettenmann H. The brain tumor microenvironment. *Glia* (2012) 60(3):502–14. doi: 10.1002/glia.21264
21. Chen Z, Hambardzumyan D. Immune Microenvironment in Glioblastoma Subtypes. *Front Immunol* (2018) 9:1004:1004. doi: 10.3389/fimmu.2018.01004
22. Miao X, Leng X, Zhang Q. The Current State of Nanoparticle-Induced Macrophage Polarization and Reprogramming Research. *Int J Mol Sci* (2017) 18(2):336. doi: 10.3390/ijms18020336
23. Komohara Y, Horlad H, Ohnishi K, Fujiwara Y, Bai B, Nakagawa T, et al. Importance of direct macrophage-tumor cell interaction on progression of human glioma. *Cancer Sci* (2012) 103(12):2165–72. doi: 10.1111/cas.12015
24. Wu T, Dai Y. Tumor microenvironment and therapeutic response. *Cancer Lett* (2017) 387:61–8. doi: 10.1016/j.canlet.2016.01.043
25. Saha D, Martuza RL, Rabkin SD. Macrophage Polarization Contributes to Glioblastoma Eradication by Combination Immunovirotherapy and Immune Checkpoint Blockade. *Cancer Cell* (2017) 32(2):253–267.e5. doi: 10.1016/j.ccell.2017.07.006
26. Gordon S. Alternative activation of macrophages. *Nat Rev Immunol* (2003) 3 (1):23–35. doi: 10.1038/nri978
27. Levy EM, Roberti MP, Mordoh J. Natural killer cells in human cancer: from biological functions to clinical applications. *J BioMed Biotechnol* (2011) 2011:676198. doi: 10.1155/2011/676198
28. Purdy AK, Campbell KS. Natural killer cells and cancer: regulation by the killer cell Ig-like receptors (KIR). *Cancer Biol Ther* (2009) 8(23):2211–20. doi: 10.4161/cbt.8.23.10455
29. Yang C, Li Y, Yang Y, Chen Z. Overview of Strategies to Improve Therapy against Tumors Using Natural Killer Cell. *J Immunol Res* (2020) 2020:8459496. doi: 10.1155/2020/8459496
30. Golán I, Rodríguez de la Fuente L, Costoya JA. NK Cell-Based Glioblastoma Immunotherapy. *Cancers (Basel)* (2018) 10(12):522. doi: 10.3390/cancers10120522
31. Castriconi R, Daga A, Dondero A, Zona G, Poliani PL, Melotti A, et al. NK cells recognize and kill human glioblastoma cells with stem cell-like properties. *J Immunol* (2009) 182(6):3530–9. doi: 10.4049/jimmunol.0802845
32. Burger MC, Zhang C, Harter PN, Romanski A, Strassheimer F, Senft C, et al. CAR-Engineered NK Cells for the Treatment of Glioblastoma: Turning Innate Effectors Into Precision Tools for Cancer Immunotherapy. *Front Immunol* (2019) 10:2683:2683. doi: 10.3389/fimmu.2019.02683
33. Curran CS, Bertics PJ. Eosinophils in glioblastoma biology. *J Neuroinflamm* (2012) 9:11. doi: 10.1186/1742-2094-9-11
34. Boix E, Torrent M, Sánchez D, Nogués MV. The antipathogen activities of eosinophil cationic protein. *Curr Pharm Biotechnol* (2008) 9(3):141–52. doi: 10.2174/138920108784567353
35. Curran CS, Evans MD, Bertics PJ. GM-CSF production by glioblastoma cells has a functional role in eosinophil survival, activation, and growth factor production for enhanced tumor cell proliferation. *J Immunol* (2011) 187 (3):1254–63. doi: 10.4049/jimmunol.1001965

36. Dustin ML. Integrins and Their Role in Immune Cell Adhesion. *Cell* (2019) 177(3):499–501. doi: 10.1016/j.cell.2019.03.038
37. Harjunpää H, Llort Asens M, Guenther C, Fagerholm SC. Cell Adhesion Molecules and Their Roles and Regulation in the Immune and Tumor Microenvironment. *Front Immunol* (2019) 10:1078:1078. doi: 10.3389/fimmu.2019.01078
38. Serezani CH, Ballinger MN, Aronoff DM, Peters-Golden M. Cyclic AMP: master regulator of innate immune cell function. *Am J Respir Cell Mol Biol* (2008) 39(2):127–32. doi: 10.1165/rcmb.2008-0091TR
39. Takano T, Lin JH, Arcuino G, Gao Q, Yang J, Nedergaard M. Glutamate release promotes growth of malignant gliomas. *Nat Med* (2001) 7(9):1010–5. doi: 10.1038/nm0901-1010
40. Savaskan NE, Seufert S, Hauke J, Tränkle C, Eyüpoglu IY, Hahnen E. Dissection of mitogenic and neurodegenerative actions of cystine and glutamate in malignant gliomas. *Oncogene* (2011) 30(1):43–53. doi: 10.1038/onc.2010.391
41. Ozawa S, Kamiya H, Tsuzuki K. Glutamate receptors in the mammalian central nervous system. *Prog Neurobiol* (1998) 54(5):581–618. doi: 10.1016/s0301-0082(97)00085-3
42. Iino M, Goto K, Kakegawa W, Okado H, Sudo M, Ishiuchi S, et al. Glia-synapse interaction through Ca2+-permeable AMPA receptors in Bergmann glia. *Science* (2001) 292(5518):926–9. doi: 10.1126/science.1058827
43. Ishiuchi S, Tsuzuki K, Yoshida Y, Yamada N, Hagimura N, Okado H, et al. Blockage of Ca(2+)-permeable AMPA receptors suppresses migration and induces apoptosis in human glioblastoma cells. *Nat Med* (2002) 8(9):971–8. doi: 10.1038/nm746
44. Beretta F, Bassani S, Binda E, Verpelli C, Bello L, Galli R, et al. The GluR2 subunit inhibits proliferation by inactivating Src-MAPK signalling and induces apoptosis by means of caspase 3/6-dependent activation in glioma cells. *Eur J Neurosci* (2009) 30(1):25–34. doi: 10.1111/j.1460-9568.2009.06804.x
45. de Groot JF, Piao Y, Lu L, Fuller GN, Yung WK. Knockdown of GluR1 expression by RNA interference inhibits glioma proliferation. *J Neurooncol* (2008) 88(2):121–33. doi: 10.1007/s11060-008-9552-2
46. Lee JH, Liu R, Li J, Zhang C, Wang Y, Cai Q, et al. Stabilization of phosphofructokinase 1 platelet isoform by AKT promotes tumorigenesis. *Nat Commun* (2017) 8(1):949. doi: 10.1038/s41467-017-00906-9
47. Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet* (2009) 10(1):57–63. doi: 10.1038/nrg2484
48. Patel AP, Tirosh I, Trombetta JJ, Shalek AK, Gillespie SM, Wakimoto H, et al. Single-cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma. *Science* (2014) 344(6190):1396–401. doi: 10.1126/science.1254257
49. Tirosh I, Suvà ML. Dissecting human gliomas by single-cell RNA sequencing. *Neuro Oncol* (2018) 20(1):37–43. doi: 10.1093/neuonc/nox126
50. Yoshioka A, Ikegaki N, Williams M, Pleasure D. Expression of N-methyl-D-aspartate (NMDA) and non-NMDA glutamate receptor genes in neuroblastoma, medulloblastoma, and other cells lines. *J Neurosci Res* (1996) 46(2):164–78. doi: 10.1002/(sici)1097-4547(19961015)46:2<164::aid-jnr4>3.0.co;2-f
51. Wainwright DA, Balyasnikova IV, Han Y, Lesniak MS. The expression of BST2 in human and experimental mouse brain tumors. *Exp Mol Pathol* (2011) 91(1):440–6. doi: 10.1016/j.yexmp.2011.04.012
52. Vinnakota K, Hu F, Ku MC, Georgieva PB, Szulzewsky F, Pohlmann A, et al. Toll-like receptor 2 mediates microglia/brain macrophage MT1-MMP expression and glioma expansion. *Neuro Oncol* (2013) 15(11):1457–68. doi: 10.1093/neuonc/not115
53. Bosone I, Cavalla P, Chiadò-Piat L, Vito ND, Schiffer D. Cyclin D1 expression in normal oligodendroglia and microglia cells: its use in the differential diagnosis of oligodendrogliomas. *Neuropathology* (2001) 21(3):155–61. doi: 10.1046/j.1440-1789.2001.00389.x
54. Yip SH, Sham PC, Wang J. Evaluation of tools for highly variable gene discovery from single-cell RNA-seq data. *Brief Bioinform* (2019) 20(4):1583–9. doi: 10.1093/bib/bby011
55. Venteicher AS, Tirosh I, Hebert C, Yizhak K, Neftel C, Filbin MG, et al. Decoupling genetics, lineages, and microenvironment in IDH-mutant gliomas by single-cell RNA-seq. *Science* (2017) 355(6332):eaai8478. doi: 10.1126/science.aai8478

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REVIEW
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Recent Advances in Immune Cell Therapy for Glioblastoma

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Glioblastoma (GBM) is the most malignant form of astrocytoma with short survival and a high recurrence rate and remains a global problem. Currently, surgery, chemotherapy, radiotherapy, and other comprehensive treatments are the main treatment modalities, but patients still have a poor prognosis mainly due to the infiltrative growth of GBM and the protective effect of the blood–brain barrier on tumor cells. Therefore, immunotherapy is expected to be a good option for GBM. In the immune system, different cells play varying roles in the treatment of GBM, so understanding the roles played by various immune cells in treating GBM and considering how to combine these effects to maximize the efficacy of these cells is important for the selection of comprehensive and optimal treatment plans and improving GBM prognosis. Therefore, this study reviews the latest research progress on the role of various types of immune cells in the treatment of GBM.

Keywords: glioblastoma, immunotherapy, immune cell, advances, mechanism

INTRODUCTION

Glioblastoma

Glioblastoma (GBM) is a rare tumor that is one of the most fatal and difficult-to-treat malignancies. Currently, the primary treatment for GBM is still based on surgery, and patients usually have a poor prognosis and poor quality of life (1). The tumor is subcortical, and most grow throughout the supratentorial cerebral hemispheres. It exhibits infiltrative growth, often invades several lobes and deep structures, and has been shown to affect the contralateral cerebral hemisphere through the corpus callosum with the frontal lobe being the most common site of occurrence (2). GBM grows rapidly with 70% to 80% of patients dying of GBM within 3 to 6 months after diagnosis and a 1-year survival rate of only 10%.

Immune Cell Therapy

There is a large body of literature demonstrating that immunotherapy is important for the treatment of GBM. Chimeric antigen receptor (CAR) T cell therapy can directly and accurately identify, localize to, and kill cancer cells. Natural killer (NK) cells control GBM expansion and inhibit tumor progression; dendritic cells (DCs) play a role in GBM immune recognition, and other immune cells play an adjuvant role in radiotherapy and chemotherapy treatments for GBM.

The main feature of GBM metastasis is extensive local invasion, which is different from the rarer event of systemic metastasis. Therefore, cancer immunotherapy at this stage focuses more on the use of immune cells to inhibit the metastasis of GBM.

Advantages of Immune Cell Therapy

Some immune cells with recognition functions can distinguish themselves from nonself cells (which present nonself antigens), providing a great opportunity for the use of immune cells to specifically recognize and kill cancer cells.

Although the metastatic spread of GBM is extremely rare, GBMs can grow in areas of the brain that are hard to access surgically. Compared with the gross removal of tissue *via* surgical resection, the specific recognition and killing ability of immune cells is more likely to remove only the cancer cells, which has great advantages over less specific treatment modalities.

Disadvantages of Immunotherapy

GBM is highly susceptible to recurrence, and most recurrent tumors have been subjected to genotoxic stress from radiotherapy and/or chemotherapy and are, thus, more immunogenic than untreated tumors (3). However, because recurrent gliomas often engage in antigen escape after immunotherapy, it is difficult to perform immunotherapy on these tumors.

CHANGES IN ASSOCIATED IMMUNE SYSTEM AFTER GBM DEVELOPMENT

Because GBM occurs in the brain, the immunosuppression of GBM involves both the tumor itself and the unique immune characteristics of the brain. The interactions of glioma stem cells (GSCs) and the tumor microenvironment play vital roles in promoting the malignant growth of GBMs. A schematic illustrating the immunosuppressive microenvironment in GBM is shown in **Figure 1**.

Brain Autoimmune Properties

The blood–brain barrier (BBB) is an important line of defense for brain immunity. The BBB is an astrocyte-supported network of tight junctions on the endothelium that prevents the diffusion of hydrophilic macromolecules into the CNS while allowing the entry of small hydrophobic molecules and the active transport of glucose and nutrients (4).

The Immune Microenvironment of GBM

Glioma Vasculature

The vasculature within gliomas shows upregulated protein expression of the macromolecules periostin and tenascin C (TNC), which can prevent T cells from moving into glioma-associated vessels and prevent their migration into the brain parenchyma (5).

Upregulation of Immunosuppressive Molecules (Immune Checkpoints)

Immune checkpoints are small molecules present on the cell surface of T lymphocytes that maintain immune homeostasis. Some immune checkpoint genes, such as CTLA-4, PD-1, LAG3, TIM, and BTLA, mediate inhibitory signals, thereby inhibiting T cell activity (6). The expression of CTLA-4 and PD-1 in GBM often rises immensely, which suppresses immunity (3).

Soluble Factors (e.g., Cytokines and Growth Factors)

The soluble factors TGFβ, IL-10, and prostaglandin 50 were the earliest immunosuppressive mediators identified in GBM patients. TGF-ßTME and IL-10 cause microglia to lose their MHC expression (5).

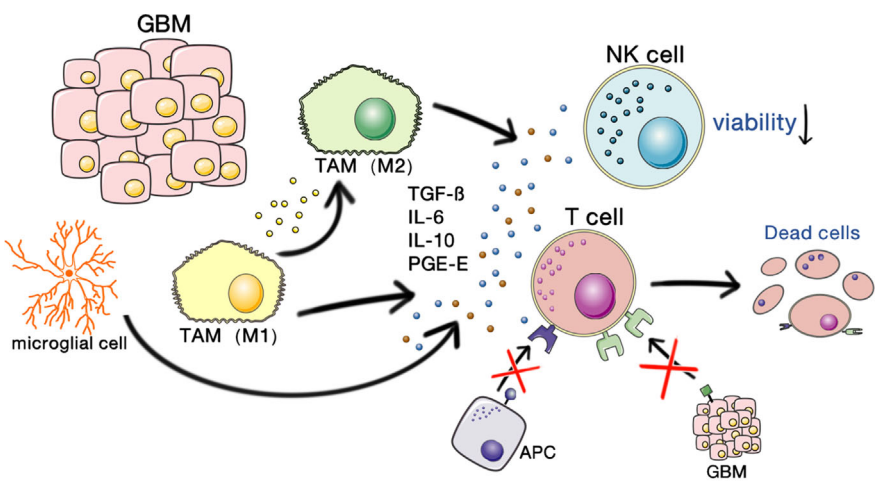

FIGURE 1 | Immunosuppressive microenvironment of GBM. GBM-associated macrophages and microglia secrete inhibitory cytokines, which decrease NK cell activity and T cell–mediated apoptosis and inhibit the binding and killing effects of T cells on antigen-presenting cells and GBM cells. This allows the tumor to escape the immune-killing effects of NK cells and T cells.

Tumor-Associated Immunosuppressive Cells

GBM is characterized by the infiltration of microglia and peripherally recruited macrophages, whereas lymphocytic infiltration is usually low (7). Tumor-associated macrophages (TAMs) secrete inhibitory cytokines, such as interleukin-6 (IL-6), IL-10, transforming growth factor β (TGF-β), and prostaglandin-E, which inhibit NK cell activity and the activation and proliferation of T cells and induce T cell apoptosis, thereby downregulating the expression of MHC and changing TAMs to the M2 phenotype, resulting in immunosuppression (3).

IMMUNE CELL THERAPY FOR GBM

Role of NK Cells in the Treatment of GBM

NK cells are the first natural line of defense against infection and antitumor immunity, and their surface inhibitory receptors recognize MHC class I molecules on the surface of normal somatic cells. When somatic cells are mutated (e.g., GBM), MHC class I expression on their surface is lost, and NK cells initiate a killing effect.

NK cells are persistent in targeting tumor cells and are difficult to escape, and current studies focus on mimicking NK cell activity to replicate their attacking and immune-killing effects (8).

The applications of NK cell therapy for GBM can be summarized as follows: 1. direct use of NK cells to kill GBM cells, 2. combined immune cell therapy regimens comprising NK cells and immune checkpoint inhibitors or drugs targeting immune-related genes or specific antibodies targeting proteins that protect against immunosuppression of NK cells, and 3. chimeric antigen-modified NK (CAR-NK) cell therapy (9). Images of NK cell–based immunotherapies are shown in **Figure 2**.

NK cells prevent systemic metastasis of GBM. If NK cells are transplanted into a GBM model, GBM death can be directly induced (11), but the difficulty of this method lies in the uncertainty of the transplantation process of the NK cells. It has been suggested that GBM occurs due to cytomegalovirus infection interfering with the immune response of NK cells (12).

Yvon et al. (13) propose an immunotherapy approach for GBM using NK cells derived from cord blood, but this method is similarly limited by immunosuppressive cytokines in the tumor microenvironment. In addition, there have been some studies proposing combination immunotherapies related to NK cells. For example, by using the addition of the immune checkpoint inhibitor PD-1 combined antibody, one group found that decreased PD-1 activity could promote the massive infiltration of NK cells and T cells as well as inhibit tumor progression (14). However, none of these combination immunotherapies have been studied with regard to the specific mechanism of action of other cells beyond NK cells to help kill GBM.

Systemic metastasis of GBM is rare thanks to the innate immunity of NK cells, and studying the combination of radiotherapy, immune cells, and immune checkpoint inhibitors is beneficial to improve the treatment of GBM. Based on the

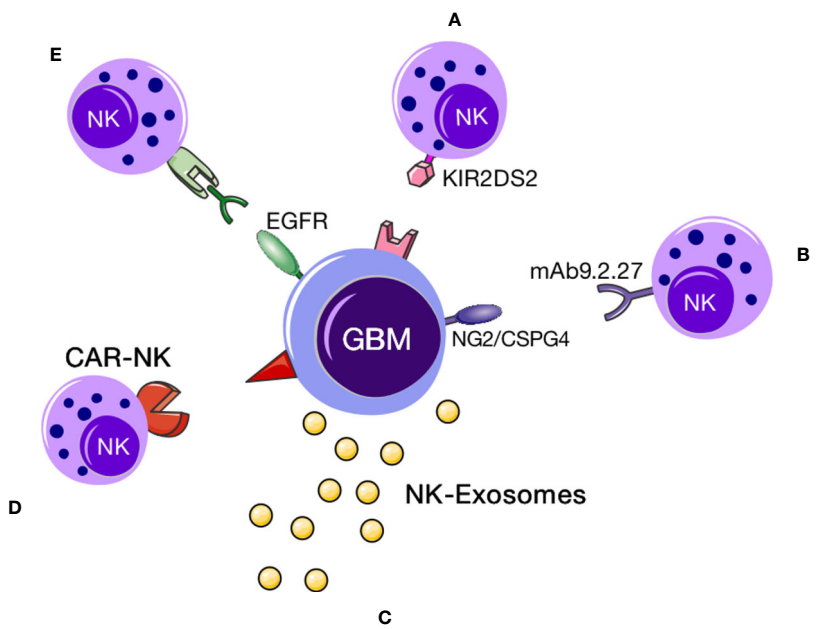

FIGURE 2 | NK cell immunotherapy. NK cell–based immunotherapy for GBM. This figure demonstrates that a variety of NK cell therapies for GBM. **(A)** KIR2DS2 immunotype NK cells could target and destroy GBM cells (10); **(B)** exosomes secreted by NK cells could specifically localize to GBM cells, upon which the cytokines within the exosomes could induce apoptotic signaling in tumor cells to promote cell death; **(C)** CAR-NK immunotherapy comprising NK cells expressing a GBM-specific CAR can target the tumor; **(D)** specific proteins (such as CD16) can bind NK cells and EGFR on the surface of GBM cells to facilitate NK cell activity. **(E)** using specific proteins (such as CD16) to bind NK cells and then specifically bind to EGFR on the surface of glioblastoma cells to exert a cytotoxic effect.

above studies, although a large amount of experimental data show that the above methods can increase immune cell infiltration, there are still many problems in the clinical application of this regimen. Therefore, the use of NK cells in the brain to kill GBM still has a series of problems, the most important and challenging of which is to prevent the inhibition of the cytotoxic effects of NK cells. NK cells are functionally inhibited in the GBM tumor microenvironment. Kozlowska et al. (15) show that GBM stem cells are highly susceptible to NK-mediated killing, but after differentiation of these stem cells, anergic NK cells fail to control GBM tumor growth because of the release of IL-6 and IL-8.

CAR-NK therapy is one of the latest developments in treating GBM. One group of investigators found that ErbB2 protein expression was elevated in a large proportion of GBM samples and used ErbB2/HER2-specific NK cells to target GBM (16), proposing sustainably expanded "CAR-NK" cells—human NK cells that express ErbB2-specific chimeric antigen receptors. The *in vitro* and *in vivo* effects of these CAR-NK cells on GBM cell culture and orthotopic GBM xenograft models as well as the therapeutic effects of NK-92/5.28 cells on endogenous antitumor immunity were also confirmed. Murakami et al. (17) also propose a method comprising a novel NK cell line carrying a chimeric immune antigen receptor (CAR-KHYG-1) to target epidermal growth factor receptor variant III (EGFRvIII) and induce antitumor effects in GBM cells. Han et al. (18) reveal that CAR-redirected NK cells effectively target wt EGFR and EGFRvIII to treat GBM and demonstrate that intracranial application of NK-92-EGFR-CAR cells can effectively inhibit tumor growth, which is a prospective clinical strategy for the treatment of GBM.

In conclusion, owing to the large number of studies on targeted NK cell therapy for GBM in progress, it seems that this treatment modality has a good chance of becoming a full-fledged immunotherapy regimen. However, most of these studies have not made any profound breakthroughs, and the safety and efficacy of adoptive immunotherapy with CAR-NK cells need to be further assessed in clinical trials. Thus, treating GBM with NK cells still has a long way to go.

Dendritic Cells

DC vaccines have been administered clinically for the treatment of GBM, but the results remain unsatisfactory. Pellegatta et al. (19) propose that DC immunotherapy for GBM might be associated with NK cells, that DC vaccines induced significant and sustained NK cell activation, and that the increase in their response had a significant correlation with prolonged patient survival. Dusoswa et al. (20) selected Siglec-9 ligands highly expressed on GBM extracellular vesicles and modified these vesicles with a receptor to promote Siglec binding on the vesicle surface, thereby achieving efficient targeting of adjuvant DCs to GBM and enhancing their potential as anticancer vaccines.

Vaccines are based on DCs containing peptides that represent one or more specific tumor antigens or whole lysates as a source of multiple antigens. However, factors such as the immunosuppressive microenvironment, lack of appropriate specific epitopes, and cancer immunoediting may limit their efficacy (21).

The activation of DCs can be driven by GBM stem cells and a mixture of monocytes, such as CD34-, CD45-, and CD56-positive cells from allogeneic umbilical cord blood (UCB) (22). Eiraku et al. (23) study the interaction of DCs with CD8+ T cells as well as with Vγ9γδ T cells and Vα24NKT cells. Immunocyte therapy based on DCs interacting with GBM lysate (24) is also a promising treatment for GBM.

A large body of literature has indicated that, because DC vaccines themselves are less toxic and do not have many adverse effects, they may become a new hope for the treatment of GBM.

Tumor-Associated Macrophages

Only a few studies have shown that macrophages directly play an immune-related role in the treatment of GBM, and Sun et al. (25) find that inhibiting Romo1 in combination with anti-PD-1 immunotherapy significantly improved the prognosis of GBM patients and particularly enhanced the function of macrophages.

Hallmark indicators of genetic alterations in GBM are amplification of EGFR and EGFRvIII, and investigators have proposed a pathway in which EGFR in combination with EGFRvIII induces macrophage infiltration by upregulating the expression of the chemokine CCL2 (26).

Most existing studies on TAMs have focused on the secretion of cytokines in the GBM microenvironment, promoting GBM progression. Herting et al. (27) find in their study that coculturing TAMs derived from bone marrow with primary GBM cells promoted the upregulation of the cytokine IL-1, which is detrimental to the tumor-killing effect of NK cells and T cells.

TAMs promote the growth of GBM by secreting pleiotropic phosphorus and promoting PTPRZ1 signaling in GBM stem cells (28). In addition, a similar study indicated that Wnt-induced signaling protein 1 (WISP1) secreted from GBM stem cells promotes the survival of both GBM stem cells and TAMs (phagocytes) to establish a tumorigenic microenvironment (29).

In addition, research on macrophages has not been consistent. Here, we simply provide examples of the following recent studies. Macrophage-associated cytokines are used as prognostic indicators of GBM, and increased IL-6 levels predict poor prognosis (30); Wei et al. (31) find that osteopontin (OPN) is an important chemokine for recruiting macrophages into GBM. Cui et al. (32) reported the importance of macrophage-associated immunosuppression in GBM angiogenesis. Although these discoveries have not been fully elaborated upon, they provide new ideas for the treatment of GBM with macrophages, indicating that macrophages play multiple roles and are expected to be applied in other aspects.

In fact, there are both advantages and disadvantages of the use of macrophages in immunotherapy. We found that many studies have proven that macrophages have an adverse effect on the prognosis of GBM. Therefore, we believe that how to apply macrophages in the future to maintain their advantages and avoiding their disadvantages will become a new research focus.

Mast Cells

Põlajeva et al. (33) propose that the accumulation of mast cells (MCs) in GBM tumors might be related to the levels of stem cell

factor and the chemokine CXCL12; Attarha et al. (34) demonstrate that MCs respond to multiple signals in a glioma grade-dependent manner to infiltrate mouse and human gliomas and induce the differentiation of glioma cells. Roy et al. (35) use the degree of recruitment of MCs as a potential biomarker for grading GBM.

APPLICATION OF IMMUNE CELL THERAPY FOR GBM

The GBM immunotherapy category includes adoptive T cell immunotherapy, CAR-T immunotherapy, DC tumor vaccines, immune checkpoint blockade, monoclonal antibodies, and cytokine therapy.

Adoptive T Cell Immunotherapy

Adoptive lymphocyte transfer (ALT) is an antigen-specific treatment during which either tumor-infiltrating lymphocytes (TILs) are obtained from tumor specimens or T cells are isolated from peripheral blood mononuclear cells (PBMCs), expanded *in vitro* against tumor antigens, and systemically applied or directly injected into the tumor site (36). Schuessler et al. (37) report the successful expansion of cytomegalovirus-specific T cells from 13 of 19 patients with recurrent GBM; moreover, 4 of the 10 patients who completed the treatment remained tumor-free during the study period.

Currently, multiple clinical trials have used ALT therapy in GBM patients (NCT01082926, NCT00331526, NCT01588769, NCT00003185, and NCT00730613), and these studies have confirmed the safety and feasibility of ALT therapy (38).

More recently, there has been progress in a clinical trial involving adoptive cellular immunotherapies (ACT), which has shown that CMV-specific ACT can effectively delay or even prevent the recurrence of GBM, which indicates that a favorable T cell gene signature is associated with the improvement in therapeutic efficacy and prolonged survival (39).

CAR-T Immunotherapy

CAR-T immunotherapy is a precisely targeted therapy for the treatment of tumors, which transduces a CAR into T cells to create CAR-T cells (40), after which they are expanded to large numbers *in vitro* and then reinjected into the patient, prompting B cells to produce antibodies and specifically recognize antigens, which, in turn, kill the tumors (41).

CAR-T immunotherapy has the capacity to cross the BBB and can safely and effectively reach tumor cells that cannot be accessed surgically (42). Brown et al. (38) treated a patient with recurrent GBM by using CAR-T cells and found that IL13R-2 was a useful immunotherapeutic target in GBM. Although this therapy has been recognized by many patients, and 4 categories have been clinically approved in China, it is still not considered a conventional treatment.

After reviewing many studies on the subject, we deduced that CAR-T therapy has not made a breakthrough in the treatment of solid tumors in recent years, and there are many unsolved issues, especially in terms of CAR-T cells entering the microenvironment of solid tumors, such as GBM, maintaining their viability and ability to rapidly and accurately identify tumor cells, and overcoming immunosuppression. Therefore, if the issue of tumor microenvironment inhibition of the CAR-T cell therapeutic effects can be solved at this stage, it will have a great impact on the immunotherapy of solid tumors.

Dendritic Cell Vaccines

The production of DC vaccines includes isolating DCs from patients, loading the cells with tumor antigens, culturing the DCs with cytokines to induce maturation, and reinjecting the cells back into the body (43). At present, vaccines are broadly divided into three categories according to different antigens: tumor-associated antigens (TAAs), tumor-specific antigens (TSAs), and tumor lysates (44).

1. *TAAs are ubiquitous but are expressed at higher levels in tumor cells than in healthy cells, so TAA vaccines are easy to develop and have good targeting*. At present, the clinical application of TAA-based DC vaccines is limited, mainly due to the following reasons: 1. There are few known TAAs, 2. the consistency of TAA expression marks its own limitations, and 3. TAA vaccines may not induce the best immune response due to immune tolerance (44). Wen et al. (45) discovered that ICT-107 vaccination in patients with newly diagnosed GBM developed good tolerance and significantly improved survival by 2.2 months.
2. *TSAs are unique to tumor cells and, unlike TAAs in tumor cells and normal cells, are usually proteins encoded by mutated genes in tumors*. They are relatively fixed in different types of cancer and patients and can be used as targets for immunotherapy (43). TSA-based DC vaccines may generate an intense targeted inflammatory response against tumor cells while avoiding potential autoimmune responses in other tissues (44). Rindopepimut (46) (Celldex Therapeutics, Hampton, New Jersey, USA), a TSA vaccine, has shown clinical benefits and significant efficacy in phase II clinical trials. However, the phase III clinical trial was terminated early because it was thought that the patients in the study might not reach their primary endpoint.
3. *Vaccination in combination with tumor lysates is usually delivered by autologous lysate-pulsed DCs, which are usually collected from the patient a few days before surgical resection, incubated with the resected tumor lysate, and then reintroduced back into patients via postoperative multiple vaccinations in combination with standardized radiotherapy administration to target residual tumor cells* (44). The phase III trial by Liau et al. (47) demonstrated that the addition of DCVax-L to the standard of care for GBM patients is feasible and safe and prolongs survival. The combination of tumor antigens and a-GalCer in anticancer vaccines can efficiently induce long-lasting immunity by activating iNK T cells (48).

Therefore, the latest research direction of DC vaccines at this stage focuses on combining tumor lysates with DCs, and the

mechanism of tumor lysate vaccines on GBM is very likely to be the combined action of multiple immune cells.

PROSPECT OF IMMUNOTHERAPY FOR GBM

Different Combination Regimens

Immunotherapy for GBM has not been used in a wide range of direct treatments due to its immaturity and our incomplete understanding of the mechanisms, so the main treatment modalities after diagnosis confirmation are surgical resection, radiotherapy, chemotherapy, and various comprehensive treatments. Conventional treatment is maximal gross resection of the tumor followed by radiotherapy and chemotherapy, and maintenance therapy with temozolomide (TMZ) is started 4 weeks after completion of the chemoradiotherapy cycle. This treatment regimen is known the standard of care (SOC). The common chemotherapeutic agent axitinib has been shown by Stephanie Du Four et al. to have a favorable effect on immune function (49).

There are currently several main GBM vaccines available: 1. autologous monocyte vaccine, 2. peptide-based tumor vaccine, 3. nucleotide-based tumor vaccine, and 4. cell line–based tumor vaccine. Each of them requires coculture of the corresponding cells with surgically resected tumor cells under different conditions to achieve immunization against tumor cells. Therefore, it is also necessary to obtain a sufficient number of tumor cells at the time of surgery.

CRS-T is a genetically modified chimera-switched receptor T cell therapy targeting PD-1. After intravenous infusion of CSR-T cells, the levels of IFN-γ and IL-6 in peripheral blood increase with the number of reinfused cells, and local intracranial injection of CSR-T cells is often more effective than intravenous injection (50).

Immunotherapy with DC vaccines has been associated with adverse effects of immunotherapy in newly diagnosed patients, and increases in tumor-specific immune responses after vaccination, including immune cell proliferation and cytokine production, can be detected (51). For patients with recurrent GBM, elevated levels of chemoresistance-associated peptides (CAPs) and/or cytoplasmic accumulation can be observed in fusion cells generated after cervical implantation of autologous glioma cells and DCs, and a specific immune response to CAPs can also be observed, which promotes an antitumor response in patients (52).

Cytokine-induced killer (CIK) cells are nonhistocompatibility (MHC)-restricted lymphotoxic cells that can be produced from PBMCs under the induction of interferon (IFN)-γ, IL-2, and CD3 monoclonal antibodies (CD3 mAb) and have a high proliferation rate and antitumor activity. Chemoradiation with CIK cells plus the standard radiotherapy-TMZ regimen shows no significant difference in survival but improves progression-free survival compared with that of TMZ alone; unfortunately, no evidence of improved overall survival was found (53).

Rindopepimut (also known as CDX-110) is a peptide-based vaccine against EGFRvIII, an EGFR variate with a deletion mutation, and the addition of rindopepimut to a standardized course did not increase survival in patients with newly diagnosed GBM in clinical trials. Further exploration of the effect of immunotherapy in future treatment combinations containing rindopepimut may be required (54).

Different vaccines are injected in different ways, and some cellular vaccines can be injected directly into the surgical cavity. After surgical resection of GBM, patients undergoing the SOC followed by local injection of an immune-stimulating oligonucleotide containing an unmethylated cytosine-guanosine motif (CpG-ODN) immediately around the surgical cavity were more likely to develop fever and postoperative hematoma after surgery than patients who received the SOC alone with similar incidences of other adverse events. Overall, this vaccine did not improve survival in patients with newly diagnosed GBM (55).

In addition, vaccines made from oncolytic viruses are also under investigation. The oncolytic virus aglatimagene besadenovec (AdV-tk) combined with valacyclovir constitutes gene-mediated cytotoxic immunotherapy (GMCI). When combined with the SOC, GMCI may stimulate immune responses in participants with GTR and subtotal resection. However, if the residual tumor burden is too large, the tumor-mediated immunosuppressive effect may mask the effect of GMCI (56).

At present, most immunotherapies can only be performed after patients undergo surgical resection, which is a limitation of immunotherapy. However, as a brain tumor, GBM is very likely to grow in sites that are not suitable for surgical resection and are more likely to recur, and treatment with immune cells or vaccines can achieve the destruction of tumors at sites that are not easily accessible *via* surgery; therefore, if the field of immunotherapy can mature, this treatment modality may replace surgical resection.

Role of Exosomes in Tumor Immunology

Exosomal vesicles naturally released by tumor cells transmit some molecules to target cells and act as an intercellular signaling pathway between the donor cytoplasm and the lumen of target cells. For example, NK cells release exosomes that express typical NK markers (CD56, etc.), killer proteins, and stimulate antitumor activity (57, 58). Exosomes carrying different RNAs and proteins can be measured in patients as biomarkers to assess disease onset and progression (59).

Barile et al. (60) demonstrate that GBM stem cells could secrete exosomes carrying active vascular endothelial growth factor A (VEGF-A), which could protect VEGF-A from cytokines, proteases, etc., in the tumor microenvironment and maintain the vascular niche. Non-GBM-stem-cell-derived VEGF-A changes with tumor size and treatment, whereas GBM-stem-cell-derived exosomes may be continuously produced and released and cross the BBB.

Skog et al. (61) and Brennan et al. (62) report that EGFRvIII could be detected in the serum exosomes of GBM patients with high diagnostic sensitivity, but EGFRvIII was present in only approximately 25% of GBM patients. Kai et al. (63) demonstrate that PTRF was also present on the exosomal membrane and that PTRF overexpression increased exosome secretion, resulting in

increased rates of tumor formation and receptor proliferation *in vitro* and *in vivo*.

A study by Liu et al. (48) shows that, in a DC vaccine, the codelivery of tumor-derived exosomes with α-galactosylceramide (α-GalCer) could effectively improve the tumor microenvironment by balancing the release of immunosuppressive factors and immunostimulatory factors. Zhu et al. (64) find that exosomes secreted by NK cells could specifically localize to GBM tumors and that NK-Exos contain FasL, perforin, granzyme B, and TNF-α, thereby inducing proapoptotic signals and triggering cell death in tumors. Chen et al. (65) propose that exosomes could interact with target cells with barrier cells. It is a new promising therapeutic agent for the treatment of refractory GBM.

Exosomal transfer of miRNAs or miRNA inhibitors to tumor cells has emerged as a new approach to deliver miRNAs that can target cancer. MiRNA-21 is overexpressed in GBM, which can improve the proliferation and malignant metastatic behavior of tumor cells (66, 67). Sponge constructs are designed to bind to their complementary miRNAs (one or more) or their seed sequences, thereby preventing miRNA binding to their biological targets.

GBM cells use exosomes to communicate with the tumor microenvironment and promote their proliferation, invasion, and metastasis (68). One of the major challenges of exosome-based therapeutic approaches is the low productivity of exosomes. Therefore, effective methods that can produce exosomes on a large scale are needed. Watson et al. (69) propose that the use of hollow fiber bioreactors could increase the yield of exosomes by 5- to 10-fold.

Although exosomes have been a research hot spot in recent years, the still-limited data on the use of exosomes as a targeted therapy for GBM is far from mature. There is no denying the fact that using GBM-derived and -targeted exosomes is a very good idea, but more research and clinical trials are needed to truly determine their effect in patients, which can take years. Thus, it is difficult to judge whether this approach is worth pursuing, but at this stage, we are optimistic that ongoing clinical trials will provide a preliminary basis for the use of exosomes in immune therapy.

Research Focuses and Prospects of Other Immune Cell Therapies

One popular direction is the implementation of combined antigen immunotherapies with the latest focus on CAR-Ts and "bispecific T cell engagers" (BiTEs) (70, 71), that is, using antibodies such as BiTEs to bind both T cells and tumor cells and create a molecular bridge that induces T cells to kill GBM cells.

In addition, following CAR-T therapy, a series of CAR immunotherapies have been investigated, such as CAR-NK cells (72) and CAR-macrophages. In the field of cancer immunotherapy, CAR-T cell therapy and PD-1 inhibitors are the most high-profile members, but in addition to T cells, the functions of NK cells and macrophages should not be ignored.

SUMMARY

In summary, it is of great significance to explore the mechanisms of action of immunotherapy in depth for the treatment of GBM and to specifically study these mechanisms of action in each type of immune cell. In addition, immunotherapy has incomparable advantages to surgical treatment, radiotherapy, and chemotherapy.

We believe that the most valuable applications of immunotherapy for the treatment of GBM are CAR-T cell therapy and DC vaccines. These two methods have a large body of preliminary data and have undergone clinical trials and applications. Although there may be some issues, any problems that arise are expected to be resolved in follow-up studies. Therefore, in the process of GBM occurrence and expansion, the use of appropriate treatment options and attempts to use immunotherapy are conducive to not only the broad killing of cancer cells but also mediating the occurrence and expansion rate of GBM. This activity may strongly contribute to the treatment of GBM and even other types of cancer.

AUTHOR CONTRIBUTIONS

XK, HT, and WG contributed to the conception and design of the study. XK, YZ, WG, XC, and WH wrote the first draft of the manuscript. HL, BH, and ZH wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Alexander BM, Cloughesy TF. Adult Glioblastoma. *J Clin Oncol* (2017) 35 (21):2402–9. doi: 10.1200/JCO.2017.73.0119
2. Chaichana KL, Parker SL, Olivi A, Quiñones-Hinojosa A. Long-term seizure outcomes in adult patients undergoing primary resection of malignant brain astrocytomas. Clinical article. *J Neurosurg* (2009) 111(2):282–92. doi: 10.3171/2009.2.JNS081132
3. Lim M, Xia YX, Bettegowda C, Weller M. Current state of immunotherapy for glioblastoma. *Nat Rev Clin Oncol* (2018) 15(7):422–42. doi: 10.1038/s41571-018-0003-5
4. Jackson CM, Choi J, Lim M. Mechanisms of immunotherapy resistance: lessons from glioblastoma. *Nat Immunol* (2019) 20(9):1100–9. doi: 10.1038/s41590-019-0433-y
5. Brown NF, Carter TJ, Ottaviani D, Mulholland P. Harnessing the immune system in glioblastoma. *Br J Cancer* (2018) 119(10):1171–81. doi: 10.1038/s41416-018-0258-8

6. Chang LS, Barroso-Sousa R, Tolaney SM, Hodi FS, Kaiser UB, Min L. Endocrine Toxicity of Cancer Immunotherapy Targeting Immune Checkpoints. *Endocr Rev* (2019) 40(1):17–65. doi: 10.1210/er.2018-00006
7. Luoto S, Hermelo I, Vuorinen EM, Hannus P, Kesseli J, Nykter M, et al. Computational Characterization of Suppressive Immune Microenvironments in Glioblastoma. *Cancer Res* (2018) 78(19):5574–85. doi: 10.1158/0008-5472.CAN-17-3714
8. Golán I, Rodríguez de la FL, Costoya JA. NK Cell-Based Glioblastoma Immunotherapy. *Cancers (Basel)* (2018) 10(12):1–32. doi: 10.3390/cancers10120522
9. Burger MC, Zhang CC, Harter PN, Romanski A, Strassheimer F, Senft C, et al. CAR-Engineered NK Cells for the Treatment of Glioblastoma: Turning Innate Effectors Into Precision Tools for Cancer Immunotherapy. *Front Immunol* (2019) 10:2683. doi: 10.3389/fimmu.2019.02683
10. Gras NA, Kmiecik J, Leiss L, Zelkowski M, Engelsen A, Bruserud Ø., et al. NK cells with KIR2DS2 immunogenotype have a functional activation advantage to efficiently kill glioblastoma and prolong animal survival. *J Immunol* (2014) 193(12):6192–206. doi: 10.4049/jimmunol.1400859
11. Lee SJ, Kang WY, Yoon Y, Jin JY, Song HJ, Her JH, et al. Natural killer (NK) cells inhibit systemic metastasis of glioblastoma cells and have therapeutic effects against glioblastomas in the brain. *BMC Cancer* (2015) 15:1–28. doi: 10.1186/s12885-015-2034-y
12. Dominguez-Valentin M, Gras NA, Rahman AM, Kumar S, Retière C, Ulvestad E, et al. Identification of a Natural Killer Cell Receptor Allele That Prolongs Survival of Cytomegalovirus-Positive Glioblastoma Patients. *Cancer Res* (2016) 76(18):5326–36. doi: 10.1158/0008-5472.CAN-16-1162
13. Yvon ES, Burga R, Powell A, Cruz CR, Fernandes R, Barese C, et al. Cord blood natural killer cells expressing a dominant negative TGF-β receptor: Implications for adoptive immunotherapy for glioblastoma. *Cytotherapy* (2017) 19(3):408–18. doi: 10.1016/j.jcyt.2016.12.005
14. Shevtsov M, Pitkin E, Ischenko A, Stangl S, Khachatryan W, Galibin O, et al. Ex vivo Hsp70-Activated NK Cells in Combination With PD-1 Inhibition Significantly Increase Overall Survival in Preclinical Models of Glioblastoma and Lung Cancer. *Front Immunol* (2019) 10:454. doi: 10.3389/fimmu.2019.00454
15. Kozlowska AK, Tseng H-C, Kaur K, Topchyan P, Inagaki A, Bui VT, et al. Resistance to cytotoxicity and sustained release of interleukin-6 and interleukin-8 in the presence of decreased interferon-γ after differentiation of glioblastoma by human natural killer cells. *Cancer Immunol Immunother* (2016) 65(9):1085–97. doi: 10.1007/s00262-016-1866-x
16. Zhang CC, Burger MC, Jennewein L, Genßler S, Schönfeld K, Zeiner P, et al. ErbB2/HER2-Specific NK Cells for Targeted Therapy of Glioblastoma. *J Natl Cancer Inst* (2016) 108(5):1–12. doi: 10.1093/jnci/djv375
17. Murakami T, Nakazawa T, Natsume A, Nishimura F, Nakamura M, Matsuda R, et al. Novel Human NK Cell Line Carrying CAR Targeting EGFRvIII Induces Antitumor Effects in Glioblastoma Cells. *Anticancer Res* (2018) 38 (9):5049–56. doi: 10.21873/anticanres.12824
18. Han JF, Chu JH, Keung CW, Zhang JY, Wang YW, Cohen JB, et al. CAR-Engineered NK Cells Targeting Wild-Type EGFR and EGFRvIII Enhance Killing of Glioblastoma and Patient-Derived Glioblastoma Stem Cells. *Sci Rep* (2015) 5:11483. doi: 10.1038/srep11483
19. Pellegatta S, Eoli M, Cuccarini V, Anghileri E, Pollo B, Pessina S, et al. Survival gain in glioblastoma patients treated with dendritic cell immunotherapy is associated with increased NK but not CD8 T cell activation in the presence of adjuvant temozolomide. *Oncoimmunology* (2018) 7(4):e1412901. doi: 10.1080/2162402X.2017.1412901
20. Dusoswa SA, Horrevorts SK, Ambrosini M, Kalay H, Paauw NJ, Nieuwland R, et al. Glycan modification of glioblastoma-derived extracellular vesicles enhances receptor-mediated targeting of dendritic cells. *J Extracell Vesicles* (2019) 8(1):1648995. doi: 10.1080/20013078.2019.1648995
21. Finocchiaro G, Pellegatta S. Immunotherapy with dendritic cells loaded with glioblastoma stem cells: from preclinical to clinical studies. Cancer Immunol. *Immunother* (2016) 65(1):101–9. doi: 10.1007/s00262-015-1754-9
22. Yurtsever A, Haydaroglu A, Biray AC, Gunduz C, Oktar N, Dalbasti T, et al. Assessment of genetic markers and glioblastoma stem-like cells in activation of dendritic cells. *Hum Cell* (2013) 26(3):105–13. doi: 10.1007/s13577-013-0065-8
23. Eiraku Y, Terunuma H, Yagi M, Deng X, Nicol AJ, Nieda M. Dendritic cells cross-talk with tumour antigen-specific CD8 T cells, Vγ9γδT cells and Vα24NKT cells in patients with glioblastoma multiforme and in healthy donors. *Clin Exp Immunol* (2018) 194(1):54–66. doi: 10.1111/cei.13185
24. Nava S, Lisini D, Pogliani S, Dossena M, Bersano A, Pellegatta S, et al. Safe and Reproducible Preparation of Functional Dendritic Cells for Immunotherapy in Glioblastoma Patients. *Stem Cells Transl Med* (2015) 4(10):1164–72. doi: 10.5966/sctm.2015-0091
25. Sun G, Cao Y, Qian C, Wan Z, Zhu J, Guo J, et al. Romo1 is involved in the immune response of glioblastoma by regulating the function of macrophages. *Aging (Albany NY)* (2020) 12:1114–27. doi: 10.18632/aging.102648
26. An Z, Knobbe-Thomsen CB, Wan X, Fan QW, Reifenberger G, Weiss WA. Correction: EGFR Cooperates with EGFRvIII to Recruit Macrophages in Glioblastoma. *Cancer Res* (2019) 79(21):5681. doi: 10.1158/0008-5472.CAN-19-2800
27. Herting CJ, Chen Z, Maximov V, Duffy A, Szulzewsky F, Shayakhmetov DM, et al. Tumour-associated macrophage-derived interleukin-1 mediates glioblastoma-associated cerebral oedema. *Brain* (2019) 142(12):3834–51. doi: 10.1093/brain/awz331
28. Shi Y, Ping YF, Zhou W, He ZC, Chen C, Bian BSJ, et al. Tumour-associated macrophages secrete pleiotrophin to promote PTPRZ1 signalling in glioblastoma stem cells for tumour growth. *Nat Commun* (2017) 8:15080. doi: 10.1038/ncomms15080
29. Tao W, Chu C, Zhou W, Huang Z, Zhai K, Fang X, et al. Dual Role of WISP1 in maintaining glioma stem cells and tumor-supportive macrophages in glioblastoma. *Nat Commun* (2020) 11(1):3015. doi: 10.1038/s41467-020-16827-z
30. Hori T, Sasayama T, Tanaka K, Koma YII, Nishihara M, Tanaka H, et al. Tumor-associated macrophage related interleukin-6 in cerebrospinal fluid as a prognostic marker for glioblastoma. *J Clin Neurosci* (2019) 68:281–9. doi: 10.1016/j.jocn.2019.07.020
31. Wei J, Marisetty A, Schrand B, Gabrusiewicz K, Hashimoto Y, Ott M, et al. Osteopontin mediates glioblastoma-associated macrophage infiltration and is a potential therapeutic target. *J Clin Invest* (2019) 129(1):137–49. doi: 10.1172/JCI121266
32. Cui X, Morales RTT, Qian W, Wang H, Gagner JP, Dolgalev I, et al. Hacking macrophage-associated immunosuppression for regulating glioblastoma angiogenesis. *Biomaterials* (2018) 161:164–78. doi: 10.1016/j.biomaterials.2018.01.053
33. Põlajeva J, Sjösten AM, Lager N, Kastemar M, Waern I, Alafuzoff I, et al. Mast cell accumulation in glioblastoma with a potential role for stem cell factor and chemokine CXCL12. *PLoS One* (2011) 6(9):e25222. doi: 10.1371/journal.pone.0025222
34. Attarha S, Roy A, Westermark B, Tchougounova E. Mast cells modulate proliferation, migration and stemness of glioma cells through downregulation of GSK3β expression and inhibition of STAT3 activation. *Cell Signal* (2017) 37:81–92. doi: 10.1016/j.cellsig.2017.06.004
35. Roy A, Attarha S, Weishaupt H, Edqvist PH, Swartling FJ, Bergqvist M, et al. Serglycin as a potential biomarker for glioma: association of serglycin expression, extent of mast cell recruitment and glioblastoma progression. *Oncotarget* (2017) 8(15):24815–27. doi: 10.18632/oncotarget.15820
36. Farber SH, Elsamadicy AA, Atik AF, Suryadevara CM, Chongsathidkiet P, Fecci PE, et al. The Safety of available immunotherapy for the treatment of glioblastoma. *Expert Opin Drug Saf* (2017) 16(3):277–87. doi: 10.1080/14740338.2017.1273898
37. Schuessler A, Smith C, Beagley L, Boyle GM, Rehan S, Matthews K, et al. Autologous T-cell therapy for cytomegalovirus as a consolidative treatment for recurrent glioblastoma. *Cancer Res* (2014) 74(13):3466–76. doi: 10.1158/0008-5472.CAN-14-0296
38. Brown CE, Alizadeh D, Starr R, Weng L, Wagner JR, Naranjo A, et al. Regression of Glioblastoma after Chimeric Antigen Receptor T-Cell Therapy. *N Engl J Med* (2016) 375(26):2561–9. doi: 10.1056/NEJMoa1610497
39. Smith C, Lineburg KE, Martins JP, Ambalathingal G, Neller MA, Morrison B, et al. Autologous CMV-specific T cells are a safe adjuvant immunotherapy for primary glioblastoma multiforme. *J Clin Invest* (2020). doi: 10.1172/JCI138649
40. Srivastava S, Riddell SR. Engineering CAR-T cells: Design concepts. *Trends Immunol* (2015) 36(8):494–502. doi: 10.1016/j.it.2015.06.004

41. Bagley SJ, Desai AS, Linette GP, June CH, O'Rourke DM. CAR T-cell therapy for glioblastoma: recent clinical advances and future challenges. *Neuro-oncology* (2018) 20(11):1429–38. doi: 10.1093/neuonc/noy032
42. Filley AC, Henriquez M, Dey M. CART Immunotherapy: Development, Success, and Translation to Malignant Gliomas and Other Solid Tumors. *Front Oncol* (2018) 8:453. doi: 10.3389/fonc.2018.00453
43. Kong Z, Wang Y, Ma W. Vaccination in the immunotherapy of glioblastoma. *Hum Vaccin Immunother* (2018) 14(2):255–68. doi: 10.1080/21645515.2017.1388481
44. Wilcox JA, Ramakrishna R, Magge R. Immunotherapy in Glioblastoma. *World Neurosurg* (2018) 116:518–28. doi: 10.1016/j.wneu.2018.04.020
45. Wen PY, Reardon DA, Armstrong TS, Phuphanich S, Aiken RD, Landolfi JC, et al. A Randomized Double-Blind Placebo-Controlled Phase II Trial of Dendritic Cell Vaccine ICT-107 in Newly Diagnosed Patients with Glioblastoma. *Clin Cancer Res* (2019) 25(19):5799–807. doi: 10.1158/1078-0432.CCR-19-0261
46. Elsamadicy AA, Chongsathidkiet P, Desai R, Woroniecka K, Farber SH, Fecci PE, et al. Prospect of rindopepimut in the treatment of glioblastoma. *Expert Opin Biol Ther* (2017) 17(4):507–13. doi: 10.1080/14712598.2017.1299705
47. Liau LM, Ashkan K, Tran DD, Campian JL, Trusheim JE, Cobbs CS, et al. First results on survival from a large Phase 3 clinical trial of an autologous dendritic cell vaccine in newly diagnosed glioblastoma. *J Transl Med* (2018) 16(1):142. doi: 10.1186/s12967-018-1507-6
48. Liu H, Chen L, Liu J, Meng HX, Zhang R, Ma L, et al. Co-delivery of tumor-derived exosomes with alpha-galactosylceramide on dendritic cell-based immunotherapy for glioblastoma. *Cancer Lett* (2017) 411:182–90. doi: 10.1016/j.canlet.2017.09.022
49. Du FS, Maenhout SK, Benteyn D, De KB, Duerinck J, Thielemans K, et al. Disease progression in recurrent glioblastoma patients treated with the VEGFR inhibitor axitinib is associated with increased regulatory T cell numbers and T cell exhaustion. *Cancer Immunol Immunother* (2016) 65 (6):727–40. doi: 10.1007/s00262-016-1836-3
50. Guo JX, Wu CX, Wang PF, Li ZJ, Han S, Jin W, et al. Bioactivity and safety of chimeric switch receptor T cells in glioblastoma patients. *Front Biosci (Landmark Ed)* (2019) 24:1158–66. doi: 10.2741/4772
51. Inogés S, Tejada S, Lopez-Diaz de Cerio Ascensión, Gállego PLJ, Espinós J, Idoate MA, et al. A phase II trial of autologous dendritic cell vaccination and radiochemotherapy following fluorescence-guided surgery in newly diagnosed glioblastoma patients. *J Transl Med* (2017) 15(1):104. doi: 10.1186/s12967-017-1202-z
52. Akasaki Y, Kikuchi T, Homma S, Koido S, Ohkusa T, Tasaki T, et al. Phase I/II trial of combination of temozolomide chemotherapy and immunotherapy with fusions of dendritic and glioma cells in patients with glioblastoma. *Cancer Immunol Immunother* (2016) 65(12):1499–509. doi: 10.1007/s00262-016-1905-7
53. Kong DS, Nam DH, Kang SH, Lee JW, Chang JH, Kim JH, et al. Phase III randomized trial of autologous cytokine-induced killer cell immunotherapy for newly diagnosed glioblastoma in Korea. *Oncotarget* (2017) 8(4):7003–13. doi: 10.18632/oncotarget.12273
54. Weller M, Butowski N, Tran DD, Recht LD, Lim M, Hirte H, et al. Rindopepimut with temozolomide for patients with newly diagnosed, EGFRvIII-expressing glioblastoma (ACT IV): a randomised, double-blind, international phase 3 trial. *Lancet Oncol* (2017) 18(10):1373–85. doi: 10.1016/S1470-2045(17)30517-X
55. Ursu R, Carpentier A, Metellus P, Lubrano V, Laigle-Donadey F, Capelle L, et al. Intracerebral injection of CpG oligonucleotide for patients with de novo glioblastoma-A phase II multicentric, randomised study. *Eur J Cancer* (2017) 73:30–7. doi: 10.1016/j.ejca.2016.12.003
56. Wheeler LA, Manzanera AG, Bell SD, Cavaliere R, McGregor JM, Grecula JC, et al. Phase II multicenter study of gene-mediated cytotoxic immunotherapy as adjuvant to surgical resection for newly diagnosed malignant glioma. *Neuro-oncology* (2016) 18(8):1137–45. doi: 10.1093/neuonc/now002
57. Parolini I, Federici C, Raggi C, Lugini L, Palleschi S, De MA, et al. Microenvironmental pH is a key factor for exosome traffic in tumor cells. *J Biol Chem* (2009) 284(49):34211–22. doi: 10.1074/jbc.M109.041152
58. Shoae-Hassani A, Hamidieh AA, Behfar M, Mohseni R, Mortazavi-Tabatabaei SA, Asgharzadeh S. NK Cell-derived Exosomes From NK Cells Previously Exposed to Neuroblastoma Cells Augment the Antitumor Activity of Cytokine-activated NK Cells. *J Immunother* (2017) 40(7):265–76. doi: 10.1097/CJI.0000000000000179
59. Treps L, Perret R, Edmond S, Ricard D, Gavard J. Glioblastoma stem-like cells secrete the pro-angiogenic VEGF-A factor in extracellular vesicles. *J Extracell Vesicles* (2017) 6(1):1359479. doi: 10.1080/20013078.2017.1359479
60. Barile L, Vassalli G. Exosomes: Therapy delivery tools and biomarkers of diseases. *Pharmacol Ther* (2017) 174:63–78. doi: 10.1016/j.pharmthera.2017.02.020
61. Skog J, Würdinger T, van RS, Meijer DH, Gainche L, Sena-Esteves M, et al. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat Cell Biol* (2008) 10(12):1470–6. doi: 10.1038/ncb1800
62. Brennan CW, Verhaak RGW, McKenna A, Campos B, Noushmehr H, Salama SR, et al. The somatic genomic landscape of glioblastoma. *Cell* (2013) 155 (2):462–77. doi: 10.1016/j.cell.2013.09.034
63. Huang K, Fang C, Yi K, Liu X, Qi H, Tan Y, et al. The role of PTRF/Cavin1 as a biomarker in both glioma and serum exosomes. *Theranostics* (2018) 8 (6):1540–57. doi: 10.7150/thno.22952
64. Zhu L, Kalimuthu S, Gangadaran P, Oh JM, Lee HW, Baek SH, et al. Exosomes Derived From Natural Killer Cells Exert Therapeutic Effect in Melanoma. *Theranostics* (2017) 7(10):2732–45. doi: 10.7150/thno.18752
65. Chen CC, Liu L, Ma F, Wong CW, Guo XE, Chacko JV, et al. Elucidation of Exosome Migration across the Blood-Brain Barrier Model In Vitro. *Cell Mol Bioeng* (2016) 9(4):509–29. doi: 10.1007/s12195-016-0458-3
66. Monfared H, Jahangard Y, Nikkhah M, Mirnajafi-Zadeh J, Mowla SJ. Potential Therapeutic Effects of Exosomes Packed With a miR-21-Sponge Construct in a Rat Model of Glioblastoma. *Front Oncol* (2019) 9:782. doi: 10.3389/fonc.2019.00782
67. Chai C, Song LJ, Han SY, Li XQ, Li M. MicroRNA-21 promotes glioma cell proliferation and inhibits senescence and apoptosis by targeting SPRY1 via the PTEN/PI3K/AKT signaling pathway. *CNS Neurosci Ther* (2018) 24(5):369–80. doi: 10.1111/cns.12785
68. Kucharzewska P, Christianson HC, Welch JE, Svensson KJ, Fredlund E, Ringnér M, et al. Exosomes reflect the hypoxic status of glioma cells and mediate hypoxia-dependent activation of vascular cells during tumor development. *Proc Natl Acad Sci U S A* (2013) 110(18):7312–7. doi: 10.1073/pnas.1220998110
69. Watson DC, Bayik D, Srivatsan A, Bergamaschi C, Valentin A, Niu G, et al. Efficient production and enhanced tumor delivery of engineered extracellular vesicles. *Biomaterials* (2016) 105:195–205. doi: 10.1016/j.biomaterials.2016.07.003
70. Choi BD, Yu X, Castano AP, Bouffard AA, Schmidts A, Larson RC, et al. CAR-T cells secreting BiTEs circumvent antigen escape without detectable toxicity. *Nat Biotechnol* (2019) 37(9):1049–58. doi: 10.1038/s41587-019-0192-1
71. Abbott RC, Cross RS, Jenkins MR. Finding the Keys to the CAR: Identifying Novel Target Antigens for T Cell Redirection Immunotherapies. *Int J Mol Sci* (2020) 21(2):1–16. doi: 10.3390/ijms21020515
72. Li Ye, Hermanson DL, Moriarity BS, Kaufman DS. Human iPSC-Derived Natural Killer Cells Engineered with Chimeric Antigen Receptors Enhance Anti-tumor Activity. *Cell Stem Cell* (2018) 23(2):181–92.e5. doi: 10.1016/j.stem.2018.06.002

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REVIEW
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Visualization of Diagnostic and Therapeutic Targets in Glioma With Molecular Imaging

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Gliomas, particularly high-grade gliomas including glioblastoma (GBM), represent the most common and malignant types of primary brain cancer in adults, and carry a poor prognosis. GBM has been classified into distinct subgroups over the years based on cellular morphology, clinical characteristics, biomarkers, and neuroimaging findings. Based on these classifications, differences in therapeutic response and patient outcomes have been established. Recently, the identification of complex molecular signatures of GBM has led to the development of diverse targeted therapeutic regimens and translation into multiple clinical trials. Chemical-, peptide-, antibody-, and nanoparticle-based probes have been designed to target specific molecules in gliomas and then be visualized with multimodality molecular imaging (MI) techniques including positron emission tomography (PET), single-photon emission computed tomography (SPECT), near-infrared fluorescence (NIRF), bioluminescence imaging (BLI), and magnetic resonance imaging (MRI). Thus, multiple molecules of interest can now be noninvasively imaged to guide targeted therapies with a potential survival benefit. Here, we review developments in molecular-targeted diagnosis and therapy in glioma, MI of these targets, and MI monitoring of treatment response, with a focus on the biological mechanisms of these advanced molecular probes. MI probes have the potential to noninvasively demonstrate the pathophysiologic features of glioma for diagnostic, treatment, and response assessment considerations for various targeted therapies, including immunotherapy. However, most MI tracers are in preclinical development, with only integrin $\alpha_V\beta_3$ and isocitrate dehydrogenase (IDH)-mutant MI tracers having been translated to patients. Expanded international collaborations would accelerate translational research in the field of glioma MI.

Keywords: glioma, molecular imaging, probes, targeted therapy, precision medicine

INTRODUCTION

Gliomas, especially glioblastoma (GBM), are the most malignant primary brain tumors in adults (1). Numerous *in vitro*, *in vivo*, and *ex vivo* studies have revealed multiple molecular fingerprints of gliomas, such as methylation of the O(6)-methylguanine-DNA methyltransferase (MGMT) promoter, mutant isocitrate dehydrogenase (IDH), platelet-derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor (VEGFR), integrin $\alpha_v\beta_3$ receptor, epidermal growth factor receptor (EGFR), c-Met, etc. These tumor-specific molecules can be used not only as targets for diagnosis and therapeutic response assessment, but also as potential targets for glioma treatment. Recently, advances in techniques for identifying new molecules of interest and the rapid development of novel molecular targeted inhibitors have given rise to new molecular imaging (MI) agents that have been developed using this highly selective approach.

Developments in MI techniques enable the visualization, characterization, and measurement of biological processes at the molecular and cellular levels in living systems (2). MI probes are introduced noninvasively to determine the expression of molecular targets of interest in tumors and, when evaluated repeatedly over time in the same subject, enable the evaluation of tumor response to a given therapy. Considering the spatial and temporal heterogeneity are inherent in gliomas, MI can serve as a useful tool for overcoming some of the limitations of routine diagnostics. For example, although pathological diagnosis is considered the gold standard, it provides molecular characterization of the glioma at a single snapshot in time (e.g., prior to chemoradiation, or in the case of recurrent disease, after multiple treatments including chemoradiation) and is limited in scope to the tumor region sampled by neurosurgeon. In addition, multiple reports have demonstrated inter-rater variability for glioma pathology diagnosis among trained experts, and the superiority of molecular and genetic profiles compared to histological analyses for prediction of overall survival (OS) in patients with glioma (3, 4). Instead, by implementing an advanced MI-based approach, the molecular marker status of tumors could be interrogated repeatedly *in vivo* over the course of the patient's treatment regimens. Accordingly, translational research involving these methods is currently underway at different stages including subcutaneous glioma animal models, orthotopic glioma animal models, and patients with glioma (e.g., NCT03539731).

Here, we searched PubMed (2000 to 2020) using the search terms "glioma" or "glioblastoma" in combination with "molecular imaging", "positron emission tomography (PET)", "fluorescence", "magnetic resonance spectroscopy (MRS)", and "single-photon emission computed tomography (SPECT)". We included only articles published in English. The articles relevant to this topic were included for analysis. Next, we address the MI tracers developed for glioma and review their current stage of clinical translation. We also discuss nonspecific tracers (e.g., ^{18}F-fluoro-2-deoxyglucose [^{18}F-FDG] and radiolabeled amino acids) that are used to monitor for treatment response to anti-glioma therapies. Additional details about the tracers routinely utilized in glioma diagnosis and therapy have been reviewed previously (5–8). The goal of this review is to narrow the gap between multidisciplinary researchers in the fields of glioma molecular diagnosis, therapy, and imaging techniques, in order to ultimately help improve targeted diagnosis and therapy in glioma.

APPLICATIONS OF CURRENT MOLECULAR IMAGING TRACERS IN TARGETED THERAPY

In **Table 1**, we summarize distinct MI modalities, and their corresponding tracers, in the context of targeted therapies against glioma. Other advanced MR imaging (MRI) techniques such as MR perfusion imaging, dynamic susceptibility contrast (DSC) MRI, and diffusion-weighted MRI are summarized elsewhere (18, 19).

The widely used oncologic and neurologic radiotracer, ^{18}F-FDG, has been employed not only for evaluating the efficacy of bevacizumab [the only U.S. Food and Drug Administration (FDA)–approved targeted inhibitor for recurrent GBM (20)] for newly diagnosed and recurrent GBM (9, 10), but also for monitoring efficacy of novel inhibitors against molecular targets of interest in glioma, such as c-Met [a receptor tyrosine kinase (RTK) whose ligand is hepatocyte growth factor] (16), phosphoinositide 3 (PI3)-kinase (21), mammalian target of rapamycin (mTOR) (22), and other RTKs (17). These studies demonstrate that ^{18}F-FDG PET/computed tomography (PET/CT) can potentially detect early metabolic changes that occur before alterations discernable on traditional anatomic MRI (e.g., tumor volume) and can thus help predict OS in these patients.

To evaluate the efficacy of novel targeted medications in glioma, other MI tracers besides ^{18}F-FDG have been used. Goggi et al. compared various PET imaging radiotracers, including ^{18}F-FDG, 3'-deoxy-3'-^{18}F-fluorothymidine (^{18}F-FLT), and 2-^{18}F-fluoroethyl-triazolyl-conjugated c(RGDyK) peptide (^{18}F-FtRGD), for early determination of tumor response to the antiangiogenic agent axitinib in mice bearing U87MG subcutaneous tumors (23). The results showed that the retention of ^{18}F-FtRGD exhibited a much earlier attenuation in the tumor by Day 7 (Day 3 for ^{18}F-FLT), compared to Day 10 for ^{18}F-FDG. Moreover, a prospective study of 16 patients with recurrent high-grade glioma (HGG) treated with bevacizumab and irinotecan concluded that both ^{18}F-FLT-avid and ^{18}F-fluoro-ethyl-tyrosine (^{18}F-FET)-avid volume reduction after two months of therapy predicted progression-free survival (PFS) and OS, and the volume-based analysis of ^{18}F-FET uptake was superior to that of ^{18}F-FLT in predicting patient survival (24).

^{18}F-FLT PET has gained traction in neuro-oncology imaging in Europe to help guide targeted therapy for gliomas. The use of this probe allows for direct and correlated quantification of proliferation rates through expression of the enzyme thymidine kinase-1 during DNA synthesis at an early stage (25, 26). Other studies have evaluated the ^{11}C-methyl-L-methionine (^{11}C-Met) radiotracer, which has been demonstrated to be an early

TABLE 1 | Widely used nonspecific molecular imaging tracers to assess glioma response to targeted inhibitor therapies.

Probe	Article	Model for test	Molecule targeted	Agents	Key details of study
^{18}F-FDG[1]	Graham et al. (9)	31 recurrent HGG patients	VEGF receptor	Bevacizumab	Prognostic of response to therapy and predictor of OS
^{18}F-FDG and MRI[1]	Omuro A et al. (10)	40 newly diagnosed GBM patients	VEGF receptor	Bevacizumab and temozolomide	Higher baseline ADC ratios and persistent 6-month FDG-PET hypermetabolism predicted poor OS
^{18}F-FET[1]	Fleischmann et al. (11)	72 recurrent HGG patients	VEGF receptor	Bevacizumab and re-irradiation	Minimal time-to-peak (TTPmin) provided a high prognostic value prior to re-irradiation
^{18}F-FDOPA	Johannes et al. (12)	30 recurrent GBM patients	VEGF receptor	Bevacizumab	Identified treatment responders as early as two weeks after treatment initiation
^{18}F-FDOPA	Robert et al. (13)	24 recurrent GBM patients	VEGF receptor	Bevacizumab	FDOPA or FLT PET uptake on parametric response maps after treatment as a useful biomarker for predicting PFS, FDOPA predicted patient OS
^{18}F-FDG PET/MRI[1]	Benjamin et al. (14)	47 recurrent GBM patients	PI3-kinase and mTOR	GDC-0084	change in PET uptake, ADC, Ktrans, and relative cerebral blood volume correlated with maximum concentration of drug and PFS
^{18}F-FLT, ^{18}F-FET and MRI	Philip et al. (15)	U87MG (orthotopically in mice)	PI3-kinase and mTOR	Bevacizumab and BEZ235	More accurately predict the clinical potential with multimodality imaging
^{18}F-FDG and ^{18}F-FLT	Rex et al. (16)	U87MG (subcutaneously in mice)	c-Met	Rilotumumab and CE-355621	Accumulation of both radiotracers reduced as early as 2 and 4 days post-initiation of therapy
^{18}F-FDG or ^{18}F-FLT	Moonshi et al. (17)	U87MG (orthotopically in mice)	RTK	Sunitinib	Longitudinal ^{18}F-FLT imaging detected therapeutic response at 7 days post-initiation of therapy, earlier than MRI (10 days) or ^{18}F-FDG PET (16 days)

[1]Clinically used in glioma patients. ADC, apparent diffusion coefficient; c-Met, one cell surface receptor tyrosine kinase; HGG, high-grade glioma; FDG, fluorodeoxyglucose; FLT, fluorothymidine; FET, fluoro-ethyl-tyrosine; GBM, glioblastoma multiforme; MRI, magnetic resonance imaging; mTOR, mammalian target of rapamycin; OS, overall survival; PFS, progression-free survival; PI3, phosphoinositide 3-kinase; RTK, receptor tyrosine kinase; U87, human GBM cell line; VEGF, vascular endothelial growth factor.

indicator, at 3 weeks, of tumor proliferation and vessel remodeling. By comparison, ^{18}F-FLT uptake correlated with positive Ki-67 staining only at 6 weeks in an analysis of the dynamic growth of angiogenesis-dependent/independent experimental GBM (27). Compared to the 110-min half-life of ^{18}F, the 20-min half-life of ^{11}C makes the latter radioisotope less amenable to practical clinical translation.

In the United States, the more commonly used amino acid-based PET radiotracer is ^{18}F-FDOPA and its uptake has been prospectively shown to be correlated with glioma grade and cellularity (28). A prospective study of 30 patients with recurrent HGG on bevacizumab therapy demonstrated that ^{18}F-FDOPA PET identified treatment responders as early as two weeks after starting treatment (12). In an earlier study of ^{18}F-FDOPA and ^{18}F-FLT PET in recurrent HGG patients treated with bevacizumab, a post-treatment increase in uptake of both radiotracers on parametric response maps (PRMs) predicted PFS, but only the ^{18}F-FDOPA PET PRMs predicted OS (13). One advantage of the amino acid-based tracers, including ^{11}C-Met, ^{18}F-FET, ^{18}F-FLT and ^{18}F-FDOPA, etc., is the fact that their uptake does not depend on blood-brain barrier (BBB) permeability.

In another study, patients treated with the indoleamine 2,3 dioxygenase 1 (IDO1) pathway inhibitor indoximod (D1-MT) and temozolomide underwent pre-treatment and on-treatment α-^{11}C-methyl-L-tryptophan (AMT) PET, and post-treatment imaging showed decreased regional uptake of the radiotracer (29). Because IDO1 metabolizes tryptophan into kynurenine, this strategy of using AMT PET to monitor therapeutic response with an IDO1 inhibitor serves as an example of a PET radiotracer "companion diagnostic" to targeted molecular therapy in GBM.

MOLECULES WITH TARGETED INHIBITORS UNDER EVALUATION IN CLINICAL TRIALS

Noninvasive imaging of the molecular events that occur in glioma has attracted increased research interest. Several promising molecular targets have been identified, including mutant IDH, PDGFR, VEGFR, integrin $\alpha_v\beta_3$ receptor, EGFR, c-Met, etc., These molecules and their specific inhibitors have been studied in multiple trials, and we summarize the MI modalities that are being used to visualize them in the context of glioma therapy. With a focus on translation from pre-clinical models to human trials, relevant studies are summarized in **Table 2**.

IDH MUTATION AND ITS INHIBITORS

IDH mutation was identified in most astrocytomas and secondary GBM as an early and inducing event in gliomagenesis (65, 66). IDH mutation status is a predictive marker of the therapeutic efficacy of alkylating chemotherapy in HGG patients (67, 68) and has also been associated with improved prognostic (i.e., OS) value in HGG and low-grade glioma (LGG) (65, 69). Therefore, IDH mutational status was introduced into the 2016 World Health Organization (WHO) classification of cancers of the central nervous system as a crucial molecular genetic feature (70). In addition, the presence of IDH mutation itself represents a therapeutic target in glioma, and several IDH1 mutation inhibitors have been evaluated in IDH-mutant glioma patients (71).

TABLE 2 | List of *in vivo* visualization of specific molecules whose targeted inhibitors are under evaluation in clinical trials.

Molecule	Article	Molecular imaging probes	Imaging instrument	Model for test	Key details of study	Targeted drugs
IDH mutation	Choi et al. (30)	None	3T Proton MRS	30 Glioma patients of all grades	Noninvasive detection of D-2HG	AGI-5198 (31), HMS-101 (32)
PDGFRβ	Tolmachev et al. (33)[2]	^{111}In-DOTA-Z09591	SPECT/CT	U87MG (subcutaneous)		Imatinib, Dasatinib (34)
VEGFR2	He et al. (35)[2]	Anti-VEGFR2-albumin-Gd-DTPA	Molecular MRI	C6 or RG2 glioma-bearing rats (orthotopic)	Angiogenesis; intratumor and intertumor heterogeneity	Bevacizumab (20)
	Chen et al. (36)[2]	^{64}Cu-DOTA-VEGF	PET	U87MG (subcutaneous in mice)	Quantitative; treatment monitoring	
	Rainer et al. (37)	^{123}I-VEGF	SPECT	23 Glioma patients	Prognostic value for overall survival	
	Jansen et al. (38)	^{89}Zr-Bevacizumab	PET	7 Children with diffuse intrinsic pontine glioma	Specific uptake in MRI contrast-enhanced areas, but with heterogeneous patterns	
Integrin $\alpha_v\beta_3$	Iagaru et al. (39)	^{18}F-FPPRGD2	PET	17 Recurrent GBM patients	Earlier identification of recurrence compared to MRI and ^{18}F-FDG PET	Cilengitide (40);
	Li et al. (41)	^{68}Ga-BNOTA-PRGD2	PET	12 Newly diagnosed glioma patients	Uptake correlated with grade	
	Schnell et al. (42)	^{18}F-Galacto-RGD	PET	12 GBM patients (newly diagnosed and recurrent)	Significant but heterogeneous tracer uptake in microvessels and glial tumor cells	
	Lee et al. (43)[2]	RGD- NaGdF4:Yb3+/Er3+ nanophosphor	PET and 3T T1-weighted MRI	U87MG (subcutaneous in mice)		
	Morales-Avila et al. (44) [2]	^{99m}Tc-HYNIC-GGC-AuNP-c [RGDfK(C)	Micro-SPECT/CT	C6-Induced tumors with blocked/nonblocked receptors (subcutaneous in mice)		
	Lanzardo et al. (45) [2]	RGD cyclic probe (DA364)	NIRF	U87MG (subcutaneous in mice)		
	Hsu et al. (46) [2]	Cy5.5-linked cyclic RGD peptide	NIRF and BLI	U87MG expressing luciferase (orthotopic in mice)	Angiogenesis	
	Ellegala et al. (47) [2]		PET	U87MG (orthotopic in mice)	Biodistribution of tracer and MET expression	
	Choi et al. (48) [2]	^{123}I- and ^{68}Ga- RGD-HSA-TIMP2	SPECT and PET	U87MG (subcutaneous in mice)	TIMP2 as an inhibitor of angiogenesis, also targets integrin $\alpha_v\beta_3$	
Integrin $\alpha_v\beta_3$ and TIMP2	Tang et al. (49) [2]	^{89}Zr-DFO-nimotuzumab	PET	U87MG expressing EGFR (subcutaneous in mice)	Assessing EGFR status	
EGFRvIII	Elliott et al. (50) [2]	ABY-029	NIRF	F98 expressing EGFR (orthotopic in mice)	Outperformed 5-ALA for fluorescence-guided surgery in EGFR+ tumors	Erlotinib (51); EGFR-retargeted oncolytic herpes simplex virus (mice) (52) CDX-110 (53)
	Fatehi et al. (55) [2]	Qd800 to an anti-EGFRvIII single domain antibody (EG2-Cys)	NIRF	U87MG (subcutaneous in mice)	Correlated with aggressiveness and resistance	
	Mishra et al. (56) [2]	EGFR conjugated metal chelates	SPECT	U-87MG and MDA-MB-468 (subcutaneous in mice)		
	Davis et al. (57) [2]	Gadolinium contrast; near-infrared fluorophore bound to EGF ligand	MRI-coupled FMT	U251 and 9L-GFP (orthotopic in mice)	Quantification of EGFR receptor	CAR-modified T (CART)-EGFRvIII cells (54)
	Zhang et al. (58) [2]	Engineered Bioluminescence Met reporter (BMR)	BLI	U87MG (subcutaneous in mice)	Pharmacokinetics and bioavailability of c-Met specific agents	

(Continued)

TABLE 2 | Continued

Molecule	Article	Molecular imaging probes	Imaging instrument	Model for test	Key details of study	Targeted drugs
c-Met	Terwisscha et al. (59) [2]	Anticalin ^{89}Zr-PRS-110	PET	U87MG (subcutaneous in mice)	Specific uptake and earlier accumulation in c-Met-expressing tumors	AMG102 (60) Crizotinib (61)
	Jun et al. (62) [2]	None	BLI	c-MET-positive and c-MET-negative luciferase-expressing primary GBM tumor cells (orthotopic in mice)	Correlating c-Met expression status with tumor growth	
	Kim et al. (63) [2]	^{125}I-labeled MET-binding peptides	SPECT/CT	U87MG (subcutaneous in mice)	Visualizing tumor but with unremarkable overall image quality	
	Jagoda et al. (64) [2]	^{89}Zr-df-Onartuzum vs. ^{76}Br-Onartuzumab	PET	U87MG (subcutaneous in mice)	Improved c-Met imaging for prognostic purposes	

[2]*Only in vivo imaging including glioma patients and animal model, but excluding in vitro imaging. 5-ALA, 5-aminolevulinic acid; 9L-GFP, rat gliosarcoma cell line expressing GFP; $\alpha_v\beta_3$, alpha(V) beta(3); BLI, bioluminescence imaging; c-Met, tyrosine-protein kinase Met or hepatocyte growth factor receptor; CT, computed tomography; D-2HG, D-2-hydroxyglutarate; DOTA, tetraxetan; DFO, desferoxamine; EGFR, epidermal growth factor receptor; FDG, fluorodeoxyglucose; FMT, fluorescence molecular tomography; GBM, glioblastoma multiforme; Gd-DTPA, gadolinium with diethylenetriaminepentacetate; HSA, human serum albumin; MRI, magnetic resonance imaging; MRS, magnetic resonance spectroscopy; NIRF, near-infrared fluorescence; NOTA, 1,4,7-triazacyclononane-1,4,7-triacetic acid; PDGF, platelet-derived growth factor; PET, positron emission tomography; RGD, tripeptide Arg–Gly–Asp; SPECT, single-photon emission computed tomography; TIMP, tissue inhibitor of metalloproteinase; U87, human GBM cell line; VEGF, vascular endothelial growth factor; U251, human GBM cell line.*

IDH mutation can be detected using various *ex vivo* methods, including direct sequencing (65, 72), allele-specific PCR (73), and immunohistochemistry (IHC) (74). Several studies have also focused on D-2-hydroxyglutarate (D-2HG). Santagata et al. used desorption electrospray-ionization mass spectrometry to detect D-2HG *ex vivo* and found that its signal overlaps with areas of tumor and correlates with the tumor contents. They further suggested that mapping the D-2HG signal onto anatomic 3D reconstructed MR images of tumors can be integrated with advanced multimodality image-guided neurosurgical procedures to enable rapid molecular analysis of surgical tissue intraoperatively (75).

In vivo imaging of IDH mutation has attracted considerable attention. However, because of the technical challenges associated with imaging the gene mutation itself, the MI approaches are currently based on D-2HG. Choi et al. estimated the concentration of D-2HG by performing spectral fitting in the case of tumors from 30 patients. Numerical and phantom analyses of MRS pulse sequences were performed, and the results were validated with mass spectrometry of *ex vivo* tissues and then successfully translated to clinic with a larger prospective trial (30, 76). Such *in vivo* MRS methods have also been shown to detect IDH mutations (**Figures 1A, B**) that were missed in IHC analyses, and the reduction in D-2HG levels has been used to monitor treatment response in patients with IDH-mutant gliomas and correlated with clinical status (82, 83). A recent clinical trial and pooled analysis demonstrated the high sensitivity and specificity of MRS compared to other imaging modalities for the detection of IDH mutational status (84, 85). MRS was used to serially monitor for a decrement of D-2HG levels in gliomas in a Phase I clinical trial of a new mutant IDH1 inhibitor (86). To date, no specific IDH-mutant-specific targeted MI probe has been developed for PET or SPECT. Nonspecific probes such as ^{18}F-FDOPA were shown to accumulate in LGG with IDH mutation (87). A more recent study suggests that dynamic ^{18}F-FDOPA uptake parameters (e.g., time to peak SUV) rather than static uptake parameters (e.g., SUVmax) may be able to discriminate between IDH mutant and IDH wild-type gliomas (88).

MI of D-2HG as a marker of IDH mutant status by MRS has achieved successful clinical translation in glioma patients and can be used to serially and noninvasively monitor for this important pathophysiologic molecular marker. Further research should be conducted to integrate this imaging modality as a neuroimaging "companion diagnostic" in clinical trials of therapies targeting the IDH1 mutation, to determine whether it can stratify patients into the responder and non-responder subsets. More novel MI techniques with higher sensitivity, higher specificity, and lower dependence on BBB permeability should be developed, in light of the low sensitivity of MRS for detecting IDH mutant status in smaller tumors due to partial-volume effects (89).

PDGFR AND SRC FAMILY KINASES (SFKs) AND THEIR INHIBITORS

PDGFR plays a critical role in HGG and synergizes with SFKs, which are nonreceptor membrane-associated tyrosine kinases.

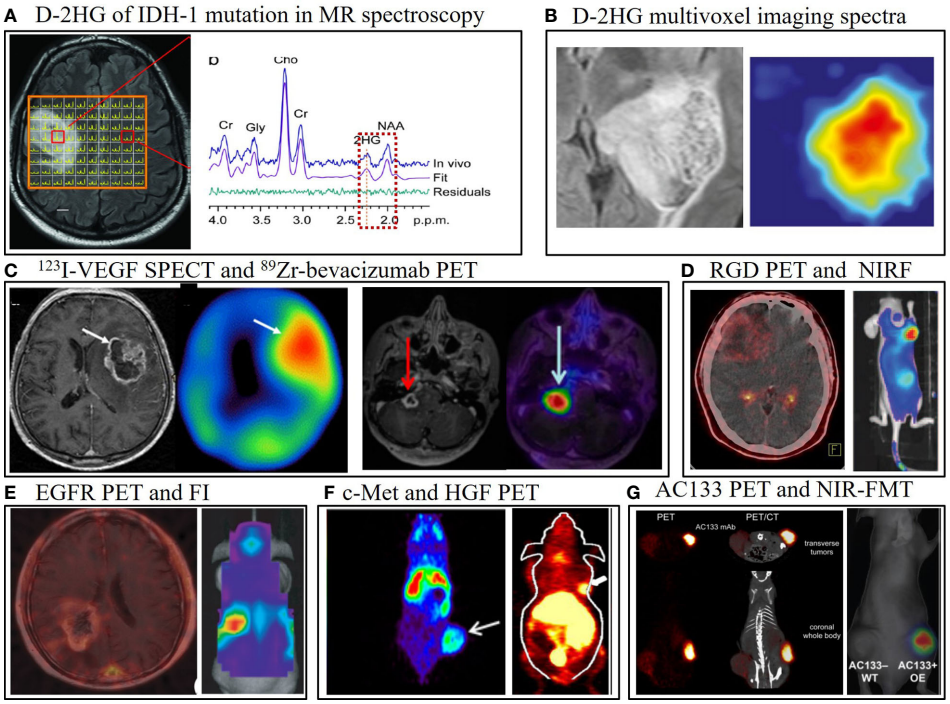

FIGURE 1 | Representative multimodality molecular imaging in glioma, including positron emission tomography (PET), single-photon emission computed tomography (SPECT), optical, and MR spectroscopy (MRS). **(A)** The major catabolite of IDH-1 mutation in gliomas, D-2-hydroxyglutarate (D-2HG), can be visualized by MRS, and this technique has been translated to clinical trials (30). **(B)** T2/FLAIR abnormal signal area in MRI is overlaid with the D-2HG multivoxel imaging spectra in MRS (76). **(C)** Glioblastoma lesion uptake with the ^{123}I-VEGF SPECT tracer (left) (37) and the ^{89}Zr-bevacizumab PET radiotracer (144 h post-injection) fused with gadolinium-enhanced T1-weighted MRI in a child with diffuse intrinsic pontine glioma (right) (38). **(D)** Integrin $\alpha_v\beta_3$ visualized in a patient with glioblastoma using ^{68}Ga-PRGD2 PET/CT by our team; RGD-Cy5.5 conjugate near-infrared fluorescence (NIRF) image showing integrin $\alpha_v\beta_3$ in a mouse bearing a subcutaneous U87MG tumor (77). **(E)** ^{11}C-PD153035 PET/CT for visualization of EGFR in human glioblastoma (78); *in vivo* optical imaging of epidermal growth factor receptor variant III (EGFRvIII)-expressing U87MG cells orthotopically implanted in a mouse identifies the tumor after intravenous injection of a EGFRvIII single-domain antibody bioconjugated to near-infrared quantum dots, with an extra cysteine for site-specific conjugation (55). **(F)** ^{89}Zr-PRS-110 PET noninvasively shows c-Met positivity in a U87MG subcutaneous tumor model (59). ^{64}Cu-labeled recombinant human hepatocyte growth factor PET also detects c-Met expression in nude mice bearing U87MG xenografted tumors (79). **(G)** Mouse bearing AC133/CD133-overexpressing U251 gliomas in a subcutaneous tumor model can be imaged with ^{64}Cu-NOTA-AC133 mAb PET/CT (80); IR700-conjugated AC133 can also identify the tumor using near-infrared fluorescence (NIRF) molecular tomography (FMT) (81). All images have been reprinted with permission; **(D)** is previously unpublished data.

PDGFR and SFKs are both associated with the invasiveness (90), self-renewal of glioma-initiating cells, and growth of tumor vasculature in HGG (91). PDGFRβ is expressed not only in vasculature, but also in GBM-associated stromal cells, which exert tumor-promoting effects on glioma cells *in vitro* and *in vivo* (92).

Specific targeted inhibitors of PDGFRβ include first-generation single-kinase inhibitors (e.g., imatinib) and second-generation inhibitors of multiple protein tyrosine kinases (e.g., dasatinib, which targets both PDGFR and SFKs). Dasatinib has been shown to inhibit bevacizumab-induced glioma cell invasion in an orthotopic xenograft model, supporting the human translation of combining dasatinib with bevacizumab in HGG (93). However, recent clinical trials showed that dasatinib in conjunction with bevacizumab did not appear to benefit patients with newly diagnosed and recurrent GBM (94, 95). MEDI-575, an immunoglobulin G2κ monoclonal antibody that selectively binds to platelet-derived growth factor receptor α (PDGFRα), also showed limited clinical efficacy in recurrent GBM in a Phase II clinical trial (96).

Developments in visualizing PDGFR expression in glioma *via* MI are relatively insufficient. Tolmachev et al. designed a PDGFRβ-binding affibody molecule, Z09591, which was labeled with ^{111}In to specifically visualize PDGFRβ expression; the affibody was used for imaging in an U87MG xenograft model by applying small-animal SPECT/CT (33). Future studies of novel PET radiotracers are warranted because they may provide increased sensitivity, specificity, and quantification accuracy. In conclusion, PDGFR can be used as a pathophysiologic marker of glioma but much work still remains for further PDGFR-based targeted therapy and imaging.

VEGFR AND BEVACIZUMAB

VEGF is the key pro-angiogenic protein that is overexpressed in and released by gliomas into their microenvironment (97). Glioma treatment with bevacizumab, an inhibitor of VEGF receptor (VEGFR) expressed on vascular endothelium, has led to increased PFS but no OS benefit in the patients with recurrent

GBM and was approved for GBM therapy in 2009 (98). However, bevacizumab failed to show a survival advantage in two large studies of patients with newly diagnosed GBM: AVaglio in Europe and RTOG-0825 in North America (99, 100).

Selecting appropriate candidates for optimal antiangiogenic therapy is critical, and this has recently attracted considerable research attention. EGFR gene amplification are associated with shorter time to progression in patients with recurrent GBM while treated with bevacizumab (101). Other tissue-based and advanced neuroimaging parameters that are used as potential biomarkers in the setting of anti-VEGFR therapy are reviewed elsewhere (102). The ^{18}F-radiolabeled FET, FLT, and FDG PET tracers mentioned earlier are based on cell proliferation and metabolism and can be used to indirectly assess anti-VEGFR treatment response (103). Here, we focus on VEGFR-specific MI, which may help in identifying suitable candidates for antiangiogenic treatment, as well as in evaluating treatment response and disease progression. An anti-VEGFR probe (anti-VEGFR-albumin-gadolinium) was designed to image VEGFR in C6 and RG2 glioma-bearing rats with MRI, and the findings were further confirmed through fluorescence staining and quantification of the fluorescence intensity of the anti-VEGFR probe (35). Moreover, a PET tracer, ^{64}Cu-DOTA-VEGF, was developed for use in small-animal PET to quantify VEGFR expression levels in animal models *in vivo* (36).A clinical research demonstrated the SPECT using recombinant human VEGF labeled with ^{123}I can visualize GBM rather than LGG and stratify patients' OS based on specific T/N ratio threshold (37) (**Figure 1C**, left). In HGG, VEGF-based radiotracer approaches used to assess response to therapy may be confounded by endogenous VEGF levels in the tumor microenvironment that compete to bind for the same VEGFR's on the vascular endothelium. Therefore, another approach would be to develop an anti-VEGFR-based radiolabeled antibody. An immunoPET tracer, ^{89}Zr-bevacizumab, was designed using a diagnostic radioisotope with the commercial antibody drug (Avastin®) to visualize the heterogeneity of binding of this drug on the vascular endothelium in diffuse intrinsic pontine glioma (DIPG) (38) (**Figure 1C**, right).

In conclusion, VEGFR has been successfully targeted with bevacizumab as an approved therapy for recurrent GBM, and its effects could be monitored with several MI techniques. Further investigation is required to correlate these VEGF- and VEGFR-targeted MI techniques with treatment efficacy in clinical trials of bevacizumab therapy for GBM, which has potential to identify the patient subset that is most likely to respond to therapy. Taking the relatively large molecular weights of VEGF or antibody into consideration, the BBB influence of these tracers should be investigated further. The newer anti-angiogenic agents in GBM, e.g., anti-VEGF therapies like TTAC-0001 (NCT03856099), could similarly be evaluated with this MI-based approach.

INTEGRIN $\alpha_v\beta_3$ AND CILENGITIDE

Integrin alpha(V)beta(3) ($\alpha_v\beta_3$) was shown to be overexpressed in neogenic vessels and glioma cells *in vitro* (104) and *ex vivo* (105); the expression of this integrin generally correlates with malignancy grade and is a negative prognostic factor (105). Several inhibitors targeting integrin $\alpha_v\beta_3$ are under development. Cilengitide, a selective $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrin inhibitor, has been shown to inhibit GBM growth in preclinical tumor models, as well as in patients with newly diagnosed and recurrent GBM in Phase I and II clinical trials (106–110). However, in the Phase III CENTRIC EORTC 26071-22072 trial, Stupp et al. reported no OS benefit when this inhibitor was combined with standard chemotherapy in newly diagnosed GBM patients with methylation of the MGMT promoter (111).

Chinot noted several possible reasons for the failure of that trial, including screening based on MGMT promoter methylation status when this biomarker may not necessarily be associated with integrin biology (112). Another reason for failure of that trial may be the heterogeneity of integrin $\alpha_v\beta_3$ expression in GBM, which was clearly demonstrated by *ex vivo* IHC (105) and *in vivo* MI studies (42). Targeted therapy is likely to be effective only when the defined target molecule is expressed at high levels. Thus, for GBM treatment, a rational MI-based approach for future clinical trials would be to (1) confirm the existence of the target as a screening inclusion criterion before initiating integrin-inhibitor treatment and (2) serially track expression of the molecular target as a physiologic surrogate for monitoring tumor response alongside traditional anatomic MRI.

Noninvasive visualization of integrins in the setting of cancer has been developed over the past decades. Sipkins et al. visualized integrin $\alpha_v\beta_3$ by using Gd-containing liposomes coated with a monoclonal antibody (mAb) in animal models of breast cancer and malignant melanoma (113). Integrin imaging for several tumor types *via* multimodality imaging including MRI, ultrasound, near-infrared fluorescence (NIRF) imaging, SPECT, and PET has been reviewed elsewhere (114).

NIRF dyes conjugated with a cyclic arginine-glycine-aspartic acid (RGD) peptide were applied to visualize subcutaneously inoculated integrin-positive gliomas (46, 77, 115). Chen et al. confirmed that the specific RGD peptide–integrin interaction which was detected using the NIRF technique could be employed to noninvasively image integrin expression in almost real-time in U87MG GBM xenografts (**Figure 1D**, right) (77). A study using ^{64}Cu-cyclam-RAFT-c(-RGDfK-)4 in a mouse model of glioma demonstrated its therapeutic efficacy and suitability for integrin imaging in the tumor (116).

The RGD-based MI tracers and techniques have been successfully translated to patients in clinical trials. ^{18}F-FPPRGD2, an RGD-dimer PET tracer, was evaluated for imaging the expression of integrin $\alpha_v\beta_3$ in healthy volunteers and in patients with GBM and other cancers requiring antiangiogenic treatment (117). ^{18}F-galacto-RGD was found to have marked yet heterogeneous uptake in microvessels and glial tumor cells (42). In another study, a relatively more specific dimer, ^{68}Ga-BNOTA-PRGD2, was utilized (**Figure 1D**, left) and a semiquantitative feature of uptake was correlated with tumor grade (41). A clinical study of ^{18}F-AlF-NOTA-PRGD2 PET/CT in newly diagnosed GBM patients showed that this integrin-

targeting PET approach predicted response to chemoradiation (84.6% sensitivity, 90.0% specificity, and 87.0% accuracy) as early as 3 weeks post-initiation of treatment when using a SUVmax threshold of 1.35 (118). How much these typical peptide-based imaging tracers depend on BBB breakdown for imaging have not thoroughly assessed in suitable models.

Although integrin $\alpha_v\beta_3$-targeted inhibitors were effective in preclinical studies and small cohorts of GBM patients in phase I and II clinical trials, they failed to demonstrate a survival benefit in a Phase III trial. However, integrin receptor imaging has been successfully translated to small pilot clinical studies of GBM patients and can be used to noninvasively demonstrate the integrin receptor distribution and expression density, which supports its use as a predictive neuroimaging biomarker during screening for prospective trial participants. Before this imaging can become a reliable predictive indicator for a specific subgroup of glioma patients, the imaging probes and techniques should be further validated for improved sensitivity and specificity in human patients.

EGFR AND ITS INHIBITORS

EGFR gene amplification and overexpression are striking features of GBM, particularly primary GBM. In approximately 50% of tumors showing EGFR amplification, a specific EGFR mutant, EGFR variant III (EGFRvIII), can be detected. EGFRvIII is specifically expressed in 31% of primary GBM patients, and compared to patients with wild-type EGFR GBM, those with EGFR-mutant GBM tend to have an older age at diagnosis, worse prognosis, and resistance to chemoradiotherapy (119, 120).

In addition to EGFR inhibitors (e.g., erlotinib), oncolytic HSV retargeted to GBM EGFR (52) and EGFRvIII vaccines have been evaluated in clinical trials. Rindopepimut (CDX-110) was designed to generate a specific immune response against EGFRvIII-expressing tumors, and the drug was demonstrated to benefit EGFRvIII-positive GBM patients in a Phase II trial, although it failed in a Phase III trial (ACT IV) of newly diagnosed, EGFRvIII-positive GBM patients (121, 122). Binder and colleagues reviewed possible reasons for failure of that trial, including loss of GBM EGFRvIII expression in ~60% of cases regardless of whether rindopepimut or control treatment was administered, and the lack of control arms in the previous promising Phase II trials (123). The incorporation of MI in such clinical trials to non-invasively detect the loss of expression of the target protein could prompt an earlier determination of lack of treatment efficacy, so a new therapy could be initiated that may lead to improved patient outcomes.

The first-in-human study of the chimeric antigen receptor modified T cell (CART)-EGFRvIII, as a cellular immunotherapy, in 10 recurrent GBM patients demonstrated on-target activity in brain. One patient had stable disease for over 18 months. However, the investigators found that the antigen expression decreased in the biopsied tissue in most patients (54). We believe that MI of antigen heterogeneity and reductions in antigen expression may provide earlier detection that the current therapy may no longer be efficacious, so that a different therapeutic strategy can be pursued earlier on.

EGFR-specific tracers were developed for multiple imaging modalities including SPECT, optical imaging, and MRI. Mishra et al. used anti-EGFR antibody-conjugated metal chelates in SPECT to image EGFR expression in mice bearing glioma cell lines (56). In another study, near-infrared imaging was performed on mice bearing orthotopic GBM by using a method in which a near-infrared quantum dot (Qd800) was conjugated to an anti-EGFRvIII single-domain (sd) antibody containing an extra cysteine to enable site-specific conjugation (EG2-Cys) (**Figure 1E**, right); this quantum dot-modified probe showed increased accumulation in tumors relative to the unconjugated quantum dot or the quantum dot conjugated to the Fc region of the antibody (EG2-hFc) (55). Another specific NIRF tracer, ABY-029, outperformed 5-ALA in detecting the tumor margin of EGFR-positive tumors and has the potential to enhance fluorescence-guided surgery (50). Lastly, ^{11}C-PD153035 PET/CT was demonstrated to be positively correlated with *ex vivo* EGFR immunostaining and Western blot analysis in the case of glioma patients (**Figure 1E**, left) (78).

Davis et al. designed a MRI-coupled fluorescence molecular tomography (FMT) system in which gadolinium (Gd)–based contrast was used and a near-infrared fluorophore was bound to EGF, the ligand of EGFR. By using this system, the EGFR expression status in animal models of U251 and 9L-GFP tumors was quantified with 100% sensitivity and specificity (57). The FMT system was particularly effective when used in combination with the anatomy-based information provided by the Gd-enhanced MRI scan data.

Therefore, specific types of EGFR mutations should be screened with MI probes to investigate their utilization as imaging biomarkers for selecting patients for oncologic vaccine-based approaches. Future studies should also examine whether targeted EGFR-mutant MI tracers can be used to direct EGFR-targeted therapy *in vivo*.

C-MET AND ITS INHIBITORS

Hepatocyte growth factor/scatter factor (HGF/SF) and its cell-surface receptor, the tyrosine kinase c-Met, were found to be closely linked with glioma cell invasion and tumor progression (124), and c-Met has been widely confirmed as a crucial predictor of GBM patient outcomes (125).

Nearly two decades ago, c-Met expression was not only demonstrated in glioma cells and tumor microvasculature, but was also shown to be associated with astrocytic tumors through immunohistochemical staining of *ex vivo* glioma samples. Elevated c-Met expression levels paralleled higher tumor grades: 21.4% positive in astrocytoma (WHO grade II) and 53.8% positive in anaplastic astrocytoma as compared with 87.5% in GBM (126). Moreover, recent research has demonstrated increased efficacy of a prognosis model that includes c-Met protein expression (127). Jun et al. found c-Met was preferentially localized in the perivascular

regions of human GBM tissues that are highly clonogenic, tumorigenic, and resistant to radiation. Bioluminescence imaging (BLI) was used to monitor tumor growth in nude mouse brains implanted with c-Met-positive and c-Met-negative luciferase-expressing GBM tumor cells, and this confirmed the relationship between c-Met expression tumor growth *in vivo* (62).

Both c-Met pathway-targeting small molecules and mAbs have been investigated in GBM, yielding promising results. AMG 102 (rilotumumab) enhanced the efficacy of temozolomide or docetaxel in U87MG cells and xenografts (60). However, in a Phase II clinical trial of rilotumumab in heavily pretreated patients with recurrent GBM, monotherapy was not associated with significant antitumor activity (128). Cabozantinib (XL184), an oral inhibitor of multiple RTKs such as c-Met and VEGFR2, yielded favorable results in the case of advanced prostate cancer (129), thyroid cancer (130), and was approved by the U.S. FDA in 2012. Interestingly, the Phase II trial of XL184 in recurrent GBM demonstrated antitumor activity, particularly in the antiangiogenic treatment-naive cohort, with a median PFS of 3.7 months in both the 140 mg/day and 100 mg/day groups (131). In the subset of patients who had received prior antiangiogenic therapy, the objective response rate was only 4.3% with a median duration of response of 4.2 months (132).

Knockdown of the c-Met protein can make tumor necrosis factor related apoptosis-inducing ligand (TRAIL)-resistant brain tumor cells sensitive to TRAIL treatment *in vitro*; moreover, in nude mice intracerebrally implanted with a c-Met-knockdown tumor cell line, the effect of stem cell-delivered S-TRAIL *in vivo* was confirmed using BLI (133). Zhang et al. monitored gene expression quantitatively and dynamically in cultured cells and in a U87MG tumor xenograft model by using a genetically engineered bioluminescent c-Met reporter gene (58). This novel MI of the reporter gene has been gradually used to visualize the crosstalk among different relevant molecular targets in glioma animal models.

Several groups have developed new radionuclide tracers to image c-Met expression in gliomas *in vivo*. With SPECT imaging, the tumor can be visualized using ^{125}I-labeled c-Met-binding peptides in human U87MG tumor-bearing mice (63). Onartuzumab, an experimental therapeutic anti-c-Met mAb, was radiolabeled with ^{76}Br or ^{89}Zr, and the resulting probes showed minimal background in normal brain (64). Terwisscha van Scheltinga et al. visualized c-Met expression by using an anticalin ^{89}Zr-PRS-110 PET radiotracer in U87MG xenografts (**Figure 1F**, left); however, nearly 40% nonspecific uptake of this probe was confirmed in the blocking experiment, and thus further investigation is necessary (59). In another study, recombinant human HGF was labeled with ^{64}Cu, and this probe had strong and specific binding to c-Met in a U87MG tumor model (**Figure 1F**, right) (79).

In summary, all the MI techniques for visualizing c-Met expression are in the preclinical phase, and they will be clinically translated after the development of targeted drugs evaluated in clinical trials.

VISUALIZATION OF SPECIFIC MOLECULES THAT DO NOT YET HAVE INHIBITORS UNDER EVALUATION IN CLINICAL TRIALS

In addition to the molecular targets for diagnosis, treatment, and imaging, other molecules exist that better characterize glioma pathophysiology including glioma stem-like cells, newly formed tumor blood vessels, etc. However, specific inhibitors against these emerging molecular biomarkers have not yet been evaluated in clinical trials. The relevant studies are summarized in **Table 3**.

CD133 AND GLIOMA STEM CELLS

Glioma cancer stem cells (CSCs) are resistant to chemoradiotherapy and have attracted the attention of multidisciplinary researchers. Gaedicke et al. developed a new imaging tracer targeting the AC133 epitope of CD133, which is a well-investigated CSC marker. An AC133-specific mAb was radiolabeled with ^{64}Cu to generate ^{64}Cu-NOTA-AC133 mAb, which was used to monitor AC133-positive GBM CSCs. High-sensitivity and high-resolution images were obtained in animal models using both PET and NIRF imaging (**Figure 1G**) (80). A novel small peptide, CBP4, was linked to gold nanoparticles and the resultant probe was shown to be suitable as an imaging agent for CD133-expressing GBM CSCs (135). Jing et al. conjugated the AC133 antibody with an IR700 dye and showed that the resulting probe can be used noninvasively to assess AC133-positive gliomas *via* near-infrared FMT; the probe was employed in near-infrared photoimmunotherapy to effectively induce cell death and tumor shrinkage in an animal model (81).

TABLE 3 | List of *in vivo* visualization of specific molecules that do not yet have inhibitors under evaluation in clinical trials.

Molecule	Article	Utilized imaging probes	Imaging modality	Model for test	Key details of study
CD133	Gaedicke et al. (80)	^{64}Cu-NOTA-AC133 mAb	MicroPET	Orthotopic glioma xenografts (subcutaneous)	Monitoring of AC133(+) glioblastoma stem cells
	Jing H et al. (81)	IR700-AC133 mAb	NIRF	Orthotopic gliomas (subcutaneous)	Non-invasive detection of AC133 and linked with photoimmunotherapy
ELTD1	Towner et al. (134)	Anti-ELTD1 SPIO-based probe	Molecular MRI	F98 (orthotopic in rat)	Signal correlated with grade and survival

CD133, promonin-1; ELTD1, epidermal growth factor, latrophilin, and 7 transmembrane domain-containing protein 1 on chromosome 1; F98, rat GBM cell line; mAb, monoclonal antibody; NIRF, near-infrared fluorescence; NOTA, 1,4,7-triazacyclononane-1,4,7-triacetic acid; PET, positron emission tomography; SPIO, superparamagnetic iron oxide.

ELTD1

EGF, latrophilin, and 7-transmembrane domain-containing protein 1 on chromosome 1 (ELTD1) was identified as a putative glioma-associated marker using a bioinformatics method and reported to be associated with glioma grade and patient survival by Towner et al. (134). An anti-ELTD1 superparamagnetic iron oxide (SPIO)-based probe was designed by coating SPIO nanoparticles with dextran and conjugating an anti-ELTD1 antibody. This probe was used to assess the *in vivo* levels of ELTD1, and further investigation revealed that the anti-ELTD1 antibody inhibited glioma growth in mouse glioma models, an effect that could be attributed to diminished vascularization (136).

PROGRESS IN CLINICAL TRANSLATION OF VARIOUS TRACERS WITH DIFFERENT MOLECULAR IMAGING TECHNIQUES

We divided the translation process (from bench to bedside) into three stages of development: (1) Preclinical stage that includes subcutaneous animal models with glioma cell lines; (2) Preclinical stage that includes orthotopic animal models with glioma cell lines; and (3) Clinical stage that involves glioma patients. In **Figure 2**, we summarize the progress from pre-clinical to clinical translation of the abovementioned targeted MI tracers. Most of the targeted tracers have only been studied in animal models. The MI studies evaluated in human glioma patients target integrin $\alpha_V\beta_3$, IDH-mutation and VEGFR, pyruvate kinase M2 and have been imaged using PET/CT, SPECT and MRI modalities. The superior molecular sensitivity of PET, the lack of radiation, and high spatial resolution of MRI render these techniques much easier to translate, along with the fact that they are routinely used in the medical field. Optical imaging (e.g., NIRF and BLI), have also been utilized to image molecular expression in glioma xenografts in subcutaneous and orthotopic animal models. Although penetration depth remains a challenge in optical imaging, intraoperative imaging could represent a promising area of research following further development in both imaging technique and tracer design. Multimodality imaging can provide a possible solution to overcome certain limitations of current methods (e.g., PET and MRI for imaging integrin $\alpha_v\beta_3$, or optical imaging and MRI for imaging EGFR and IGFBP7). This strategy could enable imaging to be performed, using a single probe, on multiple imaging platforms with diverse disease models, ranging from small animal models to large animal models and even humans.

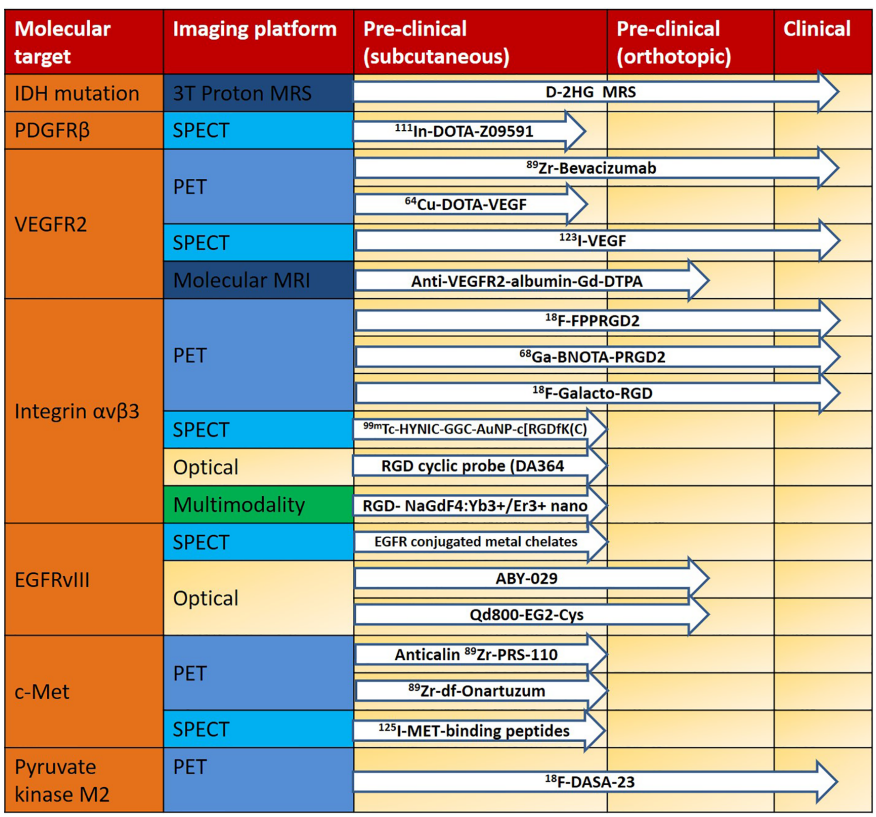

FIGURE 2 | Translational pipeline of molecular imaging probes in glioma using different imaging platforms. IDH, isocitrate dehydrogenase; MRS, magnetic resonance spectroscopy; PDGFRβ, platelet-derived growth factor receptor beta; SPECT, single-photon emission computed tomography; VEGFR2, vascular endothelial growth factor receptor 2; PET, positron emission tomography; MRI, magnetic resonance imaging; Integrin αvβ3, integrin alpha(V)beta(3); EGFRvIII, epidermal growth factor receptor variant III.

CONCLUSIONS AND PERSPECTIVES

With the discovery of multiple new molecular targets in glioma, the design and clinical translation of novel targeted diagnostics, treatments, and MI techniques have rapidly developed. MI offers several promising advantages over conventional anatomic imaging in glioma. Firstly, specific molecular expression patterns and therapeutic responses can be serially imaged *in vivo*, particularly for HGG patients, who typically undergo surgical treatment once at the time of initial diagnosis. Because of the minimal risk to patients, MI can be performed repeatedly if necessary, and can be used to evaluate tumor heterogeneity across the entire tumor, including its resected and residual components. Secondly, MI can potentially visualize prognostic and predictive biomarkers of interest to aid in selecting appropriate patients for molecular-targeted therapy. This approach would promote the evidence-based selection of patients for molecular-targeted therapeutic clinical trials and thereby possibly increase the success of improving survival in the appropriate patient cohort. Thirdly, MI can be applied routinely for the development and assessment of novel anti-glioma drugs or immunotherapy agents, because it can accurately monitor the pharmacodynamic and bioavailability of therapeutics in tumors.

Multimodality imaging probes can be designed to detect multiple biomarkers concurrently in glioma patients, and thus noninvasively map crucial molecules in this heterogenous and challenging disease. Given the advantages mentioned above, MI can represent an optimal method for achieving personalized medical care for glioma patients (137). To the previously identified "3 Rs" (right patient, right time, and right drug), MI enables us to add a fourth "R": right dosing.

Although MI offers several advantages, the use of this method in clinical research and practice currently remains at an early stage. Most MI probes are in the preclinical stage, while MI tracers targeting integrin $\alpha_V\beta_3$, VEGF receptor, and IDH-mutation have been successfully translated to pilot studies in glioma patients. Another potential limitation is that most of these studies are based on the use of peptides, proteins, and even nanoparticles. Demand exists for designing small-molecule tracers that can cross the BBB, which generally hinders the use of MI in the case of LGG with relatively more intact BBB functionality compared to HGG.

Accelerating the clinical translation of MI to benefit patients with glioma will only be achieved with deft navigation of regulatory requirements and multi-center, international cooperation. Firstly, after the potential toxicity of MI probes has been tested in small-animal models, we recommend taking advantage of early exploratory Investigational New Drug studies (138). Due to the very low concentrations of injected tracers visualized on exquisitely sensitive MI platforms, this regulatory compliance strategy is more apt for MI research in an incurable disease such as GBM. Secondly, accrual of a sufficient number of patients into MI studies to make meaningful conclusions will require international multi-center clinical trials that are guided by uniform research protocols with built-in continual quality assessment and quality control.

AUTHOR CONTRIBUTIONS

DL and CP wrote the manuscript, under the supervision of LZ and ZC. Other authors participated in some discussions. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Ostrom QT, Cioffi G, Gittleman H, Patil N, Waite K, Kruchko C, et al. CBTRUS Statistical Report: Primary Brain and Other Central Nervous System Tumors Diagnosed in the United States in 2012-2016. *Neuro Oncol* (2019) 21(Suppl 5):v1–v100. doi: 10.1093/neuonc/noz150
2. Mankoff DA. A definition of molecular imaging. *J Nucl Med* (2007) 48(6):18N, 21N.
3. Gravendeel LA, Kouwenhoven MC, Gevaert O, de Rooi JJ, Stubbs AP, Duijm JE, et al. Intrinsic gene expression profiles of gliomas are a better predictor of survival than histology. *Cancer Res* (2009) 69(23):9065–72. doi: 10.1158/0008-5472.CAN-09-2307
4. Holdhoff M, Ye X, Piotrowski AF, Strowd RE, Seopaul S, Lu Y, et al. The consistency of neuropathological diagnoses in patients undergoing surgery for suspected recurrence of glioblastoma. *J Neurooncol* (2019) 141(2):347–54. doi: 10.1007/s11060-018-03037-3
5. Ahmed R, Oborski MJ, Hwang M, Lieberman FS, Mountz JM. Malignant gliomas: current perspectives in diagnosis, treatment, and early response assessment using advanced quantitative imaging methods. *Cancer Manag Res* (2014) 6:149–70. doi: 10.2147/CMAR.S54726
6. Sharma A, McConathy J. Overview of PET tracers for brain tumor imaging. *PET Clin* (2013) 8:129–46. doi: 10.1016/j.cpet.2013.02.001
7. Albert NL, Weller M, Suchorska B, Galldiks N, Soffietti R, Kim MM, et al. Response Assessment in Neuro-Oncology working group and European Association for Neuro-Oncology recommendations for the clinical use of PET imaging in gliomas. *Neuro Oncol* (2016) 18(9):1199–208. doi: 10.1093/neuonc/now058

8. Muoio B, Giovanella L, Treglia G. Recent Developments of 18F-FET PET in Neuro-oncology. *Curr Med Chem* (2018) 25(26):3061–73. doi: 10.2174/0929867325666171123202644
9. Graham MS, Krebs S, Bale T, Domfe K, Lobaugh SM, Zhang Z, et al. Value of [(18)F]-FDG positron emission tomography in patients with recurrent glioblastoma receiving bevacizumab. *Neurooncol Adv* (2020) 2(1):vdaa050. doi: 10.1093/noajnl/vdaa050
10. Omuro A, Beal K, Gutin P, Karimi S, Correa DD, Kaley TJ, et al. Phase II Study of Bevacizumab, Temozolomide, and Hypofractionated Stereotactic Radiotherapy for Newly Diagnosed Glioblastoma. *Clin Cancer Res* (2014) 20(19):5023–31. doi: 10.1158/1078-0432.CCR-14-0822
11. Fleischmann DF, Unterrainer M, Bartenstein P, Belka C, Albert NL, Niyazi M. (18)F-FET PET prior to recurrent high-grade glioma re-irradiation-additional prognostic value of dynamic time-to-peak analysis and early static summation images? *J Neurooncol* (2017) 132(2):277–86. doi: 10.1007/s11060-016-2366-8
12. Schwarzenberg J, Czernin J, Cloughesy TF, Ellingson BM, Pope WB, Grogan T, et al. Treatment response evaluation using 18F-FDOPA PET in patients with recurrent malignant glioma on bevacizumab therapy. *Clin Cancer Res* (2014) 20(13):3550–9. doi: 10.1158/1078-0432.CCR-13-1440
13. Harris RJ, Cloughesy TF, Pope WB, Nghiemphu PL, Lai A, Zaw T, et al. 18F-FDOPA and 18F-FLT positron emission tomography parametric response maps predict response in recurrent malignant gliomas treated with bevacizumab. *Neuro Oncol* (2012) 14(8):1079–89. doi: 10.1093/neuonc/nos141
14. Ellingson BM, Yao J, Raymond C, Nathanson DA, Chakhoyan A, Simpson J, et al. Multiparametric MR-PET Imaging Predicts Pharmacokinetics and Clinical Response to GDC-0084 in Patients with Recurrent High-Grade Glioma. *Clin Cancer Res* (2020) 26(13):3135–44. doi: 10.1158/1078-0432.CCR-19-3817
15. O'Halloran PJ, Viel T, Murray DW, Wachsmuth L, Schwegmann K, Wagner S, et al. Mechanistic interrogation of combination bevacizumab/dual PI3K/mTOR inhibitor response in glioblastoma implementing novel MR and PET imaging biomarkers. *Eur J Nucl Med Mol Imaging* (2016) 43(9):1673–83. doi: 10.1007/s00259-016-3343-3
16. Rex K, Lewis XZ, Gobalakrishnan S, Glaus C, Silva MD, Radinsky R, et al. Evaluation of the antitumor effects of rilotumumab by PET imaging in a U-87 MG mouse xenograft model. *Nucl Med Biol* (2013) 40(4):458–63. doi: 10.1016/j.nucmedbio.2013.01.004
17. Moonshi SS, Bejot R, Atcha Z, Vijayaragavan V, Bhakoo KK, Goggi JL. A comparison of PET imaging agents for the assessment of therapy efficacy in a rodent model of glioma. *Am J Nucl Med Mol Imaging* (2013) 3(5):397–407.
18. Hutterer M, Hattingen E, Palm C, Proescholdt MA, Hau P. Current standards and new concepts in MRI and PET response assessment of antiangiogenic therapies in high-grade glioma patients. *Neuro Oncol* (2015) 17(6):784–800. doi: 10.1093/neuonc/nou322
19. Shukla G, Alexander GS, Bakas S, Nikam R, Talekar K, Palmer JD, et al. Advanced magnetic resonance imaging in glioblastoma: a review. *Chin Clin Oncol* (2017) 6(4):40. doi: 10.21037/cco.2017.06.28
20. Cohen MH, Shen YL, Keegan P, Pazdur R. FDA drug approval summary: bevacizumab (Avastin) as treatment of recurrent glioblastoma multiforme. *Oncologist* (2009) 14(11):1131–8. doi: 10.1634/theoncologist.2009-0121
21. Cawthorne C, Burrows N, Gieling RG, Morrow CJ, Forster D, Gregory J, et al. [18F]-FLT positron emission tomography can be used to image the response of sensitive tumors to PI3-kinase inhibition with the novel agent GDC-0941. *Mol Cancer Ther* (2013) 12(5):819–28. doi: 10.1158/1535-7163.MCT-12-0905
22. Keen HG, Ricketts SA, Maynard J, Logie A, Odedra R, Shannon AM, et al. Examining changes in [18 F]FDG and [18 F]FLT uptake in U87-MG glioma xenografts as early response biomarkers to treatment with the dual mTOR1/2 inhibitor AZD8055. *Mol Imaging Biol* (2014) 16(3):421–30. doi: 10.1007/s11307-013-0705-0
23. Goggi JL, Bejot R, Moonshi SS, Bhakoo KK. Stratification of 18F-labeled PET imaging agents for the assessment of antiangiogenic therapy responses in tumors. *J Nucl Med* (2013) 54(9):1630–6. doi: 10.2967/jnumed.112.115824
24. Martha Nowosielski MH, Putzer D, Iglseder S, Seiz M, Jacobs AH, Gobel G, et al. FET- and FLT-PET response assessment of anti-angiogenic therapy in recurrent high-grade glioma comparing tracer uptake and volume reduction. *Neuro Oncol* (2012) 14(vi):122–3. doi: 10.1093/neuonc/nos236
25. Ullrich R, Backes H, Li H, Kracht L, Miletic H, Kesper K, et al. Glioma proliferation as assessed by 3'-fluoro-3'-deoxy-L-thymidine positron emission tomography in patients with newly diagnosed high-grade glioma. *Clin Cancer Res* (2008) 14(7):2049–55. doi: 10.1158/1078-0432.CCR-07-1553
26. Backes H, Ullrich R, Neumaier B, Kracht L, Wienhard K, Jacobs AH. Noninvasive quantification of 18F-FLT human brain PET for the assessment of tumour proliferation in patients with high-grade glioma. *Eur J Nucl Med Mol Imaging* (2009) 36(12):1960–7. doi: 10.1007/s00259-009-1244-4
27. Viel T, Talasila KM, Monfared P, Wang J, Jikeli JF, Waerzeggers Y, et al. Analysis of the growth dynamics of angiogenesis-dependent and -independent experimental glioblastomas by multimodal small-animal PET and MRI. *J Nucl Med* (2012) 53(7):1135–45. doi: 10.2967/jnumed.111.101659
28. Pafundi DH, Laack NN, Youland RS, Parney IF, Lowe VJ, Giannini C, et al. Biopsy validation of 18F-DOPA PET and biodistribution in gliomas for neurosurgical planning and radiotherapy target delineation: results of a prospective pilot study. *Neuro Oncol* (2013) 15(8):1058–67. doi: 10.1093/neuonc/not002
29. Lukas RV, Juhasz C, Wainwright DA, James CD, Kennedy E, Stupp R, et al. Imaging tryptophan uptake with positron emission tomography in glioblastoma patients treated with indoximod. *J Neurooncol* (2019) 141(1):111–20. doi: 10.1007/s11060-018-03013-x
30. Choi C, Ganji SK, DeBerardinis RJ, Hatanpaa KJ, Rakheja D, Kovacs Z, et al. 2-hydroxyglutarate detection by magnetic resonance spectroscopy in IDH-mutated patients with gliomas. *Nat Med* (2012) 18(4):624–9. doi: 10.1038/nm.2682
31. Rohle D, Popovici-Muller J, Palaskas N, Turcan S, Grommes C, Campos C, et al. An inhibitor of mutant IDH1 delays growth and promotes differentiation of glioma cells. *Science* (2013) 340(6132):626–30. doi: 10.1126/science.1236062
32. Chaturvedi A, Araujo Cruz MM, Jyotsana N, Sharma A, Yun H, Gorlich K, et al. Mutant IDH1 promotes leukemogenesis in vivo and can be specifically targeted in human AML. *Blood* (2013) 122(16):2877–87. doi: 10.1182/blood-2013-03-491571
33. Tolmachev V, Varasteh Z, Honarvar H, Hosseinimehr SJ, Eriksson O, Jonasson P, et al. Imaging of platelet-derived growth factor receptor beta expression in glioblastoma xenografts using affibody molecule 111In-DOTA-Z09591. *J Nucl Med* (2014) 55(2):294–300. doi: 10.2967/jnumed.113.121814
34. Ahluwalia MS, de Groot J, Liu WM, Gladson CL. Targeting SRC in glioblastoma tumors and brain metastases: rationale and preclinical studies. *Cancer Lett* (2010) 298(2):139–49. doi: 10.1016/j.canlet.2010.08.014
35. He T, Smith N, Saunders D, Pittman BP, Lerner M, Lightfoot S, et al. Molecular MRI differentiation of VEGF receptor-2 levels in C6 and RG2 glioma models. *Am J Nucl Med Mol Imaging* (2013) 3(4):300–11.
36. Chen K, Cai W, Li ZB, Wang H, Chen X. Quantitative PET imaging of VEGF receptor expression. *Mol Imaging Biol* (2009) 11(1):15–22. doi: 10.1007/s11307-008-0172-1
37. Rainer E, Wang H, Traub-Weidinger T, Widhalm G, Fueger B, Chang J, et al. The prognostic value of [(123)I]-vascular endothelial growth factor ([(123)I]-VEGF) in glioma. *Eur J Nucl Med Mol Imaging* (2018) 45(13):2396–403. doi: 10.1007/s00259-018-4088-y
38. Jansen MH, Veldhuijzen van Zanten SEM, van Vuurden DG, Huisman MC, Vugts DJ, Hoekstra OS, et al. Molecular Drug Imaging: (89)Zr-Bevacizumab PET in Children with Diffuse Intrinsic Pontine Glioma. *J Nucl Med* (2017) 58(5):711–6. doi: 10.2967/jnumed.116.180216
39. Iagaru A, Mosci C, Mittra E, Zaharchuk G, Fischbein N, Harsh G, et al. Glioblastoma Multiforme Recurrence: An Exploratory Study of (18)F FPPRGD2 PET/CT. *Radiology* (2015) 277(2):497–506. doi: 10.1148/radiol.2015141550
40. Scaringi C, Minniti G, Caporello P, Enrici RM. Integrin inhibitor cilengitide for the treatment of glioblastoma: a brief overview of current clinical results. *Anticancer Res* (2012) 32(10):4213–23.

41. Li D, Zhao X, Zhang L, Li F, Ji N, Gao Z, et al. (68)Ga-PRGD2 PET/CT in the evaluation of Glioma: a prospective study. *Mol Pharm* (2014) 11(11):3923–9. doi: 10.1021/mp5003224
42. Schnell O, Krebs B, Carlsen J, Miederer I, Goetz C, Goldbrunner RH, et al. Imaging of integrin alpha(v)beta(3) expression in patients with malignant glioma by [18F] Galacto-RGD positron emission tomography. *Neuro Oncol* (2009) 11(6):861–70. doi: 10.1215/15228517-2009-024
43. Lee J, Lee TS, Ryu J, Hong S, Kang M, Im K, et al. RGD peptide-conjugated multimodal NaGdF4:Yb3+/Er3+ nanophosphors for upconversion luminescence, MR, and PET imaging of tumor angiogenesis. *J Nucl Med* (2013) 54(1):96–103. doi: 10.2967/jnumed.112.108043
44. Morales-Avila E, Ferro-Flores G, Ocampo-Garcia BE, De Leon-Rodriguez LM, Santos-Cuevas CL, Garcia-Becerra R, et al. Multimeric system of 99mTc-labeled gold nanoparticles conjugated to c[RGDfK(C)] for molecular imaging of tumor alpha(v)beta(3) expression. *Bioconjug Chem* (2011) 22(5):913–22. doi: 10.1021/bc100551s
45. Lanzardo S, Conti L, Brioschi C, Bartolomeo MP, Arosio D, Belvisi L, et al. A new optical imaging probe targeting alphaVbeta3 integrin in glioblastoma xenografts. *Contrast Media Mol Imaging* (2011) 6(6):449–58. doi: 10.1002/cmmi.444
46. Hsu AR, Hou LC, Veeravagu A, Greve JM, Vogel H, Tse V, et al. In vivo near-infrared fluorescence imaging of integrin alphavbeta3 in an orthotopic glioblastoma model. *Mol Imaging Biol* (2006) 8(6):315–23. doi: 10.1007/s11307-006-0059-y
47. Ellegala DB, Leong-Poi H, Carpenter JE, Klibanov AL, Kaul S, Shaffrey ME, et al. Imaging tumor angiogenesis with contrast ultrasound and microbubbles targeted to alpha(v)beta3. *Circulation* (2003) 108(3):336–41. doi: 10.1161/01.CIR.0000080326.15367.0C
48. Choi N, Kim SM, Hong KS, Cho G, Cho JH, Lee C, et al. The use of the fusion protein RGD-HSA-TIMP2 as a tumor targeting imaging probe for SPECT and PET. *Biomaterials* (2011) 32(29):7151–8. doi: 10.1016/j.biomaterials.2011.06.007
49. Tang Y, Hu Y, Liu W, Chen L, Zhao Y, Ma H, et al. A radiopharmaceutical [(89)Zr]Zr-DFO-nimotuzumab for immunoPET with epidermal growth factor receptor expression in vivo. *Nucl Med Biol* (2019) 70:23–31. doi: 10.1016/j.nucmedbio.2019.01.007
50. Elliott JT, Marra K, Evans LT, Davis SC, Samkoe KS, Feldwisch J, et al. Simultaneous In Vivo Fluorescent Markers for Perfusion, Protoporphyrin Metabolism, and EGFR Expression for Optically Guided Identification of Orthotopic Glioma. *Clin Cancer Res* (2017) 23(9):2203–12. doi: 10.1158/1078-0432.CCR-16-1400
51. Hatanpaa KJ, Burma S, Zhao D, Habib AA. Epidermal growth factor receptor in glioma: signal transduction, neuropathology, imaging, and radioresistance. *Neoplasia* (2010) 12(9):675–84. doi: 10.1593/neo.10688
52. Uchida H, Marzulli M, Nakano K, Goins WF, Chan J, Hong CS, et al. Effective treatment of an orthotopic xenograft model of human glioblastoma using an EGFR-retargeted oncolytic herpes simplex virus. *Mol Ther* (2013) 21(3):561–9. doi: 10.1038/mt.2012.211
53. Swartz AM, Li QJ, Sampson JH. Rindopepimut: a promising immunotherapeutic for the treatment of glioblastoma multiforme. *Immunotherapy* (2014) 6(6):679–90. doi: 10.2217/imt.14.21
54. O'Rourke DM, Nasrallah MP, Desai A, Melenhorst JJ, Mansfield K, Morrissette JJD, et al. A single dose of peripherally infused EGFRvIII-directed CAR T cells mediates antigen loss and induces adaptive resistance in patients with recurrent glioblastoma. *Sci Transl Med* (2017) 9(399):1–30. doi: 10.1126/scitranslmed.aaa0984
55. Fatehi D, Baral TN, Abulrob A. In vivo imaging of brain cancer using epidermal growth factor single domain antibody bioconjugated to near-infrared quantum dots. *J Nanosci Nanotechnol* (2014) 14(7):5355–62. doi: 10.1166/jnn.2014.9076
56. Mishra G, Panwar P, Mishra AK. Tumor targeting using anti-epidermal growth factor receptor (ior egf/r3) immunoconjugate with a tetraaza macrocyclic agent (DO3A-EA). *Mol Imaging* (2012) 11(5):408–16.
57. Davis SC, Samkoe KS, O'Hara JA, Gibbs-Strauss SL, Payne HL, Hoopes PJ, et al. MRI-coupled fluorescence tomography quantifies EGFR activity in brain tumors. *Acad Radiol* (2010) 17(3):271–6. doi: 10.1016/j.acra.2009.11.001
58. Zhang L, Virani S, Zhang Y, Bhojani MS, Burgess TL, Coxon A, et al. Molecular imaging of c-Met tyrosine kinase activity. *Anal Biochem* (2011) 412(1):1–8. doi: 10.1016/j.ab.2011.01.028
59. Terwisscha van Scheltinga AG, Lub-de Hooge MN, Hinner MJ, Verheijen RB, Allersdorfer A, Hulsmeyer M, et al. In vivo visualization of MET tumor expression and anticalin biodistribution with the MET-specific anticalin 89Zr-PRS-110 PET tracer. *J Nucl Med* (2014) 55(4):665–71. doi: 10.2967/jnumed.113.124941
60. Jun HT, Sun J, Rex K, Radinsky R, Kendall R, Coxon A, et al. AMG 102, a fully human anti-hepatocyte growth factor/scatter factor neutralizing antibody, enhances the efficacy of temozolomide or docetaxel in U-87 MG cells and xenografts. *Clin Cancer Res* (2007) 13(22 Pt 1):6735–42. doi: 10.1158/1078-0432.CCR-06-2969
61. Goodwin CR, Rath P, Oyinlade O, Lopez H, Mughal S, Xia S, et al. Crizotinib and erlotinib inhibits growth of c-Met(+)/EGFRvIII(+) primary human glioblastoma xenografts. *Clin Neurol Neurosurg* (2018) 171:26–33. doi: 10.1016/j.clineuro.2018.02.041
62. Jun HJ, Bronson RT, Charest A. Inhibition of EGFR induces a c-MET-driven stem cell population in glioblastoma. *Stem Cells* (2014) 32(2):338–48. doi: 10.1002/stem.1554
63. Kim EM, Park EH, Cheong SJ, Lee CM, Kim DW, Jeong HJ, et al. Characterization, biodistribution and small-animal SPECT of I-125-labeled c-Met binding peptide in mice bearing c-Met receptor tyrosine kinase-positive tumor xenografts. *Nucl Med Biol* (2009) 36(4):371–8. doi: 10.1016/j.nucmedbio.2009.01.005
64. Jagoda EM, Lang L, Bhadrasetty V, Histed S, Williams M, Kramer-Marek G, et al. Immuno-PET of the hepatocyte growth factor receptor Met using the 1-armed antibody onartuzumab. *J Nucl Med* (2012) 53(10):1592–600. doi: 10.2967/jnumed.111.102293
65. Yan H, Parsons DW, Jin G, McLendon R, Rasheed BA, Yuan W, et al. IDH1 and IDH2 mutations in gliomas. *N Engl J Med* (2009) 360(8):765–73. doi: 10.1056/NEJMoa0808710
66. Kloosterhof NK, Bralten LB, Dubbink HJ, French PJ, van den Bent MJ. Isocitrate dehydrogenase-1 mutations: a fundamentally new understanding of diffuse glioma? *Lancet Oncol* (2011) 12(1):83–91. doi: 10.1016/S1470-2045(10)70053-X
67. Leibetseder A, Ackerl M, Flechl B, Wohrer A, Widhalm G, Dieckmann K, et al. Outcome and molecular characteristics of adolescent and young adult patients with newly diagnosed primary glioblastoma: a study of the Society of Austrian Neurooncology (SANO). *Neuro Oncol* (2013) 15(1):112–21. doi: 10.1093/neuonc/nos283
68. SongTao Q, Lei Y, Si G, YanQing D, HuiXia H, XueLin Z, et al. IDH mutations predict longer survival and response to temozolomide in secondary glioblastoma. *Cancer Sci* (2012) 103(2):269–73. doi: 10.1111/j.1349-7006.2011.02134.x
69. Labussiere M, Boisselier B, Mokhtari K, Di Stefano AL, Rahimian A, Rossetto M, et al. Combined analysis of TERT, EGFR, and IDH status defines distinct prognostic glioblastoma classes. *Neurology* (2014) 83(13):1200–6. doi: 10.1212/WNL.0000000000000814
70. Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-Branger D, Cavenee WK, et al. The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. *Acta Neuropathol* (2016) 131(6):803–20. doi: 10.1007/s00401-016-1545-1
71. Schiff D, Van den Bent M, Vogelbaum MA, Wick W, Miller CR, Taphoorn M, et al. Recent developments and future directions in adult lower-grade gliomas: Society for Neuro-Oncology (SNO) and European Association of Neuro-Oncology (EANO) consensus. *Neuro Oncol* (2019) 21(7):837–53. doi: 10.1093/neuonc/noz033
72. Parsons DW, Jones S, Zhang X, Lin JC, Leary RJ, Angenendt P, et al. An integrated genomic analysis of human glioblastoma multiforme. *Science* (2008) 321(5897):1807–12. doi: 10.1126/science.1164382
73. Perizzolo M, Winkfein B, Hui S, Krulicki W, Chan JA, Demetrick DJ. IDH mutation detection in formalin-fixed paraffin-embedded gliomas using multiplex PCR and single-base extension. *Brain Pathol* (2012) 22(5):619–24. doi: 10.1111/j.1750-3639.2012.00579.x
74. Capper D, Zentgraf H, Balss J, Hartmann C, von Deimling A. Monoclonal antibody specific for IDH1 R132H mutation. *Acta Neuropathol* (2009) 118(5):599–601. doi: 10.1007/s00401-009-0595-z

75. Santagata S, Eberlin LS, Norton I, Calligaris D, Feldman DR, Ide JL, et al. Intraoperative mass spectrometry mapping of an onco-metabolite to guide brain tumor surgery. *Proc Natl Acad Sci U S A* (2014) 111(30):11121–6. doi: 10.1073/pnas.1404724111
76. Choi C, Raisanen JM, Ganji SK, Zhang S, McNeil SS, An Z, et al. Prospective Longitudinal Analysis of 2-Hydroxyglutarate Magnetic Resonance Spectroscopy Identifies Broad Clinical Utility for the Management of Patients With IDH-Mutant Glioma. *J Clin Oncol* (2016) 34(33):4030–9. doi: 10.1200/JCO.2016.67.1222
77. Chen X, Conti PS, Moats RA. In vivo near-infrared fluorescence imaging of integrin alphavbeta3 in brain tumor xenografts. *Cancer Res* (2004) 64 (21):8009–14. doi: 10.1158/0008-5472.CAN-04-1956
78. Sun J, Cai L, Zhang K, Zhang A, Pu P, Yang W, et al. A pilot study on EGFR-targeted molecular imaging of PET/CT With 11C-PD153035 in human gliomas. *Clin Nucl Med* (2014) 39(1):e20–6. doi: 10.1097/RLU.0b013e3182a23b73
79. Luo H, Hong H, Slater MR, Graves SA, Shi S, Yang Y, et al. PET of c-Met in Cancer with (6)(4)Cu-Labeled Hepatocyte Growth Factor. *J Nucl Med* (2015) 56(5):758–63. doi: 10.2967/jnumed.115.154690
80. Gaedicke S, Braun F, Prasad S, Machein M, Firat E, Hettich M, et al. Noninvasive positron emission tomography and fluorescence imaging of CD133+ tumor stem cells. *Proc Natl Acad Sci U S A* (2014) 111(6):E692–701. doi: 10.1073/pnas.1314189111
81. Jing H, Weidensteiner C, Reichardt W, Gaedicke S, Zhu X, Grosu AL, et al. Imaging and Selective Elimination of Glioblastoma Stem Cells with Theranostic Near-Infrared-Labeled CD133-Specific Antibodies. *Theranostics* (2016) 6(6):862–74. doi: 10.7150/thno.12890
82. Andronesi OC, Loebel F, Bogner W, Marjanska M, Vander Heiden MG, Iafrate AJ, et al. Treatment Response Assessment in IDH-Mutant Glioma Patients by Noninvasive 3D Functional Spectroscopic Mapping of 2-Hydroxyglutarate. *Clin Cancer Res* (2016) 22(7):1632–41. doi: 10.1158/1078-0432.CCR-15-0656
83. de la Fuente MII, Young RJ, Rubel J, Rosenblum M, Tisnado J, Briggs S, et al. Integration of 2-hydroxyglutarate-proton magnetic resonance spectroscopy into clinical practice for disease monitoring in isocitrate dehydrogenase-mutant glioma. *Neuro Oncol* (2016) 18(2):283–90. doi: 10.1093/neuonc/nov307
84. Branzoli F, Di Stefano AL, Capelle L, Ottolenghi C, Valabregue R, Deelchand DK, et al. Highly specific determination of IDH status using edited in vivo magnetic resonance spectroscopy. *Neuro Oncol* (2018) 20(7):907–16. doi: 10.1093/neuonc/nox214
85. Suh CH, Kim HS, Jung SC, Choi CG, Kim SJ. Imaging prediction of isocitrate dehydrogenase (IDH) mutation in patients with glioma: a systemic review and meta-analysis. *Eur Radiol* (2019) 29(2):745–58. doi: 10.1007/s00330-018-5608-7
86. Andronesi OC, Arrillaga-Romany IC, Ly KII, Bogner W, Ratai EM, Reitz K, et al. Pharmacodynamics of mutant-IDH1 inhibitors in glioma patients probed by in vivo 3D MRS imaging of 2-hydroxyglutarate. *Nat Commun* (2018) 9(1):1474. doi: 10.1038/s41467-018-03905-6
87. Verger A, Metellus P, Sala Q, Colin C, Bialecki E, Taieb D, et al. IDH mutation is paradoxically associated with higher 18F-FDOPA PET uptake in diffuse grade II and grade III gliomas. *Eur J Nucl Med Mol Imaging* (2017) 44 (8):1306–11. doi: 10.1007/s00259-017-3668-6
88. Ginet M, Zaragori T, Marie PY, Roch V, Gauchotte G, Rech F, et al. Integration of dynamic parameters in the analysis of (18)F-FDopa PET imaging improves the prediction of molecular features of gliomas. *Eur J Nucl Med Mol Imaging* (2020) 47(6):1381–90. doi: 10.1007/s00259-019-04509-y
89. Wenger KJ, Hattingen E, Harter PN, Richter C, Franz K, Steinbach JP, et al. Fitting algorithms and baseline correction influence the results of non-invasive in vivo quantitation of 2-hydroxyglutarate with (1) H-MRS. *NMR BioMed* (2019) 32(1):e4027. doi: 10.1002/nbm.4027
90. Lu KV, Zhu S, Cvrljevic A, Huang TT, Sarkaria S, Ahkavan D, et al. Fyn and SRC are effectors of oncogenic epidermal growth factor receptor signaling in glioblastoma patients. *Cancer Res* (2009) 69(17):6889–98. doi: 10.1158/0008-5472.CAN-09-0347
91. Jeon HM, Kim SH, Jin X, Park JB, Joshi K, Nakano I, et al. Crosstalk between Glioma-Initiating Cells and Endothelial Cells Drives Tumor Progression. *Cancer Res* (2014) 74(16):4482–92. doi: 10.1158/0008-5472.CAN-13-1597
92. Clavreul A, Etcheverry A, Chassevent A, Quillien V, Avril T, Jourdan ML, et al. Isolation of a new cell population in the glioblastoma microenvironment. *J Neurooncol* (2012) 106(3):493–504. doi: 10.1007/s11060-011-0701-7
93. Huveldt D, Lewis-Tuffin LJ, Carlson BL, Schroeder MA, Rodriguez F, Giannini C, et al. Targeting Src family kinases inhibits bevacizumab-induced glioma cell invasion. *PLoS One* (2013) 8(2):e56505. doi: 10.1371/journal.pone.0056505
94. Lassman AB, Pugh SL, Gilbert MR, Aldape KD, Geinoz S, Beumer JH, et al. Phase 2 trial of dasatinib in target-selected patients with recurrent glioblastoma (RTOG 0627). *Neuro Oncol* (2015) 17(7):992–8. doi: 10.1093/neuonc/nov011
95. Galanis E, Anderson SK, Twohy EL, Carrero XW, Dixon JG, Tran DD, et al. A phase 1 and randomized, placebo-controlled phase 2 trial of bevacizumab plus dasatinib in patients with recurrent glioblastoma: Alliance/North Central Cancer Treatment Group N0872. *Cancer* (2019) 125(21):3790–800. doi: 10.1002/cncr.32340
96. Phuphanich S, Raizer J, Chamberlain M, Canelos P, Narwal R, Hong S, et al. Phase II study of MEDI-575, an anti-platelet-derived growth factor-alpha antibody, in patients with recurrent glioblastoma. *J Neurooncol* (2017) 131 (1):185–91. doi: 10.1007/s11060-016-2287-6
97. Plate KH, Breier G, Weich HA, Mennel HD, Risau W. Vascular endothelial growth factor and glioma angiogenesis: coordinate induction of VEGF receptors, distribution of VEGF protein and possible in vivo regulatory mechanisms. *Int J Cancer* (1994) 59(4):520–9. doi: 10.1002/ijc.2910590415
98. Kreisl TN, Kim L, Moore K, Duic P, Royce C, Stroud I, et al. Phase II trial of single-agent bevacizumab followed by bevacizumab plus irinotecan at tumor progression in recurrent glioblastoma. *J Clin Oncol* (2009) 27(5):740–5. doi: 10.1200/JCO.2008.16.3055
99. Gilbert MR, Dignam JJ, Armstrong TS, Wefel JS, Blumenthal DT, Vogelbaum MA, et al. A randomized trial of bevacizumab for newly diagnosed glioblastoma. *N Engl J Med* (2014) 370(8):699–708. doi: 10.1056/NEJMoa1308573
100. Chinot OL, Wick W, Mason W, Henriksson R, Saran F, Nishikawa R, et al. Bevacizumab plus radiotherapy-temozolomide for newly diagnosed glioblastoma. *N Engl J Med* (2014) 370(8):709–22. doi: 10.1056/NEJMoa1308345
101. Hovinga KE, McCrea HJ, Brennan C, Huse J, Zheng J, Esquenazi Y, et al. EGFR amplification and classical subtype are associated with a poor response to bevacizumab in recurrent glioblastoma. *J Neurooncol* (2019) 142(2):337–45. doi: 10.1007/s11060-019-03102-5
102. Thomas AA, Omuro A. Current role of anti-angiogenic strategies for glioblastoma. *Curr Treat Options Oncol* (2014) 15(4):551–66. doi: 10.1007/s11864-014-0308-2
103. Nedergaard MK, Michaelsen SR, Perryman L, Erler J, Poulsen HS, Stockhausen MT, et al. Comparison of (18)F-FET and (18)F-FLT small animal PET for the assessment of anti-VEGF treatment response in an orthotopic model of glioblastoma. *Nucl Med Biol* (2016) 43(3):198–205. doi: 10.1016/j.nucmedbio.2015.12.002
104. Mattern RH, Read SB, Pierschbacher MD, Sze CII, Eliceiri BP, Kruse CA. Glioma cell integrin expression and their interactions with integrin antagonists: Research Article. *Cancer Ther* (2005) 3A:325–40.
105. Schittenhelm J, Schwab EII, Sperveslage J, Tatagiba M, Meyermann R, Fend F, et al. Longitudinal expression analysis of alphav integrins in human gliomas reveals upregulation of integrin alphavbeta3 as a negative prognostic factor. *J Neuropathol Exp Neurol* (2013) 72(3):194–210. doi: 10.1097/NEN.0b013e3182851019
106. MacDonald TJ, Stewart CF, Kocak M, Goldman S, Ellenbogen RG, Phillips P, et al. Phase I clinical trial of cilengitide in children with refractory brain tumors: Pediatric Brain Tumor Consortium Study PBTC-012. *J Clin Oncol* (2008) 26(6):919–24. doi: 10.1200/JCO.2007.14.1812
107. Stupp R, Hegi ME, Neyns B, Goldbrunner R, Schlegel U, Clement PM, et al. Phase I/IIa study of cilengitide and temozolomide with concomitant radiotherapy followed by cilengitide and temozolomide maintenance therapy in patients with newly diagnosed glioblastoma. *J Clin Oncol* (2010) 28(16):2712–8. doi: 10.1200/JCO.2009.26.6650
108. Gilbert MR, Kuhn J, Lamborn KR, Lieberman F, Wen PY, Mehta M, et al. Cilengitide in patients with recurrent glioblastoma: the results of NABTC 03-02, a phase II trial with measures of treatment delivery. *J Neurooncol* (2012) 106(1):147–53. doi: 10.1007/s11060-011-0650-1

109. Ishida J, Onishi M, Kurozumi K, Ichikawa T, Fujii K, Shimazu Y, et al. Integrin inhibitor suppresses bevacizumab-induced glioma invasion. *Transl Oncol* (2014) 7(2):292–302 e1. doi: 10.1016/j.tranon.2014.02.016
110. Nabors LB, Fink KL, Mikkelsen T, Grujicic D, Tarnawski R, Nam DH, et al. Two cilengitide regimens in combination with standard treatment for patients with newly diagnosed glioblastoma and unmethylated MGMT gene promoter: results of the open-label, controlled, randomized phase II CORE study. *Neuro Oncol* (2015) 17(5):708–17. doi: 10.1093/neuonc/nou356
111. Stupp R, Hegi ME, Gorlia T, Erridge SC, Perry J, Hong YK, et al. Cilengitide combined with standard treatment for patients with newly diagnosed glioblastoma with methylated MGMT promoter (CENTRIC EORTC 26071-22072 study): a multicentre, randomised, open-label, phase 3 trial. *Lancet Oncol* (2014) 15(10):1100–8. doi: 10.1016/S1470-2045(14)70379-1
112. Chinot OL. Cilengitide in glioblastoma: when did it fail? *Lancet Oncol* (2014) 15(10):1044–5. doi: 10.1016/S1470-2045(14)70403-6
113. Sipkins DA, Cheresh DA, Kazemi MR, Nevin LM, Bednarski MD, Li KC. Detection of tumor angiogenesis in vivo by alphaVbeta3-targeted magnetic resonance imaging. *Nat Med* (1998) 4(5):623–6. doi: 10.1038/nm0598-623
114. Cai W, Sam Gambhir S, Chen X. Multimodality tumor imaging targeting integrin alphavbeta3. *Biotechniques* (2005) 39(6 Suppl):S14–25. doi: 10.2144/000112091
115. Handgraaf HJM, Boonstra MC, Prevoo H, Kuil J, Bordo MW, Boogerd LSF, et al. Real-time near-infrared fluorescence imaging using cRGD-ZW800-1 for intraoperative visualization of multiple cancer types. *Oncotarget* (2017) 8 (13):21054–66. doi: 10.18632/oncotarget.15486
116. Jin ZH, Furukawa T, Degardin M, Sugyo A, Tsuji AB, Yamasaki T, et al. alphaVbeta3 Integrin-Targeted Radionuclide Therapy with 64Cu-cyclam-RAFT-c(-RGDfK-)4. *Mol Cancer Ther* (2016) 15(9):2076–85. doi: 10.1158/1535-7163.MCT-16-0040
117. Iagaru A, Mosci C, Shen B, Chin FT, Mittra E, Telli ML, et al. (18)F-FPPRGD2 PET/CT: Pilot Phase Evaluation of Breast Cancer Patients. *Radiology* (2014) 273(2):549–59. doi: 10.1148/radiol.14140028
118. Zhang H, Liu N, Gao S, Hu X, Zhao W, Tao R, et al. Can an (1)(8)F-ALF-NOTA-PRGD2 PET/CT Scan Predict Treatment Sensitivity to Concurrent Chemoradiotherapy in Patients with Newly Diagnosed Glioblastoma? *J Nucl Med* (2016) 57(4):524–9. doi: 10.2967/jnumed.115.165514
119. Heimberger AB, Crotty LE, Archer GE, Hess KR, Wikstrand CJ, Friedman AH, et al. Epidermal growth factor receptor VIII peptide vaccination is efficacious against established intracerebral tumors. *Clin Cancer Res* (2003) 9 (11):4247–54.
120. Heimberger AB, Hlatky R, Suki D, Yang D, Weinberg J, Gilbert M, et al. Prognostic effect of epidermal growth factor receptor and EGFRvIII in glioblastoma multiforme patients. *Clin Cancer Res* (2005) 11(4):1462–6. doi: 10.1158/1078-0432.CCR-04-1737
121. Schuster J, Lai RK, Recht LD, Reardon DA, Paleologos NA, Groves MD, et al. multicenter trial of rindopepimut (CDX-110) in newly diagnosed glioblastoma: the ACT III study. *Neuro Oncol* (2015) 17(6):854–61. doi: 10.1093/neuonc/nou348
122. Weller M, Butowski N, Tran DD, Recht LD, Lim M, Hirte H, et al. Rindopepimut with temozolomide for patients with newly diagnosed, EGFRvIII-expressing glioblastoma (ACT IV): a randomised, double-blind, international phase 3 trial. *Lancet Oncol* (2017) 18(10):1373–85. doi: 10.1016/S1470-2045(17)30517-X
123. Binder DC, Ladomersky E, Lenzen A, Zhai L, Lauing KL, Otto-Meyer SD, et al. Lessons learned from rindopepimut treatment in patients with EGFRvIII-expressing glioblastoma. *Transl Cancer Res* (2018) 7(Suppl 4): S510–3.
124. Moriyama T, Kataoka H, Seguchi K, Tsubouchi H, Koono M. Effects of hepatocyte growth factor (HGF) on human glioma cells in vitro: HGF acts as a motility factor in glioma cells. *Int J Cancer* (1996) 66(5):678–85. doi: 10.1002/(SICI)1097-0215(19960529)66:5<678::AID-IJC16>3.0.CO;2-0
125. Li MY, Yang P, Liu YW, Zhang CB, Wang KY, Wang YY, et al. Low c-Met expression levels are prognostic for and predict the benefits of temozolomide chemotherapy in malignant gliomas. *Sci Rep* (2016) 6:21141. doi: 10.1038/srep21141
126. Nabeshima K, Shimao Y, Sato S, Kataoka H, Moriyama T, Kawano H, et al. Expression of c-Met correlates with grade of malignancy in human astrocytic tumours: an immunohistochemical study. *Histopathology* (1997) 31(5):436–43. doi: 10.1046/j.1365-2559.1997.3010889.x
127. Bell EH, Pugh SL, McElroy JP, Gilbert MR, Mehta M, Klimowicz AC, et al. Molecular-Based Recursive Partitioning Analysis Model for Glioblastoma in the Temozolomide Era: A Correlative Analysis Based on NRG Oncology RTOG 0525. *JAMA Oncol* (2017) 3(6):784–92. doi: 10.1001/jamaoncol.2016.6020
128. Wen PY, Schiff D, Cloughesy TF, Raizer JJ, Laterra J, Smitt M, et al. A phase II study evaluating the efficacy and safety of AMG 102 (rilotumumab) in patients with recurrent glioblastoma. *Neuro Oncol* (2011) 13(4):437–46. doi: 10.1093/neuonc/noq198
129. Smith MR, Sweeney CJ, Corn PG, Rathkopf DE, Smith DC, Hussain M, et al. Cabozantinib in Chemotherapy-Pretreated Metastatic Castration-Resistant Prostate Cancer: Results of a Phase II Nonrandomized Expansion Study. *J Clin Oncol* (2014) 32(30):3391–9. doi: 10.1200/JCO.2013.54.5954
130. Elisei R, Schlumberger MJ, Muller SP, Schoffski P, Brose MS, Shah MH, et al. Cabozantinib in progressive medullary thyroid cancer. *J Clin Oncol* (2013) 31 (29):3639–46. doi: 10.1200/JCO.2012.48.4659
131. Wen PY, Drappatz J, de Groot J, Prados MD, Reardon DA, Schiff D, et al. Phase II study of cabozantinib in patients with progressive glioblastoma: subset analysis of patients naive to antiangiogenic therapy. *Neuro Oncol* (2018) 20(2):249–58. doi: 10.1093/neuonc/nox154
132. Cloughesy TF, Drappatz J, de Groot J, Prados MD, Reardon DA, Schiff D, et al. Phase II study of cabozantinib in patients with progressive glioblastoma: subset analysis of patients with prior antiangiogenic therapy. *Neuro Oncol* (2018) 20(2):259–67. doi: 10.1093/neuonc/nox151
133. Du W, Uslar L, Sevala S, Shah K. Targeting c-Met receptor overcomes TRAIL-resistance in brain tumors. *PLoS One* (2014) 9(4):e95490. doi: 10.1371/journal.pone.0095490
134. Towner RA, Jensen RL, Colman H, Vaillant B, Smith N, Casteel R, et al. ELTD1, a potential new biomarker for gliomas. *Neurosurgery* (2013) 72 (1):77–90. doi: 10.1227/NEU.0b013e318276b29d. discussion 91.
135. Cho JH, Kim AR, Kim SH, Lee SJ, Chung H, Yoon MY. Development of a novel imaging agent using peptide-coated gold nanoparticles toward brain glioma stem cell marker CD133. *Acta Biomater* (2017) 47:182–92. doi: 10.1016/j.actbio.2016.10.009
136. Ziegler J, Pody R, Coutinho de Souza P, Evans B, Saunders D, Smith N, et al. ELTD1, an effective anti-angiogenic target for gliomas: preclinical assessment in mouse GL261 and human G55 xenograft glioma models. *Neuro Oncol* (2017) 19(2):175–85. doi: 10.1093/neuonc/now147
137. Weller M, Stupp R, Hegi M, Wick W. Individualized targeted therapy for glioblastoma: fact or fiction? *Cancer J* (2012) 18(1):40–4. doi: 10.1097/PPO.0b013e318243f6c9
138. Guidance for Industry, Investigators, and Reviewers. Exploratory IND Studies. *Biotechnol Law Rep* (2005) 24(5):603–11. doi: 10.1089/blr.2006.25.167
139. *Phase III Study of Rindopepimut/GM-CSF in Patients With Newly Diagnosed Glioblastoma (ACT IV)*. NIH. U.S. National Library of Medicine, ClinicalTrials. gov (2011). Available at: http://clinicaltrials.gov/ct2/show/record/ NCT01480479.

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Chimeric Antigen Receptor T-Cell Therapy in Glioblastoma: Current and Future

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Glioblastoma (GBM) is a highly aggressive glioma with an extremely poor prognosis after conventional treatment. Recent advances in immunotherapy offer hope for these patients with incurable GBM. Our present review aimed to provide an overview of immunotherapy for GBM, especially chimeric antigen receptor T-cell (CAR T) therapy. CAR T-cell immunotherapy, which involves the engineering of T cells to kill tumors by targeting cell surface-specific antigens, has been successful in eliminating B-cell leukemia by targeting CD19. IL-13Rα2, EGFRvIII, and HER2-targeted CAR T cells have shown significant clinical efficacy and safety in phase 1 or 2 clinical trials conducted in patients with GBM; these findings support the need for further studies to examine if this therapy can ultimately benefit this patient group. However, local physical barriers, high tumor heterogeneity, and antigen escape make the use of CAR T therapy, as a treatment for GBM, challenging. The potential directions for improving the efficacy of CAR T in GBM are to combine the existing traditional therapies and the construction of multi-target CAR T cells.

Keywords: glioblastoma, brain tumor, immunotherapy, chimeric antigen receptor T cell therapy, CAR T

INTRODUCTION

Glioblastoma (GBM) is a highly aggressive, malignant, and undifferentiated glioma with a global incidence of 10/100,000, and frequently occurs in individuals aged between 55 and 60 years (1, 2). It is the most common type of astrocytoma with poor prognosis (3). After aggressive treatment, the median survival time is only 14–15 months post diagnosis, the 5-year survival rate is approximately 10%, and the final mortality rate is close to 100% (4). The etiology of GBM is poorly understood. To date, exposure to high doses of ionizing radiation is the only established risk factor (5). Cell phones, electromagnetic fields, occupational exposures, and formaldehyde have not been found to be associated with GBM (6). GBMs can be divided into primary GBMs (~90%) and secondary GBMs according to clinical and histological characteristics. Primary GBMs are without histologic or clinical evidence of a less malignant precursor change, whose majority develop quickly in elderly patients. Secondary GBMs progress from anaplastic astrocytoma or low-grade diffuse astrocytoma.

With a less degree of necrosis, they manifest in much younger patients (7). GBMs can also be classified into isocitrate dehydrogenase (IDH) wild type, which is generally equivalent to the primary GBMs, and IDH mutant type, which is mainly secondary glioblastoma based on the 2016 World Health Organization classification for tumors of the central nervous system (8). Maximum surgical resection in combination with radiotherapy and chemotherapy (temozolomide) has become the standard therapy for newly diagnosed GBM (9, 10). However, GBM recurrence is inevitable after a median survival time of 32–36 weeks. Once the disease recurs, it becomes resistant to drug treatment and is essentially incurable (11).

Due to the poor prognosis of patients treated with conventional therapies for GBM, attention has been shifted to other emerging treatments, such as immunotherapy (12). The immune system can detect and destroy tumor cells through the process of immunosurveillance. However, some tumor cells escape immunosurveillance and gradually develop into tumor lesions. The purpose of tumor immunotherapy is to overcome the immune resistance of tumor cells in order to treat the tumor. Immunotherapy includes vaccines, oncolytic virus therapies, checkpoint blockade, and adoptive T cells (13). Tumor immunotherapy has rapidly evolved in recent years and has shown promising results in a variety of tumors such as lung cancer (14), kidney cancer (15), and melanoma (16).

The most common strategy for direct recruitment of T cells is adoptive lymphocyte transfer. Autologous T cells that target tumor cells *in vitro* are trained, amplified, and activated, and then transferred to the patient's body. These genetically engineered T cells are specific to targeted cells and can strengthen tumor immunity. Adoptive T cells include tumor invasive lymphocytes, cytokine-induced killer cells, TCR engineered T cells, and chimeric antigen receptor T-cell (CAR T) therapies (17, 18). In fact, different immune therapies are often used in combination with other treatments rather than used alone for better clinical results. Among them, CAR T-cell therapies have achieved tremendous developments. Thus, we reviewed the current studies on CAR T-cell therapy for GBM, discussing the obstacles and future directions in this promising area of therapy.

OVERVIEWS OF CAR T-CELL THERAPIES IN GBM

CAR T cells are autologous or allogeneic modified T cells, which are collected from patients' peripheral blood, amplified *in vitro*, and remolded genetically to express CAR molecules on the cellular membrane *via* viral vectors or electroporation. Their extracellular domains could recognize tumor-specific antigens, while their intracellular domains contain T-cell activation signals. These modified T cells are then administered to the patient's body, where they could lyse cells that carry the relevant tumor antigens (19). The general flow of CAR T treatment in GBM is shown in **Figure 1**. Physiological antigens can recognize the TCR-CD3 complex in the extracellular region, which has six independent gene products: TCR α, β chains, and CD3 g, δ, ϵ, and ζ chains. The TCR α and β chains could bind to the HLA-peptide complex. The CD3 γ, δ, ϵ, and ζ chains can activate T cells (20). The intracellular signal domain of activated T cells usually contains a signaling domain, which could be recognized as the first-generation CAR without other signal domains. The addition of a co-stimulatory signal domain, usually CD28 or 41BB, produces a second-generation CAR. The third-generation of CAR is generated by the combination of several different co-stimulus proteins and multiple co-stimulus domains (21). This would induce the production of T cells and lead to the killing of cancer cells by cytotoxic cells (22, 23).

By 2020, the FDA has approved two CAR T-cell therapies for CD19+ B cell malignancies, named Yescarta and Kymriah (24). In a phase 2 study on patients with relapsed or refractory acute B-cell lymphoblastic leukemia, up to 81% of the patients experienced remission 3 months after CAR T-cell therapy. After 6 months, the survival rate was 73%, and the event-free rate was 90%. Moreover, after 12 months, the survival rate was 50%, and the event-free rate was 76% (25). Another multicenter phase 1–2 study, participated by 22 institutions, reached a similar conclusion (26).

In addition to successful clinical practice in above malignant hematological diseases, many clinical trials of CAR T therapy have also been carried out in other solid malignancies, including GBM (27), colorectal (28), pancreatic (29), renal cell (30), ovarian (31), and breast cancers (32). Although CAR T therapy has not yet entered clinical practice for solid tumors, it has given hope to patients with other cancer types who have few treatment options. The following chapters will focus on the progress of CAR T therapy in GBM.

CLINICAL APPLICATION OF CAR T-CELL THERAPIES IN GBM

To date, due to the lack of tumor-specific antigens expressed in GBM, the application of CAR T cells in GBM is still limited (33). However, with the emergence of the second- and third-generation CAR, it is possible to overcome the low heterogeneity of GBM tumors and achieve a better clinical effect. In order to cover the published trial results in this area, we searched PubMed and ClinicalTrials.gov (https://clinicaltrials.gov/ct2/home) for GBM trials conducted until June 2020. We found 18 clinical trials regarding CAR T-cell therapy for GBM, including trials on different CAR T-cell targets and different therapy combination strategies such as combined chemotherapy or immune checkpoint blockade. However, only three studies related to CAR T-cell targets published the clinical responses. **Table 1** shows that interleukin-13 receptor alpha 2 (IL13-Rα2) (37), human epidermal growth factor receptor 2 (HER2) (36), and epidermal growth factor receptor variant III (EGFRvIII) (35) have been clinically verified as effective and safe targets of CAR T-cell therapy for GBM.

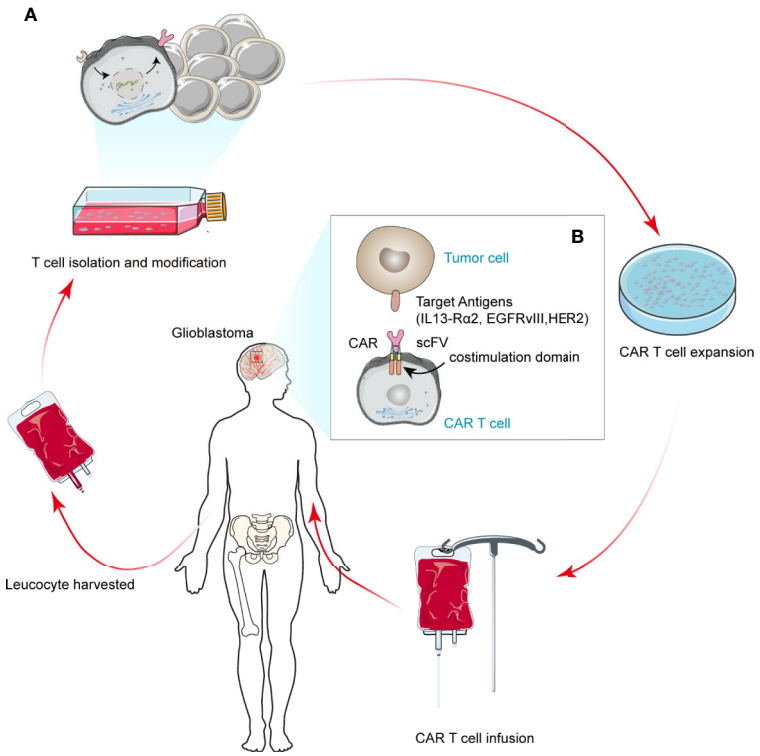

FIGURE 1 | Schematic depicting regulatory CAR T therapy in GBM. A modified CAR T cell can recognize tumor cell surface antigens in an MHC-independent manner, thus inducing tumor cell death. Currently, the antigens available for clinical trials for GBM tumor cells are IL13-Rα2, HER2, and EGFRvIII. The scFv represents a single variable region of antibody expression in T cells.

TABLE 1 | Published clinical trials of CAR T therapy in GBM.

Study	Target	Results
Goff et al. (34)	EGFRvIII	No clinically meaningful effect was evaluated in 18 patients.
O'Rourke et al. (35)	EGFRvIII	Nine patients had a stable condition for 28 days, while the rest showed disease progression at day 28.
Ahmed et al. (36)	HER2	One patient showed partial response for 9 months, seven had a stable condition for 8 weeks to 29 months, and eight experienced disease progression.
Brown et al. (37)	IL13-Rα2	One patient achieved complete response for 7.5 months.
Brown et al. (38)	IL13-Rα2	IL13-Rα2-specific CAR T cells could be used in the treatment of GBM.

IL13-Rα2

IL13-Rα2 is a monomer of IL13 with a high affinity receptor. It is overexpressed in almost all tumors related to the glomerular basement membrane, but not in healthy tissues. IL13-Rα2 is rarely expressed in normal brain cells but is highly expressed in GBMs. This specificity made it an ideal target for CAR T-cell therapy in GBM. With the increase in malignancy, the expression of IL13-Rα2 also increased. IL13-Rα2 is also considered as a prognostic indicator (39, 40).

In the immune system, IL13 is usually expressed in the sharing receptors with its homologue IL4 in several normal tissues, modulating the immune responses. A previous study showed that almost all GBMs highly express IL13 receptors. In contrast to other tissues, the IL13 receptors on GBMs are independent of IL4, leading to the discovery of IL13-Rα2 (40).

In 2004, researchers described a novel method for targeting GBM multiforme with IL13-Rα2-specific CAR T cells by their genetic alteration to express a membrane-tethered IL13 cytokine chimeric T-cell antigen receptor (also known as zetakine). The adoptive transfer of IL13-zetakine (+) CD8(+) CTL clones led to the regression of verified human GBM orthotopic xenografts *in vivo* (41). In 2015 and 2016, Brown et al. reported that several patients with recurrent and refractory GBM received CAR T cells targeting IL13Rα2. As this therapy was well tolerated, the patients' brain inflammation was temporarily managed. After treatment, the overall expression of IL13-Rα2 in some patients decreased, while the tumor necrotic volume increased at the site of IL13-zetakine(+) T-cell administration (37, 38). Subsequently, the structure of CAR T cells was optimized to achieve a better clinical effect. In 2018, Brown described the optimization of IL13-Rα2-targeted CAR T cells. They designed a 4-1BB (CD137) co-

stimulatory CAR (IL13BBzeta) and constructed a manufacturing platform using enhanced central memory T cells. This study revealed that IL13BBzeta-CAR T cells increased the T-cell persistence and anti-tumor activity. Moreover, compared with intravenous administration, the CAR T cells' local intracranial delivery elicited better anti-tumor efficacy. However, intraventricular infusions exhibited more benefits than intracranial tumor infusions in a multifocal disease model (42).

HER2

HER2 is a transmembrane tyrosine kinase receptor expressed in various normal tissues. This protein participates in the development and progression of several tumors, such as breast cancer, ovarian cancer, gastric cancer, osteosarcoma, and medulloblastoma (43, 44).

In animal models, CAR T cells targeting HER2 presented better anti-tumor activity and survival rate (45). Being a validated immunotherapy target for GBM, HER2 is expressed in nearly 80% GBM patients. Generated from GBM patients, HER2-specific T cells could target their CD133+ stem cell compartment and autologous HER2-positive GBMs (46).

Another clinical study consisting of 10 consecutive GBM patients revealed that HER2-specific T cells could stimulate T-cell proliferation and secretion of IFN-gamma and IL-2 in HER2 + autologous GBM cells. Derived from primary HER2+ GBMs, these HER2-specific T cells could killed CD133+ and CD133− cells, whereas HER2-negative tumors were not killed (46). Another study included 17 patients with progressive HER2+ GBM. Without prior lymphodepletion, they received one or more autologous HER2-CAR VST mixtures. Being well tolerated, infusions did not show any dose-limiting toxic effects. Among these 16 evaluable patients, 1 showed a partial response for over 9 months, 7 had a stable condition for 8 weeks to 29 months, 8 had disease progression after T-cell infusion during the 24–29 months of follow-up, and 3 with stable conditions survived without any signs of disease progression (36). Moreover, combined with other targets, HER2 is often applied in the study of second- or third-generation CAR T-cell therapy, which would be explained in detail in the following combined therapies.

EGFRvIII

EGFRvIII is expressed in the absence of wild-type EGFR proteins, which produces constitutionally active receptor (ligand independent) and two distantly combined epitopes from the extracellular domains. EGFRvIII was initially discovered in a primary human GBM, which was expressed in nearly 30% of GBM samples. In addition to poor prognosis, EGFRvIII could enhance proliferation, angiogenesis, and invasion of glioma cells (47). Highly tumor-heterogeneous EGFRvIII can induce phenotypic transformation. It is overexpressed in GBM cells, but not in normal cells. Considering these findings, EGFRvIII is an effective target for CAR T cell therapy in GBM (48).

In 2014, Miao observed that in areas with invasive tumor, EGFRvIII-CAR T cells were overexpressed, which suppressed the tumor growth and improved the survival time of mice (49). A study of 10 patients with recurrent GBM reported that the manufacturing and infusion of EGFRvIII-CAR T cells are safe and profitable, without evidence of cytokine release syndrome or off-tumor toxicity (35). However, other studies have shown that EGFRvIII-CAR T cells have a limited effect on GBM. In 2019, another clinical study involving 18 GBM patients who were treated with anti-EGFRvIII CAR T cells, showed a median progression-free survival time of 1.3 months with a single outlier of 12.5 months. Although cell dose would influence the persistence of CAR cells, objective responses were rarely observed. In this phase I pilot trial, the application of anti-EGFRvIII CAR T cells did not show clinically meaningful effects in GBM patients (34).

Subsequently, more trials focused on the modification of anti-EGFRvIII CAR T cells, such as BiTE-EGFR CAR T cells, PDIA3 mutant EGFRvIII CAR T cells, and EGFR806-CAR T cells. They improved the efficacy and safety of CAR T cells as a treatment for GBM (50, 51).

Novel Targets

Considering the profound tumor heterogeneity of GBM, scientists have been exploring new effective targets for GBM. Cluster of differentiation 70 (CD70) is overexpressed in glioma cells, but not in peripheral and normal brain tissues, and is associated with immune-related cell infiltration (52). This finding suggests that CD70 may be a potential new CAR T therapeutic target for GBM; however, further studies are still needed. Glioma-associated antigen ephrin type A receptor 2 (EphA2) is highly expressed primarily in the GBM cells of the brain. EphA2 has successfully exhibited an anti-tumor activity as a target for CAR T therapy in a glioma xenograft model; however, data on the duration of remission are limited (53). Chondroitin sulphate proteoglycan 4 is not only highly expressed in GBM specimens but it also has limited heterogeneity, which can be induced by TNFα. The use of this antigen has been shown to be effective against glioma cells in an *in vitro* CAR T therapy study (54). B7-H3, also known as CD206, is highly expressed in most malignant tumors, including high-grade brain tumors and sarcomas, but is rarely expressed in normal human tissues (55, 56). Although the role of B7-H3 in immune regulation remains unclear, there is no doubt that its overexpression is associated with tumor metastasis, invasion, and malignancy (57). Therefore, it is an attracting target for cancer immunotherapy. B7-H3-specific monoclonal antibodies, MGA271 (58) and 8H9 (59), have shown anti-tumor effects in preclinical mouse model studies and are well tolerated in phase I clinical trials (60). One CAR T therapy targeting B7-H3 has indicated good anti-tumor activity on GBM at both cellular and mouse levels (61). Chlorotoxin, a natural 36-amino acid peptide, has the potential bind to GBM while showing minimal cross-reactivity with normal cells in the brain (62). This provides an opportunity to expand target antigens for GBM CAR T therapy. Chlorotoxin-CAR T therapy presents a strong anti-tumor effect in patient-derived GBM cell lines and mouse xenograft models without significant toxicity to normal cells (63). In addition, the novel antigenic targets currently reported are summarized in **Table 2**.

TABLE 2 | Published novel tumor antigen targets in CAR T therapy for GBM.

Study	Target	Conclusions
Jin et al. (64)	CXCR1-or CXCR2	CXCR1 or CXCR2 modified CAR T cells were capable of tumor regression in the GBM preclinical model.
Tang et al. (61)	B7-H3	B7-H3 is overexpressed in GBM patients and can be a therapeutic target.
Yang et al. (65)	NKG2D-BBz	NKG2D CAR-T cells targeted glioblastoma cells and cancer stem cells in an NKG2D-dependent manner.
Wallstabe et al. (66)	alphavbeta3	Alphavbeta3 can enhance CAR reactivity.
Yi et al. (53)	EphA2	EphA2-CAR T cells therapy has been shown to be effective in a preclinical model.
Pellegatta et al. (54)	CSPG4	The expression level of CSPG4 in GBM was high and the heterogeneity was not obvious.
Ge et al. (67)	CD70	CD70 is associated with tumor progression.
Zhu et al. (68)	CD57	CD57 was significantly upregulated in activated human T cells.
Wang et al. (63)	Chlorotoxin	Chlorotoxin-CAR T therapy mediated potent anti-tumor activity in the orthotopic xenograft GBM models.

However, none of these targets have achieved the results of previous clinical trials.

OBSTACLES AND IMPROVE STRATEGIES IN CAR T THERAPY FOR GBM

Although several studies have been conducted and advancements have been made on CAR T for GBM, the actual clinical effect of CAR T in GBM is not promising, which is mainly caused by physical barriers, antigen escape, and tumor heterogeneity. Although GBM has been shown to be complex in immunotherapy, several strategies have shed some light on the increased efficiency of CAR T in this disease.

Blood-Brain Barrier

Historically, the brain has been recognized as an immune-privileged region. The lack of traditional lymphatic vessels and well-known antigen-presenting cells further underpinned this theory (69). Thus, GBM is an immunologically quiet tumor. In addition, the blood-brain barrier (BBB) prevents the entry of almost all large molecules and 98% of small molecules to the central nervous system, limiting the effective delivery of drugs to the tumor site (70) (**Figure 2A**).

Immune checkpoints are molecules on the surface of activated T cells that act as "brakes" to prevent the occurrence of inflammatory responses due to an immunodeficiency. The classic checkpoint of CTLA-4 or PD-1 could result in the inactivation or even death of T cells. Blockage or antagonization

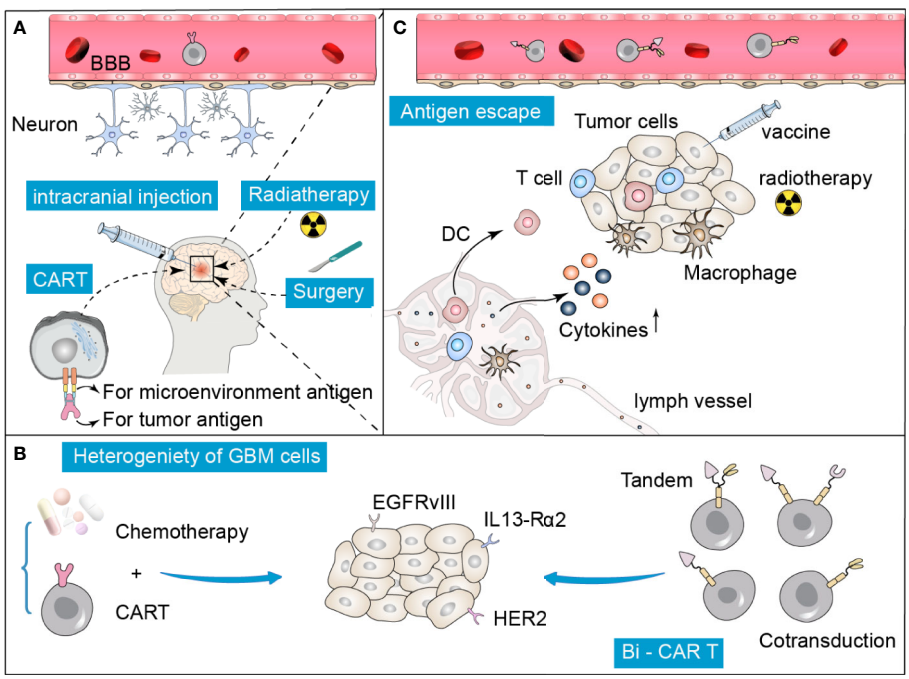

FIGURE 2 | Summary of difficulties and possible improvement strategies in CAR T treatment for GBM. **(A)** The specific anatomy of the blood-brain barrier (BBB) prevents the entry of many drugs, including CAR T cells, which can be overcome to some extent by intracranial direct injection, radiation, or surgery. **(B, C)** Tumor heterogeneity and antigen escape are the two major reasons that limit the efficacy of CAR T therapy. These obstacles can be overcome by administering a combination of traditional treatments, such as chemotherapy, radiation, and adjuvant vaccines. In addition, the construction of multi-target specific CAR T cells is also a promising approach.

of these signals can persistently activate the production of T cells (71). Checkpoint blockade showed promising clinical efficacy in many tumors; however, GBM is often resistant (72). Compared with other tumors that are responsive to immunotherapy, GBM has a notoriously low mutational burden, resulting in the less production of tumor-infiltrating T cells (73, 74). In addition, repeated immune activation in the intracranial space would promote clinical hazards, including cytokine release syndrome and autoimmune encephalitis (75).

Heterogeneity of GBM and Antigen Escape

GBM is also a remarkably heterogeneous tumor that facilitates immune escape, which may be the largest obstacle (13, 76, 77). According to the gene expression analysis, GBM can be divided into four subtypes: typical, neuroural, proneural, and mesocytic (78). Even within the same tumor specimen, GBM showed significant heterogeneity. Sottoriva et al. found that there were different subtypes of tumor fragments in different spaces of GBM (79). The presence of heterogeneity made it difficult to implement the CAR T therapy and decreased the effectiveness of immunotherapy (80, 81). CD19 CAR trials showed that a major cause of resistance to treatment is the loss of target antigen. Moreover, the loss of antigen will likely block the effect of CAR therapy in solid tumors. A potential approach to overcome this obstacle is combinational targeting of multiple GBM antigens to enhance the tumor killing activity and reduce the antigen escape.

It is difficult to identify a single universal antigen for GBM because of the highly complex tumor heterogeneity in both different GBM patients and among different GBM subtypes (82). However, multiple antigen-targeted CARs with multiple specificity, including CAR T hybrid cell populations expressing two antigens in tandem or multiple antigens to overcome tumor heterogeneity (**Figure 2B**), have shown encouraging anti-tumor efficacy and safety in preliminary studies (83). T cells co-expressing HER2 and IL13-Rα2 CARs could effectively target and kill tumor cells that express either HER2 or IL13-Rα2, and showed particularly enhanced anti-tumor activity and antigen-dependent downstream signals compared with a single targeting strategy (45). Trivalent CAR T cells, targeting HER2, IL13-Rα2, and EphA2, exhibited excellent anti-tumor activity *in vitro* in the GBM model (84). Although combination therapies have shown promise in addressing tumor heterogeneity, further optimization is needed in terms of the number and combination of target antigens.

GBM contains self-renewing and multipotent subpopulation of cells, defined as cancer stem cell (CSC) that contributed to tumorigenesis, recurrence, and high therapeutic resistance (85). GBM appears to originate not from a single cell type but from a variety of seed cells, suggesting heterogeneity in CSC themselves (86). The discovery of CSC and its role in the pathogenesis of GBM suggest that it may be a new therapeutic target. IL13-Rα2 specific CAR therapy has been shown to kill both GBM cells and CSC in animal models (87). A preclinical study of oncolytic virus therapy based on neural stem cell delivery has shown great promise in GBM (88). This suggests that therapies targeting stem cells may be beneficial in overcoming tumor heterogeneity and antigen escape. CD133 positive CSCs are present in a variety of solid tumors, including GBM (89). In a phase I clinical study of CD133 targeted CAR T therapy for 23 advanced digestive system malignancies, 3 patients achieved partial remission and 14 had stable disease, showing good anti-tumor efficacy and controlled toxicity (90). Compared to CD133 monoclonal antibody therapy and dual antigen T cell engager antibody therapy, CD133 specific CAR T had enhanced activity in patient-derived models of GBM without acute systemic toxicity (91). In addition, others such as CD15, integrin α6, CD44, and L1CAM have also been suggested as potential markers of CSCs (92). Given that most of these markers are also present in normal stem cells, targeting these markers need to be studied with caution, because it may result in a potential overlap between the CSC and the stem cells of normal cells. A more specific CSC surface marker may be one of the best treatment options in the future.

Combination With Traditional Therapy Approaches

Immune cells, including T cells, are severely restricted from entering the brain because of the BBB (93). How to efficiently transfer the modified CAR T cells to tumor lesions in the brain still needs to be explored further. Systematic and regional delivery methods have been successfully used to enhance the trafficking of CAR T cells to the tumor sites (94). Radiotherapy and surgery can damage the BBB to some extent, which provides a promising option to be combined with CAR T therapy. Immune checkpoint inhibitors combined with stereotactic radiotherapy have shown superior efficacy in preclinical glioma models (95). A study showed that direct intratumoral injection improved the anti-CAIX CAR-T potency by restricting its off-target effects (96) (**Figure 2A**). However, there may be a lower risk of off-target toxicity. Further studies are needed to describe and compare the T-cell persistence and overall therapeutic effect associated with regional and systemic delivery of CAR T-cell therapy. Pre-treatment with chemotherapy can reduce the production of regular T cells and activate the anti-tumor response. In relapsed/refractory chronic lymphocytic leukemia, CD-19-targeted CAR T therapy following conditional chemotherapy increases its efficacy (97). Bevacizumab, a vascular endothelial growth factor inhibitor, increases the lymphocyte infiltration and inhibits the occurrence of immunosuppression caused by VEGF. A previous study showed that GD2 CAR-T cell therapy combined with bevacizumab can enhance its anticancer efficacy in a preclinical model of neuroblastoma (98). In gliomas, temozolomide chemotherapy is usually associated with the occurrence of lymphotoxicity. Sequential CAR T therapy after a dose-intensified regimen of temozolomide chemotherapy has been shown to improve CAR engraftment and anticancer activity in rodent models (99). In addition, CAR T cells, with the aid of anticancer vaccines, significantly extended the survival in a GBM

mouse model, and no significant side effects were observed (100). Conventional therapies, such as chemotherapy and radiation, as well as vaccines, may help CAR T therapy overcome the problems of tumor heterogeneity and antigen escape in patients with GBM (**Figures 2B, C**).

CONCLUSION

Immunotherapy has revolutionized the overall treatment strategy for many solid tumors and is also expected to improve the response of GBM patients to treatment. In the treatment of GBM, CAR T-cell therapies, especially the second- and third-generation CAR T-cell therapies, have achieved promising preclinical efficacy to prolong the survival time of patients. However, due to the location and particularity of GBM, no phase III clinical trial results have been published. Hence, further studies are needed to modify CAR T cells and their targets to improve their clinical efficacy.

AUTHOR CONTRIBUTIONS

LL and XZ wrote the paper. LL drew the figures. YW, XH, XY, and DH designed the framework and content of the article. YQ and YD searched the literatures. YW and XH reversed the article. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Stupp R, Taillibert S, Kanner A, Read W, Steinberg D, Lhermitte B, et al. Effect of tumor-treating fields plus maintenance temozolomide vs maintenance temozolomide alone on survival in patients with glioblastoma: a randomized clinical trial. *JAMA* (2017) 318:2306–16. doi: 10.1001/jama.2017.18718
2. Thakkar JP, Dolecek TA, Horbinski C, Ostrom QT, Lightner DD, Barnholtz-Sloan JS, et al. Epidemiologic and molecular prognostic review of glioblastoma. *Cancer Epidemiol Biomarkers Prev* (2014) 23:1985–96. doi: 10.1158/1055-9965.EPI-14-0275
3. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. *CA Cancer J Clin* (2019) 69:7–34. doi: 10.3322/caac.21551
4. Stupp R, Brada M, van den Bent MJ, Tonn JC, Pentheroudakis G. High-grade glioma: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. *Ann Oncol* (2014) 25(Suppl 3):i93–i101. doi: 10.1093/annonc/mdu050
5. Hanif F, Muzaffar K, Perveen K, Malhi SM, Simjee S. Glioblastoma Multiforme: A review of its epidemiology and pathogenesis through clinical presentation and treatment. *Asian Pac J Cancer Prev* (2017) 18:3–9. doi: 10.22034/APJCP.2017.18.1.3
6. Davis ME. Glioblastoma: Overview of disease and treatment. *Clin J Oncol Nurs* (2016) 20:S2–8. doi: 10.1188/16.CJON.S1.2-8
7. Ohgaki H, Kleihues P. The definition of primary and secondary glioblastoma. *Clin Cancer Res* (2013) 19:764–72. doi: 10.1158/1078-0432.CCR-12-3002
8. Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-Branger D, Cavenee WK, et al. The 2016 World Health Organization Classification of tumors of the central nervous system: a summary. *Acta Neuropathol* (2016) 131:803–20. doi: 10.1007/s00401-016-1545-1
9. Ghiaseddin A, Peters KB. Use of bevacizumab in recurrent glioblastoma. *CNS Oncol* (2015) 4:157–69. doi: 10.2217/cns.15.8
10. Cohen MH, Johnson JR, Pazdur R. Food and Drug Administration Drug approval summary: temozolomide plus radiation therapy for the treatment of newly diagnosed glioblastoma multiforme. *Clin Cancer Res* (2005) 11:6767–71. doi: 10.1158/1078-0432.CCR-05-0722
11. Osuka S, Van Meir EG. Overcoming therapeutic resistance in glioblastoma: the way forward. *J Clin Invest* (2017) 127:415–26. doi: 10.1172/JCI89587
12. Jackson CM, Choi J, Lim M. Mechanisms of immunotherapy resistance: lessons from glioblastoma. *Nat Immunol* (2019) 20:1100–9. doi: 10.1038/s41590-019-0433-y
13. Medikonda R, Dunn G, Rahman M, Fecci P, Lim M. A review of glioblastoma immunotherapy. *J Neurooncol* (2020). doi: 10.1007/s11060-020-03448-1
14. Rochigneux P, Garcia AJ, Chanez B, Madroszyk A, Olive D, Garon EB. Medical treatment of lung cancer: can immune cells predict the response? a systematic review. *Front Immunol* (2020) 11:1036. doi: 10.3389/fimmu.2020.01036
15. Xu W, Atkins MB, McDermott DF. Checkpoint inhibitor immunotherapy in kidney cancer. *Nat Rev Urol* (2020) 17:137–50. doi: 10.1038/s41585-020-0282-3
16. Hodi FS, Chiarion-Sileni V, Gonzalez R, Grob JJ, Rutkowski P, Cowey CL, et al. Nivolumab plus ipilimumab or nivolumab alone versus ipilimumab alone in advanced melanoma (CheckMate 067): 4-year outcomes of a multicentre, randomised, phase 3 trial. *Lancet Oncol* (2018) 19:1480–92. doi: 10.1016/S1470-2045(18)30700-9
17. Kessels HW, Wolkers MC, van den Boom MD, van der Valk MA, Schumacher TN. Immunotherapy through TCR gene transfer. *Nat Immunol* (2001) 2:957–61. doi: 10.1038/ni1001-957
18. Jacobs SK, Wilson DJ, Kornblith PL, Grimm EA. Interleukin-2 or autologous lymphokine-activated killer cell treatment of malignant glioma: phase I trial. *Cancer Res* (1986) 46:2101–4. doi: 10.3171/jns.1986.64.5.0743
19. Chen D, Yang J. Development of novel antigen receptors for CAR T-cell therapy directed toward solid malignancies. *Transl Res* (2017) 187:11–21. doi: 10.1016/j.trsl.2017.05.006
20. Sadelain M, Riviere I, Riddell S. Therapeutic T cell engineering. *Nature* (2017) 545:423–31. doi: 10.1038/nature22395
21. Bagley SJ, O'Rourke DM. Clinical investigation of CAR T cells for solid tumors: lessons learned and future directions. *Pharmacol Ther* (2020) 205:107419. doi: 10.1016/j.pharmthera.2019.107419
22. Riddell SR, Sommermeyer D, Berger C, Liu LS, Balakrishnan A, Salter A, et al. Adoptive therapy with chimeric antigen receptor-modified T cells of defined subset composition. *Cancer J* (2014) 20:141–4. doi: 10.1097/PPO.0000000000000036
23. Kershaw MH, Westwood JA, Slaney CY, Darcy PK. Clinical application of genetically modified T cells in cancer therapy. *Clin Transl Immunol* (2014) 3:e16. doi: 10.1038/cti.2014.7
24. Yip A, Webster RM. The market for chimeric antigen receptor T cell therapies. *Nat Rev Drug Discov* (2018) 17:161–2. doi: 10.1038/nrd.2017.266
25. Maude SL, Laetsch TW, Buechner J, Rives S, Boyer M, Bittencourt H, et al. Tisagenlecleucel in children and young adults with B-cell lymphoblastic leukemia. *N Engl J Med* (2018) 378:439–48. doi: 10.1056/NEJMoa1709866
26. Locke FL, Ghobadi A, Jacobson CA, Miklos DB, Lekakis LJ, Oluwole OO, et al. Long-term safety and activity of axicabtagene ciloleucel in refractory large B-cell lymphoma (ZUMA-1): a single-arm, multicentre, phase 1-2 trial. *Lancet Oncol* (2019) 20:31–42. doi: 10.1016/S1470-2045(18)30864-7
27. Migliorini D, Dietrich PY, Stupp R, Linette GP, Posey AJ, June CH. CAR T-Cell Therapies in glioblastoma: a first look. *Clin Cancer Res* (2018) 24:535–40. doi: 10.1158/1078-0432.CCR-17-2871
28. Zhang C, Wang Z, Yang Z, Wang M, Li S, Li Y, et al. Phase I escalating-dose trial of CAR-T therapy targeting CEA (+) metastatic colorectal cancers. *Mol Ther* (2017) 25:1248–58. doi: 10.1016/j.ymthe.2017.03.010

29. Ko AH, Jordan AC, Tooker E, Lacey SF, Chang RB, Li Y, et al. Dual targeting of mesothelin and CD19 with chimeric antigen receptor-modified T cells in patients with metastatic pancreatic cancer. *Mol Ther* (2020). doi: 10.1016/j.ymthe.2020.07.017
30. Lamers CH, Klaver Y, Gratama JW, Sleijfer S, Debets R. Treatment of metastatic renal cell carcinoma (mRCC) with CAIX CAR-engineered T-cells-a completed study overview. *Biochem Soc Trans* (2016) 44:951–9. doi: 10.1042/BST20160037
31. Kandalaft LE, Powell DJ, Coukos G. A phase I clinical trial of adoptive transfer of folate receptor-alpha redirected autologous T cells for recurrent ovarian cancer. *J Transl Med* (2012) 10:157. doi: 10.1186/1479-5876-10-157
32. Tchou J, Zhao Y, Levine BL, Zhang PJ, Davis MM, Melenhorst JJ, et al. Safety and efficacy of intratumoral injections of chimeric antigen receptor (CAR) T cells in metastatic breast cancer. *Cancer Immunol Res* (2017) 5:1152–61. doi: 10.1158/2326-6066.CIR-17-0189
33. Bagley SJ, Desai AS, Linette GP, June CH, O'Rourke DM. CAR T-cell therapy for glioblastoma: recent clinical advances and future challenges. *Neuro Oncol* (2018) 20:1429–38. doi: 10.1093/neuonc/noy032
34. Goff SL, Morgan RA, Yang JC, Sherry RM, Robbins PF, Restifo NP, et al. Pilot trial of adoptive transfer of chimeric antigen receptor-transduced T Cells targeting EGFRvIII in patients with glioblastoma. *J Immunother* (2019) 42:126–35. doi: 10.1097/CJI.0000000000000260
35. O'Rourke DM, Nasrallah MP, Desai A, Melenhorst JJ, Mansfield K, Morrissette J, et al. A single dose of peripherally infused EGFRvIII-directed CAR T cells mediates antigen loss and induces adaptive resistance in patients with recurrent glioblastoma. *Sci Transl Med* (2017) 9. doi: 10.1126/scitranslmed.aaa0984
36. Ahmed N, Brawley V, Hegde M, Bielamowicz K, Kalra M, Landi D, et al. HER2-specific chimeric antigen receptor-modified virus-specific T cells for progressive glioblastoma: a phase 1 dose-escalation trial. *JAMA Oncol* (2017) 3:1094–101. doi: 10.1001/jamaoncol.2017.0184
37. Brown CE, Alizadeh D, Starr R, Weng L, Wagner JR, Naranjo A, et al. Regression of glioblastoma after chimeric antigen receptor T-cell therapy. *N Engl J Med* (2016) 375:2561–9. doi: 10.1056/NEJMoa1610497
38. Brown CE, Badie B, Barish ME, Weng L, Ostberg JR, Chang WC, et al. Bioactivity and safety of IL13Ralpha2-redirected chimeric antigen receptor CD8+ T cells in patients with recurrent glioblastoma. *Clin Cancer Res* (2015) 21:4062–72. doi: 10.1158/1078-0432.CCR-15-0428
39. Brown CE, Warden CD, Starr R, Deng X, Badie B, Yuan YC, et al. Glioma IL13Ralpha2 is associated with mesenchymal signature gene expression and poor patient prognosis. *PLoS One* (2013) 8:e77769. doi: 10.1371/journal.pone.0077769
40. Debinski W, Gibo DM, Hulet SW, Connor JR, Gillespie GY. Receptor for interleukin 13 is a marker and therapeutic target for human high-grade gliomas. *Clin Cancer Res* (1999) 5:985–90.
41. Kahlon KS, Brown C, Cooper LJ, Raubitschek A, Forman SJ, Jensen MC. Specific recognition and killing of glioblastoma multiforme by interleukin 13-zetakine redirected cytolytic T cells. *Cancer Res* (2004) 64:9160–6. doi: 10.1158/0008-5472.CAN-04-0454
42. Brown CE, Aguilar B, Starr R, Yang X, Chang WC, Weng L, et al. Optimization of IL13Ralpha2-targeted chimeric antigen receptor T cells for improved anti-tumor efficacy against glioblastoma. *Mol Ther* (2018) 26:31–44. doi: 10.1016/j.ymthe.2017.10.002
43. Prinzing BL, Gottschalk SM, Krenciute G. CAR T-cell therapy for glioblastoma: ready for the next round of clinical testing? *Expert Rev Anticancer Ther* (2018) 18:451–61. doi: 10.1080/14737140.2018.1451749
44. Oh DY, Bang YJ. HER2-targeted therapies -a role beyond breast cancer. *Nat Rev Clin Oncol* (2020) 17:33–48. doi: 10.1038/s41571-019-0268-3
45. Hegde M, Mukherjee M, Grada Z, Pignata A, Landi D, Navai SA, et al. Tandem CAR T cells targeting HER2 and IL13Ralpha2 mitigate tumor antigen escape. *J Clin Invest* (2016) 126:3036–52. doi: 10.1172/JCI83416
46. Ahmed N, Salsman VS, Kew Y, Shaffer D, Powell S, Zhang YJ, et al. HER2-specific T cells target primary glioblastoma stem cells and induce regression of autologous experimental tumors. *Clin Cancer Res* (2010) 16:474–85. doi: 10.1158/1078-0432.CCR-09-1322
47. Yang J, Yan J, Liu B. Targeting EGFRvIII for glioblastoma multiforme. *Cancer Lett* (2017) 403:224–30. doi: 10.1016/j.canlet.2017.06.024
48. Greenall SA, Donoghue JF, Van Sinderen M, Dubljevic V, Budiman S, Devlin M, et al. EGFRvIII-mediated transactivation of receptor tyrosine kinases in glioma: mechanism and therapeutic implications. *Oncogene* (2015) 34:5277–87. doi: 10.1038/onc.2014.448
49. Miao H, Choi BD, Suryadevara CM, Sanchez-Perez L, Yang S, De Leon G, et al. EGFRvIII-specific chimeric antigen receptor T cells migrate to and kill tumor deposits infiltrating the brain parenchyma in an invasive xenograft model of glioblastoma. *PLoS One* (2014) 9:e94281. doi: 10.1371/journal.pone.0094281
50. Ye L, Park JJ, Dong MB, Yang Q, Chow RD, Peng L, et al. In vivo CRISPR screening in CD8 T cells with AAV-sleeping beauty hybrid vectors identifies membrane targets for improving immunotherapy for glioblastoma. *Nat Biotechnol* (2019) 37:1302–13. doi: 10.1038/s41587-019-0246-4
51. Choi BD, Yu X, Castano AP, Darr H, Henderson DB, Bouffard AA, et al. CRISPR-Cas9 disruption of PD-1 enhances activity of universal EGFRvIII CAR T cells in a preclinical model of human glioblastoma. *J Immunother Cancer* (2019) 7:304. doi: 10.1186/s40425-019-0806-7
52. Jin L, Ge H, Long Y, Yang C, Chang YE, Mu L, et al. CD70, a novel target of CAR T-cell therapy for gliomas. *Neuro Oncol* (2018) 20:55–65. doi: 10.1093/neuonc/nox116
53. Yi Z, Prinzing BL, Cao F, Gottschalk S, Krenciute G. Optimizing EphA2-CAR T cells for the adoptive immunotherapy of glioma. *Mol Ther Methods Clin Dev* (2018) 9:70–80. doi: 10.1016/j.omtm.2018.01.009
54. Pellegatta S, Savoldo B, Di Ianni N, Corbetta C, Chen Y, Patane M, et al. Constitutive and TNFalpha-inducible expression of chondroitin sulfate proteoglycan 4 in glioblastoma and neurospheres: Implications for CAR-T cell therapy. *Sci Transl Med* (2018) 10. doi: 10.1126/scitranslmed.aao2731
55. Nehama D, Di Ianni N, Musio S, Du H, Patane M, Pollo B, et al. B7-H3-redirected chimeric antigen receptor T cells target glioblastoma and neurospheres. *Ebiomedicine* (2019) 47:33–43. doi: 10.1016/j.ebiom.2019.08.030
56. Seaman S, Zhu Z, Saha S, Zhang XM, Yang MY, Hilton MB, et al. Eradication of tumors through simultaneous ablation of CD276/B7-H3-positive tumor cells and tumor vasculature. *Cancer Cell* (2017) 31:501–15. doi: 10.1016/j.ccell.2017.03.005
57. Du H, Hirabayashi K, Ahn S, Kren NP, Montgomery SA, Wang X, et al. Antitumor responses in the absence of toxicity in solid tumors by targeting B7-H3 via chimeric antigen receptor T cells. *Cancer Cell* (2019) 35:221–37. doi: 10.1016/j.ccell.2019.01.002
58. Majzner RG, Theruvath JL, Nellan A, Heitzeneder S, Cui Y, Mount CW, et al. CAR T cells targeting B7-H3, a pan-cancer antigen, demonstrate potent preclinical activity against pediatric solid tumors and brain tumors. *Clin Cancer Res* (2019) 25:2560–74. doi: 10.1158/1078-0432.CCR-18-0432
59. Ahmed M, Cheng M, Zhao Q, Goldgur Y, Cheal SM, Guo HF, et al. Humanized affinity-matured monoclonal antibody 8H9 has potent antitumor activity and binds to FG loop of tumor antigen B7-H3. *J Biol Chem* (2015) 290:30018–29. doi: 10.1074/jbc.M115.679852
60. Souweidane MM, Kramer K, Pandit-Taskar N, Zhou Z, Haque S, Zanzonico P, et al. Convection-enhanced delivery for diffuse intrinsic pontine glioma: a single-centre, dose-escalation, phase 1 trial. *Lancet Oncol* (2018) 19:1040–50. doi: 10.1016/S1470-2045(18)30322-X
61. Tang X, Zhao S, Zhang Y, Wang Y, Zhang Z, Yang M, et al. B7-H3 as a novel CAR-T therapeutic target for glioblastoma. *Mol Ther Oncolytics* (2019) 14:279–87. doi: 10.1016/j.omto.2019.07.002
62. Dardevet L, Rani D, Aziz TA, Bazin I, Sabatier JM, Fadl M, et al. Chlorotoxin: a helpful natural scorpion peptide to diagnose glioma and fight tumor invasion. *Toxins (Basel)* (2015) 7:1079–101. doi: 10.3390/toxins7041079
63. Wang D, Starr R, Chang WC, Aguilar B, Alizadeh D, Wright SL, et al. Chlorotoxin-directed CAR T cells for specific and effective targeting of glioblastoma. *Sci Transl Med* (2020) 12. doi: 10.1126/scitranslmed.aaw2672
64. Jin L, Tao H, Karachi A, Long Y, Hou AY, Na M, et al. CXCR1- or CXCR2-modified CAR T cells co-opt IL-8 for maximal antitumor efficacy in solid tumors. *Nat Commun* (2019) 10:4016. doi: 10.1038/s41467-019-11869-4
65. Yang D, Sun B, Dai H, Li W, Shi L, Zhang P, et al. T cells expressing NKG2D chimeric antigen receptors efficiently eliminate glioblastoma and cancer stem cells. *J Immunother Cancer* (2019) 7:171. doi: 10.1186/s40425-019-0642-9
66. Wallstabe L, Mades A, Frenz S, Einsele H, Rader C, Hudecek M. CAR T cells targeting alphavbeta3 integrin are effective against advanced cancer in preclinical models. *Adv Cell Gene Ther* (2018) 1. doi: 10.1002/acg2.11
67. Ge H, Mu L, Jin L, Yang C, Chang YE, Long Y, et al. Tumor associated CD70 expression is involved in promoting tumor migration and macrophage infiltration in GBM. *Int J Cancer* (2017) 141:1434–44. doi: 10.1002/ijc.30830

68. Zhu X, Prasad S, Gaedicke S, Hettich M, Firat E, Niedermann G. Patient-derived glioblastoma stem cells are killed by CD133-specific CAR T cells but induce the T cell aging marker CD57. *Oncotarget* (2015) 6:171–84. doi: 10.18632/oncotarget.2767
69. Engelhardt B, Carare RO, Bechmann I, Flugel A, Laman JD, Weller RO. Vascular, glial, and lymphatic immune gateways of the central nervous system. *Acta Neuropathol* (2016) 132:317–38. doi: 10.1007/s00401-016-1606-5
70. Xu YY, Gao P, Sun Y, Duan YR. Development of targeted therapies in treatment of glioblastoma. *Cancer Biol Med* (2015) 12:223–37. doi: 10.7497/j.issn.2095-3941.2015.0020
71. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer* (2012) 12:252–64. doi: 10.1038/nrc3239
72. Hodges TR, Ott M, Xiu J, Gatalica Z, Swensen J, Zhou S, et al. Mutational burden, immune checkpoint expression, and mismatch repair in glioma: implications for immune checkpoint immunotherapy. *Neuro Oncol* (2017) 19:1047–57. doi: 10.1093/neuonc/nox026
73. Thorsson V, Gibbs DL, Brown SD, Wolf D, Bortone DS, Ou YT, et al. The immune landscape of cancer. *Immunity* (2019) 51:411–2. doi: 10.1186/s40880-019-0427-z
74. Cristescu R, Mogg R, Ayers M, Albright A, Murphy E, Yearley J, et al. Pan-tumor genomic biomarkers for PD-1 checkpoint blockade-based immunotherapy. *Science* (2018) 362(6411):eaar3593. doi: 10.1126/science.aar3593
75. Gust J, Hay KA, Hanafi LA, Li D, Myerson D, Gonzalez-Cuyar LF, et al. Endothelial activation and blood-brain barrier disruption in neurotoxicity after adoptive immunotherapy with CD19 CAR-T cells. *Cancer Discov* (2017) 7:1404–19. doi: 10.1158/2159-8290.CD-17-0698
76. Patel AP, Tirosh I, Trombetta JJ, Shalek AK, Gillespie SM, Wakimoto H, et al. Single-cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma. *Science* (2014) 344:1396–401. doi: 10.1126/science.1254257
77. Bonavia R, Inda MM, Cavenee WK, Furnari FB. Heterogeneity maintenance in glioblastoma: a social network. *Cancer Res* (2011) 71:4055–60. doi: 10.1158/0008-5472.CAN-11-0153
78. Sidaway P. CNS cancer: Glioblastoma subtypes revisited. *Nat Rev Clin Oncol* (2017) 14:587. doi: 10.1038/nrclinonc.2017.122
79. Sottoriva A, Spiteri I, Piccirillo SG, Touloumis A, Collins VP, Marioni JC, et al. Intratumor heterogeneity in human glioblastoma reflects cancer evolutionary dynamics. *Proc Natl Acad Sci U S A* (2013) 110:4009–14. doi: 10.1073/pnas.1219747110
80. Pitter KL, Tamagno I, Alikhanyan K, Hosni-Ahmed A, Pattwell SS, Donnola S, et al. Corticosteroids compromise survival in glioblastoma. *Brain* (2016) 139:1458–71. doi: 10.1093/brain/aww046
81. Giles AJ, Hutchinson M, Sonnemann HM, Jung J, Fecci PE, Ratnam NM, et al. Dexamethasone-induced immunosuppression: mechanisms and implications for immunotherapy. *J Immunother Cancer* (2018) 6:51. doi: 10.1186/s40425-018-0371-5
82. Khalafallah AM, Huq S, Jimenez AE, Serra R, Bettegowda C, Mukherjee D. "Zooming in" on glioblastoma: understanding tumor heterogeneity and its clinical implications in the era of single-cell ribonucleic acid sequencing. *Neurosurgery* (2020). doi: 10.1093/neuros/nyaa305
83. Shah NN, Maatman T, Hari P, Johnson B. Multi targeted CAR-T cell therapies for B-cell malignancies. *Front Oncol* (2019) 9:146. doi: 10.3389/fonc.2019.00146
84. Bielamowicz K, Fousek K, Byrd TT, Samaha H, Mukherjee M, Aware N, et al. Trivalent CAR T cells overcome interpatient antigenic variability in glioblastoma. *Neuro Oncol* (2018) 20:506–18. doi: 10.1093/neuonc/nox182
85. Esparza R, Azad TD, Feroze AH, Mitra SS, Cheshier SH. Glioblastoma stem cells and stem cell-targeting immunotherapies. *J Neurooncol* (2015) 123:449–57. doi: 10.1007/s11060-015-1729-x
86. Bhaduri A, Di Lullo E, Jung D, Muller S, Crouch EE, Espinosa CS, et al. Outer radial glia-like cancer stem cells contribute to heterogeneity of glioblastoma. *Cell Stem Cell* (2020) 26:48–63. doi: 10.1016/j.stem.2019.11.015
87. Brown CE, Starr R, Aguilar B, Shami AF, Martinez C, D'Apuzzo M, et al. Stem-like tumor-initiating cells isolated from IL13Ralpha2 expressing gliomas are targeted and killed by IL13-zetakine-redirected T Cells. *Clin Cancer Res* (2012) 18:2199–209. doi: 10.1158/1078-0432.CCR-11-1669
88. Ahmed AU, Thaci B, Tobias AL, Auffinger B, Zhang L, Cheng Y, et al. A preclinical evaluation of neural stem cell-based cell carrier for targeted antiglioma oncolytic virotherapy. *J Natl Cancer Inst* (2013) 105:968–77. doi: 10.1093/jnci/djt141
89. Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, et al. Identification of human brain tumour initiating cells. *Nature* (2004) 432:396–401. doi: 10.1038/nature03128
90. Wang Y, Chen M, Wu Z, Tong C, Dai H, Guo Y, et al. CD133-directed CAR T cells for advanced metastasis malignancies: a phase I trial. *Oncoimmunology* (2018) 7:e1440169. doi: 10.1080/2162402X.2018.1440169
91. Vora P, Venugopal C, Salim SK, Tatari N, Bakhshinyan D, Singh M, et al. The Rational development of CD133-targeting immunotherapies for glioblastoma. *Cell Stem Cell* (2020) 26:832–44. doi: 10.1016/j.stem.2020.04.008
92. Lathia JD, Mack SC, Mulkearns-Hubert EE, Valentim CL, Rich JN. Cancer stem cells in glioblastoma. *Genes Dev* (2015) 29:1203–17. doi: 10.1101/gad.261982.115
93. Chuntova P, Downey KM, Hegde B, Almeida ND, Okada H. Genetically engineered T-cells for malignant glioma: overcoming the barriers to effective immunotherapy. *Front Immunol* (2018) 9:3062. doi: 10.3389/fimmu.2018.03062
94. Katz SC, Point GR, Cunetta M, Thorn M, Guha P, Espat NJ, et al. Regional CAR-T cell infusions for peritoneal carcinomatosis are superior to systemic delivery. *Cancer Gene Ther* (2016) 23:142–8. doi: 10.1038/cgt.2016.14
95. Kim JE, Patel MA, Mangraviti A, Kim ES, Theodros D, Velarde E, et al. Combination therapy with anti-PD-1, anti-TIM-3, and focal radiation results in regression of murine gliomas. *Clin Cancer Res* (2017) 23:124–36. doi: 10.1158/1078-0432.CCR-15-1535
96. Cui J, Zhang Q, Song Q, Wang H, Dmitriev P, Sun MY, et al. Targeting hypoxia downstream signaling protein, CAIX, for CAR T-cell therapy against glioblastoma. *Neuro Oncol* (2019) 21:1436–46. doi: 10.1093/neuonc/noz117
97. Geyer MB, Riviere I, Senechal B, Wang X, Wang Y, Purdon TJ, et al. Safety and tolerability of conditioning chemotherapy followed by CD19-targeted CAR T cells for relapsed/refractory CLL. *JCI Insight* (2019) 5. doi: 10.1172/jci.insight.122627
98. Bocca P, Di Carlo E, Caruana I, Emionite L, Cilli M, De Angelis B, et al. Bevacizumab-mediated tumor vasculature remodelling improves tumor infiltration and antitumor efficacy of GD2-CAR T cells in a human neuroblastoma preclinical model. *Oncoimmunology* (2017) 7:e1378843. doi: 10.1080/2162402X.2017.1378843
99. Suryadevara CM, Desai R, Abel ML, Riccione KA, Batich KA, Shen SH, et al. Temozolomide lymphodepletion enhances CAR abundance and correlates with antitumor efficacy against established glioblastoma. *Oncoimmunology* (2018) 7:e1434464. doi: 10.1080/2162402X.2018.1434464
100. Ma L, Dichwalkar T, Chang J, Cossette B, Garafola D, Zhang AQ, et al. Enhanced CAR-T cell activity against solid tumors by vaccine boosting through the chimeric receptor. *Science* (2019) 365:162–8. doi: 10.1126/science.aav8692

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REVIEW
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Glioblastoma Immunotherapy Targeting the Innate Immune Checkpoint CD47-SIRPα Axis

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Glioblastoma Multiforme (GBM) is the most common and aggressive form of intracranial tumors with poor prognosis. In recent years, tumor immunotherapy has been an attractive strategy for a variety of tumors. Currently, most immunotherapies take advantage of the adaptive anti-tumor immunity, such as cytotoxic T cells. However, the predominant accumulation of tumor-associated microglia/macrophages (TAMs) results in limited success of these strategies in the glioblastoma. To improve the immunotherapeutic efficacy for GBM, it is detrimental to understand the role of TAM in glioblastoma immunosuppressive microenvironment. In this review, we will discuss the roles of CD47-SIRPα axis in TAMs infiltration and activities and the promising effects of targeting this axis on the activation of both innate and adaptive antitumor immunity in glioblastoma.

Keywords: glioblastoma, immune checkpoint, CD47-SIRPα, tumor-associated macrophages/microglia, glioblastoma microenvironment

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INTRODUCTION

Glioblastoma (GBM) is the most common primary malignant brain tumor in adults and is characterized by invasive growth and frequent recurrence. Despite of advances in surgical resection, radiotherapy, and chemotherapy, the median survival time of patients is only 12 to 15 months; the 3-year survival rate is approximately 10% (1, 2). Great progress has been made in the development of immunotherapy for extracranial tumors. However, most clinical trials of immunotherapy for GBM have shown only a moderate response and no significant improvement in over survival (OS) (3).

Currently, immunotherapy for GBM includes immune checkpoint blockade therapy, vaccination therapy, oncolytic virus therapy, and CAR-T therapy (4–6), which mainly take advantage of the adaptive anti-tumor immunity (**Figure 1**). Accumulating evidence suggests that the GBM microenvironment is characterized by high myeloid cell content, relatively few tumor-infiltrating lymphocytes (TILs) (7, 8)and T cell dysfunction (9). In contrast, tumor-associated microglia/macrophages (TAMs) account for 30% to 40% in GBM (10, 11). Approximately 85% of them are bone marrow-derived infiltrating macrophages/monocytes while the remaining fractions are locally resident microglia (12, 13), which engage in reciprocal interactions with GBM and adaptive immune

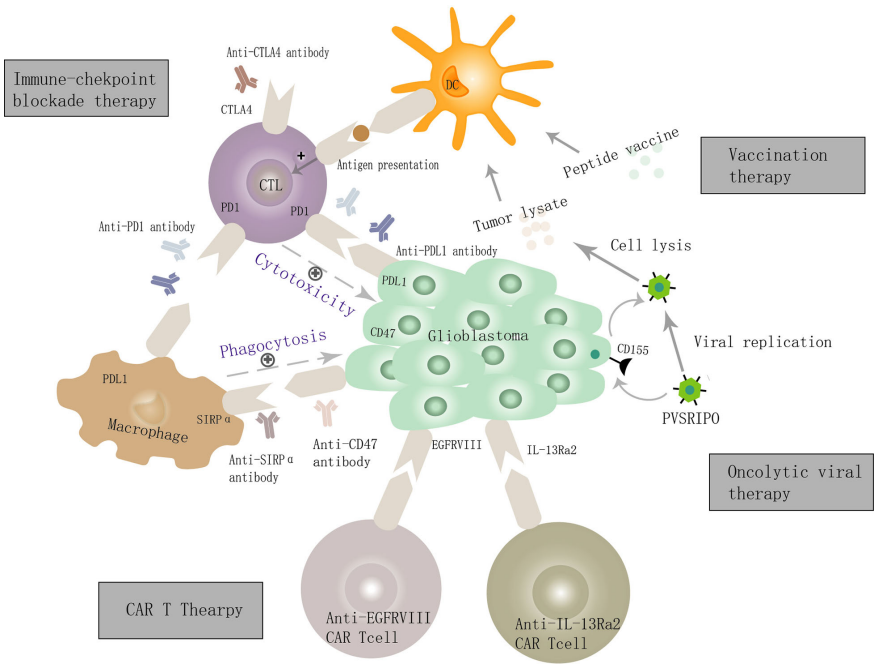

FIGURE 1 | Cellular and molecular mechanisms of GBM immunotherapy. GBM cells overexpress PDL1, CD47, and other immunosuppressive molecules and bind the ligands present on cytotoxic T lymphocytes (CTLs) and macrophage, and thereby inhibit the innate and adaptive immune function, leading to the immune escape of GBM. Targeting immune checkpoint molecules such as PDL1, CD47, and CTLA4 can activate both innate and adaptive anti-tumor immunity. The mechanism of oncolytic virus therapy is mainly *via* the creation of viruses that can selectively infect GBM cells, defeat GBM cells, and enhance adaptive anti-tumor immune responses by the dendritic cell and CTL. Several tumor-related antigens (e.g., IL-13Ra2, EGFRvIII) are expressed on the surface of GBM cells and are used as specific targets for (CAR) T cell therapy to achieve a precise treatment objective. The vaccination strategy mainly mediates the activation of CTLs by antigen-presenting cells, thus killing GBM cells.

cell to mediate tumor immune escape (14–16), promote tumor growth and progression (17–21). Therefore, reeducating, reactivating, and reconstructing the TAMs functions in GBM immunosuppressive microenvironment makes them superior again is a promising field.

The strategies targeting TAMs fall into three main groups: 1) inhibiting recruitment of the bone marrow-derived infiltrating macrophages/monocytes (22–24); 2) promoting phagocytosis of tumor cells by TAMs and restoring its innate antitumor immunity (25, 26); 3) reprogramming TAMs to antitumor macrophages/microglial either directly through tumor cell killing or by reactivating adaptive antitumor immunity (27–30). The CD47-SIRPα Axis is currently the most widely studied innate immune checkpoint (31). Interestingly, the accumulating data shows that target the CD47- SIRPα axis bridging innate and adaptive antitumor immunity (15, 32). Targeting the CD47-SIRPα axis activates both innate and adaptive antitumor immunity (33), which is promising for GBM therapies. This review will discuss in more detail about the structure and regulation of innate immune checkpoint CD47-SIRPα and their functions in the immune-suppressive microenvironment and therapeutic potential in GBM. We would like to raise awareness of immune parameters in clinical stratification schemes and encourage discussions and improvements about innate anti-tumor immunity-oriented immunotherapies.

STRUCTURE OF CD47-SIRPα

The CD47 gene is located on chromosome 3q13 and encodes an integrin-associated protein. CD47 is an important "self-labeling" molecule in the immunoglobulin superfamily that contains an immunoglobulin variable-like amino-terminal domain, five transmembrane domains, and one carboxy-terminal intracellular tail (34, 35). Signal regulatory proteins (SIRPs) are inhibitory immune receptors encoded by a cluster of genes on chromosome 20p13, including SIRPα, SIRPβ1, SIRPγ, SIRPβ2, and SIRPδ (36). SIRPα binds to CD47 with high-affinity (37). Structurally, the extracellular domain of SIRPα consists of three immunoglobulins (Ig)-like domains (the NH2-terminal V-like domain and two C1 domains), a single transmembrane segment, and the intracellular segment containing four tyrosine residues that form two typical immune-receptor tyrosine-based inhibition motifs (ITIMs). When CD47 expressed on the surface of GBM cells binds to the NH2-terminal V-like domain of SIRPα on myeloid cells, phosphorylation of the tyrosine residue in the ITIM motif results in the recruitment and activation of tyrosine phosphatase SHP1/SHP2. This process affects the levels of downstream de-phosphorylated molecules and inhibits the phagocytosis of GBM cells by macrophages (38). Hence CD47 serves as a critical "do not eat me" signal. However, the signaling mechanisms upstream and downstream of the CD47-SIRPα axis are incompletely understood.

EXPRESSION AND REGULATION OF CD47-SIRPα AXIS

CD47 has been found to be highly expressed in GBM cells, especially glioblastoma stem cells (39). Its expression levels are positively correlated with glioma grade and are associated with worse clinical outcomes (39–41). Hence It has been regarded as a critical biomarker for glioblastoma (42). Amounting studies have demonstrated that MYC (43), PKM2-β-catenin-BRG1-TCF4 complex (44), NF-Kβ (45), and NRF1 (46) may bind at the promoter of CD47 to regulate its transcription. SIRPα is expressed on myeloid cells, including macrophages, dendritic cells (DCs), neutrophils, and nerve cells (neurons, microglia) (36). Interestingly, SIRPγ is expressed on human activated T cells and also binds to CD47, albeit with a lower affinity than SIRPa (31), which may also play a pivotal role in the adaptive antitumor immunity. More comprehensive research into the dynamic control of the CD47-SIRP axis will be greatly helpful for us to understand its functions and optimize its targeting strategies.

THE FUNCTIONS OF THE CD47-SIRPα AXIS IN GLIOBLASTOMA

The exact functions of CD47 in GBM are still in debate. The increased expression of CD47 were found to promote the proliferation and invasion of GBM cells while it did not affect the proliferation ability of normal astrocytes (47, 48). However, some other studies found that CD47 could enhance the invasion ability of GBM cells through the PI3K/AKT pathway but had no effect on proliferation (49). Moreover, CD47 positive GBM cells possessed many characteristics that associate with cancer stem cells, which implies worse clinical outcomes (50). Accumulating evidence suggests that CD47 binds SIRPα on macrophages, neutrophils, and dendritic cells, subsequently inhibiting the cytotoxicity of macrophages and neutrophils, limiting the antigen-presenting function of dendritic cells, and inhibiting both innate and adaptive immune functions (38, 50, 51).

THE SIGNIFICANCE OF TARGETING CD47-SIRPα AXIS IN THE GBM MICROENVIRONMENT

Targeting the innate immune checkpoint CD47-SIRPα axis enhances the phagocytosis rate, resulting in a significant survival benefit even in the absence of peripheral macrophages (52). Therefore, when studying the effects of CD47-SIRPα immunological checkpoint inhibitors on the phagocytic function of macrophages *in vitro*, their impact on microglia function must be considered. Targeting the innate immunity checkpoint CD47-SIRPα axis exerts anti-GBM efficacy mainly through the following four pathways (**Figure 2**).

In the first pathway, it leads to enhanced tumor cell phagocytosis by both M1 and M2 macrophage subtypes and shifts the phenotype of macrophages toward the M1 subtype *in vivo* (53). And the phagocytic potential of M1 was similar to that of M2 *in vitro*. Phagocytosis by M1 increased in a CD47-dependent manner by the neutralizing antibody and siRNA against CD47 but not in M2 (54). In line with previous studies, Zhu et al. suggest that surgical resection combined with anti-CD47 immunotherapy was shown to promote the recruitment of macrophages and promote phagocytosis of glioblastoma (25). Li et al. come to a similar conclusion that humanized CD47 antibody HU5F9-G4 inhibits CD47 expression, enhanced tumor cell phagocytosis by macrophage, improves the survival time of animals, and has nontoxic effects on neurons and other tissues in a xenograft model derived from the malignant brain tumor (50).

In the second pathway, it enhances the antigen presentation ability of DC to generate potent T-cell priming and adaptive antitumor immune responses (32, 33). Christina et al. suggest that anti-CD47 treatment alone has limited anti-tumor effects and is inefficient in inducing changes within the tumor immune microenvironment or eradicating murine GBMs in immune-competent hosts. Instead, combined TMZ and CD47 blockade activates the cGAS-STING pathway, increases T-cell priming, and thereby activates both innate and adaptive immune responses *in vivo*. Hence the combination treatment is further augmented by adjuvant PD-1 blockade (33). In addition, radiotherapy was demonstrated to enhance the anti-CD47 therapeutic effects (55).

In the third pathway, glioblastoma cells may be eliminated *via* traditional antibody Fc-dependent mechanisms, including neutrophil cell-mediated antibody-dependent cellular cytotoxicity (ADCC) and macrophage-mediated antibody-dependent cellular phagocytosis (ADCP) (56, 57). Recent studies have demonstrated that neutrophil ADCC toward cancer cells occurs through a mechanism called trogocytosis, which can be further improved by targeting CD47-SIRPα interactions (58). The bispecific antibodies targeting the membrane-proximal epitope of MSLN improve ADCC activity by augmenting FcγR-IIIA activation and enhanced ADCP *via* a more efficient blockade of the CD47/SIRPα axis (59).

In the fourth pathway, it can induce apoptosis of tumor cells directly (60). It has been shown that CD47 antibody-induced apoptosis of cancer cells is due to neither ADCC nor CDC. Instead, such antitumor activity by bivalent scFv is presumably attributable to cell death caused by the ligation of CD47 (61, 62). And tumor cells may be eliminated through direct induction of apoptosis by a novel pathway involving regulation of cAMP levels by heterotrimeric Gi with subsequent effects mediated by PKA (63, 64). However, its specific functions and mechanism in GBM require further studies.

Collectively, targeting the immune checkpoint complex CD47-SIRPα has been shown as a promising anti-tumor strategy that may remodel the GBM microenvironment, restore innate and adaptive immunity functions, and improve the prognosis of patients with GBM. Notably, these promising strategies still need considerable refinement before becoming the standard clinical treatment options for GBM.

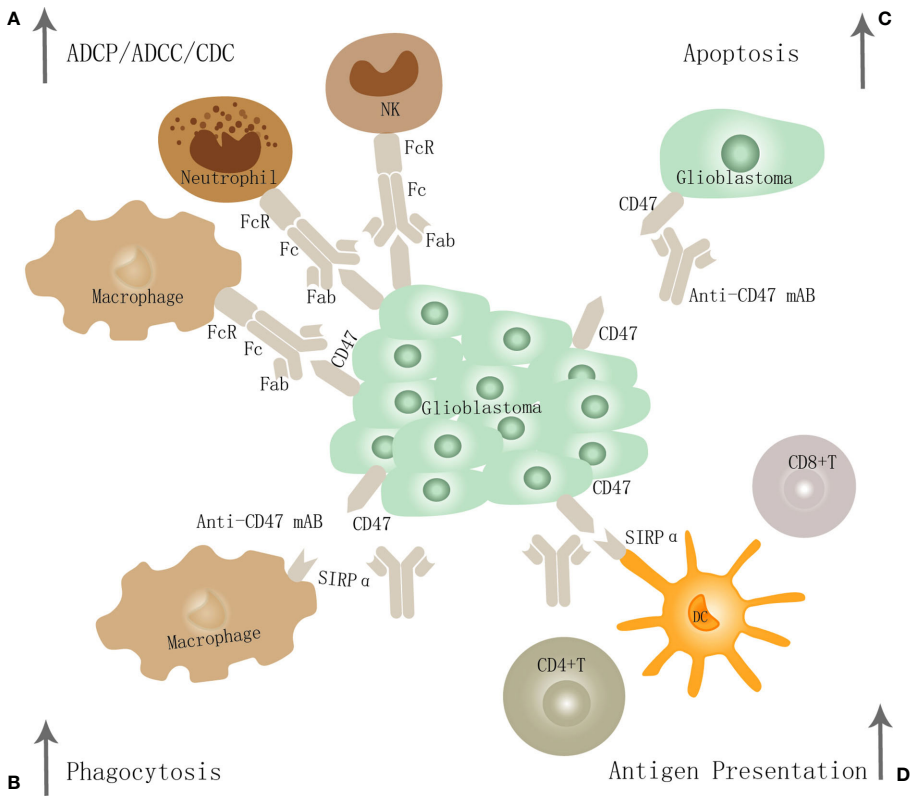

FIGURE 2 | The potential mechanism of CD47-SIRPα inhibition in GBM. Targeting the CD47-SIRPα axis may exert anti-GBM effects through the following four pathways: **(A)** Eliminate GBM cells through traditional antibody Fc-dependent mechanisms, including ADCP, ADCC, and CDC. **(B)** it leads to enhanced tumor cell phagocytosis by macrophage through disrupting the binding of CD47 to SIRPα. **(C)** Promote apoptosis of GBM cells. **(D)** Restore dendritic cells' function to present antigen to CD4+ and CD8+T cells, thereby stimulating an anti-tumor adaptive immune response.

IMMUNOLOGICAL CHECKPOINT INHIBITORS TARGETING CD47-SIRPα AXIS

Currently, inhibitors targeting CD47-SIRPα immunological checkpoints are in preclinical and clinical study phases. These inhibitors include 1) monoclonal antibodies (CD47 monoclonal antibody Hu5F9-G4, human IgG4 subclass; SIRPα monoclonal antibody FSI-189), which are mainly to block the anti-phagocytosis signal and reactive macrophages to attack and destroy tumor cells (65, 66); 2) recombinant fusion proteins (TTI-621, SIRPα-Fc fusion protein, human IgG1 subclass; TTI-622, SIRPα-Fc fusion protein, human IgG4 subclass), which are composed of the N-terminal V domain of human SIRPα and the human IgG Fc region. The N-terminal V domain of human SIRPα bind human CD47 on tumor cells and prevent it from delivering inhibitory signals to macrophages. At the same time, The IgG Fc region of SIRPαFc can bind to the high-affinity receptor FcγRI (CD64) as well as to the low-affinity receptors FcγRII (CD32) and FcγRIII (CD16) on macrophages to further enhance macrophage-mediated ADCP, tumor antigen presentation, and effective anti-tumor activity. Lower affinities for normal red blood cells and reduced side effects are important advantages of recombinant fusion protein therapies (67); 3) bispecific antibodies (NI-1701, anti-CD19/anti-CD47 bispecific antibody; NI-1801, anti-CD47/mesothelin bispecific antibody); VEGFR1D2-SIRPαD1. NI-1701 has three arms. The targeting arm binds CD19, a cell-surface antigen expressed by B-cell-origin tumors. The effector's arm destroys the CD47-mediated anti-phagocytosis signal. The Fc arm of the antibody can recruit macrophages and other innate immune killer cells. NI-1801 destroy mesothelin-positive solid tumors through the innate immune system; VEGFR1D2-SIRPαD1 consists of the second extracellular domain of VEGFR1 (VEGFR1D2) and the first extracellular domain of SIRPα (SIRPαD1), which exerted potent anti-tumor effects *via* suppressing VEGF-induced angiogenesis and activating macrophage-mediated phagocytosis (68–70). Among the immunological checkpoint inhibitors, Hu5F9-G4, TTI-621, and TTI-622 are undergoing Phase I clinical trials, although the complete data have not been published (71).

SAFETY ASSESSMENT AND FUTURE PERSPECTIVES

The main concern of CD47 inhibitors is the risk of hematological toxicity such as anemia, thrombocytopenia, and leukopenia,

given the high expression of CD47 on normal red blood cells and platelets (72, 73). Preclinical studies show that CD47 inhibitors in mice are well-tolerated, with no obvious signs of toxicity (50, 74). However, Arch Oncology and Celgene discontinued a clinical trial of the CD47 inhibitors because of possible off-target effects such as anemia (75). One of the most important issues is to reduce or avoid potential toxicity while preserving anti-tumor effects.

The toxicity of anti-CD47/SIRPα antibodies appears to be Fc-dependent. It may be desirable to block the SIRPα-CD47 interaction by antibodies devoid of the Fc portion or optimize the structure of the Fc portion. Meanwhile, targeting tumor cells for FcR-mediated phagocytosis using intact antibodies (31). For example, the macrophage checkpoint inhibitor 5F9 combined with rituximab showed promising activity in patients with aggressive and indolent lymphoma, with no clinically significant toxicity (65). SIRPα expression in normal cells is much narrower than CD47 and its targeting may result in more limited toxicity, such as recombinant fusion proteins TTI-621 and ALX148 and high-affinity monomeric SIRPα with lower affinities for normal red blood cells (67, 76, 77), which is also an ideal strategy. Red blood cells act as a "sink" binding to anti-CD47 antibodies and reduce the effective therapeutic dose. Hence, optimized initiation dose and maintenance dose to achieve an effective therapeutic blockade of CD47/SIRPα Axis is pivotal. For example, a non-human primate study revealed that the effector function competent mAb IgG1 C47B222-(CHO) showed antitumor activity *in vitro* and *in vivo* while decreased red blood cells (RBC), hematocrit and hemoglobin by >40% at 1 mg/kg (78). However, toxicokinetic studies suggest that alternative treatment regimens for Hu5F9-G4 (a low initiation dose and a higher maintenance dose) may contribute to achieving therapeutic efficacy with lower toxicity (71).

CONCLUSIONS

Preclinical studies have found that targeting the immunological checkpoint complex CD47-SIRPα can inhibit the development of glioblastoma, enhance the function of phagocytic cells, restore the function of dendritic cells and T lymphocytes, and exert anti-tumor effects by improving innate and adaptive immune responses. However, there are still a series of biosafety problems such as anemia that remain to be solved. Besides, it is incompletely understood how CD47-SIRPα blockade works at the molecular level. Further understanding of the mechanism of CD47-SIRPα inhibitors will help to improve the efficacy and reduce the side effects. Ongoing clinical trials will further clarify their efficacies as single agents or in combination therapies. Careful observations of cytotoxic T cell response, T cell exhaustion, immune gene expression signatures in GBM subtypes, immune suppression (predominant immunosuppressive cells such as TAMs) may aid in identifying patients suitable for this therapy, avoiding potential toxicities and designing optimal combination therapies.

AUTHOR CONTRIBUTIONS

HJ collected the literature and drafted the manuscript. DM modified the paper format, and WB, WX, DG, and QX guided the writing and made significant revisions to the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Bakas S, Akbari H, Pisapia J, Martinez-Lage M, Rozycki M, Rathore S, et al. In Vivo Detection of EGFRvIII in Glioblastoma via Perfusion Magnetic Resonance Imaging Signature Consistent with Deep Peritumoral Infiltration: The phi-Index. *Clin Cancer Res* (2017) 23:4724–34. doi: 10.1158/1078-0432.ccr-16-1871
2. Dunn GP, Rinne ML, Wykosky J, Genovese G, Quayle SN, Dunn IF, et al. Emerging insights into the molecular and cellular basis of glioblastoma. *Genes Dev* (2012) 26:756–84. doi: 10.1101/gad.187922.112
3. Maxwell R, Jackson CM, Lim M. Clinical Trials Investigating Immune Checkpoint Blockade in Glioblastoma. *Curr Treat Options Oncol* (2017) 18:51. doi: 10.1007/s11864-017-0492-y
4. Kamran N, Calinescu A, Candolfi M, Chandran M, Mineharu Y, Asad AS, et al. Recent advances and future of immunotherapy for glioblastoma. *Expert Opin Biol Ther* (2016) 16:1245–64. doi: 10.1080/14712598.2016.1212012
5. Lim M, Xia Y, Bettegowda C, Weller M. Current state of immunotherapy for glioblastoma. *Nat Rev Clin Oncol* (2018) 15:422–42. doi: 10.1038/s41571-018-0003-5
6. Chandramohan V, Mitchell DA, Johnson LA, Sampson JH, Bigner. Antibody DD. T-cell and dendritic cell immunotherapy for malignant brain tumors. *Future Oncol* (2013) 9:977–90. doi: 10.2217/fon.13.47
7. Berghoff AS, Kiesel B, Widhalm G, Rajky O, Ricken G, Wohrer A, et al. Programmed death ligand 1 expression and tumor-infiltrating lymphocytes in glioblastoma. *Neuro Oncol* (2015) 17:1064–75. doi: 10.1093/neuonc/nou307
8. Han S, Ma E, Wang X, Yu C, Dong T, Zhan W, et al. Rescuing defective tumor-infiltrating T-cell proliferation in glioblastoma patients. *Oncol Lett* (2016) 12:2924–9. doi: 10.3892/ol.2016.4944
9. Woroniecka K, Chongsathidkiet P, Rhodin K, Kemeny H, Dechant C, Farber SH, et al. T-Cell Exhaustion Signatures Vary with Tumor Type and Are Severe in Glioblastoma. *Clin Cancer Res* (2018) 24:4175–86. doi: 10.1158/1078-0432.Ccr-17-1846
10. Schupp J, Krebs FK, Zimmer N, Trzeciak E, Schuppan D, Tuettenberg A. Targeting myeloid cells in the tumor sustaining microenvironment. *Cell Immunol* (2019) 343:103713. doi: 10.1016/j.cellimm.2017.10.013
11. Aldape K, Brindle KM, Chesler L, Chopra R, Gajjar A, Gilbert MR, et al. Challenges to curing primary brain tumours. *Nat Rev Clin Oncol* (2019) 16:509–20. doi: 10.1038/s41571-019-0177-5
12. Chen Z, Feng X, Herting CJ, Garcia VA, Nie K, Pong WW, et al. Cellular and Molecular Identity of Tumor-Associated Macrophages in Glioblastoma. *Cancer Res* (2017) 77:2266–78. doi: 10.1158/0008-5472.CAN-16-2310
13. Zhou W, Ke SQ, Huang Z, Flavahan W, Fang X, Paul J, et al. Periostin secreted by glioblastoma stem cells recruits M2 tumour-associated macrophages and promotes malignant growth. *Nat Cell Biol* (2015) 17:170–82. doi: 10.1038/ncb3090
14. Ruffell B, Coussens LM. Macrophages and therapeutic resistance in cancer. *Cancer Cell* (2015) 27:462–72. doi: 10.1016/j.ccell.2015.02.015
15. Mantovani A, Marchesi F, Malesci A, Laghi L, Allavena P. Tumour-associated macrophages as treatment targets in oncology. *Nat Rev Clin Oncol* (2017) 14:399–416. doi: 10.1038/nrclinonc.2016.217

16. Noy R, Pollard JW. Tumor-associated macrophages: from mechanisms to therapy. *Immunity* (2014) 41:49–61. doi: 10.1016/j.immuni.2014.06.010
17. Poon CC, Sarkar S, Yong VW, Kelly JJP. Glioblastoma-associated microglia and macrophages: targets for therapies to improve prognosis. *Brain* (2017) 140:1548–60. doi: 10.1093/brain/aww355
18. Ye XZ, Xu SL, Xin YH, Yu SC, Ping YF, Chen L, et al. Tumor-Associated Microglia/Macrophages Enhance the Invasion of Glioma Stem-like Cells via TGF-beta 1 Signaling Pathway. *J Immunol* (2012) 189:444–53. doi: 10.4049/jimmunol.1103248
19. Zhu CB, Mustafa D, Zheng PP, van der Weiden M, Sacchetti A, Brandt M, et al. Activation of CECR1 in M2-like TAMs promotes paracrine stimulation-mediated glial tumor progression. *Neuro-Oncology* (2017) 19:648–59. doi: 10.1093/neuonc/now251
20. Li W, Graeber MB. The molecular profile of microglia under the influence of glioma. *Neuro-Oncology* (2012) 14:958–78. doi: 10.1093/neuonc/nos116
21. Wang N, Liang HW, Zen K. Molecular mechanisms that influence the macrophage M1-M2 polarization balance. *Front Immunol* (2014) 5:614. doi: 10.3389/fimmu.2014.00614
22. Pyonteck SM, Akkari L, Schuhmacher AJ, Bowman RL, Sevenich L, Quail DF, et al. CSF-1R inhibition alters macrophage polarization and blocks glioma progression. *Nat Med* (2013) 19:1264–+. doi: 10.1038/nm.3337
23. Stafford JH, Hirai T, Deng L, Chernikova SB, Urata K, West BL, et al. Colony stimulating factor 1 receptor inhibition delays recurrence of glioblastoma after radiation by altering myeloid cell recruitment and polarization. *Neuro Oncol* (2016) 18:797–806. doi: 10.1093/neuonc/nov272
24. Quail DF, Bowman RL, Akkari L, Quick ML, Schuhmacher AJ, Huse JT, et al. The tumor microenvironment underlies acquired resistance to CSF-1R inhibition in gliomas. *Science* (2016) 352:aad3018. doi: 10.1126/science.aad3018
25. Zhu HY, Leiss L, Yang N, Rygh CB, Mitra SS, Cheshier SH, et al. Surgical debulking promotes recruitment of macrophages and triggers glioblastoma phagocytosis in combination with CD47 blocking immunotherapy. *Oncotarget* (2017) 8:12145–57. doi: 10.18632/oncotarget.14553
26. Zhang H, Lu H, Xiang L, Bullen JW, Zhang C, Samanta D, et al. HIF-1 regulates CD47 expression in breast cancer cells to promote evasion of phagocytosis and maintenance of cancer stem cells. *Proc Natl Acad Sci U S A* (2015) 112:E6215–6223. doi: 10.1073/pnas.1520032112
27. Kaneda MM, Messer KS, Ralainirina N, Li H, Leem CJ, Gorjestani S, et al. PI3Kγ is a molecular switch that controls immune suppression. *Nature* (2016) 539:437–42. doi: 10.1038/nature19834
28. Baer C, Squadrito ML, Laoui D, Thompson D, Hansen SK, Kiialainen A, et al. Suppression of microRNA activity amplifies IFN-γ-induced macrophage activation and promotes anti-tumour immunity. *Nat Cell Biol* (2016) 18:790–802. doi: 10.1038/ncb3371
29. Guerriero JL, Sotayo A, Ponichtera HE, Castrillon JA, Pourzia AL, Schad S, et al. Class IIa HDAC inhibition reduces breast tumours and metastases through anti-tumour macrophages. *Nature* (2017) 543:428–32. doi: 10.1038/nature21409
30. Georgoudaki AM, Prokopec KE, Boura VF, Hellqvist E, Sohn S, Östling J, et al. Reprogramming Tumor-Associated Macrophages by Antibody Targeting Inhibits Cancer Progression and Metastasis. *Cell Rep* (2016) 15:2000–11. doi: 10.1016/j.celrep.2016.04.084
31. Veillette A, Chen J. SIRPα-CD47 Immune Checkpoint Blockade in Anticancer Therapy. *Trends Immunol* (2018) 39:173–84. doi: 10.1016/j.it.2017.12.005
32. Tseng D, Volkmer JP, Willingham SB, Contreras-Trujillo H, Fathman JW, Fernhoff NB, et al. Anti-CD47 antibody-mediated phagocytosis of cancer by macrophages primes an effective antitumor T-cell response. *Proc Natl Acad Sci U S A* (2013) 110:11103–8. doi: 10.1073/pnas.1305569110
33. von Roemeling CA, Wang Y, Qie Y, Yuan H, Zhao H, Liu X, et al. Therapeutic modulation of phagocytosis in glioblastoma can activate both innate and adaptive antitumour immunity. *Nat Commun* (2020) 11:1508. doi: 10.1038/s41467-020-15129-8
34. Tong B, Wang MZ. CD47 is a novel potent immunotherapy target in human malignancies: current studies and future promises. *Future Oncol* (2018) 14:2179–88. doi: 10.2217/fon-2018-0035
35. Brown EJ, Frazier WA. Integrin-associated protein (CD47) and its ligands. *Trends Cell Biol* (2001) 11:130–5. doi: 10.1016/s0962-8924(00)01906-1
36. van Beek EM, Cochrane F, Barclay AN, van den Berg TK. Signal regulatory proteins in the immune system. *J Immunol* (2005) 175:7781–7. doi: 10.4049/jimmunol.175.12.7781
37. Seiffert M, Brossart P, Cant C, Cella M, Colonna M, Brugger W, et al. Signal-regulatory protein alpha (SIRP alpha) but not SIRP beta is involved in T-cell activation, binds to CD47 with high affinity, and is expressed on immature CD34(+)CD38(-) hematopoietic cells. *Blood* (2001) 97:2741–9. doi: 10.1182/blood.V97.9.2741
38. Matlung HL, Szilagyi K, Barclay NA, van den Berg TK. The CD47-SIRP alpha signaling axis as an innate immune checkpoint in cancer. *Immunol Rev* (2017) 276:145–64. doi: 10.1111/imr.12527
39. Li F, Lv B, Liu Y, Hua T, Han J, Sun C, et al. Blocking the CD47-SIRPalpha axis by delivery of anti-CD47 antibody induces antitumor effects in glioma and glioma stem cells. *OncoImmunology* (2018) 7:139–49. doi: 10.1080/2162402X.2017.1391973
40. Willingham SB, Volkmer JP, Gentles AJ, Sahoo D, Dalerba P, Mitra SS, et al. The CD47-signal regulatory protein alpha (SIRPa) interaction is a therapeutic target for human solid tumors. *Proc Natl Acad Sci U S A* (2012) 109:6662–7. doi: 10.1073/pnas.1121623109
41. Zhao HJ, Pan F, Shi YC, Luo X, Ren RR, Peng LH, et al. Prognostic significance of CD47 in human malignancies: a systematic review and meta-analysis. *Transl Cancer Res* (2018) 7:609–74. doi: 10.21037/tcr.2018.05.31
42. Ghosh D, Funk CC, Caballero J, Shah N, Rouleau K, Earls JC, et al. A Cell-Surface Membrane Protein Signature for Glioblastoma. *Cell Syst* (2017) 4:516–529.e517. doi: 10.1016/j.cels.2017.03.004
43. Casey SC, Tong L, Li YL, Do R, Walz S, Fitzgerald KN, et al. MYC regulates the antitumor immune response through CD47 and PD-L1. *Science* (2016) 352:227–31. doi: 10.1126/science.aac9935
44. Gowda P, Patrick S, Singh A, Sheikh T, Sen E. Mutant Isocitrate Dehydrogenase 1 Disrupts PKM2-beta-Catenin-BRG1 Transcriptional Network-Driven CD47 Expression. *Mol Cell Biol* (2018) 38:18. doi: 10.1128/mcb.00001-18
45. Lo J, Lau EYT, Ching RHH, Cheng BYL, Ma MKF, Ng IOL, et al. Nuclear factor kappa B-mediated CD47 up-regulation promotes sorafenib resistance and its blockade synergizes the effect of sorafenib in hepatocellular carcinoma in mice. *Hepatology* (2015) 62:534–45. doi: 10.1002/hep.27859
46. Chang WT, Huang AM. alpha-Pal/NRF-1 regulates the promoter of the human integrin-associated protein/CD47 gene. *J Biol Chem* (2004) 279:14542–50. doi: 10.1074/jbc.M309825200
47. Sick E, Boukhari A, Deramaudt T, Ronde P, Bucher B, Andre P, et al. Activation of CD47 Receptors Causes Proliferation of Human Astrocytoma but Not Normal Astrocytes via an Akt-Dependent Pathway. *Glia* (2011) 59:308–19. doi: 10.1002/glia.21102
48. Boukhari A, Alhosin M, Bronner C, Sagini K, Truchot C, Sick E, et al. CD47 Activation-induced UHRF1 Over-expression Is Associated with Silencing of Tumor Suppressor Gene p16(INK4A) in Glioblastoma Cells. *Anticancer Res* (2015) 35:149–57. doi: 10.1002/glia.21102
49. Liu XJ, Wu X, Wang YM, Li YH, Chen XL, Yang WC, et al. CD47 Promotes Human Glioblastoma Invasion Through Activation of the PI3K/Akt Pathway. *Oncol Res* (2019) 27:415–22. doi: 10.3727/096504018x15155538502359
50. Li F, Lv BK, Liu Y, Hua T, Han JB, Sun CM, et al. Blocking the CD47-SIRP alpha axis by delivery of anti-CD47 antibody induces antitumor effects in glioma and glioma stem cells. *Oncoimmunology* (2018) 7:12. doi: 10.1080/2162402x.2017.1391973
51. Huang YT, Ma YC, Gao P, Yao Z. Targeting CD47: the achievements and concerns of current studies on cancer immunotherapy. *J Thorac Dis* (2017) 9:E168–74. doi: 10.21037/jtd.2017.02.30
52. Hutter G, Theruvath J, Graef CM, Zhang M, Schoen MK, Manz EM, et al. Microglia are effector cells of CD47-SIRP alpha antiphagocytic axis disruption against glioblastoma. *Proc Natl Acad Sci U S A* (2019) 116:997–1006. doi: 10.1073/pnas.1721434116
53. Zhang M, Hutter G, Kahn SA, Azad TD, Gholamin S, Xu CY, et al. Anti-CD47 Treatment Stimulates Phagocytosis of Glioblastoma by M1 and M2 Polarized Macrophages and Promotes M1 Polarized Macrophages In Vivo. *PLoS One* (2016) 11:21. doi: 10.1371/journal.pone.0153550

54. Sakakura K, Takahashi H, Kaira K, Toyoda M, Murata T, Ohnishi H, et al. Relationship between tumor-associated macrophage subsets and CD47 expression in squamous cell carcinoma of the head and neck in the tumor microenvironment. *Lab Invest* (2016) 96:994–1003. doi: 10.1038/labinvest.2016.70
55. Gholamin S, Youssef OA, Rafat M, Esparza R, Kahn S, Shahin M, et al. Irradiation or temozolomide chemotherapy enhances anti-CD47 treatment of glioblastoma. *Innate Immun* (2020) 26:130–7. doi: 10.1177/1753425919876690
56. Sockolosky JT, Dougan M, Ingram JR, Ho CC, Kauke MJ, Almo SC, et al. Durable antitumor responses to CD47 blockade require adaptive immune stimulation. *Proc Natl Acad Sci U S A* (2016) 113:E2646–2654. doi: 10.1073/pnas.1604268113
57. McCracken MN, Cha AC, Weissman IL. Molecular Pathways: Activating T Cells after Cancer Cell Phagocytosis from Blockade of CD47 "Don't Eat Me" Signals. *Clin Cancer Res* (2015) 21:3597–601. doi: 10.1158/1078-0432.ccr-14-2520
58. Matlung HL, Babes L, Zhao XW, van Houdt M, Treffers LW, van Rees DJ, et al. Neutrophils Kill Antibody-Opsonized Cancer Cells by Trogoptosis. *Cell Rep* (2018) 23:3946–59.e3946. doi: 10.1016/j.celrep.2018.05.082
59. Hatterer E, Chauchet X, Richard F, Barba L, Moine V, Chatel L, et al. Targeting a membrane-proximal epitope on mesothelin increases the tumoricidal activity of a bispecific antibody blocking CD47 on mesothelin-positive tumors. *mAbs* (2020) 12:1739408. doi: 10.1080/19420862.2020.1739408
60. Chao MP, Weissman IL, Majeti R. The CD47-SIRP alpha pathway in cancer immune evasion and potential therapeutic implications. *Curr Opin Immunol* (2012) 24:225–32. doi: 10.1016/j.coi.2012.01.010
61. Kikuchi Y, Uno S, Kinoshita Y, Yoshimura Y, Iida S, Wakahara Y, et al. Apoptosis inducing bivalent single-chain antibody fragments against CD47 showed antitumor potency for multiple myeloma. *Leuk Res* (2005) 29:445–50. doi: 10.1016/j.leukres.2004.09.005
62. Kikuchi Y, Uno S, Yoshimura Y, Otabe K, Iida S, Oheda M, et al. A bivalent single-chain Fv fragment against CD47 induces apoptosis for leukemic cells. *Biochem Biophys Res Commun* (2004) 315:912–8. doi: 10.1016/j.bbrc.2004.01.128
63. Manna PP, Frazier WA. CD47 mediates killing of breast tumor cells via Gi-dependent inhibition of protein kinase A. *Cancer Res* (2004) 64:1026–36. doi: 10.1158/0008-5472.can-03-1708
64. Manna PP, Frazier WA. The mechanism of CD47-dependent killing of T cells: heterotrimeric Gi-dependent inhibition of protein kinase A. *J Immunol* (2003) 170:3544–53. doi: 10.4049/jimmunol.170.7.3544
65. Advani R, Flinn I, Popplewell L, Forero A, Bartlett NL, Ghosh N, et al. CD47 Blockade by Hu5F9-G4 and Rituximab in Non-Hodgkin's Lymphoma. *N Engl J Med* (2018) 379:1711–21. doi: 10.1056/NEJMoa1807315
66. Ring NG, Herndler-Brandstetter D, Weiskopf K, Shan L, Volkmer JP, George BM, et al. Anti-SIRP alpha antibody immunotherapy enhances neutrophil and macrophage antitumor activity. *Proc Natl Acad Sci U S A* (2017) 114:E10578–85. doi: 10.1073/pnas.1710877114
67. Petrova PS, Viller NN, Wong M, Pang XL, Lin GHY, Dodge K, et al. TTI-621 (SIRP alpha Fc): A CD47-Blocking Innate Immune Checkpoint Inhibitor with Broad Antitumor Activity and Minimal Erythrocyte Binding. *Clin Cancer Res* (2017) 23:1068–79. doi: 10.1158/1078-0432.ccr-16-1700
68. Hatterer E, Barba L, Noraz N, Daubeuf B, Aubry-Lachainaye JP, von der Weid B, et al. Co-engaging CD47 and CD19 with a bispecific antibody abrogates B-cell receptor/CD19 association leading to impaired B-cell proliferation. *Mabs* (2019) 11:322–34. doi: 10.1080/19420862.2018.1558698
69. Dheilly E, Majocchi S, Moine V, Didelot G, Broyer L, Calloud S, et al. Tumor-Directed Blockade of CD47 with Bispecific Antibodies Induces Adaptive Antitumor Immunity. *Antibodies* (2018) 7:15. doi: 10.3390/antib7010003
70. Zhang XY, Wang SF, Nan YY, Fan JJ, Chen W, Luan JY, et al. Inhibition of autophagy potentiated the anti-tumor effects of VEGF and CD47 bispecific therapy in glioblastoma. *Appl Microbiol Biotechnol* (2018) 102:6503–13. doi: 10.1007/s00253-018-9069-3
71. Liu J, Wang LJ, Zhao FF, Tseng S, Narayanan C, Shura L, et al. Pre-Clinical Development of a Humanized Anti-CD47 Antibody with Anti-Cancer Therapeutic Potential. *PLoS One* (2015) 10:23. doi: 10.1371/journal.pone.0137345
72. Catani L, Sollazzo D, Ricci F, Polverelli N, Palandri F, Baccarani M, et al. The CD47 pathway is deregulated in human immune thrombocytopenia. *Exp Hematol* (2011) 39:486–94. doi: 10.1016/j.exphem.2010.12.011
73. Oldenborg PA, Zheleznyak A, Fang YF, Lagenaur CF, Gresham HD, Lindberg FP. Role of CD47 as a marker of self on red blood cells. *Science* (2000) 288:2051–62. doi: 10.1126/science.288.5473.2051
74. Gholamin S, Mitra SS, Feroze AH, Liu J, Kahn SA, Zhang M, et al. Disrupting the CD47-SIRP alpha anti-phagocytic axis by a humanized anti-CD47 antibody is an efficacious treatment for malignant pediatric brain tumors. *Sci Transl Med* (2017) 9:13. doi: 10.1126/scitranslmed.aaf2968
75. Zhang W, Huang Q, Xiao W, Zhao Y, Pi J, Xu H, et al. Advances in Anti-Tumor Treatments Targeting the CD47/SIRPα Axis. *Front Immunol* (2020) 11:18. doi: 10.3389/fimmu.2020.00018
76. Kauder SE, Kuo TC, Chen A, Harrabi O, Rocha SS, Doyle L, et al. ALX148 Is a High Affinity Sirp alpha Fusion Protein That Blocks CD47, Enhances the Activity of Anti-Cancer Antibodies and Checkpoint Inhibitors, and Has a Favorable Safety Profile in Preclinical Models. *Blood* (2017) 9:13. doi: 10.1126/scitranslmed.aaf2968
77. Weiskopf K, Ring AM, Ho CC, Volkmer JP, Levin AM, Volkmer AK, et al. Engineered SIRPα variants as immunotherapeutic adjuvants to anticancer antibodies. *Science* (2013) 341:88–91. doi: 10.1126/science.1238856
78. Pietsch EC, Dong J, Cardoso R, Zhang X, Chin D, Hawkins R, et al. Anti-leukemic activity and tolerability of anti-human CD47 monoclonal antibodies. *Blood Cancer J* (2017) 7:e536. doi: 10.1038/bcj.2017.7

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REVIEW
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Immune Checkpoint Targeted Therapy in Glioma: Status and Hopes

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Glioma is the most malignant primary tumor of the central nervous system and is characterized by an extremely low overall survival. Recent breakthroughs in cancer therapy using immune checkpoint blockade have attracted significant attention. However, despite representing the most promising (immunotherapy) treatment for cancer, the clinical application of immune checkpoint blockade in glioma patients remains challenging due to the "cold phenotype" of glioma and multiple factors inducing resistance, both intrinsic and acquired. Therefore, comprehensive understanding of the tumor microenvironment and the unique immunological status of the brain will be critical for the application of glioma immunotherapy. More sensitive biomarkers to monitor the immune response, as well as combining multiple immunotherapy strategies, may accelerate clinical progress and enable development of effective and safe treatments for glioma patients.

Keywords: brain immunology, glioma microenvironment, immune checkpoint blockade, immunotherapy resistance, immune-response monitoring biomarker

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INTRODUCTION

In recent years, novel immunotherapies targeting the immune component of the tumor microenvironment have shown great promise for the clinical management of tumors. Among various therapeutic strategies, drugs targeting immune checkpoint molecules are being heralded as a breakthrough in cancer immunotherapy.

Glioma is the most common and deadliest primary brain tumor of the central nervous system (CNS), with a 5-year survival of less than 10%. Glioblastoma multiforme (GBM) accounts for ~50% of glioma cases and is characterized by a 5-year survival rate of less than 5%, corresponding to a grade IV tumor by the World Health Organization (WHO). Unfortunately, the current gold standard of GBM treatment (total resection plus adjuvant radio-chemotherapy) represents only a palliative option for patients, and the median survival after diagnosis is less than 15 months (1).

A striking recent clinical success of checkpoint inhibitors across multiple solid tumors (2, 3) has sparked interest in immune-targeted strategies for glioma treatment. However, the CNS is commonly considered an "immunologically privileged" site as the blood-brain barrier (BBB) inhibits direct contact between the brain and immune system. Considering the unique accessibility and tissue composition of brain, it is therefore not trivial to design effective immunotherapeutic strategies. Herein, we review the unique immunology and tumor microenvironment of the brain. Furthermore, we describe various immune checkpoint blockade strategies, as well as the mechanisms of resistance to immunotherapy.

THE CNS IS IMMUNOLOGICALLY "UNIQUE" RATHER THAN "PRIVILEGED"

The term "immunologically privileged" has been commonly used to describe the failure of the brain to reject heterotopic tissue following transplantation in the past decades. Our understanding of this special characteristic of brain immunology largely originates from experiments by Peter Medawar in the 1940s (4). Although allogeneic tissue transplantation in other areas of the body can lead to immune rejection which continues to the CNS, there is a lack of convincing explanations for the fact that this systemic immune state cannot be initiated from the CNS. Medawar attributed this phenomenon to the lack of lymph nodes and lymphatic vessels in the CNS, which result in the perceived absence of efferent information of the CNS, although this view has been recently disproven (5–12). A series of studies have demonstrated that leukocyte lymphatics exist in the dura sinus and transport antigens from the dura to cervical lymph nodes (9–12). These findings propose an interesting mechanism by which cerebrospinal fluid mediates the immune communication between CNS and circulation *via* a glial-lymphatic pathway (5–8). Given the existence of an afferent system between the brain and peripheral immune system, many propose that CNS is immunologically "unique" rather than "privileged."

For the most part, the BBB is responsible for this "unique immunology" of the brain. Structurally, the BBB consist of a biomembrane between vascular endothelial cells and glial cells. Functionally, the BBB is a dynamic network between circulation and brain that blocks the diffusion of large, hydrophilic molecules or organisms while allowing the influx of small, hydrophobic molecules (13). Except for a few species, such as Neisseria meningitides and Streptococcus pneumoniae which are able to enter the brain circulation *via* specific mechanisms, the vast majority of blood-borne pathogens are excluded from the brain (14). Given that the CNS is rarely exposed to pathogens, the brain has been believed to only exhibit limited immunity due to poor tolerance of the brain tissue to inflammation. Another unique immunological characteristic of the brain is its resident immune cell population. Originating from myeloid precursors born in the yolk sac, microglial cells (MG) invade the CNS during early embryonic development and serve as the primary resident immune cells (15, 16), while most other immune cell subtypes do not exist in CNS. However, contrary to the previous view that the brain only exhibits limited immunity, recent studies have demonstrated that the systemic immune system is fully involved in the cytotoxic response to CNS antigens (17). After inflammatory stimulation, specific antigens are recognized by MG and presented to activated lymphocytes *via* the glial-lymphatic pathway, after which a large number of immune cells can easily penetrate the BBB, inducing a strong inflammatory and subsequent immune response (18–20). Despite this, both innate inflammatory and adaptive immune responses have to be tightly regulated as unrestrained inflammation-mediated intracranial hypertension can have serious consequences. Although the concept of immunological privilege of the brain has been overturned, the unique immunological environment of the CNS still represents a significant hurdle for therapies targeting immune checkpoints blockade in the brain.

THE IMMUNE MICROENVIRONMENT OF GLIOMA

The unique brain immunology leads to a particular tumor microenvironment of glioma. A variety of peripheral immune components are present in this glioma microenvironment, including myeloid derived suppressor cells (MDSCs), natural killer cells (NK cells), macrophages, neutrophils, $CD4^+$ helper T cells (Th), $CD8^+$ cytotoxic T lymphocytes (CTLs), and regulatory T (T reg) cells (21, 22), while their infiltration ratio is remarkably low numbers in gliomas compared to other tumors. Furthermore, various tumor-derived cytokines and chemokines reprogram infiltrating immune cells, which causes them to acquire unique functional phenotypes and transform into tumor-associated immune cells. These tumor-associated immune cells can therefore have profound effects on progression, recurrence, and therapeutic resistance of glioma by inducing inflammatory or anti-inflammatory responses (**Figure 1**).

Tumor-Infiltrating Lymphocytes

As the most important component of the immune response in the tumor microenvironment of most solid tumors, tumor-infiltrating lymphocytes (TILs), represented by $CD4^+$ Th, $CD8^+$ CTLs, and $CD4^+/CD25^+/FoxP3^+$ T reg (23–26), are only present in remarkably low numbers in the CNS compared to other tumor types. $CD4^+$ Th and $CD8^+$ CTL populations increase with tumor malignancy, starting at 39% in WHO grade II tumors, rising to 73% in WHO grade III, and 98% in grade IV (22). Meanwhile, a correlation between increased $CD8^+$ CTL counts and improved patient outcomes has previously been reported (27). T reg cells have a suppressive role in the adaptive immune response and inhibit the proliferation of Th cells and CTLs by secretion of suppressive cytokines (26).

The limited activity and number of TILs in the brain is predominantly caused by the unique immunological status of the brain which encourages only limited immunity in order to prevent an inflammation-mediated intracranial hypertension crisis. In response to tumor-derived inflammatory stimulation, T reg cells secrete anti-inflammatory interleukin-10 (IL-10) and transforming growth factor β (TGF-β) in order to dampen an inflammatory immune response against the tumor (28, 29). In addition to immunosuppressive mechanisms of the CNS, expression of certain genes by the tumor itself also contributes to low levels of TILs. For instance, glioma cells produce a high level of indolamine 2, 3-dioxygenase (IDO) which activates suppressor T cells by depleting tryptophan from the tumor microenvironment (30). Besides, tumor-derived Fas ligand promotes apoptosis of activated T cells and leads to an immune escape of tumor cells by inhibiting dendritic cells and maturation of T cells (31). Overexpression of

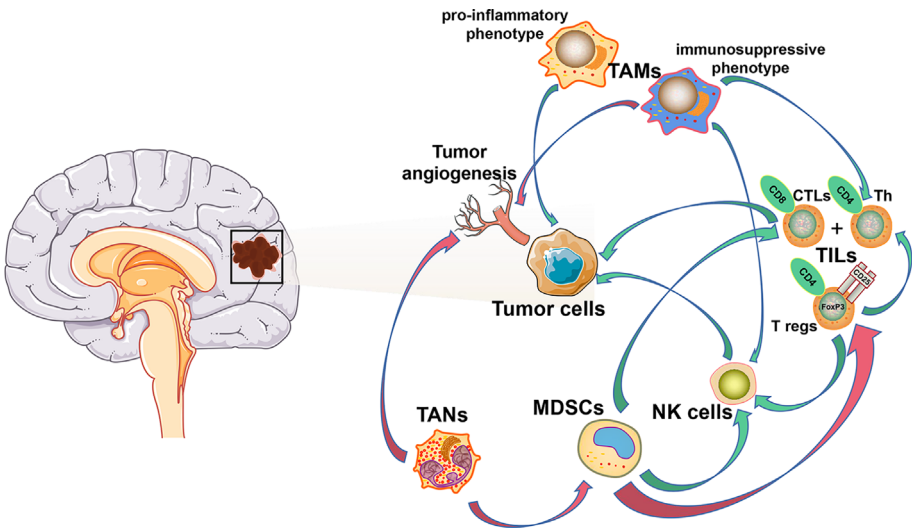

FIGURE 1 | Cellular composition of glioma immune microenvironment. The figure depicts only a general representation of all the cell types that have been reported to be associated with tumor cells in glioma immune microenvironment. Green arrow: down-regulation. Red arrow: up-regulation.

programmed cell death-ligand 1 (PD-L1) in glioma cells prevents activation of T cells and induces T cell apoptosis *via* binding to programmed death 1 (PD-1), a well-known inhibitory immune checkpoint molecule (32, 33). Moreover, an overexpression of CTLA-4 mRNA and protein, a strong $CD4^+$ T cell and $CD8^+$ CTL inhibitor, is caused by lack of CD80/86 co-stimulatory molecules (22). Therefore, a comprehensive understanding of tumor heterogeneity and the role of T cells in glioma is of critical importance for the design of therapeutic targets.

Tumor-Associated Macrophages

Tumor-associated macrophages (TAMs) are the major infiltrating immune component in the glioma microenvironment, accounting for ~50% of all immune cells, and have an important role in neoplasia, metastasis, immune escape, and tumor angiogenesis (34, 35). Several studies have reported that a majority of TAMs are derived from circulating monocyte-derived macrophages (MDMs), while the remaining proportion originates from MG (36–38). Immature monocytes migrate to the tumor microenvironment and develop into TAMs following exposure to several cytokines (34, 35). In the glioma microenvironment, tumor- or effector T cell-derived cytokines promote a change in macrophage effector mechanisms on a spectrum between a pro-inflammatory "M1" phenotype with anti-tumor responses, and an immunosuppressive "M2" phenotype with anti-inflammatory responses (39). In the early stages of glioma, TAMs inhibit tumor proliferation *via* the pro-inflammatory "M1" phenotype, while in advanced glioma, TAMs are predominantly characterized by the "M2" phenotype, which generally induces an immunosuppressive response and immune escape of the tumor. As a special type of TAMs in CNS, MG also exhibit similar plasticity to monocyte-derived macrophages (40).

Studies have demonstrated that large numbers of infiltrating TAMs are closely associated with poor prognosis (41) and the "M2" phenotype has been shown to promote tumor progression *via* secretion of immunosuppressive cytokines and factors promoting angiogenesis (41, 42). Given this evidence, it seems feasible to block the formation and phenotypic "M2" transformation of TAMs. In mouse models, CSF-1 receptor inhibition with small molecules either blocks the transformation of "M2" phenotype or depletes TAMs (43–46), both of which inhibit glioma progression and invasion. Meanwhile, some other drugs have also been shown to achieve their anti-tumor effect by depleting monocytes that serve as precursors of TAM (47). However, recent studies have expanded our understanding of macrophage polarization (48) and revealed a multifaceted response comprising classical M1 and M2 polarization, including expression changes associated with chronic inflammatory stimuli and exposure to free fatty acids, which is involved in regulation of bone marrow cell function. This indicates that the diverse transcriptional programming of TAMs in glioma extends beyond the simplified view of an "M1" versus "M2" polarization. Thus, despite the fact that both depletion of TAMs and targeting "M2" polarization can represent attractive therapeutic approaches for glioma, a more comprehensive understanding of TAM phenotypes is required for efficient and safe treatments of glioma (43, 49, 50).

Myeloid-Derived Suppressor Cells

Chronic inflammation in the tumor microenvironment is induced by overexpression of pro-inflammatory cytokines, including CSF-1, VEGF, TGF-β, and tumor necrosis factor α (TNF-α) (51, 52). These pro-inflammatory cytokines promote tumor growth, progression, and resistance to immunotherapy by inducing a transformation of immature myeloid cells into myeloid-derived suppressor cells (MDSCs). MDSCs are

recruited to peripheral lymphoid organs and the tumor microenvironment from the bone marrow, promoting tumor cell proliferation *via* various mechanisms, including suppression of cytotoxic NK cell activity, inhibition of the adaptive T cell response, induction of T cell apoptosis and T reg cell proliferation, and secretion of immunosuppressive cytokines (53–56). Therefore, MDSCs contribute to resistance to immunotherapy, and combining treatments targeting MDSCs with other immunotherapies has become a promising therapeutic strategy achieving considerable success (57–61). In glioma, related research has been focusing on strategies that either inhibit the recruitment (targeting of C-C motif chemokine ligand 2, VEGF-A, IL-8, and galectin-1) or the formation of MDSCs from myeloid precursors (targeting of M-CSF, PI3Kγ, TAM-RTKs, and COX-2). Such strategies have shown great promise in preclinical studies (62). As there is increasing evidence that the function of MDSCs is tumor type-dependent, a clear definition of this cell type in glioma remains warranted (49). Transcriptomic characterizations of MDSCs - separately from MG and MDMs – should be carried out to ascertain the suppressive function and mechanisms of differentiation into MDSCs, which could help to evaluate the clinical value of MDSCs-targeted therapies in glioma (63).

Tumor-Associated Neutrophils

Completely contrary to their pro-inflammatory function during infections, neutrophils have been frequently reported to promote tumor progression and metastasis in recent years (64–66). This unique relationship between neutrophils and tumor cells could provide a reasonable explanation for the phenomenon that circulating tumor cells often escape from immune surveillance in breast cancer as neutrophils account for the largest proportion of circulatory leukocytes (66). Besides, current study also indicated that immunosuppressive tumor-associated neutrophils (TANs) or granulocytic MDSCs are enriched in neutrophil-enriched subtypes of triple negative breast cancer and were associated with acquired immune checkpoint blockade resistance (67). In the glioma microenvironment, TANs promote tumor malignancy by mediating angiogenesis (68). Besides, TAN depletion strategies using a $Ly6G^+$ monoclonal antibody have been shown to prolong overall survival in preclinical GBM mouse models (69). However, the mechanisms underlying TAN recruitment to the tumor microenvironment and the role of TANs in tumor progression are not yet comprehensively understood and how the glioma microenvironment heterogeneity affects neutrophil reprogramming still remains to be unraveled.

Natural Killer Cells

A variety of mechanisms suppressing the activity of natural killer (NK) cells, the most efficient innate cytotoxic lymphocytes, have been identified during tumor cell progression. Similar to normal cells, glioma cells can inhibit antigen presenting cell (APC)-mediated recognition and NK cell-mediated killing through expression of MHC class I molecules (MHC I) that interact with NK cell immunoglobulin-like receptors (KIRs) (49). Besides, infiltrating NK cells in the glioma microenvironment have been reported to be commonly nonfunctional, largely owing to the combined negative regulatory effect of TAMs, MDSCs, and T reg cells (49, 70).

IMMUNE CHECKPOINT BLOCKADE STRATEGY AND INHIBITORS

There is no doubt that among various immunotherapies, despite that checkpoint blockade might not be the most promising treatment for glioma, it has been the immunotherapy most developed in clinical use. Via a combination of specific antibodies and checkpoint molecules, effector T cells can be reactivated and exert tumor cell cytotoxicity. In the next paragraphs, we describe classical immune checkpoint molecules and their inhibitors (**Table 1**).

PD-1/PD-L1

PD-1 and its ligands PD-L1/2 are the most comprehensively studied immune checkpoint molecules to date. PD-1 negatively regulates T cell receptor-mediated signaling transduction pathways and, in combination with PD-L1, inhibits activation and cytotoxic T cell effects and blocks the production of inflammatory factors, resulting in T cell inactivity. Expression of PD-1 on immune cells is tightly regulated. For instance, PD-1 expression appears on the surface of T cells shortly (less than 24 h) after T cell activation and decreases with the elimination or clearance of the antigen (49). Under chronic inflammatory conditions or in cancer, antigens repetitively stimulate CTLs to maintain high levels of PD-1 expression, eventually resulting in T cell exhaustion and depletion. Tumor-expressed PD-L1 is regulated by several mechanisms, including phosphatidylinositol 3-kinase (PI3K) signaling pathway activation and TIL-secreted interferon γ (IFN-γ) (71). In glioma, PD-L1 is predominantly expressed on tumor cells and TAMs and negatively relates to patient outcome (72–74). To date, two anti-PD-1 antibodies (Nivolumab, Pembrolizumab) and three anti-PD-L1 antibodies (Atezolizumab, Avelumab, Durvalumab) have been put into clinical application and have achieved dramatic successes against a variety of solid tumors (75–77). However, they have so far not been approved for clinical treatment of GBM despite numerous preclinical successes reported over the past decades (78–83). For instance, in the preclinical GL261 model, anti-PD-1 treatment success is dosage dependent, with the best outcome reported being a cure rate of 50% (81, 83). Anti-PD-1 monotherapy has been observed to result in an increased ratio of $CD8^+$ CTLs to T reg cells, and enhanced efficacy when combined with radiation and other checkpoint inhibitors (81, 83).

Schalper et al. (84) reported treatment of 30 GBM patients (3 primary, 27 recurrent) with preoperative and postoperative nivolumab (NCT02550249), resulting in increased transcription of chemokines, infiltration of TILs, and diversity of TCR in tumor microenvironment. While no patients with recurrent GBM benefited from treatment as measured by overall survival (OS), two of the three primary GBM patients survived for 33

TABLE 1 | Current clinical trials of immune checkpoint blockade.

Clinical trials	Stage	Targets	Drugs
Monotherapy			
NCT02017717 (CheckMate-143)	III	PD-1	Nivolumab
NCT02617589 (CheckMate-498)	III	PD-1	Nivolumab + radiation
NCT02667587 (CheckMate-548)	III	PD-1	Nivolumab + radiation + TMZ
NCT02648633	III	PD-1	Nivolumab
NCT03718767	II	PD-1	Nivolumab
NCT03797326	II	PD-1	Pembrolizumab
NCT02852655	II	PD-1	Pembrolizumab
NCT02337686	II	PD-1	Pembrolizumab
NCT02968940	II	PD-L1	Avelumab + radiation
NCT03047473	II	PD-L1	Avelumab + TMZ
NCT03341806	I	PD-L1	Avelumab
Combined with other checkpoint molecules			
NCT03707457	I	PD-1+IDO1	Nivolumab + INCB024360
NCT04047706	I	PD-1+IDO1	Nivolumab + BMS986205
NCT02658981	I	PD-1+LAG-3	Nivolumab + BMS986016
NCT03493932	I	PD-1+LAG-3	Nivolumab + BMS986016
NCT03233152	I	PD-1+CTLA-4	Nivolumab + Ipilimumab
NCT03422094	I	PD-1+CTLA-4	Nivolumab + Ipilimumab
NCT02311920	I	PD-1+CTLA-4	Nivolumab + Ipilimumab+TMZ
NCT02794883	II	PD-L1+CTLA-4	Durvalumab + Tremelimumab
Combined with VEGF/VEGFR			
NCT03743662	II	PD-1+VEGF	Nivolumab + BEV + radiation
NCT02336165	II	PD-L1+VEGF	Durvalumab + BEV
NCT03291314	I	PD-L1+VEGFR	Avelumab + Axitinib
NCT02052648	I/II	IDO1+VEGF	Indoximod + BEV + TMZ
Combined with CAR-T			
NCT03726515	I	PD-1+CAR-T	Pembrolizumab + CAR-EGFR-III-T
NCT04003649	I	PD-1+CTLA-4+CAR-T	Nivolumab + Ipilimumab + CAR-T
Combined with vaccines			
NCT02529072	I	PD-1	Nivolumab + DC vaccines
NCT02287428	I	PD-1	Pembrolizumab + NeoVax vaccines
NCT03750071	I/II	PD-L1	Avelumab + VXM01 vaccines

PD-1, programmed cell death protein 1; PD-L1, programmed cell death 1 ligand 1; IDO1, indoleamine 2,3-dioxygenase; LAG-3, lymphocyte-activation gene 3; CTLA-4, cytotoxic T-lymphocyte associated protein 4; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; CAR-T, chimeric antigen receptor T-cell; TMZ, temozolomide.

month and 28 months, respectively. CheckMate-143 phase III trial (NCT02017717) found no OS benefit when comparing nivolumab with bevacizumab (anti-VEGFA) in the treatment of recurrent GBM (median OS 9.8 vs. 10.0 months) (85). In the CheckMate-498 trial (NCT02617589), newly diagnosed GBM patients with unmethylated O6-methylguanine-DNA methytransferase (MGMT) promoter who received nivolumab plus radiotherapy did not benefit from this treatment compared with radiotherapy plus temozolomide (TMZ) as measured by OS (86). More recently, similarly disappointing results have been reported in the CheckMate-548 study (NCT02667587). Here, newly diagnosed GBM patients with methylated MGMT promoter did not show a PFS benefit with anti-PD-1 treatment; the OS effect is still pending (87). Primary results of a study by Lukas et al. (88) reporting on a clinical trial using atezolizumab, an anti-PD-L1 antibody (NCT01375842), showed that increased $CD4^+$ T cells and IDH mutation indicated better treatment efficiency of atezolizumab.

CTLA-4

Cytotoxic T-lymphocyte associated protein 4 (CTLA-4) expression on activated T cells or T reg cells was the first identified member of the immunoglobulin superfamily, and also the first immune regulation molecule used in targeted therapy. CTLA-4 inhibits T cell co-stimulatory signaling pathways by combining with ligands CD80 and CD86 expressed on APCs (89). Unlike PD-1, CTLA-4 signaling occurs at the early stages of T cell activation, and CTLA-4 is mainly expressed on T cells of the lymph node (90). In preclinical experiments, anti-CTLA-4 monotherapy prolonged OS in the GL261 syngeneic mouse model (81). Although CTLA-4 blockade strategy results in an increased median survival with 25% cure rate, the response of monotherapy was still considered limited as combined application of anti-PD-1 therapy or radiotherapy can remarkably improve efficacy (81, 90). Reardon et al. (81) also reported that combination of anti-CTLA-4 and anti-PD-1 therapy increased the cure rate to 75%. For further investigation, CTLA-4 blockade as a monotherapy or in combination with anti-PD-1 treatment is therefore currently being tested in a phase III clinical trial in patients with recurrent GBM (NCT02017717).

B7 Family

In recent years, there have been increasing numbers of studies investigating immune checkpoint molecules of the B7 family. In

addition to PD-L1 (B7-H1), studies have investigated B7-H3 (CD276), B7-H4, B7-H5 (Vista), B7-H6, and B7-H7 (HHLA2), amongst others. B7-H3 and B7-H7 have a dual function, enabling both co-stimulation and co-inhibition (91). By interacting with specific ligands, these molecules can therefore have different roles in tumor progression. For instance, recent research points out that B7-H3 positively relates to the Toll-like receptor signaling pathway and the poor survival of glioma patients, while it has also been reported to co-stimulate immunological function and be involved in anti-tumor functions (92–95). Similarly, B7-H7 shows the same phenomenon in various solid tumors (96, 97). Inhibiting NK-mediated recognition of B7-H6 is an important mechanism of the tumor immune escape. NK cells eliminate B7-H6-expressing tumor cells either directly *via* cytotoxicity or indirectly by cytokine secretion, which highlights a role for the tumor-induced "self"-molecule B7-H6 in alerting innate immunity (91). Both B7-H4 and B7-H5 have co-inhibitory functions on the immune system (91), although research on these and other members of the B7 family is still in progress. As the largest immune checkpoint family, the function and mechanisms of B7 family members in glioma remains largely unknown. Thus, a more comprehensive understating of the function of the B7 family in glioma could help to explore more effective therapeutic targets in immunotherapy.

IDO, LAG-3, and TIM-3

Indoleamine 2,3-dioxygenase (IDO) is the key enzyme of the L-tryptophan metabolism *via* the kynurenine pathway. Although IDO expressed on tumor cells and dendritic cells (DCs) is not a typical checkpoint molecule, it can inhibit T cell activation by modulation of the tryptophan metabolism which has an important role in the function of T cells (98–101). Metabolites of tryptophan also induce T cell apoptosis (101). Besides, an interaction of kynurenine and TGF-β can induce FoxP3 expression in T cells, which results in T reg cell polarization (102, 103). Preclinical models have shown that clinical trials with IDO inhibitors did not meet the expectations (104).

Lymphocyte-activation gene 3 (LAG-3) has four extracellular immunoglobulin superfamily-like domains which bind to MHC II, and is responsible for transmission of inhibitory signals (105). In addition to MHC II, another ligand for LAG-3 is Gal▪3, which is involved in the inhibition of $CD8^+$ CTLs (106). Tumor-derived antigens induce LAG-3 overexpression and thereby lead to the depletion of $CD8^+$ CTLs (107). Research in mouse xenografts revealed that co-targeting of PD-1 and LAG-3 on TILs can limit tumor growth, which is likely superior to a single inhibitory mechanism (108, 109). Given this finding, recent trials have focused on anti-PD-1 and anti-LAG-3 combination therapies rather than monotherapies. However, the vast majority of this research in still in preclinical stages.

T cell immunoglobulin domain and mucin domain protein-3 (TIM-3) is expressed on $CD4^+$ and $CD8^+$ T cells, monocytes, and macrophages (110). TIM-3 regulates T cell depletion and is involved in tumor immunosuppression and immune escape *via* binding to its ligand Gal-9 (110). Clinical trials reported that GBM patients with overexpression of TIM-3 have higher tumor malignancy, a lower quality of life, and worse prognosis (111, 112).

Although several checkpoint-related molecules have been discovered, there have been none as influential as PD-1 and CTLA-4, and the efficiency of the vast majority of checkpoint inhibitors in glioma remains doubtful. While single checkpoint inhibition is the standard of care in many tumor entities, checkpoint molecules cooperate or antagonize each other in tumor progression, making it difficult for a single checkpoint inhibitor to play a decisive role in systemic immunity. Therefore, combination of checkpoint inhibitors seems to be more efficient than monotherapy.

LESSONS FROM CLINICAL FAILURES

There is no doubt that immunotherapy holds promise for the treatment of glioma. However, even promising preclinical data are rarely translated into clinical success in glioma. Two factors complicate the clinical translation for glioma treatment. Firstly, glioma has a "cold tumor" phenotype, which is associated with a poor response to immunotherapy. Owing to the unique environment of CNS, even after inhibiting checkpoint molecules to induce T cell responses against glioma, antigen-specific TILs remain at relatively low levels. Second, current preclinical models have only limited capacity to reflect the real tumor heterogeneity of glioma. Generally, GBM can be classified into four subtypes: classical, proneural, neural, and mesenchymal, with high heterogeneity between each subtype (113, 114). There are remarkable differences in gene expression among these four subtypes, which suggests that targeting checkpoint molecules therapies may only be effective for some subpopulations expressing specific genes, but not for other subpopulations. These two factors interact to form resistance mechanisms at all phases of the antitumor immune response: intrinsic resistance prevents the initiation of a response; adaptive resistance deactivates tumor-infiltrating immune cells; and acquired resistance protects the tumor from elimination in the face of attack by the immune system. Even though dramatic immune responses have been observed in preclinical models using a variety of immunotherapy strategies, patients rarely benefit from these treatments, owing to the extensive immunosuppressive mechanisms of glioma (115, 116). However, these mechanisms render glioma a valuable model for studying how resistance allows tumors to escape immunotherapy.

Intrinsic Resistance

Intrinsic tumor resistance can be classified into three groups: patient-intrinsic factors (including sex, age, and HLA genotype), tumor-intrinsic factors (including the host immune system and tumor-associated stroma), and environmental factors (117–119). Among them, tumor-intrinsic factors, relating to the genetic, transcriptional or functional profile of the tumor cells, are the main determinants of response and resistance (116).

Several studies have demonstrated that tumors can prevent immune responses by not expressing high-quality neoantigens, and they can furthermore rapidly suppress immune responses by

expressing multiple immune checkpoint ligands and immunosuppressive cytokines (115, 116). Meanwhile, even with sufficient antigenicity, sensitivity to immune checkpoint blockade can be disrupted by tumorintrinsic genetic defects in the IFN γ signaling pathway and antigen presentation (120–122). A disruption in anti-tumor response to the IFN γ signaling pathway can inhibit the Janus kinase (JAK) and (STAT) signaling pathway, downregulating PD-L1 expression, and making anti-PD-1 treatment ineffective. Besides, the WNT–β-catenin signaling pathway has been confirmed to prevent an anti-tumor immune response by inhibiting dendritic cells and promoting the immunosuppressive cytokine IL-10 (123–126). Meanwhile, the MAPK signaling pathway also contributes to tumor immune escape by upregulation of the expression of the immunosuppressive cytokines IL6 and IL10 (127).

To date, the heterogeneity of glioma is still considered the basis for its resistance to a variety of treatments. For instance, the most extensively studied neoantigen, EGFR variant III, is a truncated EGFR neoantigen with expression in 19% of newly diagnosed GBM patients, of which 11% exhibit high levels of expression (128). Although nearly 82% of recurrent tumors do not express EGFR variant III, the vast majority of mesenchymal subtypes shows overexpression of EGFR variant III (129, 130). This characteristic makes it difficult to stably express specific antigens to induce a durable anti-tumor immune response. Besides, despite the fact that adjuvant radio-chemotherapy can enhance the efficiency of checkpoint blockade strategies, what cannot be ignored is that radio-chemotherapy has well-documented immunosuppressive functions inducing other resistance mechanisms rather than tumor-intrinsic resistance to immunotherapy, which further reduces the immune responses of the CNS (131).

Adaptive Resistance

The discovery that tumors can counter attacks of the immune system by usurping mechanisms that normally prevent autoimmunity is one of the most impactful findings in the history of oncology. Although immune checkpoint molecules may be expressed in various tumors at "baseline," a remarkable increase of their expression levels can be observed under immunological stimulation (132). Thus, immune checkpoint blockade can trigger strong anti-tumor response. In spite of the durable clinical responses that PD-1 and CTLA-4 blockade strategies have achieved in several advanced tumors, it is undeniable a large proportion of patients do not benefit from checkpoint blockade (132). One explanation for this is that TILs can exhibit severe exhaustion, similar to that observed in chronic viral infections (133). However, while the degree of immune exhaustion in GBM is severe, it does not appear to be singularly so, as other tumors that respond poorly to checkpoint inhibitors use similar adaptive resistance mechanisms (115, 134). Another explanation is that checkpoint molecules with similar mechanisms can compensate for each other. For instance, upregulation of the alternative checkpoint molecule TIM-3 has been observed in tumors resisting PD-1 blockade (135). Downregulation of one immune checkpoint generally upregulates alternative immune checkpoints, eventually leading to the durable immunosuppression and a resistance to the blockade. Given this mechanism, current clinical trials focus on overcoming adaptive resistance of PD-1 and CTLA-4 blockade strategies by targeting alternative immune checkpoints.

Acquired Resistance

Acquired resistance of tumor generally refers to the genetic alternations caused by immunological pressure (115). For instance, in non-small cell lung cancer (NSCLCs) and melanoma, significant downregulation of targeted antigens has been observed in tumor infiltrating region, resulting in the failure of immune targeted therapy (136, 137). Perhaps therapies that overcome intrinsic resistance mechanisms will also render acquired resistance inconsequential by generating a diverse repertoire of T cell clones targeting high-quality targeted antigens that rapidly eliminate a tumor before acquired resistance emerges. However, the exact effects of acquired resistance on malignant glioma remain unknown, as the low response and persistence of treatments in glioma have been considered as an important intrinsic resistance mechanism. In contrast, recent research reported 66 recurrent GBM patients who received PD-1 blockade therapy (138). Among them, 17 patients were identified as responders based on brain imaging and profiling of resected tissue. Tumors in responders were found to be enriched for alterations in the mitogen-activated protein kinase pathway and exhibited branched patterns of evolution, while non-responding tumors more frequently had mutations in the gene encoding PTEN and non-clonal evolution patterns (138). Notably, responders had a significantly longer OS than non-responders (14.3 vs. 10.1 months) (138). Given the heterogeneity of GBM mentioned above, in addition to intrinsic resistance, acquired resistance seems to play an important role in resistance to checkpoint blockade.

HYPOXIA IN THE GLIOBLASTOMA MICROENVIRONMENT

To date, research has mainly focused on the "seed's" response to therapy (i.e., tumor cells themselves), while the problem of "poor soil" (the tumor microenvironment) is often ignored. Herein, we further explored the role of hypoxia in the tumor immune microenvironment. Accumulating evidence indicates that hypoxia may protect tumors from immune responses through various mechanisms, including by inhibition of NK and CTL cell activity, promotion of immunosuppressive cytokines, and by enhancing immunosuppressive cells (T reg cells, TAMs, and neutrophils) (139).

CTLs and NK Cells

There are an increasing number of studies investigating the effects hypoxia on immune cells. For instance, IL-2, an important growth factor for T and NK cells with a pivotal role in the regulation of the host's immune response, has been reported to be exquisitely sensitive to changes in oxygen tension (140). Hypoxia can cause a prolonged reduction in IL-2 mRNA expression and inhibit NK

cell and CTL activity. Meanwhile, hypoxia has also been shown to reduce the ability of NK cells to release IFNγ, TNFα, GM-CSF, CCL3, and CCL5 (139, 141). In patients with a high risk of hypoxia, CTLs and NK cells appeared to be in resting status rather than active (139), revealing that hypoxia might lead to a state of immune suppression.

Suppressive Immune Cells and Cytokines

Hypoxia is thought to play a key role in TAM polarization. It can promote the "M2" phenotype and contribute to tumor growth, immune suppression, and tumor angiogenesis (142–144). In a bioinformatic study assessing the polarization of cells in the tumor immune microenvironment, T reg cells, neutrophils, and TAMs with an "M2" phenotype increased remarkably under hypoxia (139). Besides, hypoxia also promotes the expression of TGF-β and IL-10, two well-established suppressive cytokines (139, 142, 145).

FUTURE DIRECTIONS

The extensive immunosuppressive mechanisms in "seed" (including tumor heterogeneity and alteration of checkpoint molecules) and "soil" (hypoxia in tumor microenvironment) complicates the treatment of glioma and explains why promising preclinical data had rarely been translated into clinical success. Given this, individualized treatment and real-time monitoring of treatment response are essential.

Biomarkers

Predicting and monitoring patient responses to treatment have become an urgent requirement for the clinical development of immunotherapies. Tumor tissue biopsies remain the gold standard for diagnosis, but its application is not suitable for response monitoring. Complex and changeable signals on MRI furthermore challenge the differentiation of glioma recurrence from pseudoprogression and radiation brain necrosis. Thus, the availability of biomarkers has greatly enhanced oncological practices and is now the basis of precision medicine for many cancers. However, suitable biomarkers for immunotherapies of glioma are still unknown. Recently, studies have reported that anti-PD-1 therapy results in upregulation of T cell- and IFN γ-related gene expression in immune cells, as well as downregulation of cell-cycle-related gene expression within tumor cells (84, 138, 146). Anti-PD-1 therapy seems to result in different responses in tumors with specific genetic alternation, including increased clonal expansion of T cells, decreased expression of PD-1 in peripheral T cells, and decreased monocytes in circulation (84, 138, 146). Liquid biopsies are anticipated to become a successful strategy for biomarker response monitoring in glioma. For instance, tumor mutation burden (TMB) based on detection of circulating tumor DNA shows a high correlation with anti PD-1 response (147). Meanwhile, current studies also indicated that circulating tumor cells (CTCs) of glioma offer unique advantages for non-invasive monitoring of tumor progression which could furthermore identify pseudoprogression and radiation necrosis (148, 149). Taken together, an efficient biomarker can not only help to choose individualized treatment, but also timely reflect when patients develop resistance to adjust the treatment.

Combined Drug Therapy

Immunotherapy resistance of glioma is a result of multiple factors: intrinsic resistance and adaptive resistance in the early stages of treatment, and acquired resistance over the period of therapy mediated by genetic alternations. Owing to unique resistance mechanisms, monotherapy of checkpoint inhibitors for glioma does not seem to induce durable anti-tumor responses. Thus, combined drug therapy, to some extent, may show advantages and higher efficacy. For instance, in preclinical model, anti-PD-1 combined with anti-TIM-3 synergistically improved survival (135). Furthermore, the combination of immune checkpoint blockade and anti-tumor-associated immune cells (TAMs, MDSCs) also holds promise for the treatment of glioma. Therefore, a more comprehensive understanding of immune cell roles in the tumor microenvironment, as well as specific biomarkers for functional immune cell types and tumor response, may be necessary for individualized treatment of patients with glioma in the era of precision medicine.

AUTHOR CONTRIBUTIONS

YQ and BL contributed equally to this article. XX and QC designed this study. YQ, QS and BL performed the data collection and collation. All the authors were involved in the analysis and interpretation of data. YQ wrote the paper, with the help of the coauthors. XX and QC reviewed and revised the manuscript. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Van Meir EG, Hadjipanayis CG, Norden AD, Shu HK, Wen PY, Olson JJ. Exciting new advances in neuro-oncology: the avenue to a cure for malignant glioma. *CA Cancer J Clin* (2010) 60(3):166–93. doi: 10.3322/caac.20069
2. Emens LA, Ascierto PA, Darcy PK, Demaria S, Eggermont AMM, Redmond WL, et al. Cancer immunotherapy: Opportunities and challenges in the rapidly evolving clinical landscape. *Eur J Cancer* (2017) 81:116–29. doi: 10.1016/j.ejca.2017.01.035
3. Lipson EJ, Forde PM, Hammers HJ, Emens LA, Taube JM, Topalian SL. Antagonists of PD-1 and PD-L1 in Cancer Treatment. *Semin Oncol* (2015) 42(4):587–600. doi: 10.1053/j.seminoncol.2015.05.013
4. Medawar PB. Immunity to homologous grafted skin; the fate of skin homografts transplanted to the brain, to subcutaneous tissue, and to the anterior chamber of the eye. *Br J Exp Pathol* (1948) 29(1):58–69.
5. Goldmann J, Kwidzinski E, Brandt C, Mahlo J, Richter D, Bechmann I. T cells traffic from brain to cervical lymph nodes via the cribroid plate and the nasal mucosa. *J Leukoc Biol* (2006) 80(4):797–801. doi: 10.1189/jlb.0306176

6. Bradbury MW, Westrop RJ. Factors influencing exit of substances from cerebrospinal fluid into deep cervical lymph of the rabbit. *J Physiol* (1983) 339:519–34. doi: 10.1113/jphysiol.1983.sp014731
7. Cserr HF, Knopf PM. Cervical lymphatics, the blood-brain barrier and the immunoreactivity of the brain: a new view. *Immunol Today* (1992) 13 (12):507–12. doi: 10.1016/0167-5699(92)90027-5
8. Widner H, Jönsson BA, Hallstadius L, Wingårdh K, Strand SE, Johansson BB. Scintigraphic method to quantify the passage from brain parenchyma to the deep cervical lymph nodes in rats. *Eur J Nucl Med* (1987) 13(9):456–61. doi: 10.1007/BF00281860
9. Eide PK, Vatnehol SAS, Emblem KE, Ringstad G. Magnetic resonance imaging provides evidence of glymphatic drainage from human brain to cervical lymph nodes. *Sci Rep* (2018) 8(1):7194. doi: 10.1038/s41598-018-25666-4
10. Louveau A, Herz J, Alme MN, Salvador AF, Dong MQ, Viar KE, et al. CNS lymphatic drainage and neuroinflammation are regulated by meningeal lymphatic vasculature. *Nat Neurosci* (2018) 21(10):1380–91. doi: 10.1038/s41593-018-0227-9
11. Aspelund A, Antila S, Proulx ST, Karlsen TV, Karaman S, Detmar M, et al. A dural lymphatic vascular system that drains brain interstitial fluid and macromolecules. *J Exp Med* (2015) 212(7):991–9. doi: 10.1084/jem.20142290
12. Louveau A, Smirnov I, Keyes TJ, Eccles JD, Rouhani SJ, Peske JD, et al. Structural and functional features of central nervous system lymphatic vessels. *Nature* (2015) 523(7560):337–41. doi: 10.1038/nature14432
13. Bauer HC, Krizbai IA, Bauer H, Traweger A. "You Shall Not Pass"-tight junctions of the blood brain barrier. *Front Neurosci* (2014) 8:392. doi: 10.3389/fnins.2014.00392
14. Coureuil M, Lécuyer H, Bourdoulous S, Nassif X. A journey into the brain: insight into how bacterial pathogens cross blood-brain barriers. *Nat Rev Microbiol* (2017) 15(3):149–59. doi: 10.1038/nrmicro.2016.178
15. Hutter G, Theruvath J, Graef CM, Zhang M, Schoen MK, Manz EM, et al. Microglia are effector cells of CD47-SIRPα antiphagocytic axis disruption against glioblastoma. *Proc Natl Acad Sci U.S.A.* (2019) 116(3):997–1006. doi: 10.1073/pnas.1721434116
16. Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S, et al. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science* (2010) 330(6005):841–5. doi: 10.1126/science.1194637
17. Klein RS, Izikson L, Means T, Gibson HD, Lin E, Sobel RA, et al. IFN-inducible protein 10/CXC chemokine ligand 10-independent induction of experimental autoimmune encephalomyelitis. *J Immunol (Baltimore Md 1950)* (2004) 172(1):550–9. doi: 10.4049/jimmunol.172.1.550
18. Carson MJ, Doose JM, Melchior B, Schmid CD, Ploix CC. CNS immune privilege: hiding in plain sight. *Immunol Rev* (2006) 213:48–65. doi: 10.1111/j.1600-065X.2006.00441.x
19. Masson F, Calzascia T, Di Berardino-Besson W, de Tribolet N, Dietrich PY, Walker PR. Brain microenvironment promotes the final functional maturation of tumor-specific effector CD8+ T cells. *J Immunol (Baltimore Md 1950)* (2007) 179(2):845–53. doi: 10.4049/jimmunol.179.2.845
20. Tang Y, Le W. Differential Roles of M1 and M2 Microglia in Neurodegenerative Diseases. *Mol Neurobiol* (2016) 53(2):1181–94. doi: 10.1007/s12035-014-9070-5
21. Heimberger AB, Abou-Ghazal M, Reina-Ortiz C, Yang DS, Sun W, Qiao W, et al. Incidence and prognostic impact of FoxP3+ regulatory T cells in human gliomas. *Clin Cancer Res* (2008) 14(16):5166–72. doi: 10.1158/1078-0432.CCR-08-0320
22. Gieryng A, Pszczolkowska D, Walentynowicz KA, Rajan WD, Kaminska B. Immune microenvironment of gliomas. *Lab Investigat J Tech Methods Pathol* (2017) 97(5):498–518. doi: 10.1038/labinvest.2017.19
23. Waziri A. Glioblastoma-derived mechanisms of systemic immunosuppression. *Neurosurg Clin N Am* (2010) 21(1):31–42. doi: 10.1016/j.nec.2009.08.005
24. Lohr J, Ratliff T, Huppertz A, Ge Y, Dictus C, Ahmadi R, et al. Effector T-cell infiltration positively impacts survival of glioblastoma patients and is impaired by tumor-derived TGF-β. *Clin Cancer Res* (2011) 17(13):4296–308. doi: 10.1158/1078-0432.CCR-10-2557
25. Alexiou GA, Vartholomatos G, Karamoutsios A, Batistatou A, Kyritsis AP, Voulgaris S. Circulating progenitor cells: a comparison of patients with glioblastoma or meningioma. *Acta Neurol Belg* (2013) 113(1):7–11. doi: 10.1007/s13760-012-0097-y
26. Wainwright DA, Dey M, Chang A, Lesniak MS. Targeting Tregs in Malignant Brain Cancer: Overcoming IDO. *Front Immunol* (2013) 4:116. doi: 10.3389/fimmu.2013.00116
27. Zhang W, Wu S, Guo K, Hu Z, Peng J, Li J. Correlation and clinical significance of LC3, CD68+ microglia, CD4+ T lymphocytes, and CD8+ T lymphocytes in gliomas. *Clin Neurol Neurosurg* (2018) 168:167–74. doi: 10.1016/j.clineuro.2018.02.044
28. Gong D, Shi W, Yi SJ, Chen H, Groffen J, Heisterkamp N. TGFβ signaling plays a critical role in promoting alternative macrophage activation. *BMC Immunol* (2012) 13:31. doi: 10.1186/1471-2172-13-31
29. Vitkovic L, Maeda S, Sternberg E. Anti-inflammatory cytokines: expression and action in the brain. *Neuroimmunomodulation* (2001) 9(6):295–312. doi: 10.1159/000059387
30. Wainwright DA, Balyasnikova IV, Chang AL, Ahmed AU, Moon KS, Auffinger B, et al. IDO expression in brain tumors increases the recruitment of regulatory T cells and negatively impacts survival. *Clin Cancer Res* (2012) 18(22):6110–21. doi: 10.1158/1078-0432.CCR-12-2130
31. Jansen T, Tyler B, Mankowski JL, Recinos VR, Pradilla G, Legnani F, et al. FasL gene knock-down therapy enhances the antiglioma immune response. *Neuro Oncol* (2010) 12(5):482–9. doi: 10.1093/neuonc/nop052
32. Perng P, Lim M. Immunosuppressive Mechanisms of Malignant Gliomas: Parallels at Non-CNS Sites. *Front Oncol* (2015) 5:153. doi: 10.3389/fonc.2015.00153
33. Butte MJ, Keir ME, Phamduy TB, Sharpe AH, Freeman GJ. Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses. *Immunity* (2007) 27(1):111–22. doi: 10.1016/j.immuni.2007.05.016
34. de Groot AE, Pienta KJ. Epigenetic control of macrophage polarization: implications for targeting tumor-associated macrophages. *Oncotarget* (2018) 9(29):20908–27. doi: 10.18632/oncotarget.24556
35. Liu Y, Cao X. The origin and function of tumor-associated macrophages. *Cell Mol Immunol* (2015) 12(1):1–4. doi: 10.1038/cmi.2014.83
36. Zhou W, Ke SQ, Huang Z, Flavahan W, Fang X, Paul J, et al. Periostin secreted by glioblastoma stem cells recruits M2 tumour-associated macrophages and promotes malignant growth. *Nat Cell Biol* (2015) 17 (2):170–82. doi: 10.1038/ncb3090
37. Bowman RL, Klemm F, Akkari L, Pyonteck SM, Sevenich L, Quail DF, et al. Macrophage Ontogeny Underlies Differences in Tumor-Specific Education in Brain Malignancies. *Cell Rep* (2016) 17(9):2445–59. doi: 10.1016/j.celrep.2016.10.052
38. Chen Z, Feng X, Herting CJ, Garcia VA, Nie K, Pong WW, et al. Cellular and Molecular Identity of Tumor-Associated Macrophages in Glioblastoma. *Cancer Res* (2017) 77(9):2266–78. doi: 10.1158/0008-5472.CAN-16-2310
39. Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, et al. Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity* (2014) 41(1):14–20. doi: 10.1016/j.immuni.2014.06.008
40. Walker DG, Lue LF. Immune phenotypes of microglia in human neurodegenerative disease: challenges to detecting microglial polarization in human brains. *Alzheimers Res Ther* (2015) 7(1):56. doi: 10.1186/s13195-015-0139-9
41. Roesch S, Rapp C, Dettling S, Herold-Mende C. When Immune Cells Turn Bad-Tumor-Associated Microglia/Macrophages in Glioma. *Int J Mol Sci* (2018) 19(2):436. doi: 10.3390/ijms19020436
42. da Fonseca AC, Badie B. Microglia and macrophages in malignant gliomas: recent discoveries and implications for promising therapies. *Clin Dev Immunol* (2013) 2013:264124. doi: 10.1155/2013/264124
43. Yan D, Kowal J, Akkari L, Schuhmacher AJ, Huse JT, West BL, et al. Inhibition of colony stimulating factor-1 receptor abrogates microenvironment-mediated therapeutic resistance in gliomas. *Oncogene* (2017) 36(43):6049–58. doi: 10.1038/onc.2017.261
44. Quail DF, Bowman RL, Akkari L, Quick ML, Schuhmacher AJ, Huse JT, et al. The tumor microenvironment underlies acquired resistance to CSF-1R inhibition in gliomas. *Science* (2016) 352(6288):aad3018. doi: 10.1126/science.aad3018

45. Pyonteck SM, Akkari L, Schuhmacher AJ, Bowman RL, Sevenich L, Quail DF, et al. CSF-1R inhibition alters macrophage polarization and blocks glioma progression. *Nat Med* (2013) 19(10):1264–72. doi: 10.1038/nm.3337
46. Coniglio SJ, Eugenin E, Dobrenis K, Stanley ER, West BL, Symons MH, et al. Microglial stimulation of glioblastoma invasion involves epidermal growth factor receptor (EGFR) and colony stimulating factor 1 receptor (CSF-1R) signaling. *Mol Med (Cambridge Mass)* (2012) 18(1):519–27. doi: 10.2119/molmed.2011.00217
47. Germano G, Frapolli R, Belgiovine C, Anselmo A, Pesce S, Liguori M, et al. Role of macrophage targeting in the antitumor activity of trabectedin. *Cancer Cell* (2013) 23(2):249–62. doi: 10.1016/j.ccr.2013.01.008
48. Klemm F, Maas RR, Bowman RL, Kornete M, Soukup K, Nassiri S, et al. Interrogation of the Microenvironmental Landscape in Brain Tumors Reveals Disease-Specific Alterations of Immune Cells. *Cell* (2020) 181(7):1643–60.e17. doi: 10.1016/j.cell.2020.05.007
49. Boussiotis VA, Charest A. Immunotherapies for malignant glioma. *Oncogene* (2018) 37(9):1121–41. doi: 10.1038/s41388-017-0024-z
50. Sampson JH, Gunn MD, Fecci PE, Ashley DM. Brain immunology and immunotherapy in brain tumours. *Nat Rev Cancer* (2020) 20(1):12–25. doi: 10.1038/s41568-019-0224-7
51. Meirow Y, Kanterman J, Baniyash M. Paving the Road to Tumor Development and Spreading: Myeloid-Derived Suppressor Cells are Ruling the Fate. *Front Immunol* (2015) 6:523. doi: 10.3389/fimmu.2015.00523
52. Umansky V, Blattner C, Gebhardt C, Utikal J. The Role of Myeloid-Derived Suppressor Cells (MDSC) in Cancer Progression. *Vaccines (Basel)* (2016) 4(4):36. doi: 10.3390/vaccines4040036
53. Sceneay J, Chow MT, Chen A, Halse HM, Wong CS, Andrews DM, et al. Primary tumor hypoxia recruits CD11b+/Ly6Cmed/Ly6G+ immune suppressor cells and compromises NK cell cytotoxicity in the premetastatic niche. *Cancer Res* (2012) 72(16):3906–11. doi: 10.1158/0008-5472.CAN-11-3873
54. Gao D, Joshi N, Choi H, Ryu S, Hahn M, Catena R, et al. Myeloid progenitor cells in the premetastatic lung promote metastases by inducing mesenchymal to epithelial transition. *Cancer Res* (2012) 72(6):1384–94. doi: 10.1158/0008-5472.CAN-11-2905
55. Yan HH, Pickup M, Pang Y, Gorska AE, Li Z, Chytil A, et al. Gr-1+CD11b+ myeloid cells tip the balance of immune protection to tumor promotion in the premetastatic lung. *Cancer Res* (2010) 70(15):6139–49. doi: 10.1158/0008-5472.CAN-10-0706
56. Condamine T, Mastio J, Gabrilovich DI. Transcriptional regulation of myeloid-derived suppressor cells. *J Leukoc Biol* (2015) 98(6):913–22. doi: 10.1189/jlb.4RI0515-204R
57. Kim K, Skora AD, Li Z, Liu Q, Tam AJ, Blosser RL, et al. Eradication of metastatic mouse cancers resistant to immune checkpoint blockade by suppression of myeloid-derived cells. *Proc Natl Acad Sci U.S.A.* (2014) 111(32):11774–9. doi: 10.1073/pnas.1410626111
58. Christmas BJ, Rafie CI, Hopkins AC, Scott BA, Ma HS, Cruz KA, et al. Entinostat Converts Immune-Resistant Breast and Pancreatic Cancers into Checkpoint-Responsive Tumors by Reprogramming Tumor-Infiltrating MDSCs. *Cancer Immunol Res* (2018) 6(12):1561–77. doi: 10.1158/2326-6066.CIR-18-0070
59. Orillion A, Hashimoto A, Damayanti N, Shen L, Adelaiye-Ogala R, Arisa S, et al. Entinostat Neutralizes Myeloid-Derived Suppressor Cells and Enhances the Antitumor Effect of PD-1 Inhibition in Murine Models of Lung and Renal Cell Carcinoma. *Clin Cancer Res* (2017) 23(17):5187–201. doi: 10.1158/1078-0432.CCR-17-0741
60. Sun L, Clavijo PE, Robbins Y, Patel P, Friedman J, Greene S, et al. Inhibiting myeloid-derived suppressor cell trafficking enhances T cell immunotherapy. *JCI Insight* (2019) 4(7):e126853. doi: 10.1172/jci.insight.126853
61. Steele CW, Karim SA, Leach JDG, Bailey P, Upstill-Goddard R, Rishi L, et al. CXCR2 Inhibition Profoundly Suppresses Metastases and Augments Immunotherapy in Pancreatic Ductal Adenocarcinoma. *Cancer Cell* (2016) 29(6):832–45. doi: 10.1016/j.ccell.2016.04.014
62. Ding AS, Routkevitch D, Jackson C, Lim M. Targeting Myeloid Cells in Combination Treatments for Glioma and Other Tumors. *Front Immunol* (2019) 10:1715. doi: 10.3389/fimmu.2019.01715
63. Law AMK, Valdes-Mora F, Gallego-Ortega D. Myeloid-Derived Suppressor Cells as a Therapeutic Target for Cancer. *Cells* (2020) 9(3):561. doi: 10.3390/cells9030561
64. Khan S, Mittal S, McGee K, Alfaro-Munoz KD, Majd N, Balasubramaniyan V, et al. Role of Neutrophils and Myeloid-Derived Suppressor Cells in Glioma Progression and Treatment Resistance. *Int J Mol Sci* (2020) 21(6):1954. doi: 10.3390/ijms21061954
65. Garrido-Navas C, de Miguel-Perez D, Exposito-Hernandez J, Bayarri C, Amezcua V, Ortigosa A, et al. Cooperative and Escaping Mechanisms between Circulating Tumor Cells and Blood Constituents. *Cells* (2019) 8(11):1382. doi: 10.3390/cells8111382
66. Szczerba BM, Castro-Giner F, Vetter M, Krol I, Gkountela S, Landin J, et al. Neutrophils escort circulating tumour cells to enable cell cycle progression. *Nature* (2019) 566(7745):553–7. doi: 10.1038/s41586-019-0915-y
67. Kim IS, Gao Y, Welte T, Wang H, Liu J, Janghorban M, et al. Immuno-subtyping of breast cancer reveals distinct myeloid cell profiles and immunotherapy resistance mechanisms. *Nat Cell Biol* (2019) 21(9):1113–26. doi: 10.1038/s41556-019-0373-7
68. Fujita M, Scheurer ME, Decker SA, McDonald HA, Kohanbash G, Kastenhuber ER, et al. Role of type 1 IFNs in antiglioma immunosurveillance–using mouse studies to guide examination of novel prognostic markers in humans. *Clin Cancer Res* (2010) 16(13):3409–19. doi: 10.1158/1078-0432.CCR-10-0644
69. Liang J, Piao Y, Holmes L, Fuller GN, Henry V, Tiao N, et al. Neutrophils promote the malignant glioma phenotype through S100A4. *Clin Cancer Res* (2014) 20(1):187–98. doi: 10.1158/1078-0432.CCR-13-1279
70. Poli A, Wang J, Domingues O, Planagumà J, Yan T, Rygh CB, et al. Targeting glioblastoma with NK cells and mAb against NG2/CSPG4 prolongs animal survival. *Oncotarget* (2013) 4(9):1527–46. doi: 10.18632/oncotarget.1291
71. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer* (2012) 12(4):252–64. doi: 10.1038/nrc3239
72. Heiland DH, Haaker G, Delev D, Mercas B, Masalha W, Heynckes S, et al. Comprehensive analysis of PD-L1 expression in glioblastoma multiforme. *Oncotarget* (2017) 8(26):42214–25. doi: 10.18632/oncotarget.15031
73. Nduom EK, Wei J, Yaghi NK, Huang N, Kong LY, Gabrusiewicz K, et al. PD-L1 expression and prognostic impact in glioblastoma. *Neuro Oncol* (2016) 18(2):195–205. doi: 10.1093/neuonc/nov172
74. Liu S, Wang Z, Wang Y, Fan X, Zhang C, Ma W, et al. PD-1 related transcriptome profile and clinical outcome in diffuse gliomas. *Oncoimmunology* (2018) 7(2):e1382792. doi: 10.1080/2162402X.2017.1382792
75. Weber JS, D'Angelo SP, Minor D, Hodi FS, Gutzmer R, Neyns B, et al. Nivolumab versus chemotherapy in patients with advanced melanoma who progressed after anti-CTLA-4 treatment (CheckMate 037): a randomised, controlled, open-label, phase 3 trial. *Lancet Oncol* (2015) 16(4):375–84. doi: 10.1016/S1470-2045(15)70076-8
76. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *New Engl J Med* (2010) 363(8):711–23. doi: 10.1056/NEJMoa1003466
77. Hamid O, Robert C, Daud A, Hodi FS, Hwu WJ, Kefford R, et al. Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. *New Engl J Med* (2013) 369(2):134–44. doi: 10.1056/NEJMoa1305133
78. Agarwalla P, Barnard Z, Fecci P, Dranoff G, Curry WTJr. Sequential immunotherapy by vaccination with GM-CSF-expressing glioma cells and CTLA-4 blockade effectively treats established murine intracranial tumors. *J Immunother (Hagerstown Md 1997)* (2012) 35(5):385–9. doi: 10.1097/CJI.0b013e3182562d59
79. Fecci PE, Ochiai H, Mitchell DA, Grossi PM, Sweeney AE, Archer GE, et al. Systemic CTLA-4 blockade ameliorates glioma-induced changes to the CD4+ T cell compartment without affecting regulatory T-cell function. *Clin Cancer Res* (2007) 13(7):2158–67. doi: 10.1158/1078-0432.CCR-06-2070
80. Antonios JP, Soto H, Everson RG, Orpilla J, Moughon D, Shin N, et al. PD-1 blockade enhances the vaccination-induced immune response in glioma. *JCI Insight* (2016) 1(10):e87059. doi: 10.1172/jci.insight.87059
81. Reardon DA, Gokhale PC, Klein SR, Ligon KL, Rodig SJ, Ramkissoon SH, et al. Glioblastoma Eradication Following Immune Checkpoint Blockade in

an Orthotopic, Immunocompetent Model. *Cancer Immunol Res* (2016) 4 (2):124–35. doi: 10.1158/2326-6066.CIR-15-0151
82. Wainwright DA, Chang AL, Dey M, Balyasnikova IV, Kim CK, Tobias A, et al. Durable therapeutic efficacy utilizing combinatorial blockade against IDO, CTLA-4, and PD-L1 in mice with brain tumors. *Clin Cancer Res* (2014) 20(20):5290–301. doi: 10.1158/1078-0432.CCR-14-0514
83. Zeng J, See AP, Phallen J, Jackson CM, Belcaid Z, Ruzevick J, et al. Anti-PD-1 blockade and stereotactic radiation produce long-term survival in mice with intracranial gliomas. *Int J Radiat Oncol Biol Phys* (2013) 86(2):343–9. doi: 10.1016/j.ijrobp.2012.12.025
84. Schalper KA, Rodriguez-Ruiz ME, Diez-Valle R, López-Janeiro A, Porciuncula A, Idoate MA, et al. Neoadjuvant nivolumab modifies the tumor immune microenvironment in resectable glioblastoma. *Nat Med* (2019) 25(3):470–6. doi: 10.1038/s41591-018-0339-5
85. Reardon DA, Brandes AA, Omuro A, Mulholland P, Lim M, Wick A, et al. Effect of Nivolumab vs Bevacizumab in Patients With Recurrent Glioblastoma: The CheckMate 143 Phase 3 Randomized Clinical Trial. *JAMA Oncol* (2020) 6(7):1003–10.. doi: 10.1001/jamaoncol.2020.1024
86. Bristol-Myers Squibb. *Announces Phase 3 CheckMate-498 study did not meet primary endpoint of overall survival with opdivo (nivolumab) plus radiation in patients with newly diagnosed MGMT-unmethylated glioblastoma multiforme*. Princeton, NJ: Bristol-Myers Squibb Company (2019). 2019-11-04.
87. Bristol-Myers Squibb. *Provides Update on Phase 3 Opdivo (Nivolumab) CheckMate - 548 trial in patients with newly diagnosed MGMT - methylated glioblastoma multiforme*. Princeton, NJ: Bristol-Myers Squibb Company (2019). 2019-11- 04.
88. Lukas RV, Rodon J, Becker K, Wong ET, Shih K, Touat M, et al. Clinical activity and safety of atezolizumab in patients with recurrent glioblastoma. *J Neuro-Oncol* (2018) 140(2):317–28. doi: 10.1007/s11060-018-2955-9
89. Fong B, Jin R, Wang X, Safaee M, Lisiero DN, Yang I, et al. Monitoring of regulatory T cell frequencies and expression of CTLA-4 on T cells, before and after DC vaccination, can predict survival in GBM patients. *PloS One* (2012) 7(4):e32614. doi: 10.1371/journal.pone.0032614
90. Belcaid Z, Phallen JA, Zeng J, See AP, Mathios D, Gottschalk C, et al. Focal radiation therapy combined with 4-1BB activation and CTLA-4 blockade yields long-term survival and a protective antigen-specific memory response in a murine glioma model. *PloS One* (2014) 9(7):e101764. doi: 10.1371/journal.pone.0101764
91. Ni L, Dong C. New B7 Family Checkpoints in Human Cancers. *Mol Cancer Ther* (2017) 16(7):1203–11. doi: 10.1158/1535-7163.MCT-16-0761
92. Wang Z, Wang Z, Zhang C, Liu X, Li G, Liu S, et al. Genetic and clinical characterization of B7-H3 (CD276) expression and epigenetic regulation in diffuse brain glioma. *Cancer Sci* (2018) 109(9):2697–705. doi: 10.1111/cas.13744
93. Zhou Z, Luther N, Ibrahim GM, Hawkins C, Vibhakar R, Handler MH, et al. B7-H3, a potential therapeutic target, is expressed in diffuse intrinsic pontine glioma. *J Neuro-Oncol* (2013) 111(3):257–64. doi: 10.1007/s11060-012-1021-2
94. Wu CP, Jiang JT, Tan M, Zhu YB, Ji M, Xu KF, et al. Relationship between co-stimulatory molecule B7-H3 expression and gastric carcinoma histology and prognosis. *World J Gastroenterol* (2006) 12(3):457–9. doi: 10.3748/wjg.v12.i3.457
95. Loos M, Hedderich DM, Ottenhausen M, Giese NA, Laschinger M, Esposito I, et al. Expression of the costimulatory molecule B7-H3 is associated with prolonged survival in human pancreatic cancer. *BMC Cancer* (2009) 9:463. doi: 10.1186/1471-2407-9-463
96. Qi Y, Deng G, Xu P, Zhang H, Yuan F, Geng R, et al. HHLA2 is a novel prognostic predictor and potential therapeutic target in malignant glioma. *Oncol Rep* (2019) 42(6):2309–22. doi: 10.3892/or.2019.7343
97. Yan H, Qiu W, Koehne de Gonzalez AK, Wei JS, Tu M, Xi CH, et al. HHLA2 is a novel immune checkpoint protein in pancreatic ductal adenocarcinoma and predicts post-surgical survival. *Cancer Lett* (2019) 442:333–40. doi: 10.1016/j.canlet.2018.11.007
98. Prendergast GC, Smith C, Thomas S, Mandik-Nayak L, Laury-Kleintop L, Metz R, et al. Indoleamine 2,3-dioxygenase pathways of pathogenic inflammation and immune escape in cancer. *Cancer Immunol Immunother CII* (2014) 63(7):721–35. doi: 10.1007/s00262-014-1549-4
99. Godin-Ethier J, Hanafi LA, Piccirillo CA, Lapointe R. Indoleamine 2,3-dioxygenase expression in human cancers: clinical and immunologic perspectives. *Clin Cancer Res* (2011) 17(22):6985–91. doi: 10.1158/1078-0432.CCR-11-1331
100. Löb S, Königsrainer A, Rammensee HG, Opelz G, Terness P. Inhibitors of indoleamine-2,3-dioxygenase for cancer therapy: can we see the wood for the trees? *Nat Rev Cancer* (2009) 9(6):445–52. doi: 10.1038/nrc2639
101. Mbongue JC, Nicholas DA, Torrez TW, Kim NS, Firek AF, Langridge WH. The Role of Indoleamine 2, 3-Dioxygenase in Immune Suppression and Autoimmunity. *Vaccines (Basel)* (2015) 3(3):703–29. doi: 10.3390/vaccines3030703
102. Mellor AL, Munn DH. Physiologic control of the functional status of Foxp3+ regulatory T cells. *J Immunol (Baltimore Md 1950)* (2011) 186(8):4535–40. doi: 10.4049/jimmunol.1002937
103. Mezrich JD, Fechner JH, Zhang X, Johnson BP, Burlingham WJ, Bradfield CA. An interaction between kynurenine and the aryl hydrocarbon receptor can generate regulatory T cells. *J Immunol (Baltimore Md 1950)* (2010) 185 (6):3190–8. doi: 10.4049/jimmunol.0903670
104. Hanihara M, Kawataki T, Oh-Oka K, Mitsuka K, Nakao A, Kinouchi H. Synergistic antitumor effect with indoleamine 2,3-dioxygenase inhibition and temozolomide in a murine glioma model. *J Neurosurg* (2016) 124 (6):1594–601. doi: 10.3171/2015.5.JNS141901
105. Workman CJ, Vignali DA. The CD4-related molecule, LAG-3 (CD223), regulates the expansion of activated T cells. *Eur J Immunol* (2003) 33(4):970–9. doi: 10.1002/eji.200323382
106. Kouo T, Huang L, Pucsek AB, Cao M, Solt S, Armstrong T, et al. Galectin-3 Shapes Antitumor Immune Responses by Suppressing CD8+ T Cells via LAG-3 and Inhibiting Expansion of Plasmacytoid Dendritic Cells. *Cancer Immunol Res* (2015) 3(4):412–23. doi: 10.1158/2326-6066.CIR-14-0150
107. Andrews LP, Marciscano AE, Drake CG, Vignali DA. LAG3 (CD223) as a cancer immunotherapy target. *Immunol Rev* (2017) 276(1):80–96. doi: 10.1111/imr.12519
108. Huang RY, Eppolito C, Lele S, Shrikant P, Matsuzaki J, Odunsi K. LAG3 and PD1 co-inhibitory molecules collaborate to limit CD8+ T cell signaling and dampen antitumor immunity in a murine ovarian cancer model. *Oncotarget* (2015) 6(29):27359–77. doi: 10.18632/oncotarget.4751
109. Jing W, Gershan JA, Weber J, Tlomak D, McOlash L, Sabatos-Peyton C, et al. Combined immune checkpoint protein blockade and low dose whole body irradiation as immunotherapy for myeloma. *J Immunother Cancer* (2015) 3 (1):2. doi: 10.1186/s40425-014-0043-z
110. Das M, Zhu C, Kuchroo VK. Tim-3 and its role in regulating anti-tumor immunity. *Immunol Rev* (2017) 276(1):97–111. doi: 10.1111/imr.12520
111. Han S, Feng S, Xu L, Shi W, Wang X, Wang H, et al. Tim-3 on peripheral $CD4^+$ and $CD8^+$ T cells is involved in the development of glioma. *DNA Cell Biol* (2014) 33(4):245–50. doi: 10.1089/dna.2013.2306
112. Li G, Wang Z, Zhang C, Liu X, Cai J, Wang Z, et al. Molecular and clinical characterization of TIM-3 in glioma through 1,024 samples. *Oncoimmunology* (2017) 6(8):e1328339. doi: 10.1080/2162402X.2017.1328339
113. Verhaak RG, Hoadley KA, Purdom E, Wang V, Qi Y, Wilkerson MD, et al. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell* (2010) 17(1):98–110. doi: 10.1016/j.ccr.2009.12.020
114. The Cancer Genome Atlas Research Network. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature* (2008) 455(7216):1061–8. doi: 10.1038/nature07385
115. Jackson CM, Choi J, Lim M. Mechanisms of immunotherapy resistance: lessons from glioblastoma. *Nat Immunol* (2019) 20(9):1100–9. doi: 10.1038/s41590-019-0433-y
116. Kalbasi A, Ribas A. Tumour-intrinsic resistance to immune checkpoint blockade. *Nat Rev Immunol* (2020) 20(1):25–39. doi: 10.1038/s41577-019-0218-4
117. Chowell D, Morris LGT, Grigg CM, Weber JK, Samstein RM, Makarov V, et al. Patient HLA class I genotype influences cancer response to checkpoint blockade immunotherapy. *Science* (2018) 359(6375):582–7. doi: 10.1126/science.aao4572
118. Pitt JM, Vétizou M, Daillère R, Roberti MP, Yamazaki T, Routy B, et al. Resistance Mechanisms to Immune-Checkpoint Blockade in Cancer:

Tumor-Intrinsic and -Extrinsic Factors. *Immunity* (2016) 44(6):1255–69. doi: 10.1016/j.immuni.2016.06.001
119. Fessler J, Matson V, Gajewski TF. Exploring the emerging role of the microbiome in cancer immunotherapy. *J Immunother Cancer* (2019) 7 (1):108. doi: 10.1186/s40425-019-0574-4
120. Wilson EB, Yamada DH, Elsaesser H, Herskovitz J, Deng J, Cheng G, et al. Blockade of chronic type I interferon signaling to control persistent LCMV infection. *Science* (2013) 340(6129):202–7. doi: 10.1126/science.1235208
121. Teijaro JR, Ng C, Lee AM, Sullivan BM, Sheehan KC, Welch M, et al. Persistent LCMV infection is controlled by blockade of type I interferon signaling. *Science* (2013) 340(6129):207–11. doi: 10.1126/science.1235214
122. Benci JL, Xu B, Qiu Y, Wu TJ, Dada H, Twyman-Saint Victor C, et al. Tumor Interferon Signaling Regulates a Multigenic Resistance Program to Immune Checkpoint Blockade. *Cell* (2016) 167(6):1540–54.e12. doi: 10.1016/j.cell.2016.11.022
123. Yaguchi T, Goto Y, Kido K, Mochimaru H, Sakurai T, Tsukamoto N, et al. Immune suppression and resistance mediated by constitutive activation of Wnt/β-catenin signaling in human melanoma cells. *J Immunol (Baltimore Md 1950)* (2012) 189(5):2110–7. doi: 10.4049/jimmunol.1102282
124. Spranger S, Bao R, Gajewski TF. Melanoma-intrinsic β-catenin signalling prevents anti-tumour immunity. *Nature* (2015) 523(7559):231–5. doi: 10.1038/nature14404
125. Spranger S, Dai D, Horton B, Gajewski TF. Tumor-Residing Batf3 Dendritic Cells Are Required for Effector T Cell Trafficking and Adoptive T Cell Therapy. *Cancer Cell* (2017) 31(5):711–23.e4. doi: 10.1016/j.ccell.2017.04.003
126. Holtzhausen A, Zhao F, Evans KS, Tsutsui M, Orabona C, Tyler DS, et al. Melanoma-Derived Wnt5a Promotes Local Dendritic-Cell Expression of IDO and Immunotolerance: Opportunities for Pharmacologic Enhancement of Immunotherapy. *Cancer Immunol Res* (2015) 3(9):1082–95. doi: 10.1158/2326-6066.CIR-14-0167
127. Sumimoto H, Imabayashi F, Iwata T, Kawakami Y. The BRAF-MAPK signaling pathway is essential for cancer-immune evasion in human melanoma cells. *J Exp Med* (2006) 203(7):1651–6. doi: 10.1084/jem.20051848
128. Brennan CW, Verhaak RG, McKenna A, Campos B, Noushmehr H, Salama SR, et al. The somatic genomic landscape of glioblastoma. *Cell* (2013) 155 (2):462–77. doi: 10.1016/j.cell.2013.09.034
129. Sampson JH, Heimberger AB, Archer GE, Aldape KD, Friedman AH, Friedman HS, et al. Immunologic escape after prolonged progression-free survival with epidermal growth factor receptor variant III peptide vaccination in patients with newly diagnosed glioblastoma. *J Clin Oncol* (2010) 28(31):4722–9. doi: 10.1200/JCO.2010.28.6963
130. Wood MD, Reis GF, Reuss DE, Phillips JJ. Protein Analysis of Glioblastoma Primary and Posttreatment Pairs Suggests a Mesenchymal Shift at Recurrence. *J Neuropathol Exp Neurol* (2016) 75(10):925–35. doi: 10.1093/jnen/nlw068
131. Seifert L, Werba G, Tiwari S, Giao Ly NN, Nguy S, Alothman S, et al. Radiation Therapy Induces Macrophages to Suppress T-Cell Responses Against Pancreatic Tumors in Mice. *Gastroenterology* (2016) 150(7):1659–72.e5. doi: 10.1053/j.gastro.2016.02.070
132. Topalian SL, Drake CG, Pardoll DM. Immune checkpoint blockade: a common denominator approach to cancer therapy. *Cancer Cell* (2015) 27 (4):450–61. doi: 10.1016/j.ccell.2015.03.001
133. Woroniecka K, Chongsathidkiet P, Rhodin K, Kemeny H, Dechant C, Farber SH, et al. T-Cell Exhaustion Signatures Vary with Tumor Type and Are Severe in Glioblastoma. *Clin Cancer Res* (2018) 24(17):4175–86. doi: 10.1158/1078-0432.CCR-17-1846
134. Bauer C, Kühnemuth B, Duewell P, Ormanns S, Gress T, Schnurr M. Prevailing over T cell exhaustion: New developments in the immunotherapy of pancreatic cancer. *Cancer Lett* (2016) 381(1):259–68. doi: 10.1016/j.canlet.2016.02.057
135. Koyama S, Akbay EA, Li YY, Herter-Sprie GS, Buczkowski KA, Richards WG, et al. Adaptive resistance to therapeutic PD-1 blockade is associated with upregulation of alternative immune checkpoints. *Nat Commun* (2016) 7:10501. doi: 10.1038/ncomms10501
136. Anagnostou V, Smith KN, Forde PM, Niknafs N, Bhattacharya R, White J, et al. Evolution of Neoantigen Landscape during Immune Checkpoint Blockade in Non-Small Cell Lung Cancer. *Cancer Discovery* (2017) 7 (3):264–76. doi: 10.1158/1538-7445.AM2017-NG01
137. Rosenthal R, Cadieux EL, Salgado R, Bakir MA, Moore DA, Hiley CT, et al. Neoantigen-directed immune escape in lung cancer evolution. *Nature* (2019) 567(7749):479–85. doi: 10.1038/s41586-019-1032-7
138. Zhao J, Chen AX, Gartrell RD, Silverman AM, Aparicio L, Chu T, et al. Immune and genomic correlates of response to anti-PD-1 immunotherapy in glioblastoma. *Nat Med* (2019) 25(3):462–9. doi: 10.1038/s41591-019-0349-y
139. Lin W, Wu S, Chen X, Ye Y, Weng Y, Pan Y, et al. Characterization of Hypoxia Signature to Evaluate the Tumor Immune Microenvironment and Predict Prognosis in Glioma Groups. *Front Oncol* (2020) 10:796. doi: 10.3389/fonc.2020.00796
140. Zuckerberg AL, Goldberg LI, Lederman HM. Effects of hypoxia on interleukin-2 mRNA expression by T lymphocytes. *Crit Care Med* (1994) 22(2):197–203. doi: 10.1097/00003246-199402000-00008
141. Parodi M, Raggi F, Cangelosi D, Manzini C, Balsamo M, Blengio F, et al. Hypoxia Modifies the Transcriptome of Human NK Cells, Modulates Their Immunoregulatory Profile, and Influences NK Cell Subset Migration. *Front Immunol* (2018) 9:2358. doi: 10.3389/fimmu.2018.02358
142. Guo X, Xue H, Shao Q, Wang J, Guo X, Chen X, et al. Hypoxia promotes glioma-associated macrophage infiltration via periostin and subsequent M2 polarization by upregulating TGF-beta and M-CSFR. *Oncotarget* (2016) 7 (49):80521–42. doi: 10.18632/oncotarget.11825
143. Casazza A, Laoui D, Wenes M, Rizzolio S, Bassani N, Mambretti M, et al. Impeding macrophage entry into hypoxic tumor areas by Sema3A/Nrp1 signaling blockade inhibits angiogenesis and restores antitumor immunity. *Cancer Cell* (2013) 24(6):695–709. doi: 10.1016/j.ccr.2013.11.007
144. Henze AT, Mazzone M. The impact of hypoxia on tumor-associated macrophages. *J Clin Investigation* (2016) 126(10):3672–9. doi: 10.1172/JCI84427
145. Dennis KL, Blatner NR, Gounari F, Khazaie K. Current status of interleukin-10 and regulatory T-cells in cancer. *Curr Opin Oncol* (2013) 25(6):637–45. doi: 10.1097/CCO.0000000000000006
146. Cloughesy TF, Mochizuki AY, Orpilla JR, Hugo W, Lee AH, Davidson TB, et al. Neoadjuvant anti-PD-1 immunotherapy promotes a survival benefit with intratumoral and systemic immune responses in recurrent glioblastoma. *Nat Med* (2019) 25(3):477–86. doi: 10.1038/s41591-018-0337-7
147. Cristescu R, Mogg R, Ayers M, Albright A, Murphy E, Yearley J, et al. Pan-tumor genomic biomarkers for PD-1 checkpoint blockade-based immunotherapy. *Sci (N Y NY)* (2018) 362(6411):eaar3593. doi: 10.1126/science.aar3593
148. Macarthur KM, Kao GD, Chandrasekaran S, Alonso-Basanta M, Chapman C, Lustig RA, et al. Detection of brain tumor cells in the peripheral blood by a telomerase promoter-based assay. *Cancer Res* (2014) 74(8):2152–9. doi: 10.1158/0008-5472.CAN-13-0813
149. Gao F, Cui Y, Jiang H, Sui D, Wang Y, Jiang Z, et al. Circulating tumor cell is a common property of brain glioma and promotes the monitoring system. *Oncotarget* (2016) 7(44):71330–40. doi: 10.18632/oncotarget.11114

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Treatment Progress of Immune Checkpoint Blockade Therapy for Glioblastoma

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Glioblastoma (GBM) is a highly malignant and aggressive primary brain tumor mostly prevalent in adults and is associated with a very poor prognosis. Moreover, only a few effective treatment regimens are available due to their rapid invasion of the brain parenchyma and resistance to conventional therapy. However, the fast development of cancer immunotherapy and the remarkable survival benefit from immunotherapy in several extracranial tumor types have recently paved the way for numerous interventional studies involving GBM patients. The recent success of checkpoint blockade therapy, targeting immunoinhibitory proteins such as programmed cell death protein-1 and/or cytotoxic T lymphocyte-associated antigen-4, has initiated a paradigm shift in clinical and preclinical investigations, and the use of immunotherapy for solid tumors, which would be a potential breakthrough in the field of drug therapy for the GBM treatment. However clinical trial showed limited benefits for GBM patients. The main reason is drug resistance. This review summarizes the clinical research progress of immune checkpoint molecules and inhibitors, introduces the current research status of immune checkpoint inhibitors in the field of GBM, analyzes the molecular resistance mechanism of checkpoint blockade therapy, proposes corresponding re-sensitive strategies, and describes a reference for the design and development of subsequent clinical studies on immunotherapy for GBM.

Keywords: glioblastoma, immunotherapy, checkpoint inhibitors, checkpoint blockade therapy, resistance mechanism

INTRODUCTION

Glioblastoma (GBM) is the most advanced WHO grade IV glioma and the most common adult astrocytoma. GBM patients generally have a median survival of less than 20 months, and the 5-year survival rate is only 4–5% (1).The survival of GBM patients has not improved significantly over the past three decades. Despite aggressive standard treatments of maximum safe surgical resection, radiotherapy, and temozolomide in patients, the prognosis in newly diagnosed patients with GBM remains poor (2). GBM treatment, one of the most expensive therapy with least rewarding, is imposing a huge burden on the society. Hence, the need for a more effective antitumor treatment

has become the goal of researchers worldwide. In recent years, immune checkpoint inhibitors have been widely used as a crucial therapy for malignant tumors such as melanoma and lung cancer, leading to the provision of new research directions for the GBM treatment (3). The suppression of autoreactive T cells by immune checkpoints is a defensive measure against autoimmunity under physiological conditions. In pathological conditions, immune checkpoints protect tumor cells from immune system clearance in a similar way. Compared to the cytotoxic effects of traditional chemotherapeutics and traditional targeted therapy, immune checkpoint targeted therapy aims to regulate checkpoint molecules, change their functions, and induce the death of tumor cells (4).

The widespread application of immune checkpoint inhibitors in the field of oncology, brought new hope to humans. However, available data indicate that it is beneficial for some patients, whereas some patients progressed or relapsed after effective treatment for some time. Furthermore, some patients did not respond to immune checkpoint inhibitor treatment in the beginning, so drug resistance is the main reason for the failure of immune checkpoint blockade therapy (5). The resistance of immune checkpoint inhibitors can be divided into primary, adaptive and acquired resistance based on resistance time. Primary resistance means that the tumor does not respond to immune checkpoint inhibitor treatment in the beginning. Acquired resistance implies that the tumor is effective to immune checkpoint inhibitor treatment in the beginning, but the disease progresses or recurs after a period of treatment. Adaptive resistance means that the tumor can be recognized by the immune system, but the tumor cells adapt to the immune system without being attacked by the immune system (6). It can further be divided into endogenous resistance and exogenous. Endogenous resistance is caused by changes in tumor cells, such as alterations in immune recognition process, alterations in cell signaling pathways, alterations in gene expression, and DNA damage repair reaction. Exogenous resistance refers to external factors that might affect all the processes of T-cell activation.

This review summarized the mechanism of immune-checkpoint inhibitors, the characteristics of the GBM immune microenvironment, and the clinical research progress of immune checkpoint inhibitors in the GBM treatment. The molecular resistance mechanism of checkpoint blockade therapy is also discussed, and the corresponding re-sensitive strategies are proposed.

FUNCTION OF IMMUNE CHECKPOINTS

Immunotherapy is a therapeutic method that removes cancerous cells by improving the body's autoimmune function. T cells play a vital role in antitumor immunity. The production of effector T cells and their recognition and elimination of cancerous cells are complex multi-step processes regulated by a series of activation and inhibition signals (7). The main function of inhibition signals is to prevent the overactivation of the immune system and the occurrence of uncontrolled inflammatory response and the autoimmune disease caused by it. Suppressing T-cell antitumor immune response, however, leads to the escape of cancer cells (8). Therefore, the elimination of cancer cells depends on the balance between the activation signal and the inhibition signals. Immune checkpoint receptors, such as cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and programmed cell death protein-1 (PD-1) expressed on the T-cell surface, play a negative regulatory role during the process of T-cell activation, thereby preventing pathological over activation (9). Interfering with the immune checkpoint signals can improve the antitumor immune response by restoring T-cell function. CTLA-4 mainly acts at the early stage of immune activation, regulating the initiation and activation of T cells, and the anti-CTLA-4 antibody can activate T cells in peripheral lymphoid tissue. PD-1 mainly plays a role in the effect phase of the immune response (10). Its overexpression is observed during the activation of T cells stimulated by antigen (**Figure 1**). The interaction of PD-1 with its ligand (programmed cell death ligand protein-1) PD-L1 or (programmed cell death ligand protein-2) PD-L2 can inhibit the transduction of T-cell signals and cytokine production and reduce the number of T cells (9). These two ligands play an important role in the tumor microenvironment and are expressed in many cancer cells. Antibodies against PD-1 or PD-L1 can inhibit the transmission of this negative signal and restore cell function.

CLINICAL RESEARCH PROGRESS OF IMMUNE CHECKPOINT INHIBITORS IN GBM TREATMENT

Immune regulation depends on the balance between the activation and inhibition signals. In the physiological state, immune checkpoint molecules can inhibit cytotoxic T-cell function as an immunomodulatory mechanism (11). When the immune checkpoint is abnormal or continuously activated, the tumor immune response is suppressed, and the monoclonal antibody against the immune checkpoint can release the "immune brake," leading to the enhancement of the immunotherapy effect (12). Currently, related checkpoints are mainly focused on PD1 and CTLA-4. Although significant results (e.g., melanoma) have been obtained in clinical trials involving solid tumors, studies involving checkpoint inhibitors for GBM treatment are still being conducted.

Clinical trials on immune checkpoint inhibitors are mainly divided into the following categories: immune checkpoint inhibitor monotherapy and combination therapy. Combination therapy includes immune checkpoint inhibitors combined with chemotherapy, stereotactic radiosurgery therapy, or targeting other immune targets. Clinical trials of GBM immune checkpoint inhibitors are still in the early stage. Most trials are in the recruitment stage or in progress (**Table 1**), with only a few published preliminary results. Currently, there are seven immune checkpoint inhibitors approved for sale in the United States, including one monoclonal antibody against CTLA-4 (Ipilumumab), three monoclonal antibodies against PD-1 (Nivolumab, pembrolizumab, and Cemiplimab), and three PD-L1 monoclonal antibodies (atezolizumab, Devaru, and

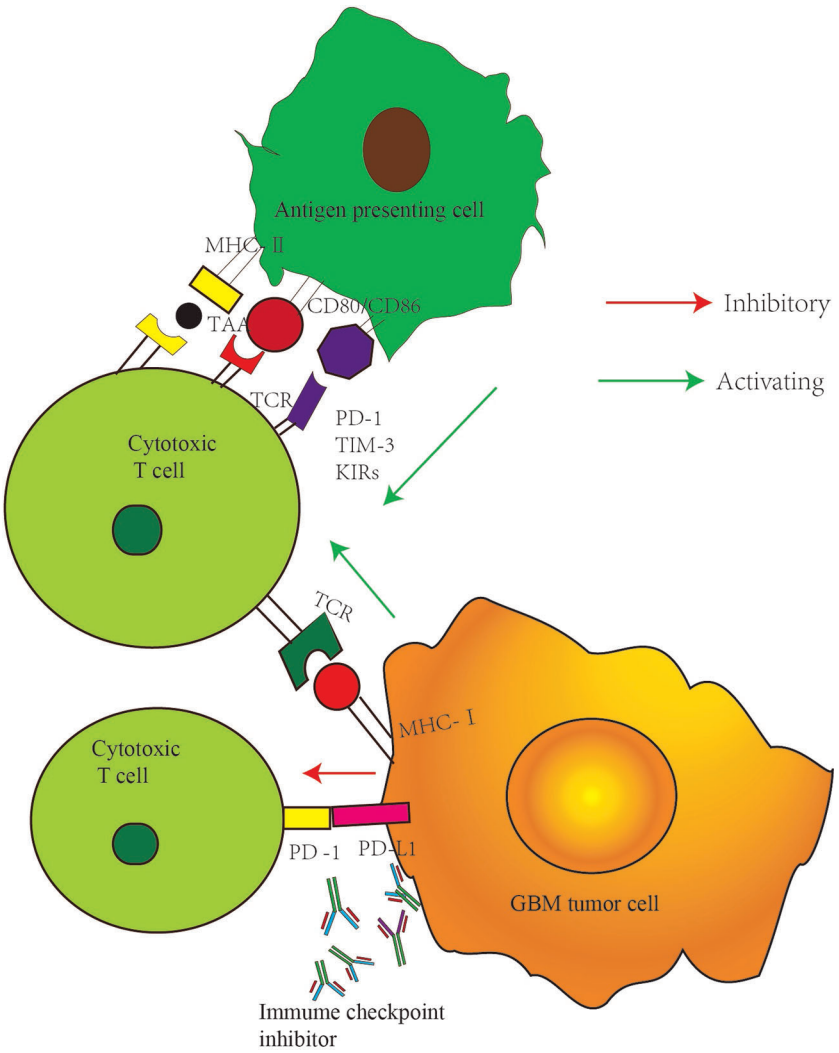

FIGURE 1 | Major checkpoint inhibition pathway in GBM cancer cells. MHC II, major histocompatibility complex II; TAA, tumor associated antigen; TCR, T cell receptor; MHCI, major histocompatibility complex I; PD-1, programmed cell death protein 1; PD-L1, programmed cell death ligand protein 1; TIM-3, T cell immunoglobulin mucin molecule 3; KIRs, killer immunoglobulin-like receptors.

Avelumab). Some of them have already been measured in some clinical trials. Schalper et al. treated 30 patients with GBM (3 cases of primary GBM and 27 cases of recurrent GBM) with Nivolumab (trial no. NCT02550249) before and after operation (13). Adjuvant Nivolumab therapy can enhance chemokine transcription, increase immune cells infiltration, and increase T-cell receptor Crohn-like in the tumor microenvironment. However, there was no significant survival benefit in 27 patients with recurrent GBM, but 2 of 3 patients with primary GBM survived for 28 and 33 months, respectively. Another phase III clinical trial of Ipilumumab combined with Nivolumab (trial no. NCT02017717) is also under way, Forty recurrent GBM patients were randomly divided into two groups: Nivolumab group and Nivolumab + Ipilimumab group. The results showed that the tolerance of patients in Nivolumab group was good, whereas the tolerance of patients in Nivolumab + Ipilimumab group was affected by an excessive dose of Ipilimumab (14). The subsequent phase III trial of CheckMate 143 showed that Nivolumab did not show more significant survival benefits than bevacizumab (median overall survival time 9.8 vs. 10 months) (15). The trail's failure may be related to the low expression level of PD-L1 in the included patients (16). Although few clinical trials on GBM immune checkpoint inhibitors have been successful, researchers have never given up. New potential immune checkpoints such as dioxygenase, CD47, and CD137 have been found, providing the possibility of successful immunotherapy in the future. A recently published phase I single-arm clinical trial (trial no. NCT02658981) included 44 GBM patients treated with lymphocyte activation gene (LAG) inhibitor, CD137 inhibitor, and the combination with a checkpoint inhibitor, to explore treatment strategies for potential targets. The results have not yet been published yet.

TABLE 1 | Currently ongoing clinical trials based on immune checkpoint inhibitors*.

Clinical Trial	Phase	Study population	Target	Experimental design
NCT02017717 (Check Mate-143)	III	Recurrent GBM	PD-1 VEGF	Nivolumab vs. bevacizumab (phase III), nivolumab vs. ipilimumab + nivolumab (phase I)
NCT02617589 (Check Mate-498)	III	Primary diagnosed GBM *MGMT*-unmethylated	PD-1	Nivolumab + radiotherapy VS. TMZ+ radiotherapy
NCT02667587 (Check Mate-548)	III	Primary diagnosed GBM *MGMT*-unmethylated	PD-1	Nivolumab + TMZ+ radiotherapy VS TMZ+ radiotherapy
NCT03726515	I	Newly diagnosed GBM *MGMT*-unmethylated	PD-1	CAR-EGFRvIII-T cell + Pembrolizumab
NCT02550249	III	Primary GBM Recurrent BGM	PD-1	Nivolumab group vs. Nivolumab + Ipilimumab group
NCT03707457	I	Recurrent BGM	PD-1 IDO1	Nivolumab Anti-GITRantibody MK-4166 IDO1inhibitory INCB024360 Ipilimumab
NCT02852655	II	Recurrent GBM	PD-1	Neoadjuvant and postsurgical pembrolizumab vs. postsurgical pembrolizumab alone
NCT03743662	II	Recurrent GBM *MGMT*-methylated	PD-1 VEGF	Nivolumab BEV
NCT02658981	I	Recurrent GBM	PD-1 LAG-3 CD137	Nivolumab BMS986016(anti-LAG-3antibody) Urelumab(anti-CD137antibody)
NCT03233152	I	Recurrent GBM	PD-1 CTLA-4	Nivolumab + Ipilimumab
NCT02287428	I	Primary diagnosed GBM *MGMT*-unmethylated	PD-1	Pembrolizumab + Personalized neoantigen vaccine (NeoVax) vs. radiotherapy +NeoVax
NCT02335918	II	Recurrent GBM	PD-1 CD27	Anti-CD27antibody Varlilumab + Nivolumab
NCT03493932	I	Recurrent GBM	PD1 LAG-3	Nivolumab BMS986016
NCT02968940	II	Recurrent IDH mutant GBM	PD1	Avelumab
NCT03422094	I	Primary diagnosed GBM *MGMT*-unmethylated	PD-1 CTLA-4	NeoVax Nivolumab Ipilimumab
NCT03491683	I/II	Primary diagnosed GBM	PD-1	IN0-5401+ IN0-9012 + Nivolumab + Cemiplimab + TMZ
NCT03718767	II	Recurrent IDH mutant GBM	PD-1	Nivolumab
NCT02798406	II	Recurrent GBM	PD-1	Oncolytic virus DNX-2401 Pembrolizumab
NCT03341806	I	Recurrent GBM	PD-L1	Avelumab
NCT03291314	I	Recurrent GBM	PD-L1 VEGFR	Avelumab + Axitinib
NCT02794883	II	Recurrent GBM	PD-L1 CTLA-4	Durvalumab Anti-CTLA-4 antibody Tremelimumab
NCT02336165	II	GBM	PD-L1 VEGF	Durvalumab + radiotherapy (newly diagnosed GBM), durvalumab monotherapy (recurrent GBM), durvalumab + bevacizumab (recurrent GBM)
NCT03047473	II	Primary diagnosed GBM	PD-L1	Avelumab +TMZ
NCT02311920	I	Primary diagnosed GBM	PD-1 CTLA-4	Nivolumab Ipilimumab TMZ
NCT04003649	I	Recurrent BGM	PD-1 CTLA-4	CAR-T cell + Nivolumab + Ipilimumab vs. CAR-T cell + Nivolumab
NCT04047706	I	Primarydiagnosed GBM	PD-1 IDO1	IDO1inhibitory BMS986205+Nivolumab + TMZ + radiotherapy vs. IDO1inhibitory BMS986205+ Nivolumab + radiotherapy

GBM, glioblastoma; PD1, programmed cell death protein 1; VEGF, vascular endothelial growth factor; BEV, bevacizumab; TMZ, temozolomide; EGFRvⅢ, epidermal growth factor receptor variant Ⅲ; IDO1, indoleamine-2, 3-dioxygenase 1; GITR, glucocorticoid-induced tumor necrosis factor receptor; LAG-3, lymphocyte-activation gene 3; DC, dendritic cells; CTLA-4, cytotoxic T lymphocyte-associated antigen 4; PDL1, programmed cell death protein ligand 1; IDH, isocitrate debydrogenase; CAR, chimeric antigen receptor.

**All the data come from ClinicalTrials.gov.*

ANALYSIS OF RESISTANCE MECHANISM IN IMMUNE CHECKPOINT INHIBITOR TREATMENT

T-cells' activity can be inhibited by some small molecular proteins. Tumor cells use this mechanism to suppress T-cells and survive by escaping from the human immune system. Immune checkpoint inhibitors can relieve this inhibition, reactivate T-cells and destroy cancer cells. Thus, T-cells play a vital role in this process, not only T-cells themselves, but also factors secreted by multiple originated tumor associate microenvironment (TAM) (17). TAM consist by tumor-associated myeloid cells, cancer stem cells, fibroblasts, other permeable immune cells and cells which form vessels or lymphatics. The immune suppressive microenvironment of GBM patients is a comprehensive and self-sufficient system (18). The drug resistance of immune checkpoint inhibitor is complex program, which can be divided into endogenous resistance and exogenous resistance. Endogenous resistance refers to drug resistance caused by changes in tumor cells, such as changes in the immune recognition process, cell signaling pathway, gene expression, and DNA damage repair. Exogenous resistance means that all processes of T-cell activation are affected by

external factors (19). The mechanisms and strategies of overcoming various resistance are described below (**Figure 2**).

Intrinsic factors refer to tumor cells expressing certain genes or inhibiting certain signal transduction pathways, preventing immune killer cells from infiltrating or playing a role in the tumor microenvironment, leading to immunotherapy resistance (20). The recognition of tumor antigens by effector T cells is particularly important in immunotherapy. When the mutation load of the tumor and the ratio of DNA mismatch repair and genomic microsatellite instability-high(MSI-H) are low, the production of tumor-associated antigens is reduced, which may cause drug resistance (21). Generally, low mutation burden is one reason why GBM is insensitive to immunotherapy. However, high hypermutation is observed in some gliomas cases, and chemotherapy can drive the acquisition of hypermutated populations without promoting a response to PD-1 blockade. In lung cancer and melanoma, the high tumor mutation burden(TMB) was originated from the accumulation of clonal mutations during the longstanding process of tumor generating. However, the use of TMZ in newly diagnosed gliomas with low TMB resulted in the selection pressure, further induced the resistant clones generation (MMR-deficient clones) with high TMB in a short period. Although these TMZ resistant gliomas cells obtained hypermutation, few clonal antigens per cell developed, thus no stronger immunogenic response was induced. So MMR-deficient hypermutation gliomas are characterized by a lack of prominent T cell infiltrates, extensive intratumoral heterogeneity, poor patient survival, and a low rate of response to PD-1 blockade (22).

The activation of the signaling pathway of mitogen-activated protein kinase, and the vascular endothelial growth factor and interleukin (IL) producing can inhibit the recruitment and function of T cells leading to the prevention of T-cell infiltration in the tumor (23). The deletion of the PTEN gene can increase the expression of immunosuppressive cytokines, leading to the reduction of T cell infiltration in tumors, and reducing T-cell-mediated tumor cell death. Thus, the PTEN gene deletion may promote immune tolerance (24). The interferon-γ (INF-γ) pathway plays a role in primary (25), adaptive and acquired resistance. INF-γ produced by tumor-specific T cells can recognize tumor cells and their homologous antigens and promote the increased expression of some protein molecules such as major histocompatibility complex (MHC) molecules and molecules involved in antigen presentation, molecules recruiting immune cells, and the effector molecules that inhibit tumor proliferation or promote tumor apoptosis. Therefore, tumor cells lacking the INF-γ signaling pathway are not vulnerable to T cells, leading to immune checkpoint inhibitor resistance (26).

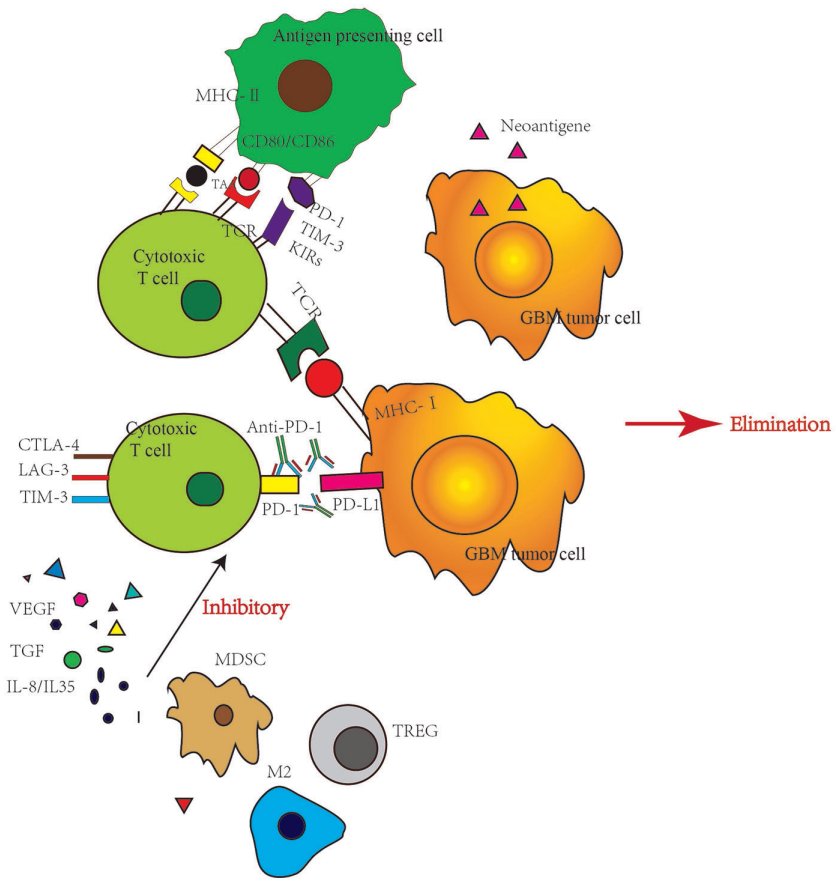

FIGURE 2 | Immune checkpoint inhibitor resistance mechanisms. VEGF, vascular endothelial growth factor; TGF, transforming growth factor; MDSC, myeloid-derived suppressor cell; M2, M2 type macrophage; TREG, regulatory T cell; LAG-3, Lymphocyte activation gene-3.

The most important external factor is the immune microenvironment (27). Many immune cells are often gathered inside and around tumor cells, and these immune cells form a protective barrier against tumor (28). However, once this barrier is broken, there is an acceleration in the tumor occurrence and development. For example, regulatory T-cell (Tregs) play a major role in maintaining self-tolerance (29). Tregs can secrete inhibitory cytokines such as IL-10, IL-35 and transforming growth factor-β (TGF- β), or directly inhibit Teff (CD4+CD25-effector T-cells) response. Tregs can infiltrate various tumor cells (30). An experimental study has shown that the therapeutic effect of a CTLA-4 inhibitor is related to the ratio of Teffs to Tregs (31). The higher the ratio, the better is the therapeutic effect. Myelogenic inhibitory cells (myeloid-derived suppressor cells, MDSCs) represent a group of heterogeneous myeloid cells (32), which can strongly inhibit the antitumor activity of T cells, natural killer cells and some bone marrow cells such as dendritic cells, and stimulate the increase of Tregs. MDSCs also effect on neovascularization, tumor cell infiltration and metastasis, leading to tumor progression (33).

To improve the efficacy of immune checkpoint inhibitors in clinical treatment, there is an urgent need to find biomarkers that can predict treatment sensitivity and screen the population suitable for this therapeutic procedure. The key to the efficacy of immune checkpoint inhibitors lies in the effector immune cells reaching the tumor area. The immune checkpoint pathway plays a leading role in the mechanism of inhibiting anti-tumor immunity (34). The former is often judged by the expression of tumor-infiltrating lymphocytes (TILs) or the ratio of immune effector cells to immunosuppressive cells. However, the criteria for the latter are not clear enough, as no accurate biomarkers have been found. Currently, the most promising approach is the prediction of sensitivity toanti-PD-1/PD-L1 therapy (35). Clinical studies on melanoma have shown that the density of TILs and the proportion of T cells expressing PD-1 or PD-L1 are related to the sensitivity to treatment. According to these indicators, tumors are divided into four types. Type I tumors (TILs +, PD-L1+) which exhibit adaptive immune resistance, are most likely to be sensitive to immune checkpoint inhibitors. Type II tumor (TILs-,PD-L1-) are characterized by immunological ignorance and likely to be insensitive to immune checkpoint inhibitors due to the absence of an obvious immune response. Type III tumors (TILs-,PD-L1+) show intrinsic induction, which is the tumor intrinsic expression of PD-L1 in the absence of immune response. This type of tumor is ineffective when immune checkpoint inhibitors are used alone. It also emphasizes that PD-L1expression cannot be used alone as an index to predict the efficacy of PD-1/PD-L1 inhibitors. Type IV tumors (TILs +, PD-L1-) are characterized by immune infiltration tolerance. It does not depend onPD-L1 expression. Other immunosuppressive signals may exist in this type of tumor, so that the inhibition of other immune checkpoints may have a therapeutic effect. Although this classification is based on the study of melanoma, it provides a theoretical basis for understanding the tumor immune microenvironment of GBM and the rational use of immune checkpoint inhibitors. However, the efficiency and reliability of predicting the sensitivity of GBM to immune checkpoint inhibitors need further research. In addition, the combination of immune checkpoint inhibitors and other antineoplastic drugs is also under study (36). Chemotherapy, radiotherapy, kinase inhibitors, and epigenetic modified drugs may have a synergistic effect on immunotherapy by improving tumor immunogenicity (27).

CONCLUSIONS

There is no U.S. Food and Drug Administration-approved immunotherapy for GBM despite numerous unique therapies currently tested in clinical trials. GBM is a highly immunosuppressive tumor and there are limitations to the extent of a safe immune response in the central nervous system. To date, many trials of targeted therapies comprising single components have not demonstrated any significant efficacy in GBM treatment. The advent of immune checkpoint inhibitors has led to the improved prognosis of many patients with solid tumors, such as malignant melanoma, non-small cell lung cancer, and renal cell carcinoma. However, it has only limited efficacy in clinical trials of GBM. To improve the efficacy of immune checkpoint inhibitors in treatment GBM, there is a need for biomarkers that can effectively predict the effect of immunotherapy to screen the adaptive patients to achieve "individualized immunotherapy" (37). Immune checkpoint inhibitors may have a lasting clinical effect in a small number of patients. To reduce or delay drug resistance, the combination of multiple treatment strategies is encouraged. The main therapeutic markers currently used include PD-L1 expression, tumor mutation burden, TILs, and MSI-H. However, due to the complexity of the antitumor immune response and the huge heterogeneity of tumors, the prediction of a curative effect and the screening of markers are still difficult and challenging. Whole-genome sequencing and epigenetic analysis help select the dominant population and perform an accurate, individualized treatment. Conversely, the combination of other anticancer therapies is also expected to produce a synergistic effect. In combination with tumor gene analysis and immune characteristic analysis, making full use of the synergistic effect of different treatment strategies to carry out combination therapy is a feasible measure in reducing or delaying immune resistance in checkpoint inhibitor drugs.

AUTHOR CONTRIBUTIONS

NZ, LW, MY, CK, and HY conceived the hypothesis, did the literature search, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Reuss DE, Mamatjan Y, Schrimpf D, Capper D, Hovestadt V, Kratz A, et al. IDH mutant diffuse and anaplastic astrocytomas have similar age at presentation and little difference in survival: a grading problem for WHO. *Acta Neuropathol* (2015) 129(6):867–73. doi: 10.1007/s00401-015-1438-8
2. Sanai N, Berger MS. Surgical oncology for gliomas: the state of the art. *Nat Rev Clin Oncol* (2018) 15(2):112–25. doi: 10.1038/nrclinonc.2017.171
3. Bersanelli M, Buti S. From targeting the tumor to targeting the immune system: Transversal challenges in oncology with the inhibition of the PD-1/PD-L1 axis. *World J Clin Oncol* (2017) 8(1):37–53. doi: 10.5306/wjco.v8.i1.37
4. Romani M, Pistillo MP, Carosio R, Morabito A, Banelli B. Immune Checkpoints and Innovative Therapies in Glioblastoma. *Front Oncol* (2018) 8:464. doi: 10.3389/fonc.2018.00464
5. Cabrita R, Mitra S, Sanna A, Ekedahl H, Lövgren K, Olsson H, et al. The Role of PTEN Loss in Immune Escape, Melanoma Prognosis and Therapy Response. *Cancers* (2020) 12(3):742. doi: 10.3390/cancers12030742
6. Sharma P, Hu-Lieskovan S, Wargo JA, Ribas A. Primary, Adaptive, and Acquired Resistance to Cancer Immunotherapy. *Cell* (2017) 168(4):707–23. doi: 10.1016/j.cell.2017.01.017
7. Lim M, Xia Y, Bettegowda C, Weller M. Current state of immunotherapy for glioblastoma. *Nat Rev Clin Oncol* (2018) 15(7):422–42. doi: 10.1038/s41571-018-0003-5
8. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer* (2012) 12(4):252–64. doi: 10.1038/nrc3239
9. Fife BT, Bluestone JA. Control of peripheral T-cell tolerance and autoimmunity via the CTLA-4 and PD-1 pathways. *Immunol Rev* (2008) 224:166–82. doi: 10.1111/j.1600-065X.2008.00662.x
10. Sharpe AH, Pauken KE. The diverse functions of the PD1 inhibitory pathway. *Nat Rev Immunol* (2018) 18(3):153–67. doi: 10.1038/nri.2017.108
11. McGranahan T, Therkelsen KE, Ahmad S, Nagpal S. Current State of Immunotherapy for Treatment of Glioblastoma. *Curr Treat Options Oncol* (2019) 20(3):24. doi: 10.1007/s11864-019-0619-4
12. Ampie L, Woolf EC, Dardis C. Immunotherapeutic advancements for glioblastoma. *Front Oncol* (2015) 5:12. doi: 10.3389/fonc.2015.00012
13. Schalper KA, Rodriguez-Ruiz ME, Diez-Valle R, López-Janeiro A, Porciuncula A, Idoate MA, et al. Neoadjuvant nivolumab modifies the tumor immune microenvironment in resectable glioblastoma. *Nat Med* (2019) 25(3):470–6. doi: 10.1038/s41591-018-0339-5
14. Omuro A, Vlahovic G, Lim M, Sahebjam S, Baehring J, Cloughesy T, et al. Nivolumab with or without ipilimumab in patients with recurrent glioblastoma: results from exploratory phase I cohorts of CheckMate 143. *Neuro-oncology* (2018) 20(5):674–86. doi: 10.1093/neuonc/nox208
15. Reardon DA, Brandes AA, Omuro A, Mulholland P, Lim M, Wick A, et al. Effect of Nivolumab vs Bevacizumab in Patients With Recurrent Glioblastoma: The CheckMate 143 Phase 3 Randomized Clinical Trial. *JAMA Oncol* (2020) 6(7):1003–10. doi: 10.1001/jamaoncol.2020.1024
16. Kurz SC, Wen PY. Quo Vadis-Do Immunotherapies Have a Role in Glioblastoma? *Curr Treat Options Neurol* (2018) 20(5):14. doi: 10.1007/s11940-018-0499-0
17. Woroniecka K, Chongsathidkiet P, Rhodin K, Kemeny H, Dechant C, Farber SH, et al. T-Cell Exhaustion Signatures Vary with Tumor Type and Are Severe in Glioblastoma. *Clin Cancer Res* (2018) 24(17):4175–86. doi: 10.1158/1078-0432.CCR-17-1846
18. Chongsathidkiet P, Jackson C, Koyama S, Loebel F, Cui X, Farber SH, et al. Sequestration of T cells in bone marrow in the setting of glioblastoma and other intracranial tumors. *Nat Med* (2018) 24(9):1459–68. doi: 10.1038/s41591-018-0135-2
19. Riaz N, Morris L, Havel JJ, Makarov V, Desrichard A, Chan TA. The role of neoantigens in response to immune checkpoint blockade. *Int Immunol* (2016) 28(8):411–9. doi: 10.1093/intimm/dxw019
20. McGranahan N, Furness AJ, Rosenthal R, Ramskov S, Lyngaa R, Saini SK, et al. Clonal neoantigens elicit T cell immunoreactivity and sensitivity to immune checkpoint blockade. *Sci (N Y NY)* (2016) 351(6280):1463–9. doi: 10.1126/science.aaf1490
21. Anagnostou V, Smith KN, Forde PM, Niknafs N, Bhattacharya R, White J, et al. Evolution of Neoantigen Landscape during Immune Checkpoint Blockade in Non-Small Cell Lung Cancer. *Cancer Discov* (2017) 7(3):264–76. doi: 10.1158/1538-7445.AM2017-NG01
22. Touat M, Li YY, Boynton AN, Spurr LF, Iorgulescu JB, Bohrson CL, et al. Mechanisms and therapeutic implications of hypermutation in gliomas. *Nature* (2020) 580(7804):517–23. doi: 10.1038/s41586-020-2209-9
23. Liu C, Peng W, Xu C, Lou Y, Zhang M, Wargo JA, et al. BRAF inhibition increases tumor infiltration by T cells and enhances the antitumor activity of adoptive immunotherapy in mice. *Clin Cancer Res* (2013) 19(2):393–403. doi: 10.1158/1078-0432.CCR-12-1626
24. Peng W, Chen JQ, Liu C, Malu S, Creasy C, Tetzlaff MT, et al. Loss of PTEN Promotes Resistance to T Cell-Mediated Immunotherapy. *Cancer Discov* (2016) 6(2):202–16. doi: 10.1158/2159-8290.CD-15-0283
25. Takeda K, Nakayama M, Hayakawa Y, Kojima Y, Ikeda H, Imai N, et al. IFN-γ is required for cytotoxic T cell-dependent cancer genome immunoediting. *Nat Commun* (2017) 8:14607. doi: 10.1038/ncomms14607
26. Benci JL, Xu B, Qiu Y, Wu TJ, Dada H, Twyman-Saint Victor C, et al. Tumor Interferon Signaling Regulates a Multigenic Resistance Program to Immune Checkpoint Blockade. *Cell* (2016) 167(6):1540–54 e12. doi: 10.1016/j.cell.2016.11.022
27. Galon J, Bruni D. Tumor Immunology and Tumor Evolution: Intertwined Histories. *Immunity* (2020) 52(1):55–81. doi: 10.1016/j.immuni.2019.12.018
28. Quail DF, Joyce JA. The Microenvironmental Landscape of Brain Tumors. *Cancer Cell* (2017) 31(3):326–41. doi: 10.1016/j.ccell.2017.02.009
29. Xie F, Zhou X, Fang M, Li H, Su P, Tu Y, et al. Extracellular Vesicles in Cancer Immune Microenvironment and Cancer Immunotherapy. *Advanced Sci (Weinheim Baden-Wurttemberg Germany)* (2019) 6(24):1901779. doi: 10.1002/advs.201901779
30. Chaudhary B, Elkord E. Regulatory T Cells in the Tumor Microenvironment and Cancer Progression: Role and Therapeutic Targeting. *Vaccines* (2016) 4(3):28. doi: 10.3390/vaccines4030028
31. Quezada SA, Peggs KS, Curran MA, Allison JP. CTLA4 blockade and GM-CSF combination immunotherapy alters the intratumor balance of effector and regulatory T cells. *J Clin Investigation* (2006) 116(7):1935–45. doi: 10.1172/JCI27745
32. Weber R, Fleming V, Hu X, Nagibin V, Groth C, Altevogt P, et al. Myeloid-Derived Suppressor Cells Hinder the Anti-Cancer Activity of Immune Checkpoint Inhibitors. *Front Immunol* (2018) 9:1310. doi: 10.3389/fimmu.2018.01310
33. Meyer C, Cagnon L, Costa-Nunes CM, Baumgaertner P, Montandon N, Leyvraz L, et al. Frequencies of circulating MDSC correlate with clinical outcome of melanoma patients treated with ipilimumab. *Cancer Immunol Immunother CII* (2014) 63(3):247–57. doi: 10.1007/s00262-013-1508-5
34. Liu D, Li X, Chen X, Sun Y, Tang A, Li Z, et al. Neural regulation of drug resistance in cancer treatment. *Biochim Biophys Acta Rev Cancer* (2019) 1871(1):20–8. doi: 10.1016/j.bbcan.2018.10.001
35. Teng MW, Ngiow SF, Ribas A, Smyth MJ. Classifying Cancers Based on T-cell Infiltration and PD-L1. *Cancer Res* (2015) 75(11):2139–45. doi: 10.1158/0008-5472.CAN-15-0255
36. Topalian SL, Drake CG, Pardoll DM. Immune checkpoint blockade: a common denominator approach to cancer therapy. *Cancer Cell* (2015) 27(4):450–61. doi: 10.1016/j.ccell.2015.03.001
37. Jackson CM, Choi J, Lim M. Mechanisms of immunotherapy resistance: lessons from glioblastoma. *Nat Immunol* (2019) 20(9):1100–9. doi: 10.1038/s41590-019-0433-y

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Redox Regulator *GLRX* Is Associated With Tumor Immunity in Glioma

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Glutaredoxin is central to cellular redox chemistry and regulates redox homeostasis and malignant progression of many cancers. In glioma, the role of its coding gene (*GLRX*) remains unclear. We aimed to elucidate the role of glutaredoxin at the transcriptome level and its clinical prognostic value in glioma. In total, we evaluated 1,717 glioma samples with transcriptome data and corresponding clinical data as well as single-cell sequencing data from 6 glioma patients from publicly available databases. Gene set variation analysis and gene ontology analysis were performed to reveal the biological function of *GLRX*. The immune cell enrichment score was calculated by GSVA analysis. Single-cell sequencing data was visualized by *t*-distributed stochastic neighbor embedding analysis. The prognostic value of *GLRX* in glioma was verified by the Kaplan-Meier curve and multivariate COX analysis. *GLRX* was found to be highly enriched in gliomas of higher grades with wild-type *IDH*, without 1p/19q co-deletion, and with a methylated *MGMT* promoter. Moreover, *GLRX* could be a potential marker for the mesenchymal molecular subtype of gliomas. The expression of *GLRX* was closely related to the tumor immune process, immune checkpoints, and inflammatory factors with *GLRX* being specifically expressed in M0 macrophages. *GLRX* is also shown to be an independent prognostic factor in glioma. Altogether, our study outcomes show that *GLRX* is highly enriched in malignant gliomas and is closely related to the tumor immune microenvironment. Therefore, *GLRX*-targeted cell redox regulatory therapy may enhance the efficacy of glioma immunotherapy.

Keywords: glioma, *GLRX*, macrophage, prognosis, tumor immunity

INTRODUCTION

Glioma is the most common malignant tumor affecting the central nervous system, and it is mainly characterized by a high recurrence rate and short survival time (1). To date, the most effective treatment for glioma is surgical resection to maximum safety extent (2), which can be followed by additional individualized treatments such as radiotherapy and chemotherapy. Even with aggressive

treatment, the prognosis for glioma patients remains very poor. Therefore, finding novel therapeutic targets and molecular targeted drugs may pave the way for an improved prognosis for these patients.

Glutaredoxin (Grx), also known as thiol transferase, is ubiquitously expressed in bacteria, viruses, and mammals. It has a relative molecular weight of approximately 12 kDa and comprises 106–107 amino acids (3). Grx is an important component of the thiol-disulfide bond oxidoreductase family and catalyzes the redox reaction between glutathione (GSH) and protein disulfide bonds that are necessary for optimal protein activity (4). Several studies have reported that Grx performs a variety of biological functions in cancer related to relieving oxidative stress, transcription regulation, and control of DNA synthesis by modulating the activity of ribonucleotide reductase (3, 5, 6). However, there are few reports on the role of Grx in glioma.

The GSH system is an essential regulator of redox balance in the brain (7), and Grx acts as a central "antioxidant" in neurons to protect them from oxidative stress injury. Previous studies have reported that Grx is also involved in glioma and metastasis development as well as in drug resistance (6, 8). Therefore, understanding the role of Grx in the context of glioma is pivotal for the development of novel therapeutic approaches targeting malignant gliomas.

We investigated the expression and function of the Grx coding gene (*GLRX*) at the transcriptome level using publicly available data sets from the Chinese Glioma Genome Atlas (CGGA) and The Cancer Genome Atlas (TCGA), which included RNA sequencing (RNA-seq) data and corresponding clinical details about the cancer patients. We found that *GLRX* is associated with high tumor grade and malignant phenotypes. Moreover, gene ontology analysis and gene set variation analysis revealed, for the first time, that *GLRX* can function as a mediator of the immune response. Further CIBERSORT analysis revealed that a higher expression level of GLRX is correlated with enrichment of macrophages in glioma tissue. Single-cell analysis, immunohistochemical (IHC) staining, and immunofluorescent staining (IF) validated that GLRX may be specifically expressed in M0 macrophages. Last, we found that *GLRX* is an independent prognostic factor in glioma. Altogether, these findings suggest that *GLRX* is highly enriched in malignant gliomas and is closely related to the tumor immune microenvironment. Therefore, *GLRX*-targeted cell redox regulatory therapy may enhance response to immunotherapy in patients with glioma.

MATERIALS AND METHODS

Data Collection

This study was approved by the Capital Medical University Institutional Review Board. We collected transcriptome sequencing data generated by the Illumina HiSeq platform that was publicly available from the CGGA and CGGA (2019) databases (https://www.cgga.org.cn) for 325 and 693 samples, respectively. We evaluated the status of isocitrate dehydrogenase (IDH) mutation, 1p/19q, and MGMT promoter methylation as described in previous studies (9–11). Overall patient survival was estimated from the date of diagnosis to the reported date of death or last follow-up. RNA-seq data were obtained from TCGA (https://tcgadata.nci.nih.gov), and single-cell sequencing data were retrieved from the GSE89567 data set of the Gene Expression Omnibus database. All clinical and molecular information on the samples evaluated in the present study is presented in **Table 1**. We used the online software GEPIA (http://gepia.cancer-pku.cn) (12) to evaluate expression differences between glioblastoma multiform (GBM) and normal brain tissues.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Analyses

The biological functions and signaling pathways related to *GLRX* were explored by GO and KEGG analyses using the DAVID bioinformatics resource (version 6.7) (13). After Spearman correlation analysis, GO results on the most correlated genes were visualized by heat map.

Gene Set Variation Analysis (GSVA)

GSVA was performed with the GSVA package (from R Project 3.5.1) of R software with default parameters. The list of GO terms was obtained from the Gene Set Enrichment Analysis database (https://www.gsea-msigdb.org/gsea/msigdb/index.jsp). Relationships between genes and biological functions were determined using Pearson correlation analysis.

Immune Function Analysis

The relationship between *GLRX* expression and immune function was evaluated by Pearson correlation analysis. Immune function scores (14) were calculated by GSVA analysis, and the immune function gene set was downloaded from AmiGO 2 (http://amigo.geneontology.org/amigo/landing). The classification of immune functions was done according to the guidelines of AmiGO 2.

CIBERSORT

RNA-seq data were evaluated using the CIBERSORT software (https://cibersort.stanford.edu). The signature gene profile of 22 immune cell types was used in CIBERSORT to estimate the proportion of tumor-infiltrating immune cell types (15).

T-Distributed Stochastic Neighbor Embedding (T-SNE) Analysis

T-SNE analysis was performed with the Rtsne package from R Project (version 3.5.1); perplexity was set to 20. Identification of cell types was based on the specific cell markers obtained from the CellMarker database (http://biocc.hrbmu.edu.cn/CellMarker/).

Prognostic Analysis

Patient survival distribution and significance was evaluated by Kaplan-Meier survival curve and log-rank test. Kaplan-Meier

TABLE 1 | Sample information.

Characteristics (CGGA)	No. of Patients (n=325)
Age	
<45	191
≥45	134
Gender	
Male	203
Female	122
WHO Grade	
Grade II	103
Grade III	79
Grade IV	139
NA	4
TCGA Subtypes	
Proneural	102
Neural	81
Classical	74
Mesenchymal	68
Radiotherapy+TMZ Chemotherapy	
Yes	154
No	24
Radiotherapy	
Yes	258
No	51
NA	16
TMZ Chemotherapy	
Yes	178
No	124
NA	23
IDH1/2 mutation	
Mutation	175
Wild type	149
NA	1
1p/19q codeletion	
Co-deletion	67
Non-co-deletion	250
NA	8
MGMT methylation	
Methylation	130
Unmethylation	112
NA	64
Characteristics [CGGA(2019)]	**No. of Patients (n=693)**
Age	
<45	382
≥45	310
NA	1
Gender	
Male	398
Female	295
WHO Grade	
Grade II	188
Grade III	255
Grade IV	249
NA	1
TCGA Subtypes	
Proneural	296
Neural	167
Classical	83
Mesenchymal	147
Radiotherapy+TMZ Chemotherapy	
Yes	413
No	67
Radiotherapy	
Yes	509
No	113
NA	71
TMZ Chemotherapy	
Yes	457
No	151
NA	85
IDH1/2 mutation	
Mutation	356
Wild type	286
NA	51
1p/19q co-deletion	
Co-deletion	145
Non-co-deletion	478
NA	70
MGMT methylation	
Methylation	127
Unmethylation	73
NA	492
Characteristics (TCGA)	**No. of Patients (n=699)**
Age	
<45	296
≥45	340
NA	63
Gender	
Male	368
Female	268
NA	63
WHO Grade	
Grade II	223
Grade III	245
Grade IV	168
NA	63
TCGA Subtypes	
Proneural	250
Neural	115
Classical	92
Mesenchymal	105
NA	137
IDH1/2 mutation	
Mutation	443
Wild type	246
NA	10
1p/19q co-deletion	
Co-deletion	172
Non-co-deletion	520
NA	7
MGMT methylation	
Methylation	492
Unmethylation	168
NA	39

The number of glioma patients engaged in our study is listed. All patients were stratified with age, clinicopathological characteristics, and treatment options, respectively.

analysis was performed using R software (version 3.5.1, http://www.r-project.org). The prognostic value of *GLRX* was estimated by univariate and multivariate Cox proportional hazard model analysis using SPSS statistical software (version 25.0; IBM, Armonk, NY, USA). Patients with missing information were excluded from the analysis.

IHC Staining

Paraffin-embedded samples were obtained from the CGGA sample bank. First, 5-μm sections were cut for IHC staining.

Samples were deparaffinized in an oven at 65°C for 1 h. Then the samples were rehydrated in decreasing concentrations of alcohol. IHC analysis with GLRX1 (Abcam, ab45953,1:1000) and CD11b antibodies (Proteintech, 66519-1-lg, 1:1000) was conducted according to standard procedures. Photos were taken with an optical microscope.

Cell Culture and Reagents

THP-1 cells (purchased from National Infrastructure of Cell Line Resource, http://www.cellresource.cn/) were maintained in RPMI1640 media supplemented with L-glutamine, 1% penicillin and streptomycin, β-mercaptoethanol (Gibco, 2169148, 0.055 mM) and 10% fetal bovine serum (FBS, Gibco) at 37°C under a humidified, 5% CO_2 atmosphere (16). THP-1 cells were differentiated to M0 macrophages by treatment with 25 nM phorbol 12-myristate 13-acetate (MCE, HY-18739) for 48 h, washed and incubated with normal RPMI1640 media for 24 h, and then incubated with recombinant human GM-CSF (50 ng/ml, Peprotech, 300-03) for 96 h. For M2 polarization, 50% of complete RPMI1640 medium was added, and it was incubated for 48 h. Then the M2 macrophage was obtained by removing the culture medium and culturing cells for an additional 48 h in M2 medium with recombinant human M-CSF (100 ng/ml, Peprotech, 300-25) (17).

IF Staining

Macrophages were washed with PBS three times. Four percent fixative solution (Solarbio, P1110) was added to the Petri dish for 10 min. Then, the solution was removed and cells washed three times. Next, 0.5% Triton X-100 (Solarbio, T8200) was added to the dish for 10 min. The solution was removed and cells washed three times. Five percent BSA (Solarbio, A8010) was added to the dish, and it was incubated for 1 h at room temperature. Then, primary antibodies were added to the M0 (GLRX1: 1:500, Abcam, ab45953; CD11b: 1:100, Proteintech, 66519-1-lg) and M2 macrophages (GLRX1: 1:500, Abcam, ab45953; CD163: 1:100, Abcam, ab156769) (18), respectively, and they were incubated overnight at 4°C. The solution was removed and cells washed three times. Secondary antibodies (DyLight 488 goat antirabbit polyclonal antibody, Abcam, ab96899, 1:200; DyLight 594 goat antimouse polyclonal antibody, Abcam, ab96881, 1:200) were used for 1 h at room temperature. The solution was removed and cells washed three times. Prolong™ Diamond Antifade Mountant with DAPI (Invitrogen, P36962) was added to the dish, and photos were taken with confocal microscopy.

Other Immune Biological Analysis

Pearson's correlation analysis was used to evaluate the relationship between *GLRX* and immune checkpoints. Inflammation-related metagenes were described as before (19).

Statistical Analysis

A multiple group comparison was performed using Tukey's test. Other statistical computations and figure drawing were performed with several R packages, including ggplot2, pheatmap, pROC, and corrgram. All statistical tests were two-sided, and a p-value < 0.05 was considered statistically significant in all analyses.

RESULTS

Association of *GLRX* Expression With Clinical and Molecular Pathological Characteristics in Glioma

To investigate the role of *GLRX* in gliomas, we compared the expression levels of *GLRX* between normal brain tissue and GBM (grade IV, according to the World Health Organization [WHO]). The analysis revealed that *GLRX* expression was significantly enriched in GBM samples ($p < 0.05$, **Figure 1A**). Due to the histopathological heterogeneity of gliomas, RNA-seq data of glioma samples from three independent databases were analyzed according to WHO guidelines, and the analysis included *IDH* mutation status, 1p/19q co-deletion status, and MGMT promoter status. Among samples from the CGGA database, *GLRX* expression was higher in GBM (grade IV) compared with glioma (grades II and III) (**Figure 1B**). This result was further validated in the RNA-seq data from TCGA and CGGA (2019) databases (**Figure 1F** and **Supplementary Figure S1A**). In addition, IHC staining was conducted to explore the expression of *GLRX* in glioma tissues. Consistent with the RNA-seq data, we found that *GLRX* was enriched in GBM tissues (**Figures 1J, K**). The *IDH* mutation status, 1p/19q co-deletion status, and MGMT promoter status play important roles in the prognosis and chemotherapy outcomes of glioma patients and vary significantly among glioma patients (20). Therefore, we explored the correlation between *GLRX* expression and these three molecular pathologic statuses. We found that *GLRX* expression was highly enriched in *IDH* wild-type glioma patients compared with those harboring *IDH* mutations (**Figures 1C, G** and **Supplementary Figure S1B**). Moreover, patients with 1p/19q non-co-deletion had a higher expression of *GLRX* in all three databases (**Figures 1D, H** and **Supplementary Figure S1C**). Regarding the MGMT promoter status in the CGGA database, we found that gliomas with a methylated MGMT promoter had lower *GLRX* expression compared to those in the unmethylated group (**Figure 1E**). A similar trend was observed in the two other databases (**Figure 1I** and **Supplementary Figure S1D**). These findings indicate that *GLRX* expression is enriched in GBM and is tightly correlated with the malignant phenotype of glioma.

GLRX Is a Potential Marker for Mesenchymal Molecular Subtype Glioma

Next, we investigated the molecular expression pattern of *GLRX* in different molecular subtypes defined by TCGA network (21). *GLRX* was significantly upregulated in the mesenchymal subtype of glioma compared with the other three subtypes in the CGGA (**Figure 2A**), TCGA (**Figure 2C**), and CGGA (2019) databases (**Supplementary Figure S2A**). The IHC staining of *GLRX* in GBM tissues also verified this finding (**Figures 3E–G**). To further validate this finding, we evaluated the receiver operating characteristic (ROC) curve for *GLRX* expression and

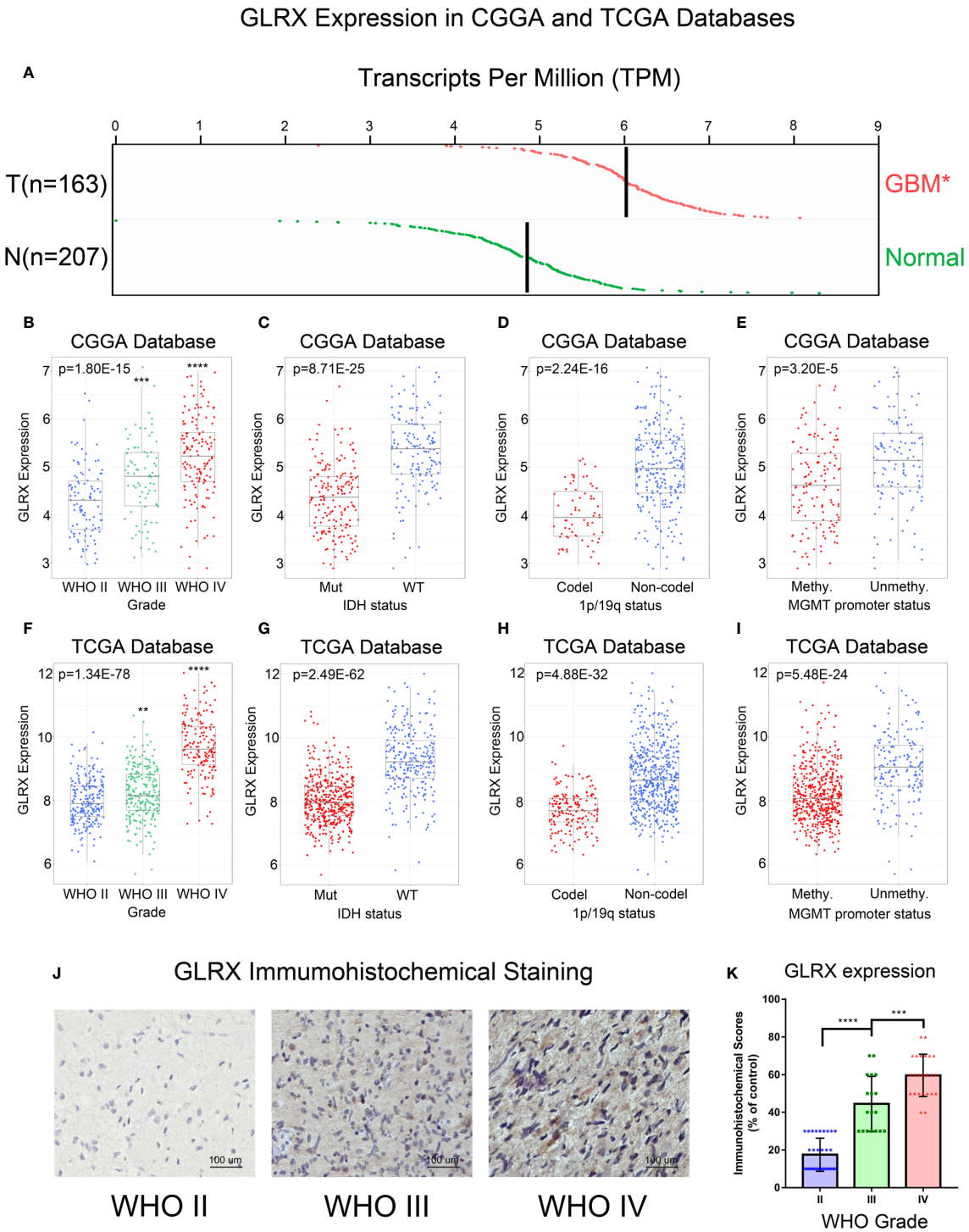


FIGURE 1 | *GLRX* is correlated with relative malignant molecular pathological characteristics of gliomas. **(A)** *GLRX* showed a significantly higher expression in GBM (WHO grade IV) compared with normal brain tissue in online analysis of GEPIA (T: GBM tumor; N: normal brain tissue). **(B, F)** *GLRX* was significantly increased in GBM (WHO grade IV) in the CGGA and TCGA databases. **(C, G)** *GLRX* was significantly increased in IDH wild-type gliomas in the CGGA and TCGA databases (Mut: IDH mutation; WT: IDH wildtype). **(D, H)** *GLRX* was significantly increased in 1p/19q non-co-deletion gliomas in the CGGA and TCGA databases (Codel: 1p/19q co-deletion; Non-codel: 1p/19q non-co-deletion). **(E, I)** *GLRX* was significantly increased in the MGMT unmethylated group in the CGGA and TCGA databases. **(J)** The representative photos of IHC staining of GLRX in different glioma grades. Scale bar is 100 um. **(K)** The immunohistochemical scores of GLRX were measured in different grades. Respectively, 32, 18, and 24 patients were from grade II, grade III and grade IV. *, **, ***, and **** indicate $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively.

mesenchymal subtype for gliomas of all grades. Surprisingly, the area under the curve (AUC) of *GLRX* expression was up to 90.9%, 90.2%, and 78.0% for the CGGA, TCGA, and CGGA (2019) data sets, respectively (**Figures 2B, D** and **Supplementary Figure S2B**). These results suggest that GLRX is highly expressed in mesenchymal subtype glioma and may play an oncogenic role in glioma progression. BMI1 and CD44 were reported to differentiate the mesenchymal molecular subtype from other gliomas (22, 23). Thus, we took these two well-studied biomarker genes as positive controls to performed ROC curve analysis (**Supplementary Figure S2C–H**). Through comparing the AUC of these three genes, we inferred that *GLRX* may serve as a biomarker for mesenchymal subtype gliomas.

GLRX Is Strongly Associated With Immune Functions in Glioma

We performed GO analysis to identify the *GLRX*-related biological functions in gliomas. At first, we screened genes that were strongly correlated with *GLRX* (Pearson $R > 0.55$ and $p < 0.0001$) in all three databases. The analysis revealed a total of 479 genes in CGGA, 877 genes in TCGA, and 537 genes in CGGA (2019) that were significantly correlated with *GLRX* expression. The genes positively correlated with *GLRX* expression were mostly involved in immune response, defense response, and inflammatory response in all databases (**Figures 3A, C** and **Supplementary Figure S3A**). Additionally, we performed KEGG pathway analysis to further explore the signaling pathways associated with the abovementioned genes. As expected, the KEGG analysis identified these genes to be associated with immune response pathways, including FcγR-mediated phagocytosis, the toll-like receptor signaling pathway, and complementary and coagulation cascades in the three databases (**Figures 3B, D** and **Supplementary Figure S3B**). The heat map representation of the genes (shown in **Table 2**) within each biological process exhibits a clear positive correlation with *GLRX* expression and the landscape of corresponding clinical patient features (**Figures 3E, F** and **Supplementary Figure S3C**). These findings suggest that *GLRX* takes part in the immune response process and may be a marker for predicting immune-related biological processes in gliomas.

Special Immune Function of *GLRX*

Tumor-infiltrated immune cells, including T cells, NK cells, macrophages, and other cells, mount the immune response to kill or induce apoptosis of cancer cells (24). To further clarify the role of *GLRX* in the immune response in gliomas, we first assessed the correlation between *GLRX* and GO terms downloaded from the AmiGO2 web portal (http://amigo.geneontology.org/). We found 84.69%, 78.90%, and 87.07% biofunction of the immune system to be positively correlated with *GLRX* in the CGGA, TCGA, and CGGA (2019) data sets, respectively (**Figures 4A, B** and **Supplementary Figure S4A**). Overall, more immune-related GO terms were positively correlated with *GLRX* than any other kind of GO term. This further illustrates that *GLRX* has a strong correlation with the immune system. Last, to understand the role of *GLRX* in the immune system, we performed a correlation coefficient analysis on data from the three databases (**Figure 4C** and **Supplementary Figure S4B**). We observed that the majority of immune functions showed positive correlation with *GLRX*; only the term "T cell-mediated immune response to tumor cell (T cell response)" was found to be negatively correlated with *GLRX*.

GLRX Is Associated With Inhibitory Immune Checkpoints and Inflammatory Responses

As mentioned above, most immune functions had positive correlation with *GLRX* with the exception of T cell responses. In a previous study, we reported that glioma patients with a stronger immune response had a much poorer prognosis (25). This abnormal phenomenon suggests that depletion of immune components in gliomas triggered by immune checkpoints could contribute to a malignant tumor phenotype. To validate this hypothesis, we investigated the relationship between *GLRX* and known immune checkpoint genes, including PD-1, TIM-3, PD-L1, and PD-L2, in the CGGA, TCGA, and CGGA (2019) databases (**Figures 5A–H** and **Supplementary Figure S5A-D**). The results indicated that *GLRX* had a strong positive correlation with these inhibitory immune checkpoint molecules and that *GLRX* may influence their expression to support glioma cells escaping immunological surveillance. Additionally, we also analyzed the role of *GLRX* in the glioma inflammatory response in these databases as described previously (19) (**Figures 5I, J** and **Supplementary Figure S5E**). We found that *GLRX* was positively associated with HCK, interferons, LCK, MHC-I, MHC-II, STAT1, and STAT2 expression, and it was negatively associated with IgG expression. These results suggest that upregulation of *GLRX* is involved in the activation of signal transduction in T cells, macrophages, and antigen-presenting cells, but it is negatively associated with B lymphocytes related metagenes. All these findings collectively confirm that *GLRX* plays an important role in immune response in gliomas.

GLRX Is Associated With M0 Macrophages

An activated immune response may promote the infiltration of immune cells into the tumor microenvironment and change its dynamics. To investigate whether *GLRX* was associated with infiltrated immune cells, we used CIBERSORT software to analyze the CGGA, TCGA, and CGGA (2019) databases (**Figure 6A** and **Supplementary Figure S6**). The analysis revealed that higher *GLRX* expression was positively correlated with enrichment of macrophages in glioma tissue. Moreover, single-cell sequencing data (**Figures 6B–G**) demonstrated that *GLRX* may be specifically expressed in M0 macrophages compared to other types of macrophages. To verify this finding, IHC co-localization staining was performed to explore the expression of GLRX in macrophages in tumor specimens. A previous study reported that CD11b was a biomarker of M0 macrophages (17). The results showed that GLRX was expressed in most M0 macrophages (**Figures 7A, B**). Furthermore, GM-CSF and PMA were used to induce THP-1 cells to differentiate

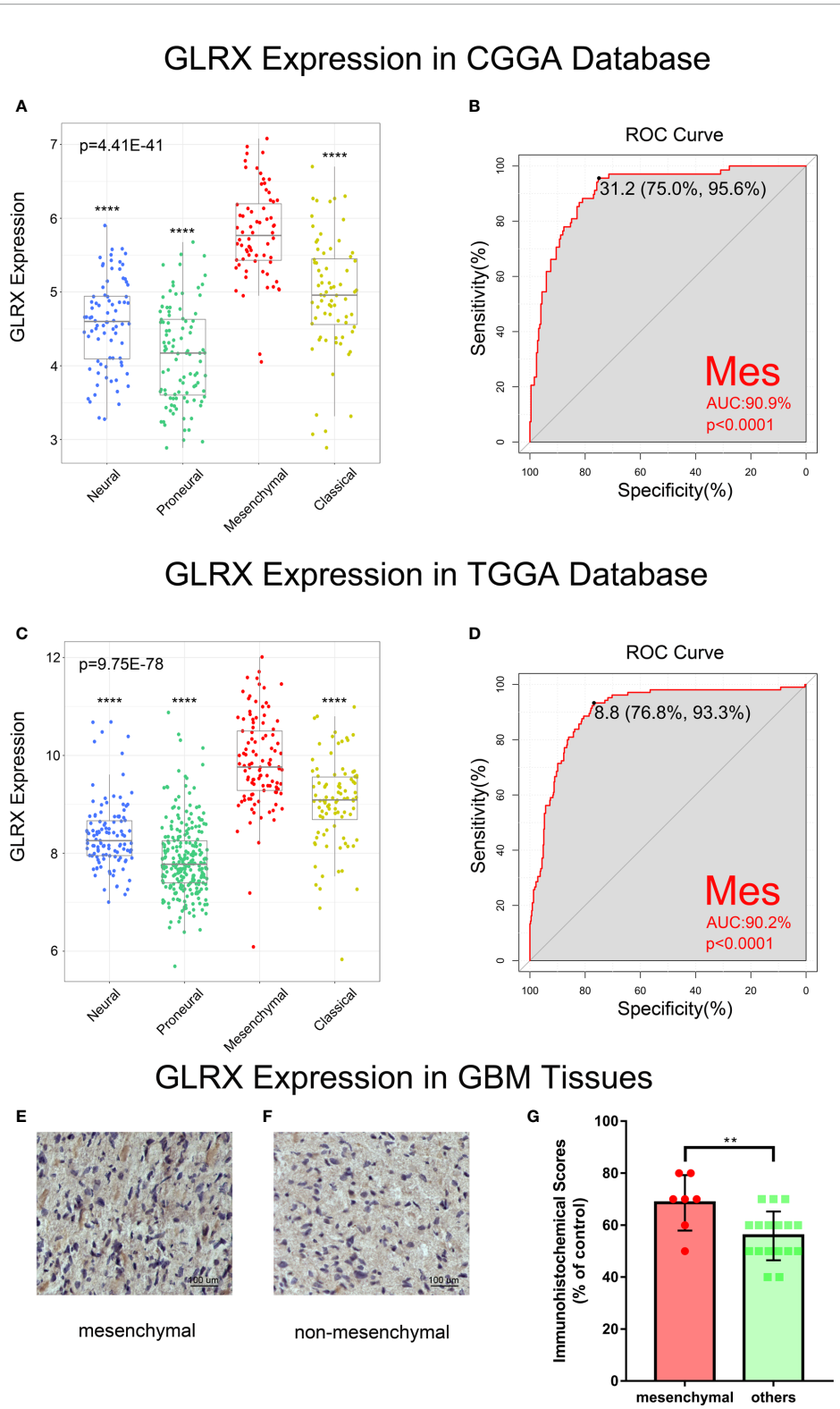


FIGURE 2 | *GLRX* is a potential marker for malignant subtypes of gliomas. **(A, C)** *GLRX* was highly expressed in the mesenchymal subtype in the CGGA and TCGA databases. **(B, D)** ROC curve analysis showed that *GLRX* was highly sensitive and specific to predict the mesenchymal subtype in the CGGA and TCGA databases. **(E, F)** The representative photos of IHC staining of GLRX in different TCGA molecular subtypes. Scale bar is 100 um. **(G)** The immunohistochemical scores of GLRX were measured in different TCGA molecular subtypes. Respectively, 7 and 17 patients were from GBM. Differences between groups were tested by Tukey's multiple comparisons test. ** and **** indicate p<0.01 and p < 0.0001, respectively.

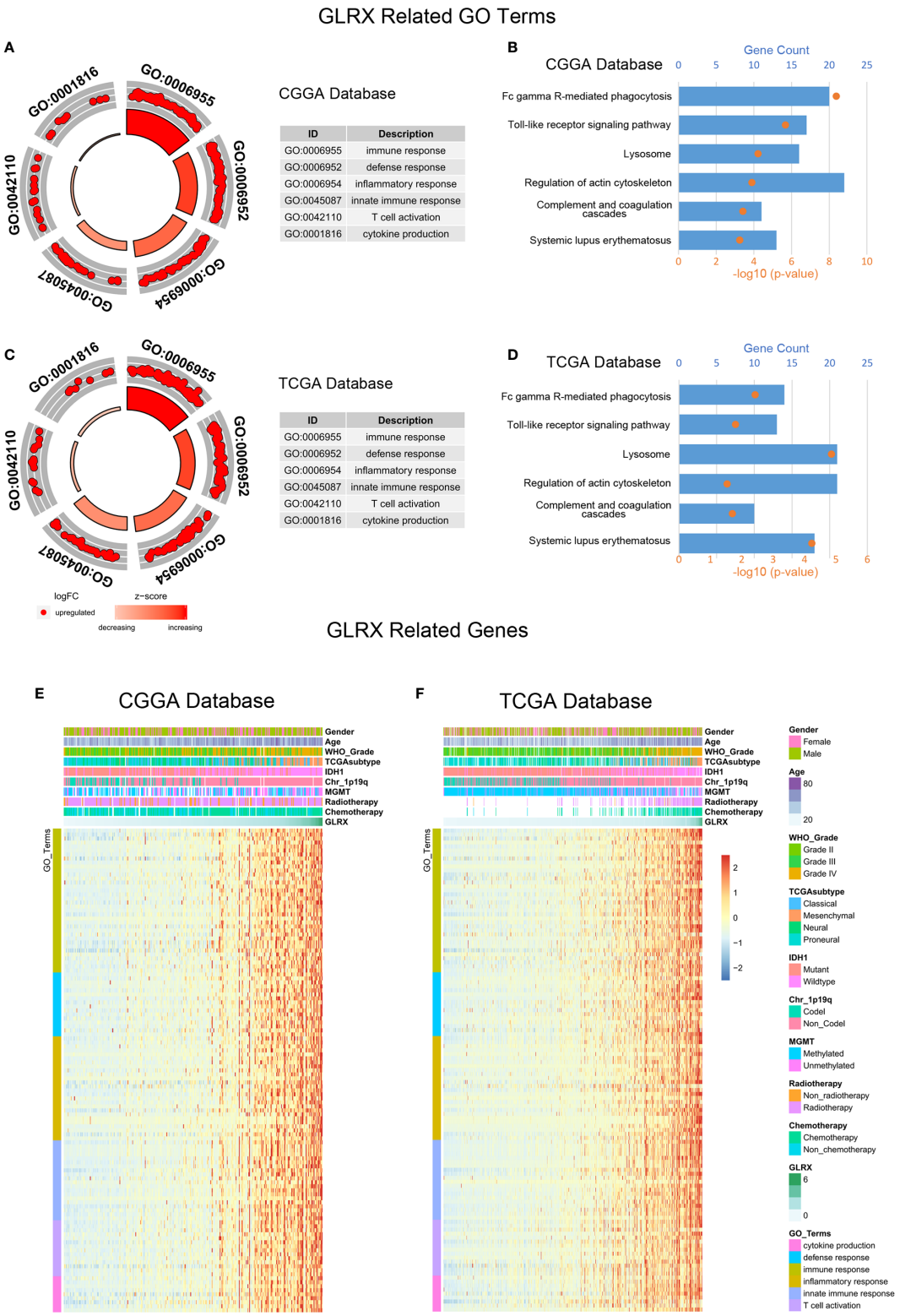


FIGURE 3 | *GLRX* is strongly associated with immune processes in gliomas. **(A, C)** GO analysis showed that *GLRX* was mostly associated with immune, defense, and inflammatory responses in both the CGGA and TCGA databases. **(B, D)** KEGG pathway analysis showed that *GLRX* was mostly involved in the immune response–related pathway in the CGGA and TCGA databases. **(E, F)** Most immune process–related genes were significantly positively correlated with *GLRX* expression in the CGGA and TCGA databases.

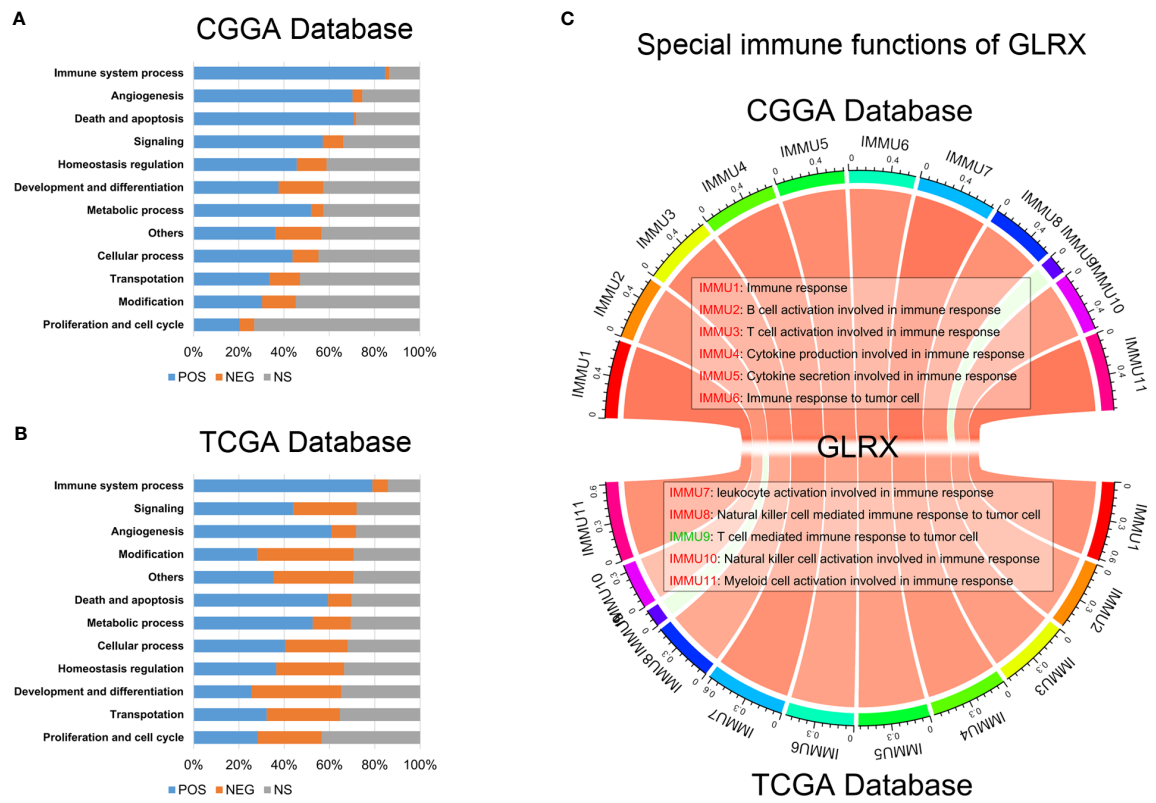


FIGURE 4 | *GLRX* is closely related to the state of tumor immune functions. **(A, B)** *GLRX* had a positive correlation with 84.69% and 78.90% of the biological functions of immune system processes in the CGGA and TCGA databases, respectively. The scale values in the graph represent the proportions of significantly correlated biological functions in each biological function classification. **(C)** The correlation coefficient between *GLRX* and immune function scores in CGGA and TCGA databases. The red words represent a positive correlation. The green words represent a negative correlation.

toward M0 macrophages *in vitro*. Under this circumstance, M-CSF was added to medium to induce M0 macrophages to polarize to M2 macrophages (16, 17). IF staining results showed that GLRX was highly expressed by M0 macrophages compared to M2 macrophages (**Figures 7C, D**). These findings suggest that the effect of *GLRX* on the immune system is mediated by M0 macrophages, further validating that *GLRX* plays a pivotal role in the immune response.

GLRX Predicts Survival Outcome in Gliomas

Because *GLRX* showed a robust negative correlation with the T cell response, we further investigated the prognostic value of *GLRX* by Kaplan-Meier and Cox proportional hazard model analyses. We found that patients with a higher expression of *GLRX* had a significantly shorter overall survival compared with those with lower *GLRX* expression (**Figures 8A, B** and **Supplementary Figure S7A**). Moreover, *GLRX* expression, WHO grade, age at diagnosis, *IDH* status, 1p/19q status, and MGMT promoter status were significantly associated with overall patient survival in all the three data sets that were evaluated. Multivariate analysis further confirmed *GLRX* as a significant predictor after adjusting for the clinical factors mentioned above (**Figures 8C, D** and **Supplementary Figure S7B**). These findings reveal that *GLRX* may serve as an indicator for the poor prognosis in gliomas due to its suppressive effects on the T cell immune response against tumor cells.

DISCUSSION

Glioma is one of the most fatal malignancies to afflict human health (1). Although temozolomide was approved for the treatment of gliomas by the U.S. Food and Drug Administration in 2005, researchers have continued to search for novel chemotherapeutic drugs with improved efficacy to treat gliomas (26). Nevertheless, no significant results have been achieved so far. Therefore, novel therapeutic approaches against gliomas remain an urgent requirement. In recent years, targeted drugs and immunotherapeutic approaches have exhibited extraordinary prospects (27, 28). Based on high-throughput sequencing, our team built the CGGA database and screened the *PTPRZ1-MET* fusion gene, which is expressed almost exclusively in secondary glioblastomas. The targeted drug PLB-1001 showed a good response rate in phase 2 clinical trials (29). Moreover, immune checkpoint blockade therapy also achieved success in treating

TABLE 2 | Representative genes of each biological function.

Gene	GO_Terms
NFKB2	immune response
B2M	immune response
IL4R	immune response
LILRA6	immune response
FCGR3A	immune response
LAIR1	immune response
DBNL	immune response
NCF4	immune response
STXBP2	immune response
TNFRSF14	immune response
CTSS	immune response
PDCD1LG2	immune response
BCAP31	immune response
LILRB1	immune response
LAT2	immune response
CTSC	immune response
GBP2	immune response
GALNT2	immune response
SBNO2	immune response
IFITM3	immune response
GPSM3	immune response
GPR65	immune response
FCGRT	immune response
FTH1	immune response
SQSTM1	immune response
FCER1G	immune response
MR1	immune response
ARHGDIB	immune response
PSMB8	immune response
TNFSF8	immune response
PSMB9	immune response
IKBKE	immune response
FCGR2B	immune response
CD300A	immune response
CD274	immune response
RNF19B	immune response
TNIP1	defense response
C5AR1	defense response
CLIC1	defense response
SP140	defense response
MNDA	defense response
CLEC5A	defense response
TYROBP	defense response
TCIRG1	defense response
HCK	defense response
MAP2K3	defense response
CD300C	defense response
APOL2	defense response
CYBB	defense response
STAB1	defense response
ALOX5	defense response
CD14	defense response
CCL2	inflammatory response
NMI	inflammatory response
ADORA3	inflammatory response
S100A8	inflammatory response
AIF1	inflammatory response
CCR1	inflammatory response
S100A9	inflammatory response
ITGB2	inflammatory response
TNFRSF1B	inflammatory response
IL10RB	inflammatory response
HMOX1	inflammatory response
TICAM2	inflammatory response
SERPINA3	inflammatory response
C2	inflammatory response
SPP1	inflammatory response
B4GALT1	inflammatory response
NFKBIZ	inflammatory response
CEBPB	inflammatory response
LY96	inflammatory response
PDPN	inflammatory response
LYZ	inflammatory response
NFAM1	inflammatory response
IL6R	inflammatory response
CD40	inflammatory response
CD163	inflammatory response
CCR5	inflammatory response
KYNU	innate immune response
IL1R1	innate immune response
TLR1	innate immune response
NCF1C	innate immune response
TLR2	innate immune response
C1R	innate immune response
APOBEC3G	innate immune response
C1S	innate immune response
C1QC	innate immune response
GCH1	innate immune response
SP100	innate immune response
NCF2	innate immune response
NCF1	innate immune response
SERPING1	innate immune response
C1QA	innate immune response
CYBA	innate immune response
C1QB	innate immune response
CORO1A	innate immune response
C1RL	innate immune response
VSIG4	innate immune response
PTPRC	T cell activation
STAT5A	T cell activation
RELB	T cell activation
PTPN22	T cell activation
VAV1	T cell activation
ITGAM	T cell activation
DOCK2	T cell activation
CD86	T cell activation
IRF1	T cell activation
CLEC7A	T cell activation
FAS	T cell activation
LCP1	T cell activation
RAB27A	T cell activation
SYK	T cell activation
SLC11A1	cytokine production
NLRC4	cytokine production
MYD88	cytokine production
LYN	cytokine production
CD4	cytokine production
CD226	cytokine production
PRKCD	cytokine production
PTAFR	cytokine production
LCP2	cytokine production

(Continued)

TABLE 2 | Continued

The representative genes of each biological function are which obtained from GO analysis in heat map are listed.

gliomas. Cloughesy et al. reported that neoadjuvant anti-PD-1 immunotherapy, which enhances T cell–mediated antitumor immunity, could significantly extend overall survival of patients

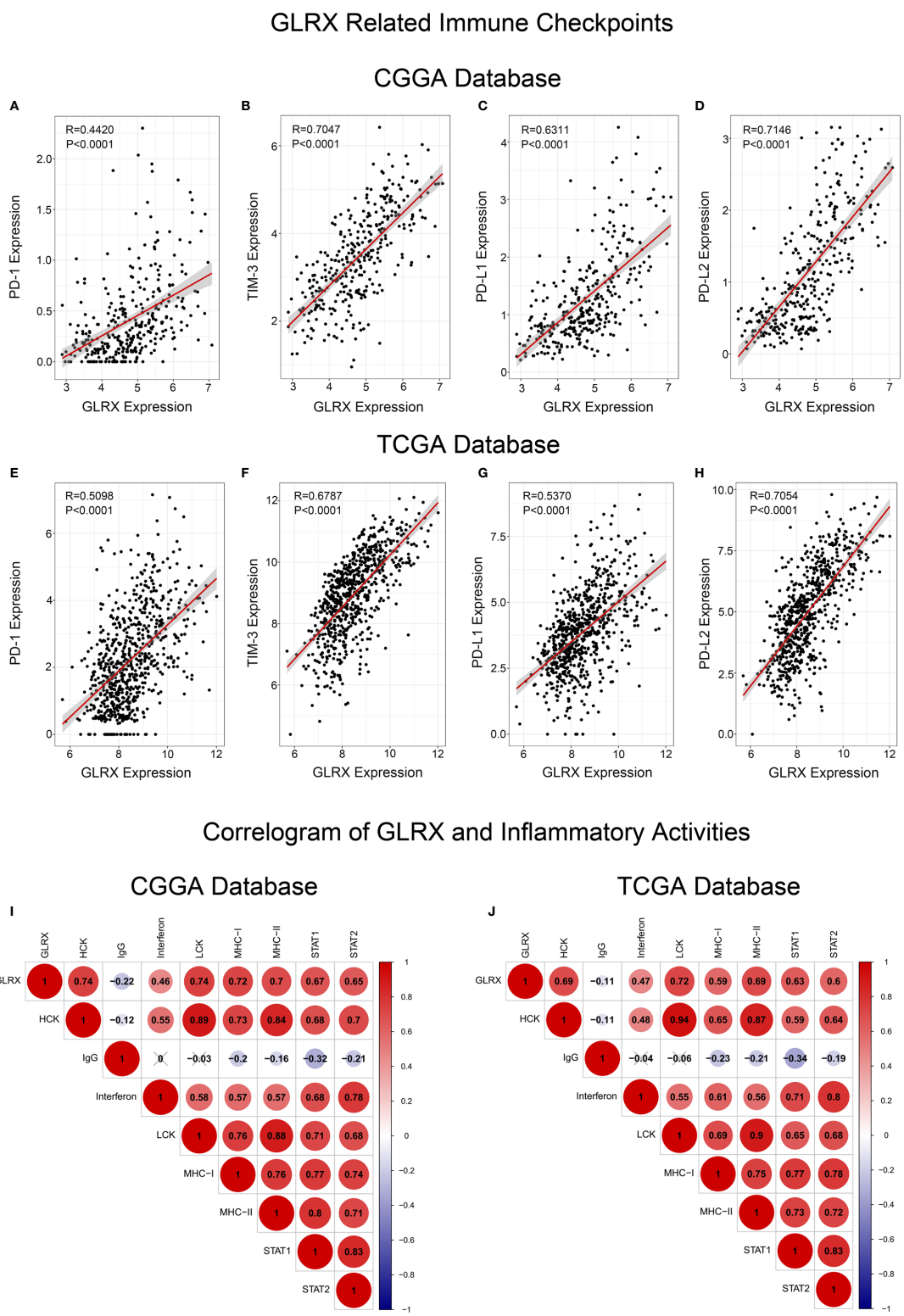


FIGURE 5 | *GLRX* is associated with inhibitory immune checkpoints and inflammatory activities. **(A–H)** *GLRX* was synergistic with inhibitory immune checkpoints in tumor-induced immune responses. A strong correlation between *GLRX* and inhibitory immune checkpoint expression was found in both the CGGA and TCGA databases. **(I, J)** The correlation coefficient between *GLRX* and inflammatory activity function scores in gliomas. The red circle represents a positive correlation. The blue circle represents a negative correlation. The grey "×" represents no significant correlation. Similar results are found in both CGGA and TCGA databases.

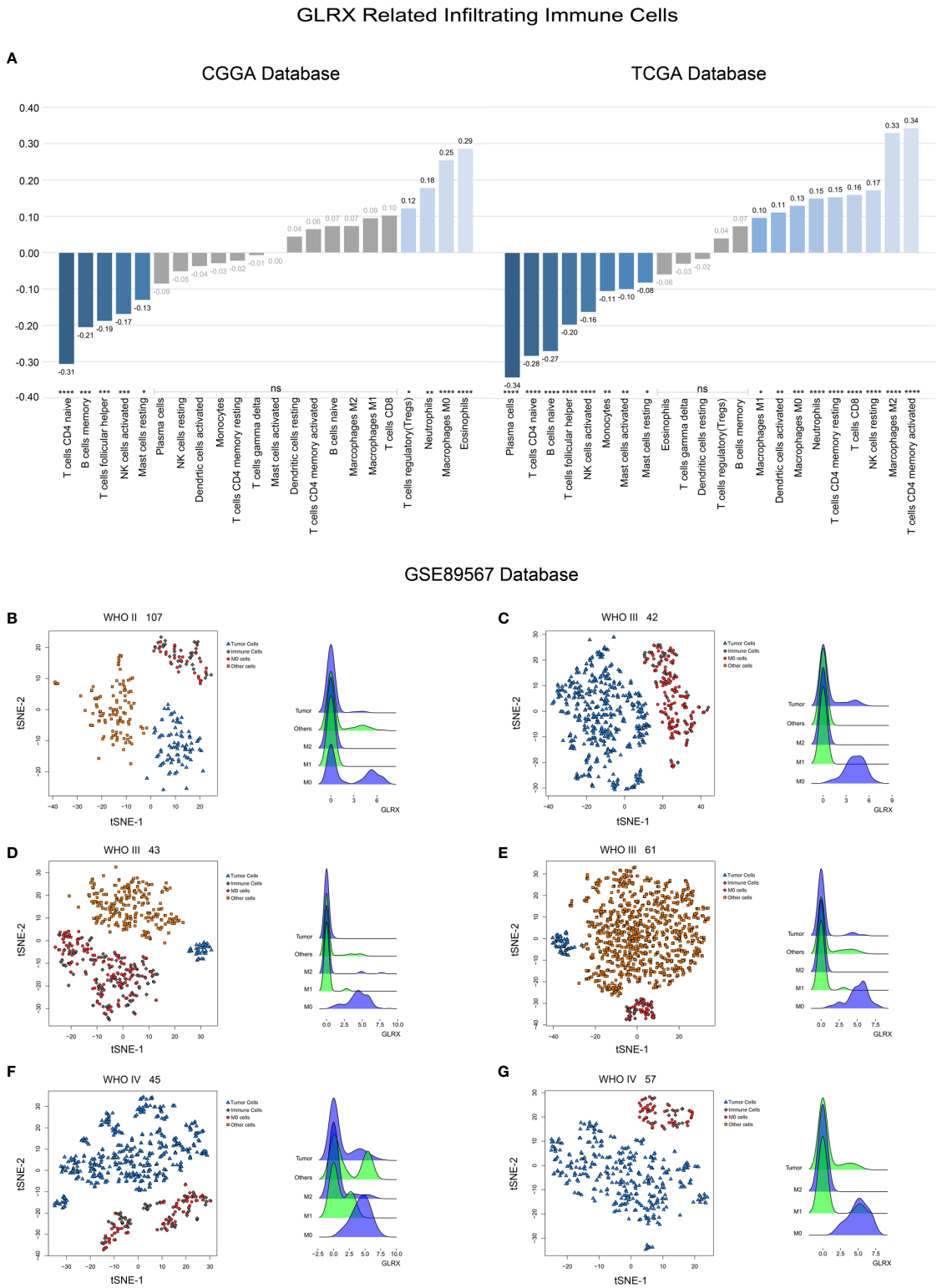


FIGURE 6 | *GLRX* is expressed by immune cells. **(A)** The relationship between *GLRX* and infiltrated immune cells in both CGGA and TCGA databases. **(B–G)** The relationship between *GLRX* and macrophages in the GSE89567 database. The correlation between *GLRX* and infiltrated immune cells was analyzed by Pearson correlation analysis. ns, *, **, ***, and **** indicate no statistical difference, $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively. M0, M1, and M2 indicate M0, M1, and M2 macrophages, respectively.

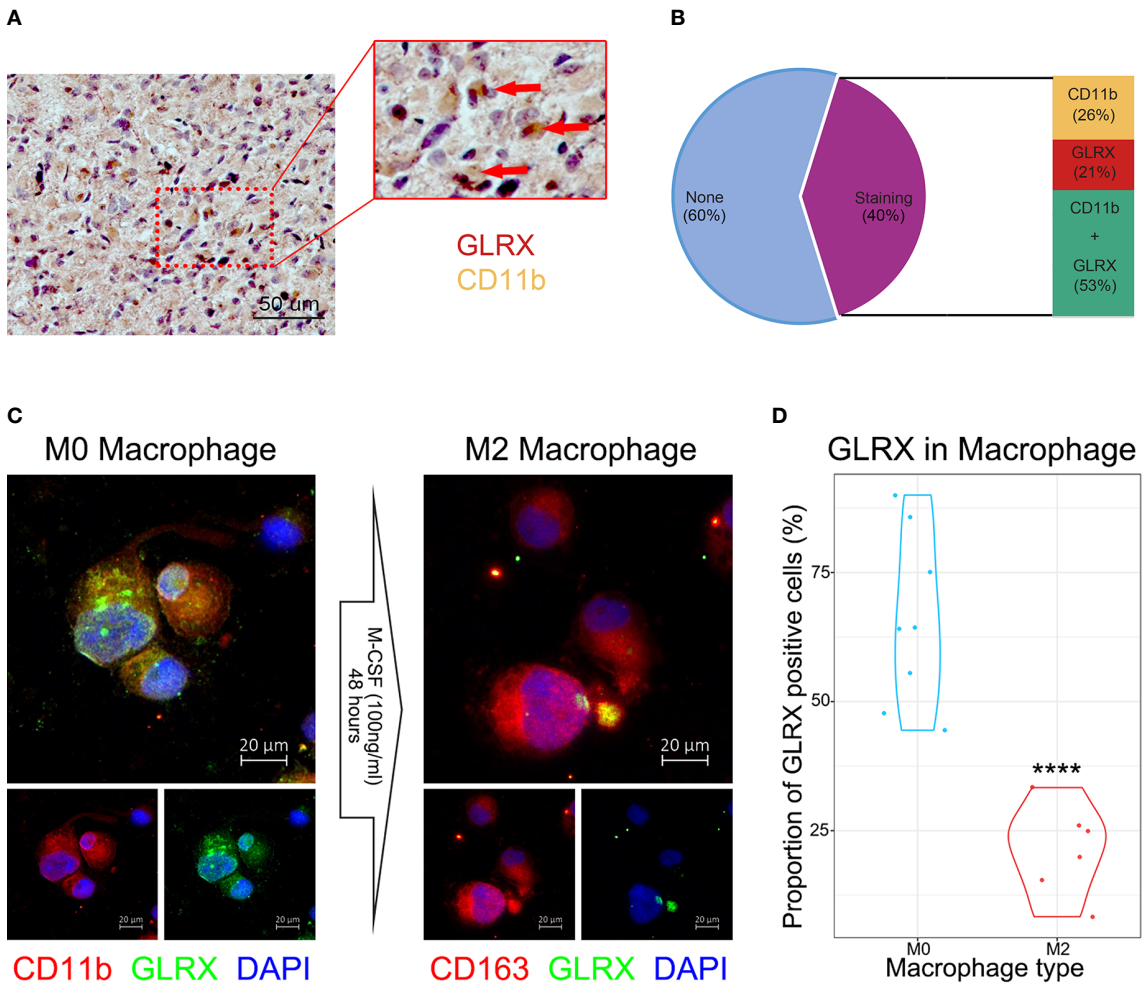
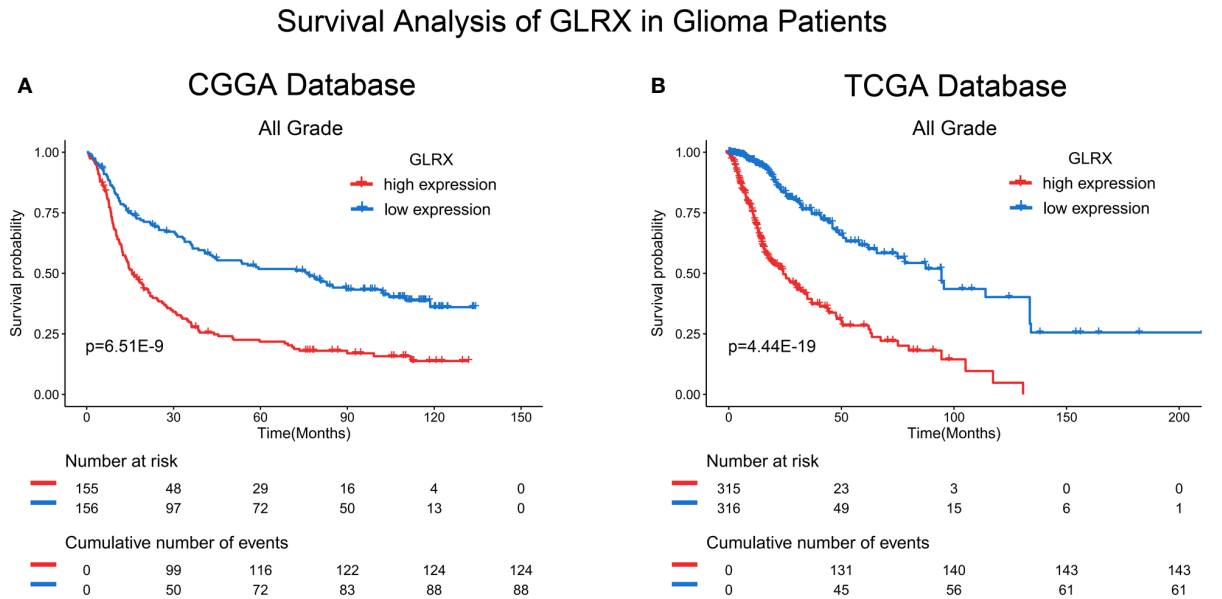


FIGURE 7 | *GLRX* is specifically expressed by M0 macrophages. **(A)** The IHC co-localization staining of CD11b and GLRX. The red point represents GLRX, and the yellow area represents CD11b. Scale bar is 50 um **(B)** The proportion of stained cells. None represents cells without expression of CD11b and GLRX. Staining represents cells expressing CD11b and/or GLRX. CD11b represents cells expressing CD11b. GLRX represents cells expressing GLRX. CD11b + GLRX represents cells expressing CD11b and GLRX. **(C)** The left three photos are IF staining of M0 macrophages. Green fluorescence is GLRX, and red fluorescence is CD11b. Above is the merged photo of the below two. The right three photos are IF staining of M2 macrophages. Green fluorescence is GLRX, and red fluorescence is CD163. Above is the merged photo of the below two. Cell nuclei are stained with DAPI. Scale bar is 20 um. **(D)** The violin graph shows the proportion of GLRX-positive cells in M0 or M2 macrophage IF images. ****indicates $p < 0.0001$.

with recurrent glioblastomas (30). Despite the promising clinical results, these therapeutic approaches can benefit just a fraction of patients with gliomas. Therefore, exploring therapeutic approaches or a multifunctional small molecule that could benefit most glioma patients is of great interest.

The thioredoxin and glutathione systems are the key cellular redox systems involved in gliomas (8, 31, 32). Glioma proliferation is associated with parenchymal alterations and oxidative stress that further leads to the impairment of brain homeostasis (4). Tumor cellular respiration produces hyperoxides, such as H_2O_2, and reactive oxygen species (ROS), which, when present at high levels, damage the DNA. This process is considered to be a pernicious factor in malignant glioma development (33). Hence, functional antioxidant systems that can scavenge these hyperoxides hold promise to keep the cell cycle of glioma cells under control. Simultaneously, a better understanding of the antioxidant system can pave the way for finding new therapeutic approaches to fight gliomas. Because glioma cells are more susceptible to oxidative stress induced by hyperoxides, inhibition of antioxidant systems or their components can prevent them from performing oxidative scavenging, thereby exposing the glioma cells to intense



C

CGGA Database

Variable	Univariate analysis		Multivariate analysis	
	HR (95% CI)	p Value	HR (95% CI)	p Value
GLRX	1.018 (1.013-1.023)	< 0.0001	1.007 (1.000-1.013)	0.041
WHO Grade	2.741 (2.278-3.298)	< 0.0001	2.48 (2.011-3.059)	< 0.0001
Gender	0.931 (0.707-1.227)	0.613		
Age	1.031 (1.018-1.044)	< 0.0001	1.008 (0.995-1.021)	0.216
IDH Status	0.382 (0.289-0.505)	< 0.0001	1.001 (0.696-1.440)	0.994
1p/19q Status	0.168 (0.101-0.277)	< 0.0001		
MGMT Promoter Status	0.679 (0.503-0.919)	0.012		

D

TCGA Database

Variable	Univariate analysis		Multivariate analysis	
	HR (95% CI)	p Value	HR (95% CI)	p Value
GLRX	3.628 (2.613-5.038)	< 0.0001	1.866 (1.243-2.803)	0.003
WHO Grade	4.962 (3.881-6.346)	< 0.0001	1.835 (1.345-2.504)	< 0.0001
Gender	1.029 (0.769-1.377)	0.847		
Age	1.074 (1.062-1.087)	< 0.0001	1.057 (1.041-1.073)	<0.0001
IDH Status	0.093 (0.066-0.132)	< 0.0001	0.369 (0.208-0.656)	0.001
1p/19q Status	0.214 (0.126-0.363)	< 0.0001	0.629 (0.329-1.201)	0.16
MGMT Promoter Status	0.305 (0.221-0.421)	<0.0001	0.925 (0.633-1.351)	0.686

FIGURE 8 | *GLRX* is a prognostic factor in glioma patients. **(A, B)** Clinical outcomes of patients with gliomas of low or high expression of *GLRX*. Kaplan-Meier survival analysis was performed in both CGGA and TCGA databases. **(C, D)** Univariate and multivariate analyses of clinical prognostic parameters in both CGGA and TCGA databases.

oxidative stress and blocking their proliferation, leading to their death. The endogenous antioxidative molecule Grx plays an important role in the glutathione system (7, 34). Grx expression is associated with tumor proliferation and therapy resistance in several cancers. Previous studies report that Grx is overexpressed in pancreatic ductal carcinoma compared to normal pancreatic tissue and that Grx overexpression increases MCF-7 adenocarcinoma cell resistance to doxorubicin (35, 36). However, little is known about the role of Grx in gliomas. Therefore, as a potential therapeutic target, it is imperative to explore the unique role of Grx and how it works in gliomas.

We started by checking the expression of *GLRX* in glioblastoma tissue compared with normal brain tissue. Our results showed that, similar to other tumor types, glioblastoma samples exhibited higher expression of *GLRX*. Next, we analyzed RNA-seq data of 1,717 glioma patients compiled from the CGGA, CGGA (2019), and TCGA databases. As expected, *GLRX* expression was significantly upregulated in higher malignant pathological grades of gliomas. Moreover, we also found that *GLRX* expression was significantly higher in glioma patients with malignant molecular phenotypes, including those harboring the *IDH* wild-type state, 1p/19q non-codeletion state, and MGMT unmethylated promoters. Furthermore, *GLRX* was highly enriched in mesenchymal subtype gliomas. The mesenchymal subtype is characterized by stronger immunosuppression, and aggressive phenotype, and malignant

proliferation due to the mesenchymal differentiation triggered by *NF1* mutations (21). A previous study has reported enhanced expression of immune checkpoints in mesenchymal subtype cancers compared with the other three transcriptional characteristic subtypes (37). Therefore, *GLRX* may be upregulated and involved in the immunosuppressive microenvironment of gliomas *via* modulation of the cellular component of the immune system. These findings suggest that *GLRX* expression is associated with the malignant behavior of gliomas. Thus, unraveling the mechanism of *GLRX* in gliomas may pave the way for the development of novel therapeutic approaches to fight this deadly malignancy.

To gain an in-depth understanding of the biological functions of *GLRX*, a series of analyses were performed. GO analysis revealed that *GLRX* plays a crucial role in immune and inflammatory responses in gliomas. Consistent with these results, KEGG and GSVA analyses also show that *GLRX* and related genes are involved in several immune response pathways, and *GLRX* is positively correlated with most immune functions with the exception of T cell response. Furthermore, *GLRX* was found to be significantly enriched in the mesenchymal glioma subtype with *GLRX* negatively mediating the T cell response and playing a suppressive role in the antitumor immune response. Taken together, these results suggest that *GLRX* may upregulate the expression of immune checkpoints to perform these functions. Upon analysis of the relationship between *GLRX* and known immune checkpoint genes, we confirm that *GLRX* is positively correlated with most inhibitory immune checkpoints, including PD-1, TIM-3, PD-L1, and PD-L2, which are involved in the regulation of the PD-1/PD-L1 pathway. These immune checkpoints are major negative immune regulators and are involved in regulating T cell activation, tolerance, and exhaustion (19). Our findings demonstrate that *GLRX* may exert antiglioma immune roles by affecting the expression of these inhibitory immune checkpoints. Additionally, *GLRX* is involved in inflammatory activities known to promote glioma progression *via* activation of tumor-associated macrophages (14, 38). To further validate the role of *GLRX* in the immune response, we used CIBERSORT software to calculate the percentage of each type of infiltrated immune cell. Our results show that *GLRX* is positively correlated with macrophages but negatively correlated with different subgroups of T cells. This further confirms the conclusions of our study. Last, single-cell sequencing analysis and IHC co-localization staining were performed to identify the exact components of the immune system that express *GLRX*. RNA-seq data and cellular molecular biomarkers reveal that *GLRX* is enriched in immune cells, particularly in M0 macrophages. M0 macrophages are a subgroup of resting immune cells that can undergo a directional polarization (17) to classically activated M1 macrophages and alternatively activated M2 macrophages. Macrophages in glioma tissue are prone to M2-like phenotypes, which are considered to be tumor-supporting macrophages (39). A previous study also reported that patients with higher expression of M0 macrophages had a poorer prognosis (17). Thus, we suspected that *GLRX* may play a role in M0 polarization and have an immuno-suppressive function. Based on the results of our present study, we hypothesize that *GLRX* is a potential target for redox and immunotherapy of gliomas.

Importantly, high levels of *GLRX* were associated with poor patient prognosis. Univariate and multivariate analyses indicated that high expression of *GLRX* predicted significantly lower survival. As a result, *GLRX* may serve as a potential prognostic predictor for glioma patients.

Redox therapy is being increasingly explored in tumor therapy (40, 41). Studies on breast, liver, pancreatic, and non-small cell lung cancers report that blocking the glutathione system could prevent tumor cell proliferation *in vitro* and *in vivo* (33, 35, 36, 42). As gliomas have access to abundant oxygen as well as to cellular respiration products, glioma cells become more dependent on the antioxidant system to survive and proliferate. Even a slight reduction in antioxidant levels could lead to glioma cell death (43). Meanwhile, cancer immunotherapy has also shown potential benefits for glioma patients. CAR-T, anti-PD-1, and anti-PD-L1 immunotherapies have shown higher immune response rates and longer survival in patients with brain metastases (2, 24, 27, 30). Our study suggests that *GLRX* is a key regulator of immune checkpoints and the immune response. Therefore, as a co-regulator of both redox and immune systems, inhibiting Grx could not only kill glioma cells through directly enhancing oxidative stress, but also downregulate the expression of inhibitory immune checkpoints and enhance the immune response. Thus, our study establishes *GLRX* as a novel potential target to enhance the efficacy of anticancer therapies, thereby paving the way for novel therapeutic approaches for treating gliomas.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.cgga.org.cn, CGGA.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Capital Medical University Institutional Review Board (IRB). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YC, GL: data analysis and editing the manuscript. YZ, LH: data collection and organization of CGGA database. YF, DW: data collection and organization of TCGA database. WZ, HH: conception, supervision, and design of the manuscript.

All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.580934/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | GLRX is correlated with the relative malignant molecular pathological characteristics of glioma. **(A)** GLRX was significantly increased in GBM (WHO grade IV) in the CGGA (2019) database. **(B)** GLRX was significantly increased in IDH wild-type gliomas in the CGGA (2019) database (Mut: IDH mutation; WT: IDH wild type). **(C)** GLRX was significantly increased in 1p/19q non-co-deletion gliomas in the CGGA (2019) database (Codel: 1p/19q co-deletion; Non-codel: 1p/19q non-co-deletion). **(D)** GLRX was significantly increased in the MGMT unmethylated group in the CGGA (2019) database. ns and **** indicate no statistical difference and $p < 0.0001$, respectively.

SUPPLEMENTARY FIGURE 2 | GLRX is a potential marker for malignant subtypes of gliomas. **(A)** GLRX was highly expressed in the mesenchymal subtype in the CGGA (2019) database. **(B)** ROC curve analysis showed that GLRX was highly sensitive and specific to predict the mesenchymal subtype in the CGGA (2019) database. **(C–E)** ROC curve analysis showed that BMI1 was less sensitive and specific to predict the mesenchymal subtype in the CGGA, TCGA, and CGGA (2019) databases, respectively. **(F–H)** ROC curve analysis showed that CD44 was highly sensitive and specific to predict the mesenchymal subtype in the CGGA, TCGA, and CGGA (2019) databases, respectively. Differences between groups were tested by Tukey's multiple comparisons test. *** and **** indicate $p < 0.001$ and $p < 0.0001$, respectively.

SUPPLEMENTARY FIGURE 3 | GLRX is strongly associated with immune processes in gliomas. **(A)** GO analysis showed that GLRX was mostly associated with immune, defense, and inflammatory responses in the CGGA (2019) database. **(B)** KEGG pathway analysis showed that GLRX was mostly involved in the immune response–related pathway in the CGGA (2019) database. **(C)** Most immune process–related genes were significantly positively correlated with GLRX expression in the CGGA (2019) database.

SUPPLEMENTARY FIGURE 4 | GLRX is closely related to the state of tumor immune functions. **(A, B)** GLRX had positive correlation with 87.07% of the biological functions of the immune system process in the CGGA (2019) database. The scale values in the graph represent the proportions of significantly correlated biological functions in each biological function classification. **(B)** The correlation coefficient between GLRX and the immune function scores in the CGGA (2019) database. The red words represent a positive correlation. The green words represent a negative correlation.

SUPPLEMENTARY FIGURE 5 | GLRX is associated with immune checkpoints and inflammatory activities. **(A–D)** GLRX was synergistic with inhibitory immune checkpoints in tumor-induced immune responses. A strong correlation between GLRX and inhibitory immune checkpoint expression was found in the CGGA (2019) database. **(E)** The correlation coefficient between GLRX and inflammatory activity function scores in the CGGA (2019) database. The red circle represents a positive correlation. The blue circle represents a negative correlation. The grey "×" represents no significant correlation.

SUPPLEMENTARY FIGURE 6 | The relationship between GLRX and infiltrated immune cells in the CGGA (2019) database. ns, *, **, ***, and **** indicate no statistical difference, $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p<0.0001$, respectively.

SUPPLEMENTARY FIGURE 7 | GLRX is a prognostic factor in glioma patients. **(A)** Clinical outcomes of patients with gliomas of low or high expression of GLRX. Kaplan-Meier survival analysis was performed in the CGGA database. **(B)** Univariate and multivariate analyses of clinical prognostic parameters in CGGA database.

REFERENCES

1. Jiang T, Mao Y, Ma W, Mao Q, You Y, Yang X, et al. CGCG clinical practice guidelines for the management of adult diffuse gliomas. *Cancer Lett* (2016) 375(2):263–73. doi: 10.1016/j.canlet.2016.01.024
2. Van Meir EG, Hadjipanayis CG, Norden AD, Shu HK, Wen PY, Olson JJ. Exciting new advances in neuro-oncology: the avenue to a cure for malignant glioma. *CA Cancer J Clin* (2010) 60(3):166–93. doi: 10.3322/caac.20069
3. Ahn BY, Moss B. Glutaredoxin Homolog Encoded by Vaccinia Virus Is a Virion-Associated Enzyme with Thioltransferase and Dehydroascorbate Reductase Activities. *Proc Natl Acad Sci U S A* (1992) 89(15):7060–4. doi: 10.1073/pnas.89.15.7060
4. Branco V, Pimentel J, Brito MA, Carvalho C. Thioredoxin, Glutathione and Related Molecules in Tumors of the Nervous System. *Curr Med Chem* (2020) 27(12):1878–900. doi: 10.2174/0929867326666190201113004
5. Du YT, Zhang HH, Lu J, Holmgren A. Glutathione and Glutaredoxin Act as a Backup of Human Thioredoxin Reductase 1 to Reduce Thioredoxin 1 Preventing Cell Death by Aurothioglucose. *J Biol Chem* (2012) 287 (45):38210–9. doi: 10.1074/jbc.M112.392225
6. Backos DS, Franklin CC, Reigan P. The role of glutathione in brain tumor drug resistance. *Biochem Pharmacol* (2012) 83(8):1005–12. doi: 10.1016/j.bcp.2011.11.016
7. Ren XY, Zou LL, Zhang X, Branco V, Wang J, Carvalho C, et al. Redox Signaling Mediated by Thioredoxin and Glutathione Systems in the Central Nervous System. *Antioxid Redox Signaling* (2017) 27(13):989–1010. doi: 10.1089/ars.2016.6925
8. Zhang PX, Gao JX, Wang X, Wen WH, Yang HW, Tian YJ, et al. A novel indication of thioredoxin-interacting protein as a tumor suppressor gene in malignant glioma. *Oncol Lett* (2017) 14(2):2053–8. doi: 10.3892/ol.2017.6397
9. Chai RC, Zhang KN, Chang YZ, Wu F, Liu YQ, Zhao Z, et al. Systematically characterize the clinical and biological significances of 1p19q genes in 1p/19q non-codeletion glioma. *Carcinogenesis* (2019) 40(10):1229–39. doi: 10.1093/carcin/bgz102
10. Chai RC, Zhang KN, Liu YQ, Wu F, Zhao Z, Wang KY, et al. Combinations of four or more CpGs methylation present equivalent predictive value for MGMT expression and temozolomide therapeutic prognosis in gliomas. *CNS Neurosci Ther* (2019) 25(3):314–22. doi: 10.1111/cns.13040
11. Wang HY, Tang K, Liang TY, Zhang WZ, Li JY, Wang W, et al. The comparison of clinical and biological characteristics between IDH1 and IDH2 mutations in gliomas. *J Exp Clin Cancer Res* (2016) 35:86. doi: 10.1186/s13046-016-0362-7
12. Tang ZF, Li CW, Kang BX, Gao G, Li C, Zhang ZM. GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses. *Nucleic Acids Res* (2017) 45(W1):W98–W102. doi: 10.1093/nar/gkx247
13. Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* (2009) 4 (1):44–57. doi: 10.1038/nprot.2008.211
14. Li GZ, Wang Z, Zhang CB, Liu X, Cai JQ, Wang ZL, et al. Molecular and clinical characterization of TIM-3 in glioma through 1,024 samples. *Oncoimmunology* (2017) 6(8):e1328339. doi: 10.1080/2162402X.2017.1328339

15. Newman AM, Steen CB, Liu CL, Gentles AJ, Chaudhuri AA, Scherer F, et al. Determining cell type abundance and expression from bulk tissues with digital cytometry. *Nat Biotechnol* (2019) 37(7):773–+. doi: 10.1038/s41587-019-0114-2
16. Knowles LM, Kagiri D, Bernard M, Schwarz EC, Eichler H, Pilch J. Macrophage Polarization is Deregulated in Haemophilia. *Thromb Haemostasis* (2019) 119(2):234–45. doi: 10.1055/s-0038-1676796
17. Gabrusiewicz K, Rodriguez B, Wei J, Hashimoto Y, Healy LM, Maiti SN, et al. Glioblastoma-infiltrated innate immune cells resemble M0 macrophage phenotype. *Jci Insight* (2016) 1(2):e85841. doi: 10.1172/jci.insight.85841
18. Wang Q, He Z, Huang M, Liu T, Wang Y, Xu H, et al. Vascular niche IL-6 induces alternative macrophage activation in glioblastoma through HIF-2alpha. *Nat Commun* (2018) 9(1):559. doi: 10.1038/s41467-018-03050-0
19. Wang Z, Zhang CB, Liu X, Wang ZL, Sun LH, Li GZ, et al. Molecular and clinical characterization of PD-L1 expression at transcriptional level via 976 samples of brain glioma. *OncoImmunology* (2016) 5(11):e1196310. doi: 10.1080/2162402X.2016.1196310
20. Hartmann C, Meyer J, Balss J, Capper D, Mueller W, Christians A, et al. Type and frequency of IDH1 and IDH2 mutations are related to astrocytic and oligodendroglial differentiation and age: a study of 1,010 diffuse gliomas. *Acta Neuropathol* (2009) 118(4):469–74. doi: 10.1007/s00401-009-0561-9
21. Verhaak RGW, Hoadley KA, Purdom E, Wang V, Qi Y, Wilkerson MD, et al. Integrated Genomic Analysis Identifies Clinically Relevant Subtypes of Glioblastoma Characterized by Abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell* (2010) 17(1):98–110. doi: 10.1016/j.ccr.2009.12.020
22. Behnan J, Finocchiaro G, Hanna G. The landscape of the mesenchymal signature in brain tumours. *Brain* (2019) 142:847–66. doi: 10.1093/brain/awz044
23. Jin X, Kim L, Wu Q, Wallace LC, Prager B, Sanvoranart T, et al. Targeting Glioma Stem Cells through Combined Bmi1 and Ezh2 Inhibition. *Neuro-Oncology* (2017) 19:80–0. doi: 10.1093/neuonc/nox168.329
24. Zhao XD, Subramanian S. Oncogenic pathways that affect antitumor immune response and immune checkpoint blockade therapy. *Pharmacol Ther* (2018) 181:76–84. doi: 10.1016/j.pharmthera.2017.07.004
25. Wang ZL, Wang Z, Li GZ, Wang QW, Bao ZS, Zhang CB, et al. Immune Cytolytic Activity Is Associated With Genetic and Clinical Properties of Glioma. *Front Immunol* (2019) 10:1756. doi: 10.3389/fimmu.2019.01756
26. Herrlinger U, Tzaridis T, Mack F, Steinbach JP, Schlegel U, Sabel M, et al. Lomustine-temozolomide combination therapy versus standard temozolomide therapy in patients with newly diagnosed glioblastoma with methylated MGMT promoter (CeTeG/NOA-09): a randomised, open-label, phase 3 trial. *Lancet* (2019) 393(10172):678–88. doi: 10.1016/S0140-6736(18)31791-4
27. Sharma P, Allison JP. The future of immune checkpoint therapy. *Science* (2015) 348(6230):56–61. doi: 10.1126/science.aaa8172
28. Zhu XF, Zhou H, Liu YN, Wen YY, Wei CF, Yu QQ, et al. Transferrin/aptamer conjugated mesoporous ruthenium nanosystem for redox-controlled and targeted chemo-photodynamic therapy of glioma. *Acta Biomater* (2018) 82:143–57. doi: 10.1016/j.actbio.2018.10.012
29. Hu H, Mu Q, Bao Z, Chen Y, Liu Y, Chen J, et al. Mutational Landscape of Secondary Glioblastoma Guides MET-Targeted Trial in Brain Tumor. *Cell* (2018) 175(6):1665–1678 e1618. doi: 10.1016/j.cell.2018.09.038
30. Cloughesy TF, Mochizuki AY, Orpilla JR, Hugo W, Lee AH, Davidson TB, et al. Neoadjuvant anti-PD-1 immunotherapy promotes a survival benefit with intratumoral and systemic immune responses in recurrent glioblastoma. *Nat Med* (2019) 25(3):477–+. doi: 10.1038/s41591-018-0337-7
31. Branco V, Coppo L, Sola S, Lu J, Rodrigues CMP, Holmgren A, et al. Impaired cross-talk between the thioredoxin and glutathione systems is related to ASK-1 mediated apoptosis in neuronal cells exposed to mercury. *Redox Biol* (2017) 13:278–87. doi: 10.1016/j.redox.2017.05.024
32. Yokomizo A, Ono M, Nanri H, Makino Y, Ohga T, Wada M, et al. Cellular levels of thioredoxin associated with drug sensitivity to cisplatin, mitomycin C, doxorubicin, and etoposide. *Cancer Res* (1995) 55(19):4293–6.
33. Zhang QQ, Chen WQ, Lv XL, Weng QY, Chen MJ, Cui R, et al. Piperlongumine, a Novel TrxR1 Inhibitor, Induces Apoptosis in Hepatocellular Carcinoma Cells by ROS-Mediated ER Stress. *Front Pharmacol* (2019) 10:1180. doi: 10.3389/fphar.2019.01180
34. Okuda M, Inoue N, Azumi H, Seno T, Sumi Y, Hirata K, et al. Expression of glutaredoxin in human coronary arteries: its potential role in antioxidant protection against atherosclerosis. *Arterioscler Thromb Vasc Biol* (2001) 21 (9):1483–7. doi: 10.1161/hq0901.095550
35. Meyer EB, Wells WW. Thioltransferase overexpression increases resistance of MCF-7 cells to adriamycin. *Free Radical Biol Med* (1999) 26(5-6):770–6. doi: 10.1016/S0891-5849(98)00247-0
36. Nakamura H, Bai J, Nishinaka Y, Ueda S, Sasada T, Ohshio G, et al. Expression of thioredoxin and glutaredoxin, redox-regulating proteins, in pancreatic cancer. *Cancer Detection Prev* (2000) 24(1):53–60.
37. Doucette T, Rao G, Rao A, Shen L, Aldape K, Wei J, et al. Immune Heterogeneity of Glioblastoma Subtypes: Extrapolation from the Cancer Genome Atlas. *Cancer Immunol Res* (2013) 1(2):112–22. doi: 10.1158/2326-6066.Cir-13-0028
38. Guan XD, Zhang CB, Zhao JY, Sun G, Song QK, Jia W. CMTM6 overexpression is associated with molecular and clinical characteristics of malignancy and predicts poor prognosis in gliomas. *Ebiomedicine* (2018) 35:233–43. doi: 10.1016/j.ebiom.2018.08.012
39. Xiang W, Shi RC, Kang X, Zhang X, Chen P, Zhang LL, et al. Monoacylglycerol lipase regulates cannabinoid receptor 2-dependent macrophage activation and cancer progression. *Nat Commun* (2018) 9 (1):2574. doi: 10.1038/s41467-018-04999-8
40. Salazar-Ramiro A, Ramirez-Ortega D, de la Cruz VP, Hernandez-Pedro NY, Gonzalez-Esquivel DF, Sotelo J, et al. Role of Redox Status in Development of Glioblastoma. *Front Immunol* (2016) 7:156. doi: 10.3389/fimmu.2016.00156
41. Trachootham D, Alexandre J, Huang P. Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? *Nat Rev Drug Discovery* (2009) 8(7):579–91. doi: 10.1038/nrd2803
42. Peltoniemi MJ, Rytila PH, Harju TH, Soini YM, Salmenkivi KM, Ruddock LW, et al. Modulation of glutaredoxin in the lung and sputum of cigarette smokers and chronic obstructive pulmonary disease. *Respir Res* (2006) 7:133. doi: 10.1186/1465-9921-7-133
43. Durgadoss L, Nidadavolu P, Valli RK, Saeed U, Mishra M, Seth P, et al. Redox modification of Akt mediated by the dopaminergic neurotoxin MPTP, in mouse midbrain, leads to down-regulation of pAkt. *FASEB J* (2012) 26 (4):1473–83. doi: 10.1096/fj.11-194100

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PD-L1-Mediated Immunosuppression in Glioblastoma Is Associated With the Infiltration and M2-Polarization of Tumor-Associated Macrophages

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There has been no significant improvements for immune checkpoint inhibitors since its first use. Tumour-associated macrophages (TAMs) are critical mediators in the PD-1/PD-L1 axis, contributing to the immunosuppressive tumour microenvironment. This study aims to investigate the potential role of PD-L1 in regulating TAMs in glioblastoma. Gene expression data and clinical information of glioma patients were collected from TCGA (n = 614) and CGGA (n = 325) databases. Differentially expressed genes between PD-L1^{high} and PD-L1^{low} groups were identified and subjected to bioinformatical analysis. We found that PD-L1 was frequently expressed in gliomas with a grade-dependent pattern. Higher PD-L1 expression predicted shorter overall survival. Moreover, PD-L1 was positively correlated with immunosuppressive cells (macrophage, neutrophil and immature DC) and negatively correlated with cytocidal immune cells (CD8$^+$ T cell and Th1). Importantly, PD-L1 high expression was significantly correlated with M2-polarization of macrophages (M2-TAMs). We conclude that PD-L1 is an unfavourable prognostic marker for patients with glioblastoma; PD-L1-mediated immunosuppression may attribute to the infiltration and M2-polarization of TAMs.

Keywords: immune checkpoint inhibitor, PD-1/PD-L1 axis, glioblastoma tumor microenvironment, immunosuppression, tumor-associated macrophages

INTRODUCTION

Glioblastoma multiforme (GBM) is the most common malignant primary brain tumor in adults. It accounts for 30% of all the brain tumors and more than 50% of gliomas (1, 2). The current standard of care for GBM patients includes surgical tumor resection followed by radiotherapy with concomitant and adjuvant temozolomide (3). Even with aggressive and comprehensive treatment, tumor recurrence is inevitable. The median overall survival of patients diagnosed with GBM is less than

two years (1). Given the poor survival of GBM patients and inefficiency of the current therapy regimen, alternative treatment strategies, and novel therapeutic targets are clearly needed.

Immunotherapy is a revolutionary anti-cancer therapy in the past decade. Various immunotherapy modalities have been established, including immune checkpoint inhibitors, CAR-T, vaccine and oncolytic virus (4). The most well-known checkpoint inhibitors are antibodies of cytotoxic T lymphocyte antigen 4 (CTLA-4), programmed cell death protein 1 (PD-1), and its ligand programmed death-ligand 1 (PD-L1) (5). Upon activation, the checkpoints induce inhibitory or even apoptotic effects within immune cells (mainly effector T cells) (6, 7). Immune-checkpoint inhibitors can block the interaction between the ligands and the immune-repressive receptors, thus overcome the inhibition of immune cells and reactivate the cytocidal immune response (8). Currently, immune-checkpoint inhibitors have shown remarkable benefits in prolonging survival in many cancers such as lung cancer, breast cancer, and melanoma (9–11).

In GBM, however, therapeutic response to checkpoint inhibitors is variant and mostly ineffective. All the large phase 3 clinical studies on PD-1 inhibitors in GBM have failed to show survival benefits (12, 13). Less than 10% of patients with recurrent GBM respond to PD-1 inhibitors (14). It is recognized that GBM is highly genetically heterogeneous and unresponsive to immunotherapy approaches (15). The underlying mechanisms remain elusive and can be multifaceted, wherein the immunosuppressive tumor microenvironment (TME) represents a critical factor (13). The engagement of PD-1 and PD-L1 is an essential mechanism that contributes to the immune-suppressive TME. Multiple suppressive effects can be triggered, such as the induction of cellular apoptosis, the impairment of T lymphocyte proliferation, and the inhibition of "effector" cytokine generation (7). The majority of PD-L1 expression is contributed by tumor-infiltrating myeloid cells (TIMs, including TAM, tumor associated neutrophils and myeloid-derived suppressor cells) (16). Recent studies reported that PD-L1 inhibitor could skew TAMs towards a pro-inflammatory M1 status (17, 18), expanding its canonical T cell suppression function. Currently, the relationship between PD-L1 and TAMs in GBM remains poorly understood.

The dissatisfied efficacy of anti-PD-1 antibodies in GBM necessitates the basic research on PD1/PD-L1 axis-mediated immune resistance. This study aims to delineate the role of PD-L1 in the immunosuppressive TME, focusing on its relationship with TAMs. By using transcriptional gene expression data from CGGA (The Chinese Glioma Genome Atlas) and TCGA (The Cancer Genome Atlas) databases, we depicted the immune landscape associated with PD-L1 in GBM and proposed that PD-L1-mediated immunosuppression may correlate with the infiltration and M2-polarization of macrophages.

METHODS AND MATERIALS

Data Acquisition

Glioma gene expression profile and patients' clinical information were downloaded from TCGA (http://cancergenome.nih.gov) and CGGA (http://www.cgga.org.cn) databases. In the TCGA dataset, the transcriptome expression data of 150 GBM and 464 lower gliomas were collected. In the CGGA dataset, the total number of samples is 325, including 144 GBM and 181 lower gliomas. Patients' information on age, gender, World Health Organization (WHO) grade, diagnosis with molecular characteristics, treatment, and patient prognoses were organized (**Table S1**). PD-L1 and other gene expression profiles were obtained as described previously (19, 20). Gene expression level was presented as PRKM (reads per kilobase transcriptome per million reads).

Bioinformatic Analysis

Genes that showed consistent differential expression in both TCGA and CGGA cohorts were extracted. Overlapped highly expressed genes in the PD-L1^{high} (807 over-expressed genes) and PD-L1^{low} (559 over-expressed genes) GBMs were subjected to KEGG enrichment analysis (**Table S2**). Gene annotation and pathway enrichment analysis were performed by Database for Annotation, Visualization, and Integrated Discovery (DAVID), and Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.kegg.jp/kegg/pathway.html). Gene Set Variation Analysis (GSVA package of R http://www.bioconductor.org/) was used to explore the correlation between PD-L1 and the predefined, highly distinctive transcriptional profile of each immune cell type (21–24). The classical chemokines and surface markers of both M1- and M2- macrophages were also included (25–28). Twenty-six types of immune cells with corresponding gene signatures were utilized for analyses (**Table S3**).

Statistical Analysis

R language (v. 3.4.3, AT&T BellLaboratories, Lucent Technologies), SPSS software (v. 22.0, IBM company), and GraphPad Prism (v. 8.0, LLC) for Windows were used for statistical analyses and figure generation. Samples from the TCGA and CGGA datasets were analysed separately. Genes differentially expressed between PD-L1 high and low groups (PD-L1^{high} and PD-L1^{low}, stratified by median value) were estimated by a two-tailed Student's t-test. The Significance Analysis of Microarrays (SAM) package of R was performed to control the FDR (False Discovery Rate). Statistically significant was considered when FDR <0.01 and norm p <0.05. The prognostic value of these differentially expressed genes was evaluated by the survival package of R. A multivariate Cox proportional hazard model was performed to evaluate the independent prognostic variables. Kaplan-Meier curves were utilized to depict the survival distributions. PD-L1-correlated genes and immune cells were explored by Pearson's correlation coefficient (r) using R. A significant correlation was indicated by an absolute r-value greater than 0.3 and a p-value less than 0.05.

RESULTS

PD-L1 Is Frequently Expressed in Gliomas and Predicts Unfavorable Overall Survival in GBM

Firstly, we found that PD-L1 mRNA was frequently expressed in different grades of gliomas with a grade-dependent pattern in TCGA cohorts (**Figure 1A**). A similar trend was found in CGGA

cohort, although the difference was of no statistical significance (**Figure S1A**). Of note, when compared with other GBM subtypes, the proneural subtype has particularly lower PD-L1 expression whereas the mesenchymal subtype has a relatively higher level (**Figure 1B**, **S1B**). In both cohorts, GBM patients with higher PD-L1 expression had shorter overall survival (**Figure 1C** TCGA, median survival: PD-L1^{high} vs PD-L1^{low}, 375 vs 453 days, p = 0.0272; **Figure 1D** CGGA, median survival: PD-L1^{high} vs PD-L1^{low}, 315 vs 567 days, p = 0.0021). Cox regression analysis further showed that PD-L1 was an independent prognostic marker in GBM (**Table S4**). The results suggested that PD-L1 was frequently expressed in gliomas and could serve as a prognostic biomarker in GBM.

PD-L1 Is Positively Correlated With Immunosuppressive M2-Macrophage and Suppresses Effector T Cells in GBM

PD-L1 is the ligand of the well-known immune checkpoint PD-1, which mediates the suppression on effector T cell (7). To investigate the immune cells that may correlate with PD-L1 in GBM, we identified PD-L1-associated immune components in GBM microenvironment by utilizing GSVA analysis (**Figure 2** and **Table S5**). The results showed that PD-L1 expression was positively correlated with the infiltration of macrophages (CGGA r = 0.3024, p = 0.0002; TCGA r = 0.30818, p = 0.0001), M1-macrophage (CGGA r = 0.25567, p = 0.002; TCGA r = 0.36842,

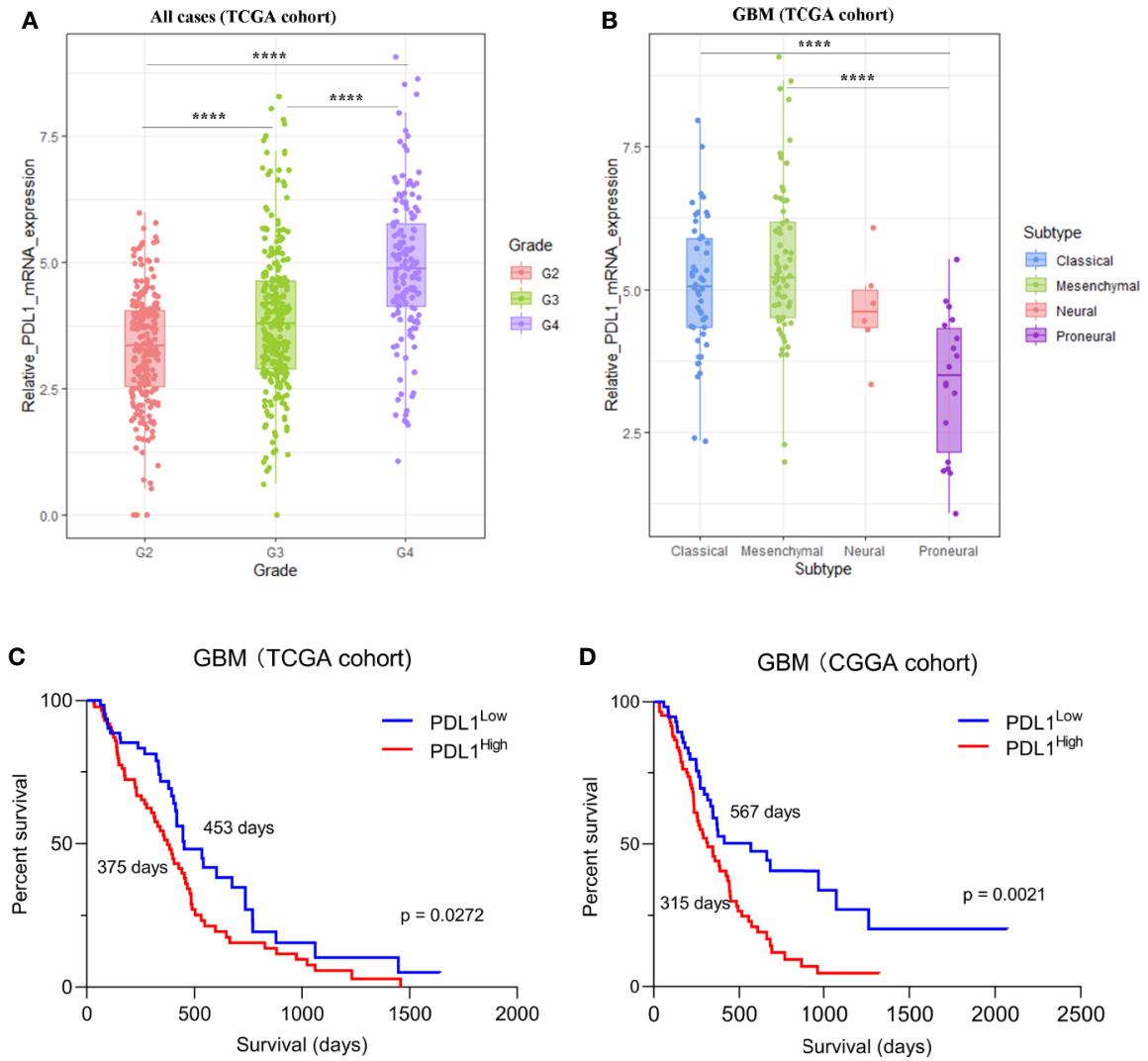

FIGURE 1 | The expression of PD-L1 in gliomas in TCGA dataset. **(A)**. The expression of PD-L1 in each grade of glioma, G2 (Grade II) mean = 3.321, G3 (Grade III) mean = 3.870, G4 (Grade IV) mean = 4.915. **(B)**. Expression of PD-L1 in GBM subtypes, proneural mean = 3.365, neural mean = 4.669, classical mean = 5.078, mesenchymal mean = 5.442; one-way ANOVA followed by Tukey's multiple comparisons test was used. **(C, D)**. Kaplan-Meier analysis showed the prognosis value of PD-L1 in GBM, median survival days of PD-L1^{high} vs. PD-L1^{low}: 375 days vs 453 days (C, TCGA cohort) and 315 days vs 567 days (D, CGGA cohort); Log-rank test was used; ****p < 0.0001.

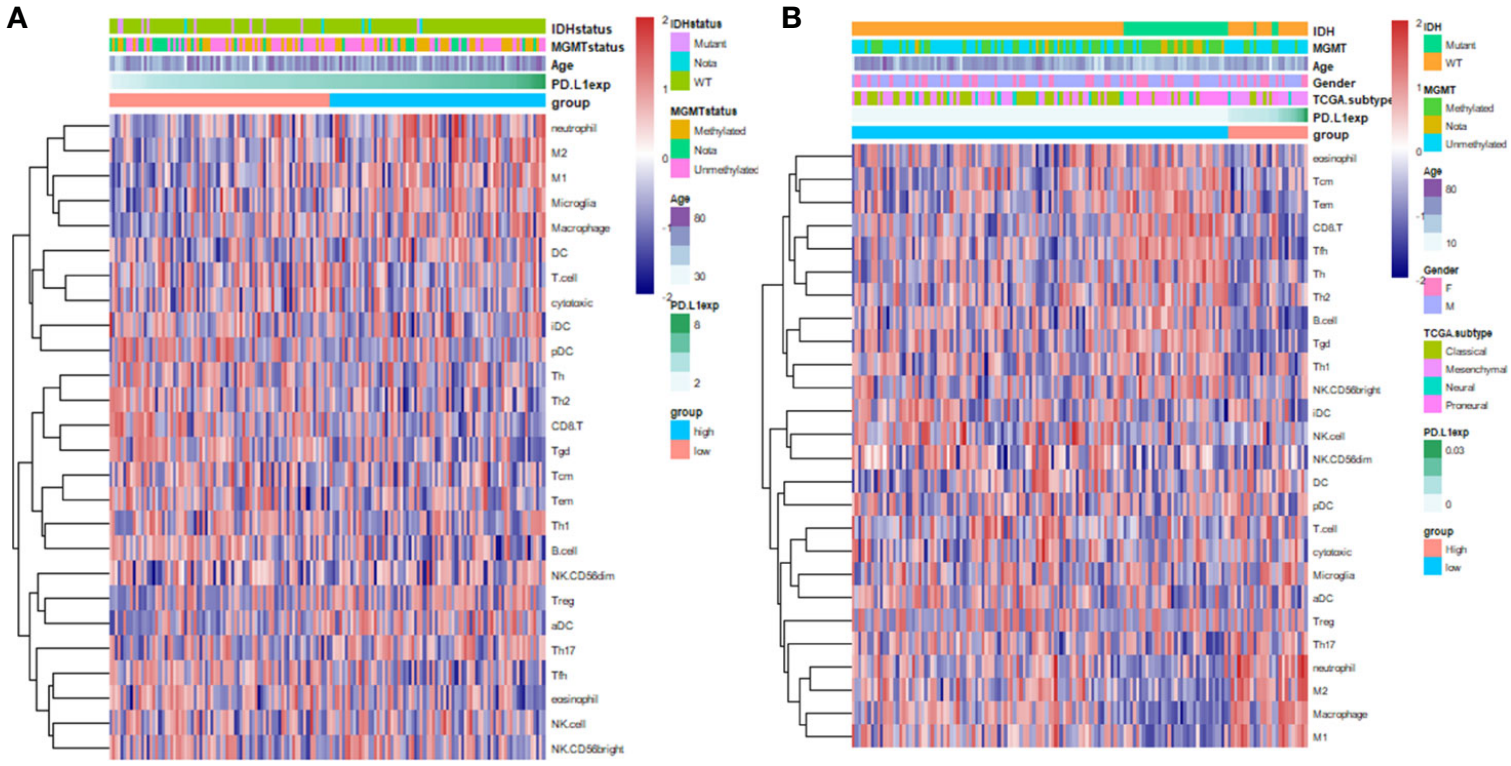

FIGURE 2 | Heatmap indicates the correlation of PD-L1 with 26 immune cell subpopulations in CGGA dataset **(A)** and TCGA dataset **(B)**. Each coloured square illustrates the correlation between PD-L1 and the transcriptional profile of the corresponding immune cell type. Red colour illustrates a positive correlation (r = 1), white indicates no correlation (r = 0), and blue a negative correlation (r = −1). Immune cells in the ranking list: **(A)**, eosinophil, Tcm, Tem, $CD8^+$T cell, Tfh, Th, Th2, B cell, Tgd (γδT cell), Th1, NK CD56bright cell, iDC, NK cell, NK CD56dim cell, DC, pDC, T cell, Cytotoxic T cell, Microglia, aDC, Treg, Th17, neutrophil, M2-like-Macrophage, Macrophage,M1-like-Macrophage; **(B)**, neutrophil, M2-like-Macrophage, M1-like-Macrophage, Microglia, Macrophage, DC, T cell, Cytotoxic T cell, iDC, pDC, Th, Th2, $CD8^+$T cell, Tgd, Tcm, Tem, Th1, B cell, NK CD56dim cell, Treg, aDC, Th17, Tfh, eosinophil.

$p < 0.0001$), M2-macrophage (CGGA $r = 0.30057$, $p = 0.0003$; TCGA $r = 0.36069$, $p < 0.0001$), neutrophil (CGGA $r = 0.33619$, $p < 0.0001$; TCGA $r = 0.26032$, $p = 0.0013$), and Treg (CGGA, $r = 0.02204$, $p > 0.05$; TCGA, $r = 0.21973$, $p = 0.0069$). In contrast, PD-L1 was negatively correlated with the infiltration of $CD8^+$ T cell (CGGA, $r = -0.19901$, $p = 0.0168$; TCGA, $r = -0.35739$, $p < 0.0001$), Tfh (CGGA, $r = -0.3092$, $p = 0.0002$; TCGA, $r = -0.25058$, $p = 0.0020$), Tgd (CGGA, $r = -0.30892$, $p = 0.0002$; TCGA, $r = -0.36625$, $p < 0.0001$), and B cell (CGGA $r = -0.32434$, $p < 0.0001$; TCGA $r = -0.26846$, $p = 0.0009$). These results indicated that PD-L1 was positively correlated with the infiltration of macrophages in GBM and negatively correlated with effector T immune cells.

PD-L1 Correlates With M2-Macrophages-Related Chemokines

The M2-polarization of TAM is recognized as an immune-suppressive phenotype (29). It is striking to notice the correlation between PD-L1 and M2-macrophages. To verified the above findings, we investigated the correlation between PD-L1 and canonical chemokines of both M1-macrophages (IL-12, IL-23, TNF, IFNG) and M2 macrophages (IL-10, TGF-β, IL-4, IL-13). In TCGA cohort (**Figure 3**), PD-L1 was positively correlated with M2-macrophage chemokines, such as TGF-β1 ($r = 0.34433$, $p < 0.0001$), TGF-β3 ($r = 0.22328$, $p = 0.0049$), and IL-10 ($r = 0.18208$, $p = 0.0225$). However, no significant correlation was found between PD-L1 and M1-macrophage chemokines ($|r| < 0.1$, $p > 0.05$). In CGGA cohort, PD-L1 also showed positive correlation with M2 chemokines, although the differences were not statistically significant (**Figure S2B**). These findings supported that PD-L1 was intimately correlated with M2-macrophages in the microenvironment of GBM.

PD-L1 Is Associated With Signalling Pathways That Modulate Macrophage Polarization

In order to investigate the function of PD-L1 in modulating TAM M2 polarization, genes highly expressed in PD-L1^{high} and PD-L1^{low} GBMs were used to identify potential biological pathways (**Table S2**). As shown in **Figure 4**, the pathways enriched in PD-L1^{high} GBM include (1) NF-kappa B signalling pathway; (2) apoptotic process; (3) positive regulation of interferon-gamma production; (4) Fc gamma R-mediated phagocytosis; (5) positive regulation of interleukin-10 production; (6) response to interferon-gamma; (7) negative regulation of ERK1 and ERK2 cascade; (8) mTOR signalling pathway; (9) innate immune response; (10) Cytokine-cytokine receptor interaction. Enriched pathways in PD-L1^{low} GBM contain (1) nervous system development; (2) regulation of transcription, DNA-templated; (3) spinal cord oligodendrocyte cell fate specification; (4) negative regulation of transcription from RNA polymerase II promoter; (5) neurogenesis; (6) mRNA splicing, *via* spliceosome; (7) negative regulation of neuron differentiation; (8) Oxidative phosphorylation. In sum, critical pathways involved in macrophage polarization were enriched in PD-L1^{high} GBMs, while biological pathways enriched in PD-L1^{low} GBMs were less relevant with macrophages functions.

DISCUSSION

The continuous failure of clinical trials on PD-1 antibodies in GBM necessitates basic researches on the mechanism of immunotherapies resistance. This study depicts the immune features associated with PD-L1 in the TME of GBM. Firstly, the PD-L1 mRNA expression shows a grade-dependent pattern in gliomas. Higher PD-L1 expression predicted a poorer outcome in patients with GBM. Moreover, PD-L1 expression is associated with the infiltration of immune-suppressive macrophages and neutrophils. We further found that PD-L1 high expression was positively correlated with the M2-polarization of TAMs, evidenced by the increased M2-related gene signatures and canonical chemokines. Signalling pathways that correlated with macrophage polarization were enriched in PD-L1^{high} GBMs, indicating a critical role of PD-L1 in modulating macrophage activation. The present study provides preliminary evidence on the intimate correlation between PD-L1 and M2-TAMs, supporting the notion that PD-L1 inhibitors could enhance the efficacy of prevalent PD-1 antibodies for GBM therapy.

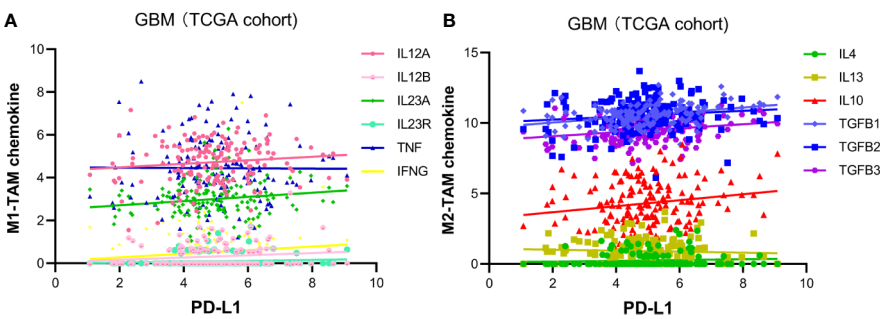

FIGURE 3 | Pearson's correlation of PD-L1 and TAM-related chemokines in TCGA glioblastoma dataset. **(A)** Chemokine of M1-TAM: IL12A ($r = 0.117796$, $p > 0.05$), IL12B ($r = 0.140694$, $p > 0.05$), IL23A ($r = 0.149971$, $p > 0.05$), IL23R ($r = 0.107516$, $p > 0.05$), TNF ($r = -0.00741$, $p > 0.05$), IFNG ($r = 0.129588$, $p < 0.0001$). **(B)** Chemokine of M2-TAM: TGF-β1 ($r = 0.34433$, $p < 0.0001$), TGF-β2 ($r = 0.12637$, $p = 0.1148$), TGF-β3 ($r = 0.22328$, $p < 0.0049$), IL-10 ($r = 0.18208$, $p = 0.0225$), IL-4 ($r = 0.0748$, $p > 0.05$), IL-13 ($r = -0.05977$, $p > 0.05$).

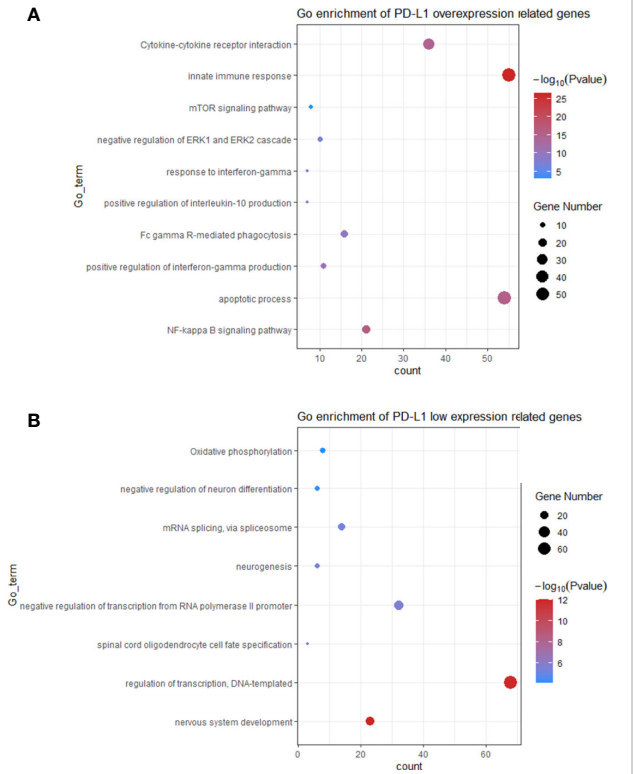

FIGURE 4 | The top bioinformatics hits of biological pathways derived from genes enriched in GBM patients with PD-L1^{high} **(A)** and PD-L1^{low} **(B)**. Plot sizes show gene counts enriched in the enrichment of pathway. Colour depth indicates the p value from low (red) to high level (blue). The p values of all presented hits are less than 0.05.

It is important to determine the expression pattern of PD-L1 in GBM. The protein level of PD-L1 has been considered as a critical predictive marker for therapeutic response to PD-1/PD-L1 antibody in multiple types of cancer (30). However, the positive rate and expression level of PD-L1 in GBM can be influenced by many factors, such as the selected anti-PD-L1 antibody; the positive criteria; and the intrinsic tumoral heterogeneity (31). For instance, the percentage of GBM patients with detectable PD-L1 protein expression level varies from 61 to 88% according to different reports (32, 33), while the median percentage of PD-L1-expression cells in GBM is only 2.77% (32). Thus, a more comprehensive landscape of PD-L1 expression in glioma is needed. In this study, we found that PD-L1 mRNA was frequently expressed in all grades of gliomas and exhibited a grade-dependent manner. This finding is in line with previous studies that PD-L1 is positively correlated with glioma grades (34). We also noticed that the proneural GBM subtype had lower PD-L1 expression among all the GBM subgroups whereas the mesenchymal subtype had a relatively higher level. These findings are in agreement with other reports that the proneural subtype has a better outcome and the immunosuppressive genes are predominant in mesenchymal subtype (35, 36).

Whether PD-L1 represents a stable prognosis predictor in glioma is still under debate. Over half of the published reports proposed the negative correlation of PD-L1 expression and survival time of glioma patients, while other studies showed no significant correlation between PD-L1 and patient survival (31, 33, 37). This study shows that higher PD-L1 mRNA expression is correlated with shorter overall survival. The Cox regression analysis further indicates that PD-L1 is an independent unfavourable prognostic marker in GBMs.

Intra-tumor heterogeneity and unresponsive to immunotherapy represent the major obstacles for immune-checkpoint antibodies in GBM. The WHO 2016 glioma diagnosis scheme based on molecular characteristics represents a big step towards precise diagnosis and tailored therapy for patients with diffused glioma (38). GBMs are well-known insensitive "cold" tumors with relatively low tumor mutation burden and quiescent immune reactivity (13, 39). The highly immune-suppressive TME with a paucity of infiltrating CTLs has been considered a pivotal mediator of the insensitivity (40), wherein TAMs play an indispensable role (16). Classically, TAMs can polarize to M1 macrophages (the classical activation) which exhibit pro-inflammatory and cancer-inhibiting effects. Alternatively, stimuli such as IL-4, IL-14, IL-10 can induce macrophages towards an anti-inflammatory and cancer-promoting M2 phenotype (41, 42). In GBM, TAMs were the predominant infiltrating immune cells and usually polarized to an immunosuppressive M2-like phenotype (43, 44). This study shows that PD-L1 may be an important regulator of macrophages in GBM, supported by the positive correlation between them. Moreover, PD-L1 is positively correlated with the canonical markers of M2 macrophages, while has insignificant correlation with M1 markers. Thus, PD-L1 may participate in the induction and maintenance of macrophage M2-polarization.

The tumor infiltrating neutrophils could also inhibit T cells activity *via* the PD-1/PD-L1 pathway in hepatocellular carcinoma (45) and was associated with pro-inflammatory cytokine in lung cancer (46). The positive correlation between PD-L1 and neutrophil in the study implies that neutrophil-mediated immunosuppression may also occur in GBM. Regulatory T cells (Treg) are emerging as a mediator of immunosuppression in glioblastoma by inhibiting the generation of IL-12 and IFN-γ and suppressing tumor infiltrating T cells (47). PD-L1-mediated immunosuppression may also involve Treg.

In contrast, PD-L1 shows negative correlations with CD8$^+$ cytotoxic T cells, T follicular helper cells (Tfh), gamma delta (γδ) T cells (Tgd), and B cells. CD8$^+$ T cells represent the major tumoricidal T lymphocyte. The immunosuppressive TME is feathered by the exhaustion, anergy, and apoptosis of CD8$^+$ T cells (48). Tfh belongs to CD4$^+$ T cells which play a critical immune protective role *via* facilitating B cell generating antibody (49). Tgd produces various cytokines and chemokines (IFN-γ, TNF-α, IL-17) and can lysate infected or malignant cells (50). Thus, the immunosuppression mediated by PD-L1 may involve in a broader range of anti-tumor lymphocytes.

Genes that highly expressed in PD-L1^{high} GBMs enriched in multiple critical polarizing-related signalling pathways. For

instance, The classical activation of macrophages (M1) is induced by LPS and interferon-gamma (IFN-γ) (51). NF-kappa B is a critical transcription factor that transduces activation signals from LPS/TLR4 (52). Meanwhile, Shen J *et al.* also showed that NF-kappa B mediated IL-17 induced M2 macrophage polarization (53). IL-10 represents one of the canonical M2 stimuli. Upon ligating with its receptor IL-10R, IL-10 activates the transcription factor STAT3, which suppresses the production of pro-inflammatory cytokine (54, 55). The key role of mTORC1 in macrophage polarization has been reported, constitutive mTORC1 activation impaired the M2 polarization and increase pro-inflammatory response (56); depletion of mTORC1 decreased inflammation and pathogenic immune response during infection (57).

Translationally, targeting PD-L1 may represent a promising approach to re-educate TAM towards the anti-tumor M1 phenotype. Inhibition of PD-L1 would, therefore, abrogate the immune-suppression induced by M2 macrophage. It has been reported that PD-L1 involved in constitute negative signals pathways and led to immune-suppressive phenotypes in macrophages. Inhibition of PD-L1 promoted the proliferation, survival, and activation of macrophages (18). A similar remodeling of intratumoral macrophages was observed in a murine sarcoma model treated with anti-PD1/anti-CTLA-4 antibodies (17). Our study and others imply that targeting the PD-1/PD-L1 axis may yield additional anti-cancer effects mediated by TAM polarization. This notion can be exploited in the treatment of GBM since the combinations of PD-L1 and PD-1 antibodies have shown potent anti-GBM efficacy in pre-clinical studies (58–60).

Experimental studies are needed to validate the findings of this study. *Ex vivo* profiling of the cell components in GBM tissues would provide valuable information of TME. In addition, the correlation analysis can only provide preliminary evidence of the relationship rather than determine the causal relationship between PD-L1 and M2 macrophages. One of the major challenges of exploiting PD-L1 for prognosis prediction in GBM is to determine the positive criteria and cut-off value of PD-L1 expression, which necessitates large-scale studies. Lastly, it remains elusive whether M2-TAM polarization causally induces PD-L1 expression or vice versa, further studies are needed to delineate the relationship between PD-L1 and macrophage polarization.

This study provides preliminary evidence that PD-L1 is intimately correlated the infiltration and M2-polarization of macrophages. This notion represents an underappreciated immunosuppressive mechanism in the context of GBM. One brave but reasonable speculation is that PD-L1 inhibitors may enhance the efficacy of the prevalent PD-1 antibodies in GBM. It would be worthy to evaluate the efficacy of combining PD-L1 and PD-1 antibodies in future clinical trials.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

ZYZ and MG designed the study and wrote the manuscript. HZ designed the study, interpreted the results, and revised the manuscript. XL and MG performed the analysis and prepared the figures. BC, SZ, and ZTZ designed and supervised the study. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Ostrom QT, Cioffi G, Gittleman H, Patil N, Waite K, Kruchko C, et al. CBTRUS Statistical Report: Primary Brain and Other Central Nervous System Tumors Diagnosed in the United States in 2012-2016. *Neuro Oncol* (2019) 21:v1–v100. doi: 10.1093/neuonc/noz150
2. Tan AC, Ashley DM, Lopez GY, Malinzak M, Friedman HS, Khasraw M. Management of glioblastoma: State of the art and future directions. *CA Cancer J Clin* (2020) 70(4):299–312. doi: 10.3322/caac.21613
3. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med* (2005) 352:987–96. doi: 10.1056/NEJMoa043330
4. Dougan M, Dranoff G. Immune therapy for cancer. *Annu Rev Immunol* (2009) 27:83–117. doi: 10.1146/annurev.immunol.021908.132544
5. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer* (2012) 12:252–64. doi: 10.1038/nrc3239
6. Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med* (2002) 8:793–800. doi: 10.1038/nm730
7. Zou W, Wolchok JD, Chen L. PD-L1 (B7-H1) and PD-1 pathway blockade for cancer therapy: Mechanisms, response biomarkers, and combinations. *Sci Transl Med* (2016) 8:328rv4. doi: 10.1126/scitranslmed.aad7118
8. Wei SC, Duffy CR, Allison JP. Fundamental Mechanisms of Immune Checkpoint Blockade Therapy. *Cancer Discovery* (2018) 8:1069–86. doi: 10.1158/2159-8290.CD-18-0367
9. Iams WT, Porter J, Horn L. Immunotherapeutic approaches for small-cell lung cancer. *Nat Rev Clin Oncol* (2020) 17:300–12. doi: 10.1038/s41571-019-0316-z
10. Emens LA. Breast Cancer Immunotherapy: Facts and Hopes. *Clin Cancer Res* (2018) 24:511–20. doi: 10.1158/1078-0432.CCR-16-3001
11. Hodi FS, Chiarion-Sileni V, Gonzalez R, Grob JJ, Rutkowski P, Cowey CL, et al. Nivolumab plus ipilimumab or nivolumab alone versus ipilimumab alone in advanced melanoma (CheckMate 067): 4-year outcomes of a

multicentre, randomised, phase 3 trial. *Lancet Oncol* (2018) 19:1480–92. doi: 10.1016/S1470-2045(18)30700-9
12. Reardon DA, Brandes AA, Omuro A, Mulholland P, Lim M, Wick A, et al. Effect of Nivolumab vs Bevacizumab in Patients With Recurrent Glioblastoma: The CheckMate 143 Phase 3 Randomized Clinical Trial. *JAMA Oncol* (2020) 6(7):1003–10. doi: 10.1001/jamaoncol.2020.1024
13. Lim M, Xia Y, Bettegowda C, Weller M. Current state of immunotherapy for glioblastoma. *Nat Rev Clin Oncol* (2018) 15:422–42. doi: 10.1038/s41571-018-0003-5
14. Le DT, Uram JN, Wang H, Bartlett BR, Kemberling H, Eyring AD, et al. PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. *N Engl J Med* (2015) 372:2509–20. doi: 10.1056/NEJMoa1500596
15. Wang X, Guo G, Guan H, Yu Y, Lu J, Yu J. Challenges and potential of PD-1/PD-L1 checkpoint blockade immunotherapy for glioblastoma. *J Exp Clin Cancer Res* (2019) 38:87. doi: 10.1186/s13046-019-1085-3
16. Antonios JP, Soto H, Everson RG, Moughon D, Orpilla JR, Shin NP, et al. Immunosuppressive tumor-infiltrating myeloid cells mediate adaptive immune resistance via a PD-1/PD-L1 mechanism in glioblastoma. *Neuro Oncol* (2017) 19:796–807. doi: 10.1093/neuonc/now287
17. Gubin MM, Esaulova E, Ward JP, Malkova ON, Runci D, Wong P, et al. High-Dimensional Analysis Delineates Myeloid and Lymphoid Compartment Remodeling during Successful Immune-Checkpoint Cancer Therapy. *Cell* (2018) 175:1014–30.e19. doi: 10.1016/j.cell.2018.09.030
18. Hartley GP, Chow L, Ammons DT, Wheat WH, Dow SW. Programmed Cell Death Ligand 1 (PD-L1) Signaling Regulates Macrophage Proliferation and Activation. *Cancer Immunol Res* (2018) 6:1260–73. doi: 10.1158/2326-6066.CIR-17-0537
19. Zhao Z, Meng F, Wang W, Wang Z, Zhang C, Jiang T. Comprehensive RNA-seq transcriptomic profiling in the malignant progression of gliomas. *Sci Data* (2017) 4:170024. doi: 10.1038/sdata.2017.24
20. Gao M, Lin Y, Liu X, Li Y, Zhang C, Wang Z, et al. ISG20 promotes local tumor immunity and contributes to poor survival in human glioma. *Oncoimmunology* (2019) 8:e1534038. doi: 10.1080/2162402X.2018.1534038
21. Bindea G, Mlecnik B, Tosolini M, Kirilovsky A, Waldner M, Obenauf AC, et al. Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. *Immunity* (2013) 39:782–95. doi: 10.1016/j.immuni.2013.10.003
22. Chtanova T, Newton R, Liu SM, Weininger L, Young TR, Silva DG, et al. Identification of T cell-restricted genes, and signatures for different T cell responses, using a comprehensive collection of microarray datasets. *J Immunol* (2005) 175:7837–47. doi: 10.4049/jimmunol.175.12.7837
23. Hyrcza MD, Kovacs C, Loutfy M, Halpenny R, Heisler L, Yang S, et al. Distinct transcriptional profiles in ex vivo CD4+ and CD8+ T cells are established early in human immunodeficiency virus type 1 infection and are characterized by a chronic interferon response as well as extensive transcriptional changes in CD8+ T cells. *J Virol* (2007) 81:3477–86. doi: 10.1128/JVI.01552-06
24. Wendt K, Wilk E, Buyny S, Buer J, Schmidt RE, Jacobs R. Gene and protein characteristics reflect functional diversity of CD56dim and CD56bright NK cells. *J Leukoc Biol* (2006) 80:1529–41. doi: 10.1189/jlb.0306191
25. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol* (2004) 25:677–86. doi: 10.1016/j.it.2004.09.015
26. Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, et al. Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity* (2014) 41:14–20. doi: 10.1016/j.immuni.2014.06.008
27. Li W, Graeber MB. The molecular profile of microglia under the influence of glioma. *Neuro Oncol* (2012) 14:958–78. doi: 10.1093/neuonc/nos116
28. Gabrusiewicz K, Rodriguez B, Wei J, Hashimoto Y, Healy LM, Maiti SN, et al. Glioblastoma-infiltrated innate immune cells resemble M0 macrophage phenotype. *JCI Insight* (2016) 1(2):e85841,1–19. doi: 10.1172/jci.insight.85841
29. Chen Y, Song Y, Du W, Gong L, Chang H, Zou Z. Tumor-associated macrophages: an accomplice in solid tumor progression. *J BioMed Sci* (2019) 26:78. doi: 10.1186/s12929-019-0568-z
30. Herbst RS, Soria JC, Kowanetz M, Fine GD, Hamid O, Gordon MS, et al. Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. *Nature* (2014) 515:563–7. doi: 10.1038/nature14011
31. Chen RQ, Liu F, Qiu XY, Chen XQ. The Prognostic and Therapeutic Value of PD-L1 in Glioma. *Front Pharmacol* (2018) 9:1503. doi: 10.3389/fphar.2018.01503
32. Nduom EK, Wei J, Yaghi NK, Huang N, Kong LY, Gabrusiewicz K, et al. PD-L1 expression and prognostic impact in glioblastoma. *Neuro Oncol* (2016) 18:195–205. doi: 10.1093/neuonc/nov172
33. Berghoff AS, Kiesel B, Widhalm G, Rajky O, Ricken G, Wohrer A, et al. Programmed death ligand 1 expression and tumor-infiltrating lymphocytes in glioblastoma. *Neuro Oncol* (2015) 17:1064–75. doi: 10.1093/neuonc/nou307
34. Xue S, Hu M, Li P, Ma J, Xie L, Teng F, et al. Relationship between expression of PD-L1 and tumor angiogenesis, proliferation, and invasion in glioma. *Oncotarget* (2017) 8:49702–12. doi: 10.18632/oncotarget.17922
35. Wang Z, Zhang C, Liu X, Wang Z, Sun L, Li G, et al. Molecular and clinical characterization of PD-L1 expression at transcriptional level via 976 samples of brain glioma. *Oncoimmunology* (2016) 5:e1196310. doi: 10.1080/2162402X.2016.1196310
36. Doucette T, Rao G, Rao A, Shen L, Aldape K, Wei J, et al. Immune heterogeneity of glioblastoma subtypes: extrapolation from the cancer genome atlas. *Cancer Immunol Res* (2013) 1:112–22. doi: 10.1158/2326-6066.CIR-13-0028
37. Jan CI, Tsai WC, Harn HJ, Shyu WC, Liu MC, Lu HM, et al. Predictors of Response to Autologous Dendritic Cell Therapy in Glioblastoma Multiforme. *Front Immunol* (2018) 9:727. doi: 10.3389/fimmu.2018.00727
38. Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-Branger D, Cavenee WK, et al. The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. *Acta Neuropathol* (2016) 131:803–20. doi: 10.1007/s00401-016-1545-1
39. Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SA, Behjati S, Biankin AV, et al. Signatures of mutational processes in human cancer. *Nature* (2013) 500:415–21. doi: 10.1038/nature12477
40. Hao C, Parney IF, Roa WH, Turner J, Petruk KC, Ramsay DA. Cytokine and cytokine receptor mRNA expression in human glioblastomas: evidence of Th1, Th2 and Th3 cytokine dysregulation. *Acta Neuropathol* (2002) 103:171–8. doi: 10.1007/s004010100448
41. Cheng H, Wang Z, Fu L, Xu T. Macrophage Polarization in the Development and Progression of Ovarian Cancers: An Overview. *Front Oncol* (2019) 9:421. doi: 10.3389/fonc.2019.00421
42. Guadagno E, Presta I, Maisano D, Donato A, Pirrone CK, Cardillo G, et al. Role of Macrophages in Brain Tumor Growth and Progression. *Int J Mol Sci* (2018) 19(4):1005,1–19. doi: 10.3390/ijms19041005
43. Fu W, Wang W, Li H, Jiao Y, Huo R, Yan Z, et al. Single-Cell Atlas Reveals Complexity of the Immunosuppressive Microenvironment of Initial and Recurrent Glioblastoma. *Front Immunol* (2020) 11:835. doi: 10.3389/fimmu.2020.00835
44. Hussain SF, Yang D, Suki D, Aldape K, Grimm E, Heimberger AB. The role of human glioma-infiltrating microglia/macrophages in mediating antitumor immune responses. *Neuro Oncol* (2006) 8:261–79. doi: 10.1215/15228517-2006-008
45. He G, Zhang H, Zhou J, Wang B, Chen Y, Kong Y, et al. Peritumoural neutrophils negatively regulate adaptive immunity via the PD-L1/PD-1 signalling pathway in hepatocellular carcinoma. *J Exp Clin Cancer Res* (2015) 34:141. doi: 10.1186/s13046-015-0256-0
46. Koyama S, Akbay EA, Li YY, Aref AR, Skoulidis F, Herter-Sprie GS, et al. STK11/LKB1 Deficiency Promotes Neutrophil Recruitment and Proinflammatory Cytokine Production to Suppress T-cell Activity in the Lung Tumor Microenvironment. *Cancer Res* (2016) 76:999–1008. doi: 10.1158/0008-5472.CAN-15-1439
47. Humphries W, Wei J, Sampson JH, Heimberger AB. The role of tregs in glioma-mediated immunosuppression: potential target for intervention. *Neurosurg Clin N Am* (2010) 21:125–37. doi: 10.1016/j.nec.2009.08.012
48. Mirzaei R, Sarkar S, Yong VW. T Cell Exhaustion in Glioblastoma: Intricacies of Immune Checkpoints. *Trends Immunol* (2017) 38:104–15. doi: 10.1016/j.it.2016.11.005
49. Qin L, Waseem TC, Sahoo A, Bieerkehazhi S, Zhou H, Galkina EV, et al. Insights Into the Molecular Mechanisms of T Follicular Helper-Mediated Immunity and Pathology. *Front Immunol* (2018) 9:1884. doi: 10.3389/fimmu.2018.01884
50. Zhao Y, Niu C, Cui J. Gamma-delta (gammadelta) T cells: friend or foe in cancer development? *J Transl Med* (2018) 16:3. doi: 10.1186/s12967-018-1491-x
51. Orecchioni M, Ghosheh Y, Pramod AB, Ley K. Macrophage Polarization: Different Gene Signatures in M1(LPS+) vs. Classically and M2(LPS-) vs.

Alternatively Activated Macrophages. *Front Immunol* (2019) 10:1084. doi: 10.3389/fimmu.2019.01084
52. Lawrence T, Natoli G. Transcriptional regulation of macrophage polarization: enabling diversity with identity. *Nat Rev Immunol* (2011) 11:750–61. doi: 10.1038/nri3088
53. Shen J, Sun X, Pan B, Cao S, Cao J, Che D, et al. IL-17 induces macrophages to M2-like phenotype via NF-kappaB. *Cancer Manag Res* (2018) 10:4217–28. doi: 10.2147/CMAR.S174899
54. Lang R, Patel D, Morris JJ, Rutschman RL, Murray PJ. Shaping gene expression in activated and resting primary macrophages by IL-10. *J Immunol* (2002) 169:2253–63. doi: 10.4049/jimmunol.169.5.2253
55. Wang N, Liang H, Zen K. Molecular mechanisms that influence the macrophage m1-m2 polarization balance. *Front Immunol* (2014) 5:614. doi: 10.3389/fimmu.2014.00614
56. Byles V, Covarrubias AJ, Ben-Sahra I, Lamming DW, Sabatini DM, Manning BD, et al. The TSC-mTOR pathway regulates macrophage polarization. *Nat Commun* (2013) 4:2834. doi: 10.1038/ncomms3834
57. Haloul M, Oliveira ERA, Kader M, Wells JZ, Tominello TR, El Andaloussi A, et al. mTORC1-mediated polarization of M1 macrophages and their accumulation in the liver correlate with immunopathology in fatal ehrlichiosis. *Sci Rep* (2019) 9:14050. doi: 10.1038/s41598-019-50320-y
58. Saha D, Martuza RL, Rabkin SD. Macrophage Polarization Contributes to Glioblastoma Eradication by Combination Immunovirotherapy and Immune Checkpoint Blockade. *Cancer Cell* (2017) 32:253–67.e5. doi: 10.1016/j.ccell.2017.07.006
59. Reardon DA, Gokhale PC, Klein SR, Ligon KL, Rodig SJ, Ramkissoon SH, et al. Glioblastoma Eradication Following Immune Checkpoint Blockade in an Orthotopic, Immunocompetent Model. *Cancer Immunol Res* (2016) 4:124–35. doi: 10.1158/2326-6066.CIR-15-0151
60. Wainwright DA, Chang AL, Dey M, Balyasnikova IV, Kim CK, Tobias A, et al. Durable therapeutic efficacy utilizing combinatorial blockade against IDO, CTLA-4, and PD-L1 in mice with brain tumors. *Clin Cancer Res* (2014) 20:5290–301. doi: 10.1158/1078-0432.CCR-14-0514

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An Immune-Related Signature for Predicting the Prognosis of Lower-Grade Gliomas

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Background: Lower-grade gliomas (LGGs) have more favorable outcomes than glioblastomas; however, LGGs often progress to process glioblastomas within a few years. Numerous studies have proven that the tumor microenvironment (TME) is correlated with the prognosis of glioma.

Methods: LGG RNA-Sequencing (RNA-seq) data from The Cancer Genome Atlas (TCGA) and the Chinese Glioma Genome Atlas (CGGA) were extracted and then divided into training and testing cohorts, respectively. Immune-related differentially expressed genes (DEGs) were screened to establish a prognostic signature by a multivariate Cox proportional hazards regression model. The immune-related risk score and clinical information, such as age, sex, World Health Organization (WHO) grade, and isocitrate dehydrogenase 1 (IDH1) mutation, were used to independently validate and develop a prognostic nomogram. GO and KEGG pathway analyses to DEGs between immune-related high-risk and low-risk groups were performed.

Results: Sixteen immune-related genes were screened for establishing a prognostic signature. The risk score had a negative correlation with prognosis, with an area under the receiver operating characteristic (ROC) curve of 0.941. The risk score, age, grade, and IDH1 mutation were identified as independent prognostic factors in patients with LGGs. The hazard ratios (HRs) of the high-risk score were 5.247 [95% confidence interval (CI) = 3.060–8.996] in the multivariate analysis. A prognostic nomogram of 1-, 3-, and 5-year survival was established and validated internally and externally. Go and KEGG pathway analyses implied that immune-related biological function and pathways were involved in the TME.

Conclusion: The immune-related prognostic signature and the prognostic nomogram could accurately predict survival.

Keywords: lower-grade gliomas, immune, risk score, prognostic signature, glioma

INTRODUCTION

Glioma is a type of cancer that originates in glial cells, which support the nerve cells of the brain and keep the cells healthy. It is the most common primary malignant brain tumor (1). Glioma has various symptoms, including seizures, personality changes, movement difficulty, headache, problems with understanding or speaking, and vision problems. The symptoms that occur mainly depend on the tumor location as well as other tumors. According to the standards set by the World Health Organization (WHO), glioma is classified into grades I, II, III, and IV. Gliomas with histological grades II and III are identified as lower-grade gliomas (LGGs) and have highly variable clinical behaviors (2). The outcomes of LGGs are more favorable than those of grade IV gliomas. Unfortunately, the progression of LGGs occurs in almost 70% of patients within ten years (1). Aggressive high-grade gliomas have an inferior prognosis despite the treatment management with surgical resection plus radiation therapy and chemotherapy (3, 4). Because of this highly offensive ability, LGGs cannot be completely cured. Thus, delaying tumor onset and reducing tumor progression are the most challenging issues. Garcia et al. claimed that the prognosis of glioma is associated with age, sex, comorbidities, socioeconomic state, and ethnicity (5). Moreover, the study found that the absence of isocitrate dehydrogenase 1 (IDH1) mutation in LGGs was similar to glioblastoma regarding molecular and clinical characteristics (2). IDH mutation, which is considerably associated with improved prognosis, is sporadic in glioblastoma, while it is common in LGGs (6). In the analysis of the single nucleotide variation from aggregated somatic mutation of LGGs from The Cancer Genome Atlas (TCGA), we found that the mutation rate of IDH1 was 77%, and the survival rate was significantly improved in the IDH1 mutation population. Furthermore, the IDH mutation rate of CGGA was 74% with a survival protection in IDH-mutated group, which was similar to the conclusion of TCGA.

Therapeutic resistance does ultimately develop despite effective targeted therapies for tumor cells (7). Recently, research on identifying the mechanisms of resistance to therapies showed that substantial alteration occurred, not only in tumor cells but also in the tumor environment (TME). These alterations imply the importance of the extrinsic compartments of tumor cells in tumor development (8). Malignancy formation is a co-evolution of neoplastic cells together with the TME surrounded by immune cells, tumor vasculature, and extracellular matrix. The TME always dictates aberrant cellular function and affects the subsequent development of more advanced and refractory malignancies (9). Increasing evidence has extensively indicated that immune infiltrates are correlated with the prognosis of the glioma (10, 11). Indeed, immunotherapy is a novel approach utilizing the immune system against tumor progression with few short-term side effects. Thus, establishing a scientific immune-related model derived from LGG samples to predict prognosis is important.

In the current study, we screened for immune-related genes by using deep-sequencing technologies for transcriptome profiling correlated with the immune system. Univariate Cox proportional hazards regression was carried out to identify prognostic biomarkers followed by an L1 penalized least absolute shrinkage and selection operator (Lasso) Cox analysis. Multivariate Cox regression analysis was used to establish a prognostic signature to calculate the immune-related risk score that was independent of various clinical factors. A nomogram that can be utilized to personalize prognosis predictions was constructed based on age, sex, IDH1 mutation, and risk score. In addition, the prognostic signature and its independence were validated internally in TCGA and externally in Chinese Glioma Genome Atlas (CGGA). We believe that the immune-related prognostic signature will contribute to identification of potential the therapeutic biomarkers and the development of an individualized therapy guide for LGG patients.

MATERIAL AND METHODS

Patient Datasets

We extracted LGG gene information on the transcriptome in fragment per kilobase per million (FPKM) from the TCGA project (https://portal.gdc.cancer.gov/). RNA-Sequencing (RNA-Seq) data from 529 LGG tumor tissue samples and five normal brain tissue samples were screened for differentially expressed genes (DEGs). DEGs were defined as a significant difference in the expression levels of genes between in glioma and normal tissues. This procedure was implemented by R software (version 3.6.1) with the "limma" package, and we set the significance threshold as $\log_2$Foldchange ($\log_2$FC) >1 and adjusted $p<0.05$ for screening the DEGs with Wilcox test. The immune-related gene list was provided by the IMMPORT website (https://www.immport.org/). The intersecting gene set of DEGs and immune-related genes was used to construct the prognostic signature. The survival curve of each included gene that divided into high expression group and low expression group was mapped by R. In addition, we also downloaded the corresponding clinical information of patients from the TCGA database, including survival time, vital status, sex, age, the emergence of IDH1 mutation, and tumor grade. Samples with

Abbreviations: LGGs, Lower-grade gliomas; TME, the tumor microenvironment; TCGA, The Cancer Genome Atlas; CGGA, Chinese Glioma Genome; DEGs, differentially expressed genes; IDH1, isocitrate dehydrogenase 1; ROC, receiver operating characteristic; SNPs, single nucleotide polymorphisms; Lasso, least absolute shrinkage and selection operator; FPKM, fragment per kilobase per million; RNA-Seq, RNA-Sequencing; log2FC, log2Foldchange; OS, overall survival; HRs, hazard ratios; CIs, confidence intervals; C-index, concordance index; TIMER, Tumor IMmune Estimation Resource; TP53, tumor protein p53; ATRX, ATRX, chromatin remodeler; AUC, area under ROC curve; TERT, telomerase reverse transcriptase; LCIs, lower confidence intervals; UCIs, upper confidence intervals; TMSB15A, thymosin beta 15a; MAVS, mitochondrial antiviral signaling protein; S100A16, S100 calcium binding protein A16; FABP6, fatty acid binding protein; PLTP, phospholipid transfer protein; IFIH1, interferon induced with helicase C domain 1; F2R, coagulation factor II thrombin receptor; CSRP1, cysteine and glycine rich protein 1; APOBEC3C, apolipoprotein B mRNA editing enzyme catalytic subunit 3C; SEMA5A, semaphorin 5A; GDNF, glial cell derived neurotrophic factor; NMB, neuromedin B; BMPR1A, bone morphogenetic protein receptor type 1A; EGFR, epidermal growth factor receptor; BID, BH3 interacting domain death agonist; CDK4, cyclin dependent kinase 4; GO, gene ontology; ECM, extracellular matrix; not applicable; IQR, interquartile range.

missing information or with a survival time less than 90 days were excluded. The dataset from TCGA was used be the training cohort, while the RNA-Seq data from the CGGA project (http://www.cgga.org.cn) was used as the testing cohort to validate the prognostic signature.

Screening for Immune-Related Prognostic Genes and Establishing a Prognostic Signature

Univariate Cox proportional hazards regression was conducted based on the data of the training cohort for candidate genes associated with overall survival (OS). A novel algorithm, Lasso regression was applied to screen parameters in high-dimensional data (12). Lasso regression was performed on 126 genes with an adjusted p-value of less than 0.05 and further screened 25 candidate genes. Subsequently, we established a multivariate Cox proportional hazards regression model to predict prognosis based on the candidate immune-related genes. Sixteen genes with its coefficients (β), hazard ratios (HRs), and 95% confidence intervals (CIs) were ultimately estimated using the maximum likelihood ratio method. The risk score is a sum value that is calculated as β multiplied by each immune-related gene expression as follows: risk score = (expression of gene A*β_A) + (expression of gene B*β_B + (expression of gene C*β_C) + … (expression of gene N*β_N) (13). The median risk score value of the training cohort was taken as a cutoff point for dichotomization into high- and low-risk groups (14). With the R package "survminer", Kaplan–Meier plots and the log-rank test were used to estimate the survival rate between the low- and high-risk groups (14). A time-dependent receiver operating characteristic (ROC) curve was calculated to assess the predictive value of the multivariate Cox model (15, 16). To rule out the factors that cause accidental death in patients, such as death from postoperative complications, we excluded samples with a follow-up or OS time shorter than 90 days. The survival rate curve and ROC curve were also drawn based on the data from CGGA to validate the prognostic ability of the model.

Independent Prognostic Role of the Immune-Related Prognostic Signature

To determine the impact of the immune-related risk score on prognosis, we need to assess whether the risk score is independent of other clinical factors, including sex, age, IDH1 mutation stage, and tumor WHO grade. Thus, univariate and multivariate Cox proportional hazards regression analyses were performed to determine the independent prognostic role of the immune-related risk score with the forward stepwise procedure. The immune-related risk score and clinical factors were deemed as independent factors if the adjusted p value was less than 0.05.

Development and Validation of the Prognostic Nomogram

To develop an individual prognostic signature for the 1-, 3- and 5-year survival rates, a nomogram was formulated according to the significant results of the multivariate Cox proportional hazards regression model (17). We constructed this prognostic model using a backward step-down selection process with the Akaike information criterion (18). Finally, four corresponding clinical factors, including age, WHO grade, IDH mutation, and immune-related risk, were used to the develop the nomogram. The calculation of the concordance index (C-index) and the construction of a calibration curve plot were performed for the internal and external validations to check the predictive accuracy and or stability capacity of the nomogram (19). The C-index of the nomogram was observed by bootstraps with 1,000 resamples (1). The value of the C-index ranged from 0.5 to 1.0, and the size of the value determined the predictive performance of the nomogram (18). Calibration curves are used to determine the survival of the unknown sample by comparing it with the actual survival and provide a visual plot to determine the predictive of a model. A perfect calibration curve would have an R^2 value of 1. The larger the slope of the steeper line, the more sensitive the measurement is.

GO and KEGG Pathway Analyses of DEGs

The DEGs between immune-related high-risk and low-risk groups were screened with a log2FC >1 and adjusted p <0.05. GO analysis with functions including molecular function (MF), biological pathways (BP), cellular component (CC), and KEGG pathway analyses were performed to the DEGs by using R software at the functional level. P <0.05 and q <0.05 were considered to have a significance.

RESULTS

Characteristics of the Datasets

There were 5,009 DEGs between 529 LGG samples and five normal brain tissues, 239 of which were immune-related DEGs. In the univariate Cox proportional hazards regression, 126 immune-related genes were retained for Lasso regression. Finally, 25 candidate genes were used to conduct a multivariate Cox proportional hazards regression (**Supplementary Material 1**). 459 LGG samples in training set and 362 LGG samples in the testing set with corresponding clinical information were included for the prognostic signature. In the Cox regression, we took the IDH1 mutation state into the model because we found that the most susceptible genes in LGG were IDH1, tumor protein p53 (TP53) and ATRX, chromatin remodeler (ATRX), and only the IDH1 mutation is closely associated to prognosis (**Figure 1**). The same rules were utilized to extract data from the CGGA database.

The Immune-Related Prognostic Signature and Predictability Assessment

According to the relationship between the expression of significant and independent genes and OS, the risk score model with 16 immune-related genes was established by multivariate Cox proportional hazards regression. The β value, HR, 95CI% and p-value of each included gene in the model are shown in **Table 1**. Survival analysis revealed that 11 of the 16 immune-related genes in the signature were related to prognosis. Seven of these genes (S100A16, PLTP, IFIH1, F2R, CSRP1,

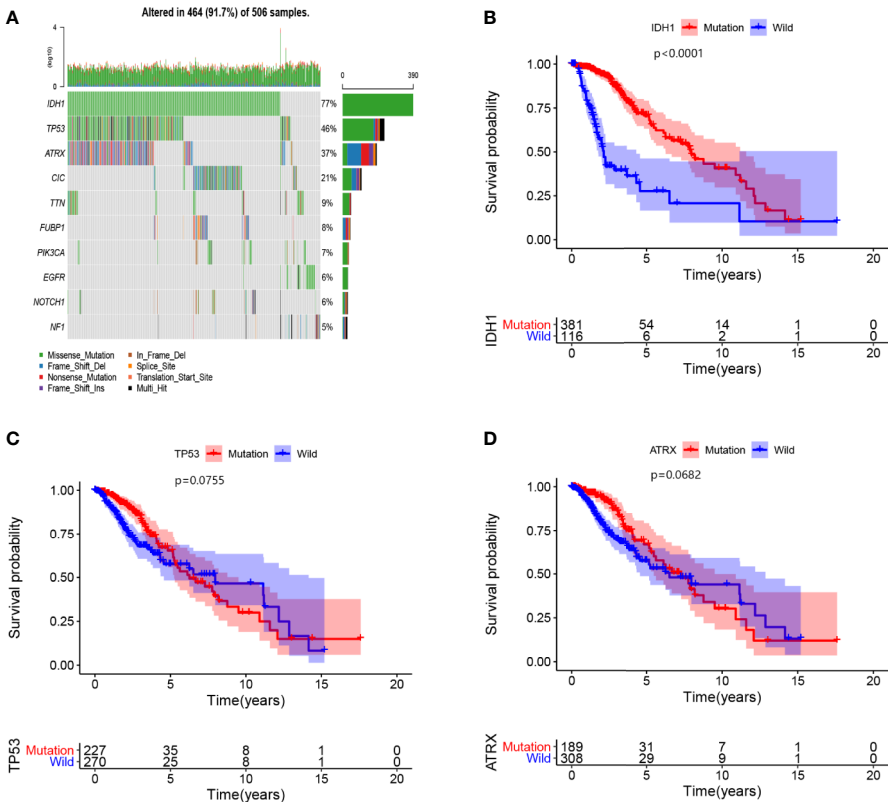

FIGURE 1 | **(A)** The 10 most frequently mutated genes are displayed in the LGG samples of TCGA. The mutated rates of IDH1, TP53, and ATRX exceed 20%, and the types of IDH1 mutations are all missense mutations. **(B–D)** Survival curves of IDH1, TP53, and ATRX mutant genes, of which only IDH1 mutation affects the prognosis of LGGs.

APOBEC3C, SEMA5A) are considered tumor-promoting genes, and four genes (GDNF, NMB, BMPR1A, EGFR) are considered tumor-protecting genes. The immune-related risk score of each sample in the training cohort and the testing cohort was calculated in accordance with the model formula. The median risk score of the training cohort was 0.645, which was deemed as the cutoff point for dichotomizing the risk of a sample as either low- or high-risk in the training cohort (**Supplementary Material 2**) and the testing cohort (**Supplementary Material 3**). 459 LGG samples from the TCGA were divided into high-risk group with 229 samples and low-risk group with 230 samples according to the immune-related risk score. In CGGA, the samples were split into 175 samples in a high-risk group and 187 samples in a low-risk group. In addition, the risk scores of the grade II group are lower than the grade III group, as well as that in the IDH1 mutation and IDH1 wild type groups. In the training set, the low-risk patients had a much-improved prognosis (**Figures 2A, C**), and the area under ROC curve (AUC) value was 0.941 (**Figure 2D**). Moreover, a similar result was statistically significant in the testing set. Additionally, the ROC curve achieved an AUC value of 0.712 (**Figures 2B, E**). Except for FABP6, the genes incorporated into the model have a significant difference in expression between the low-risk and high-risk groups (**Supplemental Material 4**).

Independent Predictive Role of the Immune-Related Prognostic Signature

As reported before, we included the corresponding clinical information to validate the independent predictive role of the model. Sample with missing clinical information for independent prediction analysis were further excluded, and additional information can be found in **Table 2**. Univariate and multivariate Cox regression analyses were sequentially used to identify the independence of various clinical factors. Finally, the results showed that age, WHO grade, IDH1 mutation state, and the risk score calculated from the above immune-related risk score model were independent prognostic factors associated with OS. Among these independent factors, the risk score value was the most critical and played a vital role. The risk of adverse events in the high-risk group was 6.947 times that of the low-risk group in the univariate Cox regression and 5.247 times that of the low-risk group in the multivariate Cox regression (**Figures 3A, B**).

Establishing and Validating an Individualized Nomogram

A nomogram derived from routine pretreatment parameters used in the multivariable analysis was established. The establishment of a nomogram is a crucial step in determining the likelihood of individualized predicted 1-, 3-, and 5-year

TABLE 1 | Multivariate Cox proportional hazard regression.

Gene	β	HR	95%LCI	95%UCI	P value
TMSB15A	0.035	1.035	1.024	1.047	<0.001
MAVS	0.058	1.060	0.985	1.140	0.122
S100A16	0.006	1.006	1.000	1.012	0.042
FABP6	0.044	1.045	1.016	1.075	0.002
PLTP	0.004	1.004	1.002	1.006	<0.001
IFIH1	0.044	1.044	1.006	1.085	0.025
F2R	0.015	1.015	0.999	1.032	0.064
CSRP1	0.010	1.010	1.003	1.017	0.003
APOBEC3C	0.036	1.036	0.994	1.080	0.093
SEMA5A	0.049	1.050	1.033	1.066	<0.001
GDNF	−0.507	0.602	0.449	0.807	<0.001
NMB	−0.003	0.997	0.995	0.999	0.004
BMPR1A	−0.109	0.897	0.774	1.039	0.148
EGFR	0.001	1.001	1.000	1.002	0.101
BID	−0.059	0.943	0.910	0.978	0.002
CDK4	0.002	1.002	1.001	1.004	0.003

HR, hazard ratios; LCI, lower confidence intervals; UCI, upper confidence intervals; TMSB15A, thymosin beta 15a; MAVS, mitochondrial antiviral signaling protein; S100A16, S100 calcium binding protein A16; FABP6, fatty acid binding protein; PLTP, phospholipid transfer protein; IFIH1, interferon induced with helicase C domain 1; F2R, coagulation factor II thrombin receptor; CSRP1, cysteine and glycine rich protein 1; APOBEC3C, apolipoprotein B mRNA editing enzyme catalytic subunit 3C; SEMA5A, semaphorin 5A; GDNF, glial cell derived neurotrophic factor; NMB, neuromedin B; BMPR1A, bone morphogenetic protein receptor type 1A; EGFR, epidermal growth factor receptor; BID, BH3 interacting domain death agonist; CDK4, cyclin dependent kinase 4.

survival prognoses for LGG patients (**Figure 3C**). Then, the nomogram was validated internally and externally by calculating the C-index and calibration curve, and the prediction achieved a reasonable accuracy. The C-index was 0.878 for the internal validation and 0.680 for the external validation, which indicates a consistent prediction capability. In addition, as seen from the graph in **Figure 4**, each calibration curve had goodness-off-fit.

GO and KEGG Pathway Analyses

There are 1,263 DEGs screened between immune-related high-risk group and low-risk group in TCGA. A bubble chart in the **Figure 5** shows the GO analysis of the top 10 listed based on the adjusted p value in BP, CC, and MF. GO functions, for example, extracellular matrix (ECM) function, immune response, and cytokine secretion which were related to TME in cancer were screened (**Supplemental Material 5**). KEGG analysis indicated that these DEGs were included in ECM–receptor interaction, proteoglycans in cancer, PI3K–Akt signaling pathway, cell cycle, cytokine–cytokine receptor interaction, and so on (**Figure 6**, **Supplemental Material 6**), which were related to the biological function in malignant tumors.

DISCUSSION

Until now, the prognosis of glioma patients varies greatly and depends on the characteristics of clinical outcomes according to the clinical practice guidelines (20). However, studies have claimed that some essential clinical characteristics, such as the WHO grade (III *vs.* IV) and resection, chemotherapy, and radiation therapy strategies, have little prognostic value (21, 22). Moreover, Parks et al. (23) did not recommend an individual prognostic model focusing on only clinical information to predict the prognosis of patients due to its imprecision. In the present study, we first screened out the immune-related DEGs in LGG. Then, 16 genes were used to establish a prognostic signature according to a multivariate Cox proportional hazards regression followed by a Lasso Cox analysis, which avoided overfitting to the greatest extent. A sixteen gene signature was identified as a prognostic signature in LGG and validated in the CGGA. Subsequently, the independent predictive role of the signature was confirmed. Finally, a personalized predicted nomogram taking risk score combined with age, IDH1 mutation, and WHO grade was formed to predict prognosis.

Clinical outcomes have been considered as the most important indicators for predicting the prognosis of malignant tumor patients. However, studies have deemed that the prognostic assessments based on clinical factors, are adequate for prognostic prediction, even for pathology classification (24, 25). However, beyond that, mutations in some favorable genes, such as IDH, TP53, and telomerase reverse transcriptase (TERT), are often used for prognostic predictions. However, the conclusion remains to be elucidated. TP53 mutations seem to have a critical effect on altering the survival time of tumor patients (26), but there was no similar effect of TP53 mutations in LGG from our survival analysis as shown in **Figure 1A**. Nonoguchi et al. (27) found that TERT mutations had a vital correlation with survival in glioblastoma, but the relationship was absent following multivariate analysis. Two years later, Simon et al. (28) suggested that TERT mutations have a predictive role in only patients with an incomplete resection and no history of temozolomide therapy. In the current study, we found that older age, WHO grade III and IDH1 mutation absence were independent factors for poor outcomes in the univariate analysis as well as in the multivariate analysis. When the patient's age increases by one year, the unfavorable event risk increases by 5.5%. The IDH1 mutation was the only protective factor, and the risk of patients with this mutation was 0.464 times that of the patients without

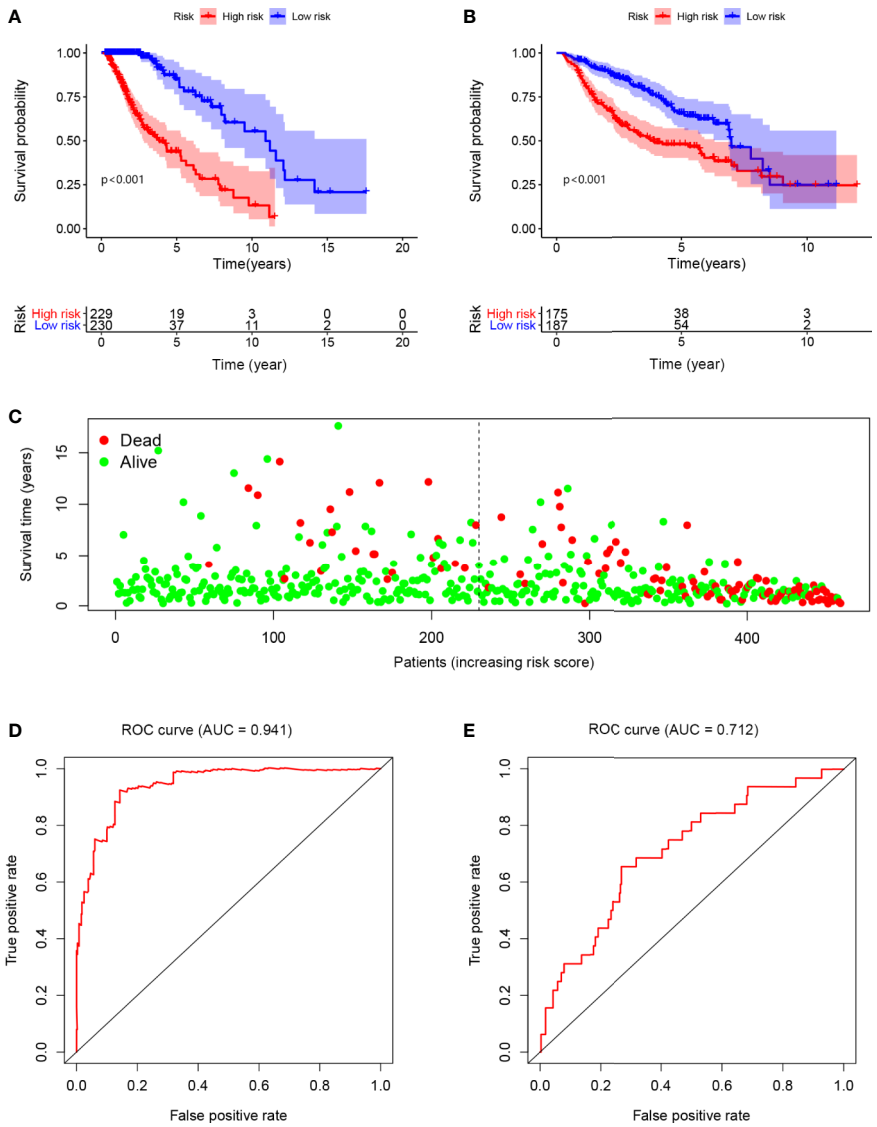

FIGURE 2 | The low risk group has a better prognosis both in the training cohort **(A)** and testing cohort **(B)**. As the risk score increases, the patients' survival rates visually decreased as well as the survival time **(C)**. The AUC values are 0.941 and 0.712 in the training cohort **(D)** and testing cohort **(E)**, respectively.

this mutation (**Figure 3B**). Similar results regarding older age and IDH1 mutation were put forward by Jones et al. (29). A multivariate analysis from **Figure 3B** indicated that grade III has been shown to lead to an elevated risk when compared with grade II.

As reported above, the impact of important clinical factors on the prognosis of gliomas has been known for a long time, but the influence of the gene set as a group on the prognosis of gliomas may have amazing significance. Recently, a novel approach was proposed by calculating the gene expression from RNA-Seq data, which is a far more precise measurement using next-generation sequencing technologies for transcriptome profiling than other methods (1, 13, 14, 30, 31). Studies have mainly focused on genes (30), pseudogenes (14), microRNAs (13), and lncRNAs (32) in glioma when establishing a prognostic signature. There have been some breakthrough outcomes in the treatment of gliomas, and immunological research has a pivotal position. Nevertheless, fewer studies had explored the effect of immune-related genes in a prognosis prediction model. After all, the immune system and tumor cells affect each other in prognosis (33). The immune-related risk score calculated by the prognostic signature in our study illustrates that the HR sharply increased to 5.247 in multivariate analysis. As shown in the **Figure 2D** of the ROC curve, the AUC value was 0.941, indicating that the model was accurate. The validation results from CGGA were the same as those from TCGA. Moreover, the risk score was higher in the grade III group and the IDH1 mutation group, representing a poor prognosis. Despite the lack of success of the individual

TABLE 2 | Clinical information of TCGA and CGGA.

Variables	TCGA (459 samples)	CGGA (362 samples)	P value
Survival time(days)			<0.001
Median (IQR)	609(407–1120)	1031(560–1826)	
Survival State			
Alive	353	218	<0.001
Dead	106	144	
Age (years)			
Median (IQR)	41(33–53)	40(33–47)	0.001
Gender			
Female	207	157	0.621
Male	252	205	
Grade			
G2	220	159	0.253
G3	239	203	
IDH1 Mutation			
NO	102	87	0.647
YES	348	275	
NA	9	0	
High Risk			0.659
NO	230	187	
YES	229	175	

G2, WHO II; G3, WHO III; NA, not applicable; IQR, interquartile range.

prognostic calculator for glioblastoma (23), we established a prognostic nomogram for predicting the 1-, 3- and 5-years survival rate of LGG patients that was internally and externally validated and revealed that the nomogram could provide an individual prediction. The reasons why our results were different from those of Park et al. (23) could be that our subjects were patients with LGG rather than glioblastoma; moreover, another reason could be that we included the risk score in the analysis in addition to some clinical information such as age and sex. From the nomogram, we can clearly see that a high risk score accounts for a large proportion of the total points. Overall, our data lead us to the conclusion that the immune-related prognostic signature shows a powerful predictive ability in LGG.

The immune system is famous for its protection against illness and infection related to bacteria, viruses, fungi, or parasites. Interestingly, immune system is a complicated synthesis which contains stromal cells, ECM, extracellular molecules and so on, can initiate an immune response to malignant tumor. Tumorigenesis is related to the aberrant innate and adaptive immune response by selecting aggressive clones, stimulating malignant cell proliferation and metastasis, and inducing immunosuppression (34, 35). Furthermore, brain ECM was modulated in the process of glioma infiltration and it was probably a novel therapeutic target to control glioma infiltration (36). In our study, GO and KEGG pathway analyses to the DEGs between immune-related high-risk and low-risk groups implied that many biological function and pathways, for example, ECM organization, immune response,

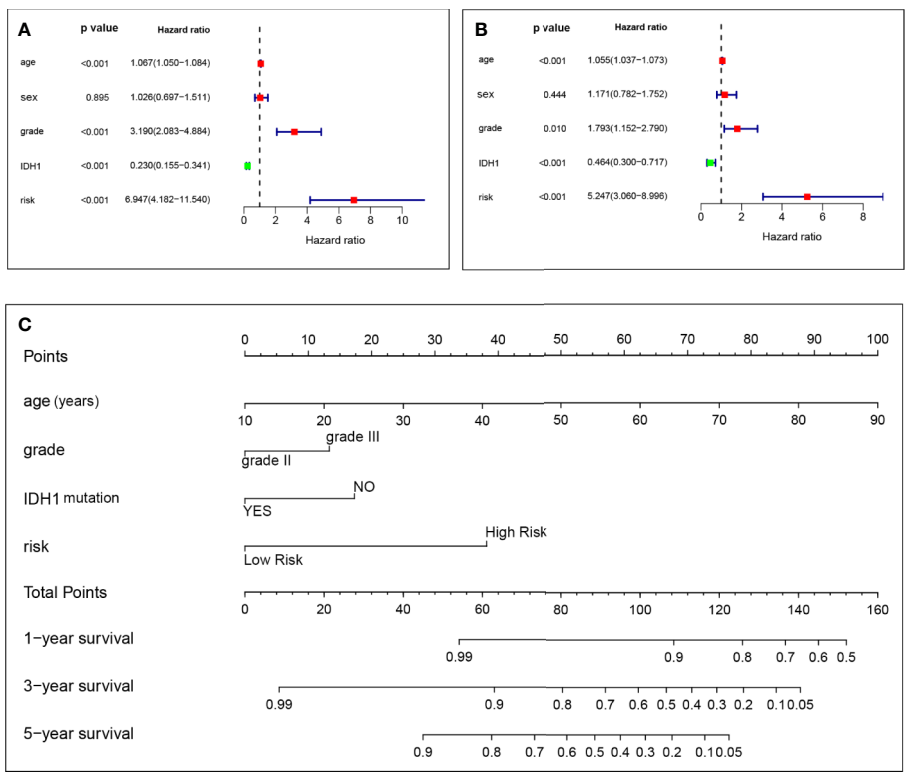

FIGURE 3 | Age, grade, IDH1, and immune-related risk are independent factors in the univariate **(A)** and multivariate **(B)** Cox proportional hazards regression. The nomogram for predicting the overall survival of an individual patient. The values of age, grade, IDH1, and risk are acquired from each variable axis. The total points on the axis are the sum values of these four factors, which can predict the 1-, 3-, and 5-year survival **(C)**.

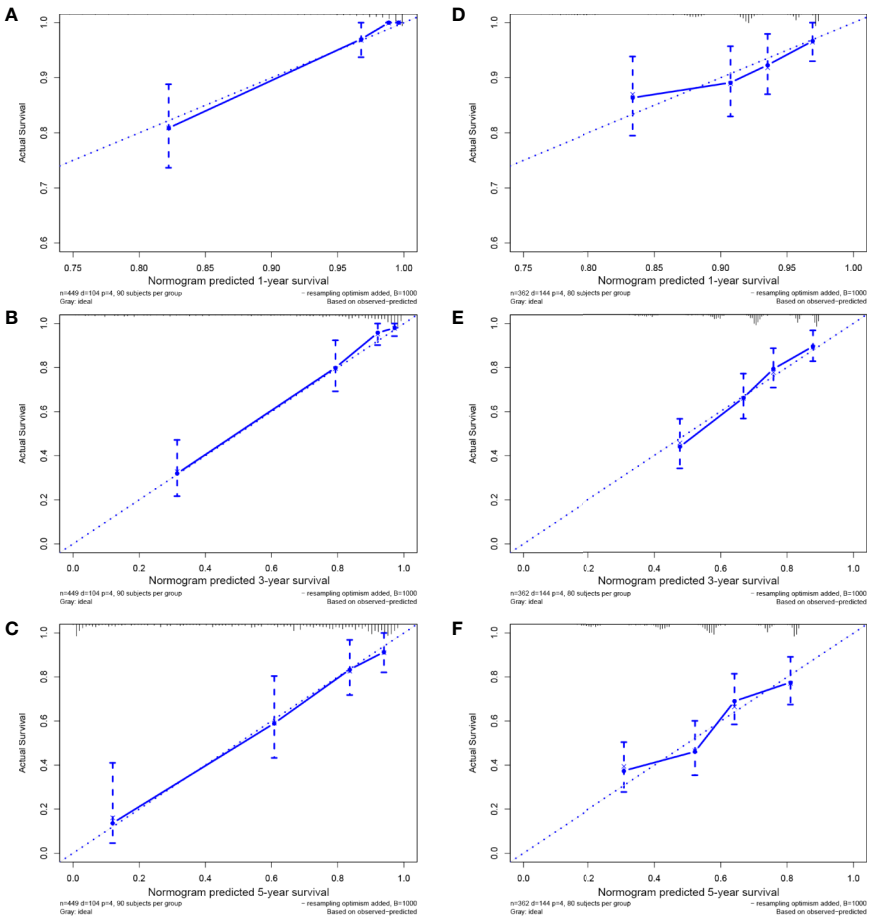

FIGURE 4 | Internal and external validations in the TCGA and CGGA groups. The calibration curves for predicting 1-, 3-, and 5-year survival in the training cohort **(A–C)** and in the testing cohort **(D–F)**.

ECM–receptor interaction, cytokine–cytokine receptor interaction, and so on, probably have a significant role in immune-related tumor growth procedure. Thus, the immune system of the host in the TME plays a critical role in dictating aberrant cellular function in advanced and refractory malignancies. How do the immune system and cancer cells affect each other? The answer to this question might be explained as follows: the immune system helps to fight against cancer, while cancer can weaken the immune system, and treatments may sometimes weaken the immune system. Immune cells, including B cells, CD4 T cells, CD8 T cells, neutrophil, macrophage, and dendritic cells, are the primary functional elements in the immune system. For example, high levels of macrophage infiltration had both positive and negative correlations with tumor growth. A positive effect of macrophage infiltration on prognosis was shown in colorectal cancer, while adverse effects were displayed in breast cancer, ovarian cancer, bladder cancer, and gastric cancer (37). A high density of tumor-infiltrated T cells correlated with a good prognosis in breast cancer (38), while an elevated level of neutrophils was associated with poor outcomes in renal cell carcinoma, colorectal cancer, and glioblastoma (39). Tumor-related immune escape is achieved by avoiding immune recognition and instigating an immunosuppressive environment. The mechanism of avoiding immune recognition by cytotoxic T cells involves losing tumor antigen expression (40). On the other hand, immune tolerant is instigated by secreting suppressive molecules (41), expressing inhibitory checkpoint molecules (42, 43), and inducing the recruitment of macrophages to drive chemokines (44).

In summary, the role of the immune system in LGG has not been fully elucidated, and this study provided available information about the immune system in the tumor formation process. We believe that the prognostic signature could provide insights into predictive biomarkers or therapeutic targets for patients with LGG. Furthermore, we look forward to using the nomogram for individual prognostic assessments. However, it should be noted that the signature was established based on 16 immune-related genes and has not been proven to be the best prognostic signature. Furthermore, we used the IDH1/2 mutation

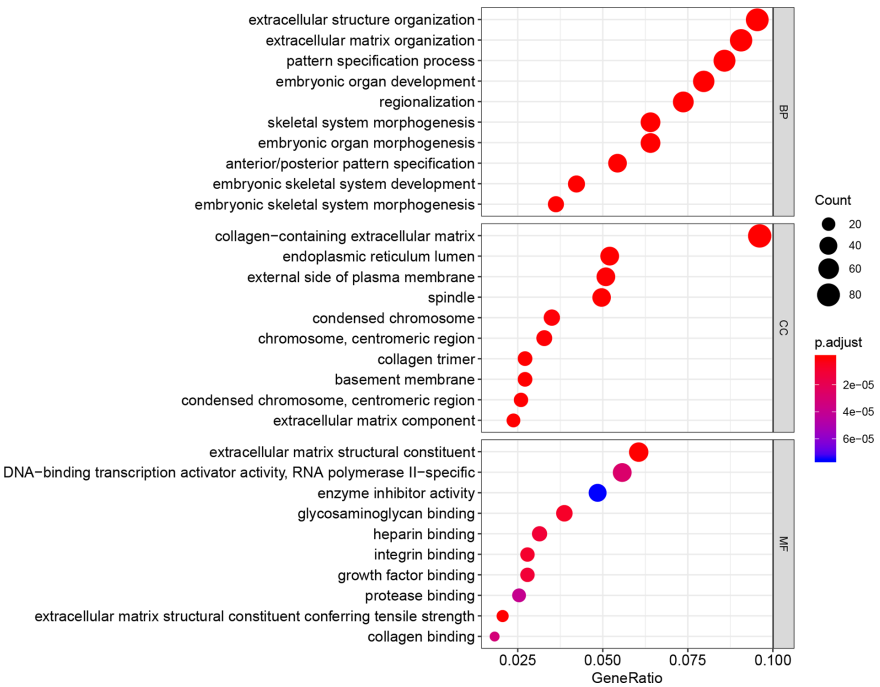

FIGURE 5 | GO analysis to the 1,263 DEGs between immune-related high-risk and low-risk groups shows the top 10 listed biological function in BP, CC, and MF.

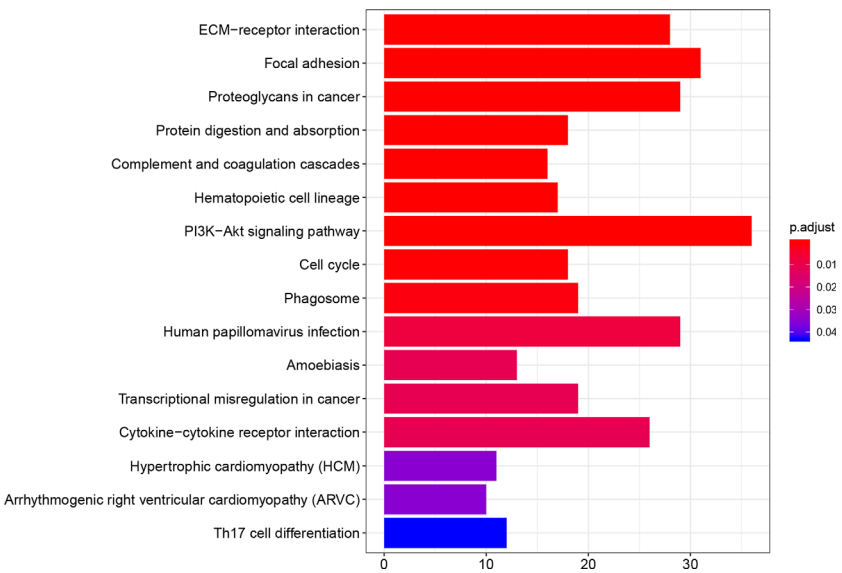

FIGURE 6 | KEGG pathway analysis to the 1,263 DEGs between immune-related high-risk and low-risk groups shows the probable pathways, and some of them were immune-related.

for the IDH1 mutation when validating in CGGA, which may lead to an imprecise validation. However, the incidence of IDH2 mutations in LGG is scarce; it was only 3.95% in TCGA, which can be neglected when validating the model.

CONCLUSIONS

The immune-related prognostic signature and the prognostic nomogram could accurately predict the survival.

AUTHOR'S NOTE

The mechanisms of the resistance to therapies should be identified not only in tumor cells but also in the tumor environment (TME).

Prognostic signature can be used as a novel approach predicting the prognosis of patients.

DATA AVAILABILITY STATEMENT

The RNA-seq data and corresponding clinical information were observed from the TCGA (https://portal.gdc.cancer.gov/) and CGGA (http://www.cgga.org.cn). The immune-related gene list was got from the IMMPORT website (https://www.immport.org/).

AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by HZ, XL, YL ZZ, and LS. The first draft of the manuscript was written by HZ, XL, and BC and all authors commented on previous versions of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.603341/full#supplementary-material

REFERENCES

1. Kiran M, Chatrath A, Tang X, Keenan DM, Dutta A. A Prognostic Signature for Lower Grade Gliomas Based on Expression of Long Non-Coding RNAs. *Mol Neurobiol* (2019) 56(7):4786–98. doi: 10.1007/s12035-018-1416-y
2. Cancer Genome Atlas Research N, Brat DJ, Verhaak RG, Aldape KD, Yung WK, Salama SR, et al. Comprehensive, Integrative Genomic Analysis of Diffuse Lower-Grade Gliomas. *New Engl J Med* (2015) 372(26):2481–98. doi: 10.1056/NEJMoa1402121
3. Huang J, Samson P, Perkins SM, Ansstas G, Chheda MG, DeWees TA, et al. Impact of concurrent chemotherapy with radiation therapy for elderly patients with newly diagnosed glioblastoma: a review of the National Cancer Data Base. *J Neuro Oncol* (2017) 131(3):593–601. doi: 10.1007/s11060-016-2331-6
4. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *New Engl J Med* (2005) 352(10):987–96. doi: 10.1056/NEJMoa043330
5. Garcia CR, Slone SA, Dolecek TA, Huang B, Neltner JH, Villano JL. Primary central nervous system tumor treatment and survival in the United States, 2004-2015. *J Neuro Oncol* (2019) 144(1):179–91. doi: 10.1007/s11060-019-03218-8
6. Ducray F, Marie Y, Sanson M. IDH1 and IDH2 mutations in gliomas. *New Engl J Med* (2009) 360(21):2248–2249; author reply 2249. doi: 10.1056/NEJMc090593
7. Wagle N, Emery C, Berger MF, Davis MJ, Sawyer A, Pochanard P, et al. Dissecting therapeutic resistance to RAF inhibition in melanoma by tumor genomic profiling. *J Clin Oncol Off J Am Soc Clin Oncol* (2011) 29(22):3085–96. doi: 10.1200/JCO.2010.33.2312
8. Meads MB, Gatenby RA, Dalton WS. Environment-mediated drug resistance: a major contributor to minimal residual disease. *Nat Rev Cancer* (2009) 9(9):665–74. doi: 10.1038/nrc2714
9. Junttila MR, de Sauvage FJ. Influence of tumour micro-environment heterogeneity on therapeutic response. *Nature* (2013) 501(7467):346–54. doi: 10.1038/nature12626
10. Gieryng A, Pszczolkowska D, Walentynowicz KA, Rajan WD, Kaminska B. Immune microenvironment of gliomas. *Lab Invest* (2017) 97(5):498–518. doi: 10.1038/labinvest.2017.19
11. Jia D, Li S, Li D, Xue H, Yang D, Liu Y. Mining TCGA database for genes of prognostic value in glioblastoma microenvironment. *Aging (Albany NY)* (2018) 10(4):592–605. doi: 10.18632/aging.101415
12. Goeman JJ. L1 penalized estimation in the Cox proportional hazards model. *Biom J* (2010) 52(1):70–84. doi: 10.1002/bimj.200900028
13. Qian Z, Li Y, Fan X, Zhang C, Wang Y, Jiang T, et al. Prognostic value of a microRNA signature as a novel biomarker in patients with lower-grade gliomas. *J Neuro Oncol* (2018) 137(1):127–37. doi: 10.1007/s11060-017-2704-5
14. Liu B, Liu J, Liu K, Huang H, Li Y, Hu X, et al. A prognostic signature of five pseudogenes for predicting lower-grade gliomas. *Biomed Pharmacother = Biomed Pharmacother* (2019) 117:109116. doi: 10.1016/j.biopha.2019.109116
15. Kamarudin AN, Cox T, Kolamunnage-Dona R. Time-dependent ROC curve analysis in medical research: current methods and applications. *BMC Med Res Methodol* (2017) 17(1):53. doi: 10.1186/s12874-017-0332-6
16. Michael H, Tian L, Ghebremichael M. The ROC curve for regularly measured longitudinal biomarkers. *Biostatistics* (2019) 20(3):433–51. doi: 10.1093/biostatistics/kxy010
17. Long J, Wang A, Bai Y, Lin J, Yang X, Wang D, et al. Development and validation of a TP53-associated immune prognostic model for hepatocellular carcinoma. *EBioMedicine* (2019) 42:363–74. doi: 10.1016/j.ebiom.2019.03.022
18. Wang Y, Li J, Xia Y, Gong R, Wang K, Yan Z, et al. Prognostic nomogram for intrahepatic cholangiocarcinoma after partial hepatectomy. *J Clin Oncol Off J Am Soc Clin Oncol* (2013) 31(9):1188–95. doi: 10.1200/JCO.2012.41.5984
19. Duan J, Xie Y, Qu L, Wang L, Zhou S, Wang Y, et al. A nomogram-based immunoprofile predicts overall survival for previously untreated patients with esophageal squamous cell carcinoma after esophagectomy. *J Immunother Cancer* (2018) 6(1):100. doi: 10.1186/s40425-018-0418-7
20. Park CK, Kim JH, Nam DH, Kim CY, Chung SB, Kim YH, et al. A practical scoring system to determine whether to proceed with surgical resection in recurrent glioblastoma. *Neuro Oncol* (2013) 15(8):1096–101. doi: 10.1093/neuonc/not069
21. Varlet P, , Le Teuff G, Le Deley MC, Giangaspero F, Haberler C, Jacques TS, et al. WHO grade has no prognostic value in the pediatric high-grade glioma

included in the HERBY trial. *Neuro Oncol* (2019) 22(1):116–27. doi: 10.1093/neuonc/noz142
22. Carlsson SK, Brothers SP, Wahlestedt C. Emerging treatment strategies for glioblastoma multiforme. *EMBO Mol Med* (2014) 6(11):1359–70. doi: 10.15252/emmm.201302627
23. Parks C, Heald J, Hall G, Kamaly-Asl I. Can the prognosis of individual patients with glioblastoma be predicted using an online calculator? *Neuro Oncol* (2013) 15(8):1074–8. doi: 10.1093/neuonc/not033
24. Razzak M. Genetics: new molecular classification of gastric adenocarcinoma proposed by The Cancer Genome Atlas. *Nat Rev Clin Oncol* (2014) 11(9):499. doi: 10.1038/nrclinonc.2014.138
25. Galon J, Pages F, Marincola FM, Thurin M, Trinchieri G, Fox BA, et al. The immune score as a new possible approach for the classification of cancer. *J Transl Med* (2012) 10:1. doi: 10.1186/1479-5876-10-1
26. Waszak SM, Northcott PA, Buchhalter I, Robinson GW, Sutter C, Groebner S, et al. Spectrum and prevalence of genetic predisposition in medulloblastoma: a retrospective genetic study and prospective validation in a clinical trial cohort. *Lancet Oncol* (2018) 19(6):785–98. doi: 10.1016/S1470-2045(18)30242-0
27. Nonoguchi N, Ohta T, Oh JE, Kim YH, Kleihues P, Ohgaki H. TERT promoter mutations in primary and secondary glioblastomas. *Acta Neuropathol* (2013) 126(6):931–7. doi: 10.1007/s00401-013-1163-0
28. Simon M, Hosen I, Gousias K, Rachakonda S, Heidenreich B, Gessi M, et al. TERT promoter mutations: a novel independent prognostic factor in primary glioblastomas. *Neuro Oncol* (2015) 17(1):45–52. doi: 10.1093/neuonc/nou158
29. Jones PS, Carroll KT, Koch M, DiCesare JAT, Reitz K, Frosch M, et al. Isocitrate Dehydrogenase Mutations in Low-Grade Gliomas Correlate With Prolonged Overall Survival in Older Patients. *Neurosurgery* (2019) 84(2):519–28. doi: 10.1093/neuros/nyy149
30. Zhang GH, Zhong QY, Gou XX, Fan EX, Shuai Y, Wu MN, et al. Seven genes for the prognostic prediction in patients with glioma. Clin Trans Oncol Off Publ Fed Spanish Oncol Societies Natl Cancer Institute Mexico (2019) 21 (10):1327–35. doi: 10.1007/s12094-019-02057-3
31. Guo XX, Su J, He XF. A 4-gene panel predicting the survival of patients with glioblastoma. *J Cell Biochem* (2019) 120(9):16037–43. doi: 10.1002/jcb.28883
32. Zhou M, Zhang Z, Zhao H, Bao S, Cheng L, Sun J. An Immune-Related Six-lncRNA Signature to Improve Prognosis Prediction of Glioblastoma Multiforme. *Mol Neurobiol* (2018) 55(5):3684–97. doi: 10.1007/s12035-017-0572-9
33. Shembrey C, Huntington ND, Hollande F. Impact of Tumor and Immunological Heterogeneity on the Anti-Cancer Immune Response. *Cancers (Basel)* (2019) 11:(9). doi: 10.3390/cancers11091217
34. Palucka AK, Coussens LM. The Basis of OncoImmunology. *Cell* (2016) 164 (6):1233–47. doi: 10.1016/j.cell.2016.01.049
35. Fridman WH, Pages F, Sautes-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer* (2012) 12 (4):298–306. doi: 10.1038/nrc3245
36. Ferrer VP, Moura Neto V, Mentlein R. Glioma infiltration and extracellular matrix: key players and modulators. *Glia* (2018) 66(8):1542–65. doi: 10.1002/glia.23309
37. Zhang QW, Liu L, Gong CY, Shi HS, Zeng YH, Wang XZ, et al. Prognostic significance of tumor-associated macrophages in solid tumor: a meta-analysis of the literature. *PloS One* (2012) 7(12):e50946. doi: 10.1371/journal.pone.0050946
38. Oldford SA, Robb JD, Codner D, Gadag V, Watson PH, Drover S. Tumor cell expression of HLA-DM associates with a Th1 profile and predicts improved survival in breast carcinoma patients. *Int Immunol* (2006) 18(11):1591–602. doi: 10.1093/intimm/dxl092
39. Donskov F. Immunomonitoring and prognostic relevance of neutrophils in clinical trials. *Semin Cancer Biol* (2013) 23(3):200–7. doi: 10.1016/j.semcancer.2013.02.001
40. Gonzalez H, Hagerling C, Werb Z. Roles of the immune system in cancer: from tumor initiation to metastatic progression. *Genes Dev* (2018) 32(19-20):1267–84. doi: 10.1101/gad.314617.118
41. Bottcher JP, Bonavita E, Chakravarty P, Blees H, Cabeza-Cabrerizo M, Sammicheli S, et al. NK Cells Stimulate Recruitment of cDC1 into the Tumor Microenvironment Promoting Cancer Immune Control. *Cell* (2018) 172(5):1022–1037 e1014. doi: 10.1016/j.cell.2018.01.004
42. Boger C, Behrens HM, Kruger S, Rocken C. The novel negative checkpoint regulator VISTA is expressed in gastric carcinoma and associated with PD-L1/PD-1: A future perspective for a combined gastric cancer therapy? *Oncoimmunology* (2017) 6(4):e1293215. doi: 10.1080/2162402X.2017.1293215
43. Dominguez-Soto A, Sierra-Filardi E, Puig-Kroger A, Perez-Maceda B, Gomez-Aguado F, Corcuera MT, et al. Dendritic cell-specific ICAM-3-grabbing nonintegrin expression on M2-polarized and tumor-associated macrophages is macrophage-CSF dependent and enhanced by tumor-derived IL-6 and IL-10. *J Immunol* (2011) 186(4):2192–200. doi: 10.4049/jimmunol.1000475
44. Weitzenfeld P, Ben-Baruch A. The chemokine system, and its CCR5 and CXCR4 receptors, as potential targets for personalized therapy in cancer. *Cancer Lett* (2014) 352(1):36–53. doi: 10.1016/j.canlet.2013.10.006

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Six Immune Associated Genes Construct Prognostic Model Evaluate Low-Grade Glioma

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Background: The immunotherapy of Glioma has always been a research hotspot. Although tumor associated microglia/macrophages (TAMs) proves to be important in glioma progression and drug resistance, our knowledge about how TAMs influence glioma remains unclear. The relationship between glioma and TAMs still needs further study.

Methods: We collected the data of TAMs in glioma from NCBI Gene Expression Omnibus (GEO) that included 20 glioma samples and 15 control samples from four datasets. Six genes were screened from the Differential Expression Gene through Gene ontology (GO) analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, protein–protein interaction (PPI) network and single-cell sequencing analysis. A risk score was then constructed based on the six genes and patients' overall survival rates of 669 patients from The Cancer Genome Atlas (TCGA). The efficacy of the risk score in prognosis and prediction was verified in Chinese Glioma Genome Atlas (CGGA).

Results: Six genes, including CD163, FPR3, LPAR5, P2ry12, PLAUR, SIGLEC1, that participate in signal transduction and plasma membrane were selected. Half of them, like CD163, FPR3, SIGLEC1, were mainly expression in M2 macrophages. FPR3 and SIGLEC1 were high expression genes in glioma associated with grades and IDH status. The overall survival rates of the high risk score group was significantly lower than that of the low risk score group, especially in LGG.

Conclusion: Joint usage of the 6 candidate genes may be an effective method to diagnose and evaluate the prognosis of glioma, especially in Low-grade glioma (LGG).

Keywords: glioma, tumor associated macrophage, single cell sequence, prognosis, biomarker

INTRODUCTION

Glioma is the most common primary tumor in central nervous system, accounting for 80% of all malignant brain tumors (1). Current glioma treatment frequently involves many ways, including surgery, radiation therapy, chemotherapy, immunotherapy (2), targeted therapy (3), and tumor treating fields (TTF) (4). Although modern aggressive comprehensive treatments are improving, the outcome for glioma remains quite poor. Gliomas are complexly composed of diverse malignant cells and nonmalignant cells, whose development in a special environment called tumor microenvironment (TME) (5). Among the myriad cell types, microglia, and infiltrating macrophages are known as tumor associated microglia/macrophages (TAMs), accounting for 30%~50% of the glioma mass (6). Through interactions with neoplastic cells, TAMs provide a tumor-favorable microenvironment that enable glioma to escape immune surveillance, consequently promoting glioma proliferation and metastasis (6). Therefore it is important to improve our understanding of the interactions between glioma and TAMs and then to develop more effective treatment strategies.

The TAMs of glioma are composed of two distinct populations, including tissue-resident microglia and bone marrow-derived macrophages (BMDMs) (7). According to the cell markers and functions of TAMs, they are divided into two phenotypes: the M1 macrophages phenotype is associated with inflammation playing a role in anti-tumor, while the M2 macrophages phenotype mediate the tumor growth by promoting the secretion of angiogenesis factor and immunosuppressive cytokine (8). *In vitro*, the similar dual phenotypes have been induced by exposure either to LPS/IFNγ or IL10/IL4 (9). More recently, the complex situation of TAMs had been discussed extensively and discovered the current M1 and M2 classification schemes are not absolute, other classifications based on the specific pathways or molecules are used to identify the phenotypes of TAMs (10). Whereas many research have revealed that the strategies converting M2 macrophages to M1 macrophages or inhibiting M2-polarization of TAMs suppressed tumor growth (11). However, the communication between glioma and TAMs is still unclear. To understand the glioma comprehensively and deeply, the study of TAMs is essential.

Here, we screened bulk-RNA sequencing and Single-cell-RNA sequencing data that compare TAMs of glioma with normal microglia collected from non-tumor samples from GEO database, analyzed the differential expression genes (DEGs) and then tested the relationship between DEGs and prognosis of glioma by using data from TCGA and CCGA. We found most of the DEGs between TAMs and non-tumor microglia are also the different genes between M1 and M2 macrophages. However the prognosis of low-grade glioma cannot be predicted by single gene from the DEGs passed through screening.Finally, we constructed a risk score based on the six genes by using TCGA database and verified it in CGGA database. Meanwhile we explored the role of SIGLEC1 (also known as CD169) and FPR3 in the prognosis and immunotherapy of glioma and thought them would be new biomarkers and targets in diagnoses and treatment of glioma.

MATERIALS AND METHODS

Patient Samples

The Ethics Committee of Wuhan University approved this study, and all experiments complied with the current laws of PR China. In total, three control samples from patients with cerebral hemorrhage and six glioma samples were collected during May 2020 and October 2020, including both low-grade glioma (grade I, one case; grade II, two cases) and glioblastoma multiform (grade IV, three cases). All patients provided written informed consent. Samples of tumor tissue were collected during surgery, snap-frozen in liquid nitrogen, and stored until experimental use. Patients were not treated with chemotherapy or radiotherapy before surgery.

Data Acquisition

This study acquired 20 glioma samples and 15 control samples from four datasets downloaded from NCBI Gene Expression Omnibus(GEO)(https://www.ncbi.nlm.nih.gov/geo/), including GSE80338, GSE115397, GSE135437, and GSE84465. The gene expression data and clinical data including grades, IDH status and survival time are downloaded from TCGA (669 patients) (https://www.cancer.gov/) and CGGA (1018 patients) (http://www.cgga.org.cn/) database.

Analysis of Differential Expression Gene

The bulk-RNASeq data was analyzed by limma package, while the scRNASeq data was analyzed by FindMarkers function of Seurat package. The DEGs in each of the three datasets were defined by using fold-change filtering (fold change >1) and padj <0.05, and then the up-regulated genes and down-regulated genes from each datasets were intersected, respectively.

GO and KEGG Pathway Analysis

The functions of the 64 DEGs were uploaded to DAVID database (https://david.ncifcrf.gov/) to be analyzed. Hierarchical clustering of the DEGs was performed according to the biological process, cell component, molecular function and KEGG pathways. The terms were in rank according to the counts and p-value <0.05 was thought significance.

Identification of Cell Types

Two scRNASeq data were pretreatment through the standard analysis process of Single cell analysis R package Seurat. Identification of cell types used specific cell markers acquired from the official CellMarker website (http://biocc.hrbmu.edu.cn/CellMarker/).

ICH Images Acquisition

The ICH images of normal brain and glioma were acquired from THE HUMAN PROTEIN ATLAS (https://www.proteinatlas.org/), Due to the lack of protein expression data of FPR3 in brain and glioma, we acquired the proteins expression data of the rest five genes and the four levels are distinguished according to the degree of staining, including High, Medium, Low, and Not detected. The number of patients with staining also acquired.

Quantitative Real-Time PCR and RNA Extraction

The extraction of total RNA from tissues and cells was carried out using the Trizol reagent (Invitrogen, USA). For the reverse transcription of RNA, the PrimeScript RT Reagent Kit (RR047A, Takara, Japan) was used to synthesize cDNA. Using SYBR Premix Ex Taq II (RR820A, Takara), we performed qPCR to detect mRNA levels following the specifications provided by the manufacturers. qPCR was performed on a 2.1 Real-Time PCR System using Bio-Rad CFX Manager (Bio-Rad, USA). The relative Ct method was adopted to compare the data of the experimental group and the control group, and β-actin was set as internal control. The primer sequences are listed in **Supplementary Table S2**.

Statistical Analysis

mRNA expression, $2^{-\Delta\Delta CT}$, as measured using RT-PCR in the different samples, was compared using One-way analysis of variance (ANOVA). Statistical analyses and visualization were performed in R 3.6.0. All the packages used in R were listed below: Cairo, ggplot2, ggplotify, Seurat, cowplot, survminer, survival, glmnet, ROCR, estimate, ggcorrplot, and ggpubr. The log-rank test was used in Kaplan-Meier survival analysis. Lasso regression was used to constructed prognostic model. Statistical significance was indicated in the figures as follows: ns $p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p <= 0.0001$.

RESULTS

Sixty-Four Genes Were Associated With the TAMs of Glioma

We first screened the GEO database and collected three datasets of TAMs in glioma, the GSE80338 and GSE115397 collected CD11b+ microglia/macrophages from glioma and normal brain tissue and sequenced using RNA sequencing, while the GSE135437 was using FACS sorted on lineage-negative live CD45-positive cells and sequenced using the mCEL-Seq2 protocol. The DEGs in each of the three datasets were defined by using fold-change filtering (fold change >1) and padj <0.05, the up-regulated genes and down-regulated genes from each datasets were intersected respectively. Finally, we got 43 up-regulated genes and 21 down-regulated genes (**Figure 1A**). A heatmap showed the expression of all this 64 DEGs in three datasets (**Figure 1B**). Among the 64 DEGs, we found many oncogenes such as HIF1A, VEGFA, TGFBI, and HBEGF. Meanwhile many immune cell markers were also included, like MAF, SALL1, MCF2L, CD83, CD163, and MSR1. A PPI network plot showed the interaction of the 64 DEGs (**Figure 1C**).

GO and KEGG Pathway Analysis of the 64 DEGs and the Relationship With M1/M2 Macrophage

To explore the function of the 64 DEGs, we performed GO and KEGG Pathway Analysis by uploading the DEGs into DAVID database. GO analysis showed the screened genes are involved in many important functions and structures. In BP category, most genes mainly enriched in signal transduction, rest of the DEGs enriched in the cell adhesion and angiogenesis that associated with the invasion and migration of glioma. In CC category, more than a third of the DEGs enriched in plasma membrane and integral component of plasma membrane. In the MF category, enriched terms included protein homodimerization activity, sequence-specific DNA binding, receptor binding, scavenger receptor activity, virus receptor activity, and glucocorticoid receptor binding (**Figure 2A**). The KEGG Pathway analysis revealed three pathways were involved such as Mineral absorption, HIF-1 signaling pathway and Cell adhesion molecules (CAMs) (**Figure 2B**). Due to the interaction between TAMs and glioma were mainly related to the signal transduction through the proteins in the plasma membrane and affect the invasion and migration of glioma, we narrowed the candidate genes down to 38 genes, subsequently choose the most interacted node genes, FPR3, and its interacted genes to explored further (**Figure 2C**).

According to the different biomarkers of M1/M2 macrophages, we defined the cell types of GSE135437 and studied the distribution of M1/M2 macrophages in GBM and control samples. Almost all M2 macrophages were in the GBM cells, while M1 macrophages were in the control cells (**Figures 2D, E**). Furthermore we explored the relationship between the biological process and cell types and found that the signal transduction and angiogenesis enriched in a subgroup of M2 macrophages, however the cell adhesion widely distributed in both control and GBM cells (**Figure 2F**). To verify this relationship, we used another GBM scRNASeq dataset GSE84465 that including neoplastic cells, TAMs and many other types of cells in glioma. In contrast, the signal transduction mainly distributed in M1 macrophages, though part of M2 macrophages also expressed the signal transduction proteins. Meanwhile the cell adhesion signal was in the neoplastic cells and M2 macrophages. The angiogenesis signal was still in the M2 macrophages (**Supplementary Figure 1A**).

The Distribution and Expression of Six Genes and the Relationship With M1/M2 Macrophage

Microglia and macrophages take a major proportion of GBM. According to the cell annotation of GSE84465, nearly half of the cells were immune cells. We redefined the immune cells to subdivide into M1/M2 macrophages and found that 18.47% of the GBM cells were M1 macrophages, 34.77% of the GBM cells were M2 macrophages and 28.92% of the GBM cells were neoplastic cells (**Figure 3A**). Consistently with GSE135437, In GSE84465, almost all the M2 macrophages were in the GBM cells, while the M1 macrophages were in the periphery cells (**Figures 3B, C**). Then we analyzed the distribution and expression of six genes, the results showed that CD163, FPR3, and SIGLEC1 were expressed almost exclusively in M2 macrophages, while LPAR5 was widely expressed in M1/M2 macrophages. In GSE84465, P2RY12 was mainly expression in M1 macrophages, but in another dataset, it expressed in both M1

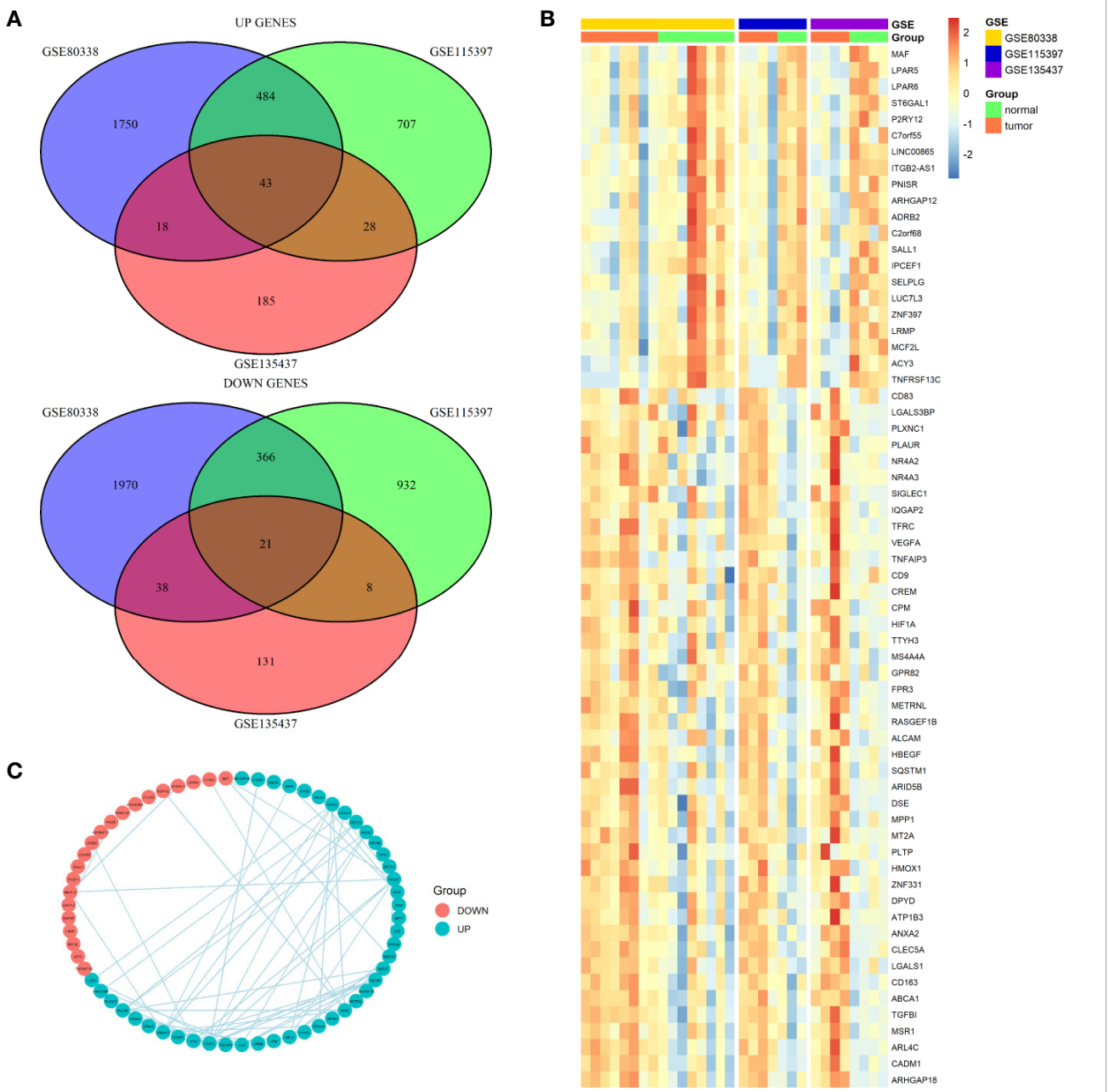

FIGURE 1 | Differential expression genes profiles in microglia/macrophage from glioma and normal brain tissue. **(A)** The overlapping significantly differentially expressed genes in microglia/macrophage of glioma vs. normal. There were significantly 43 upregulated and 21 downregulated genes in microglia/macrophage of glioma vs. normal. **(B)** Hierarchical clustering of the differentially expressed genes in three datasets. **(C)** PPI network map showed the interaction of the 64 DEGs.

and M2 macrophages. PLAUR was also not expressed in only one cell type (**Figure 3D** and **Supplementary Figure 1B**).

In consideration of the heterogeneity of GBM, each scRNASeq dataset only contained four couples of samples, we could not determine whether the difference between the two datasets reflected real features of the three genes. We determined to test the six genes in the TCGA and CCGA database.

FPR3 and SIGLEC1, Two Novel Potential Diagnostic Biomarkers for Glioma

The six genes were analyzed by using TCGA and CGGA database respectively. According to the tumor grades, IDH states, we tested all the six genes and found that the expression of LPAR5 had no differences in both tumor grades and IDH states in CGGA database, while in TCGA database, the expression of

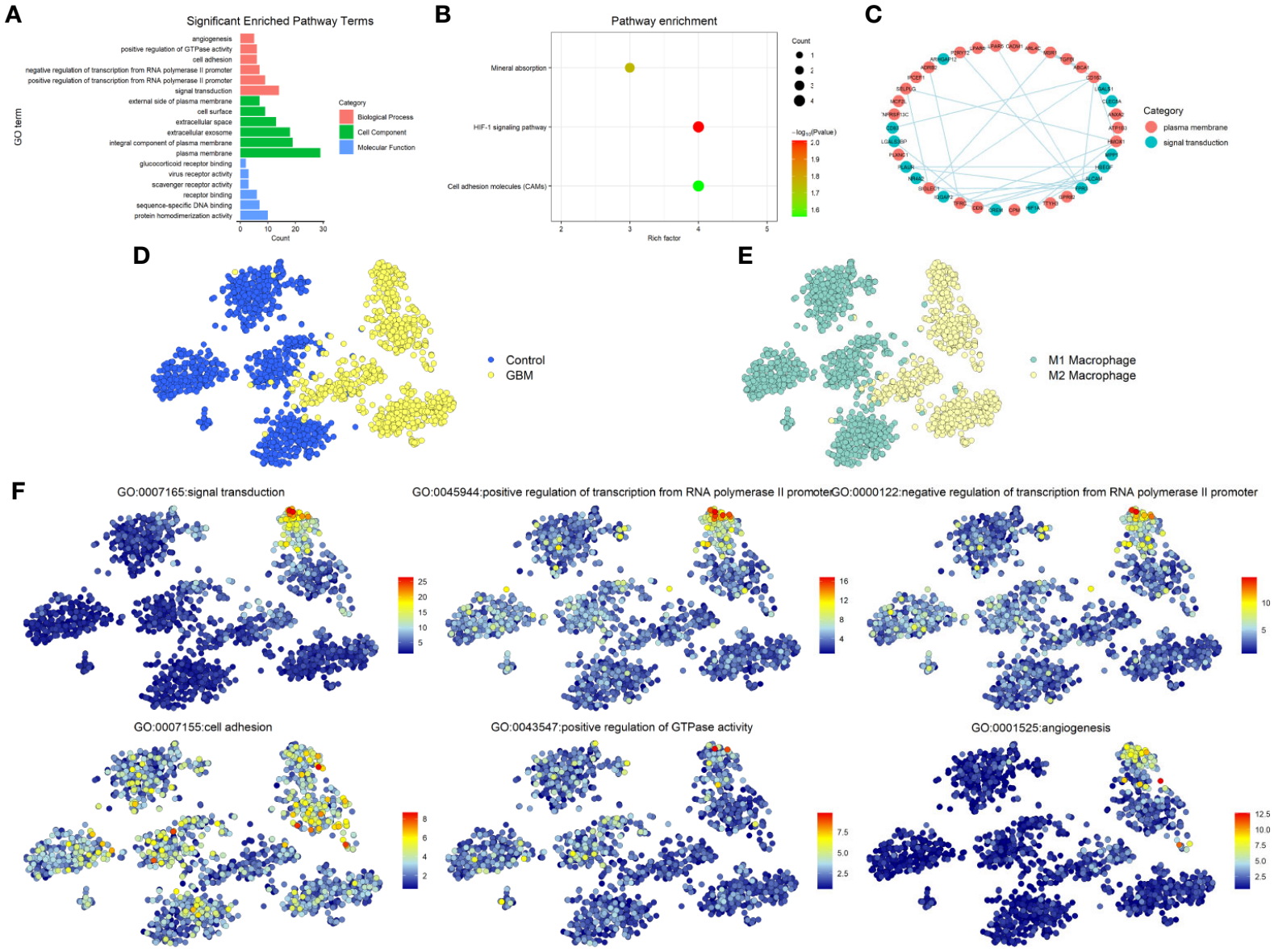

FIGURE 2 | Functional enrichment analysis. **(A)** Gene ontology (GO) analysis for 64 DEGs. Pink indicates biological process (BP), green indicates molecular function (MF), and blue indicates cellular component (CC). **(B)** KEGG Pathway analysis for 64 DEGs and shows significantly enriched signaling pathways. **(C)** PPI network map showed the interaction of the 38 genes from cell adhesion, plasma membrane and Cell adhesion molecules. **(D)** The distribution of GBM and Control cells in GSE135437. **(E)** The distribution of status of macrophages in GSE135437. **(F)** The distribution of top-six biological processes in GSE135437.

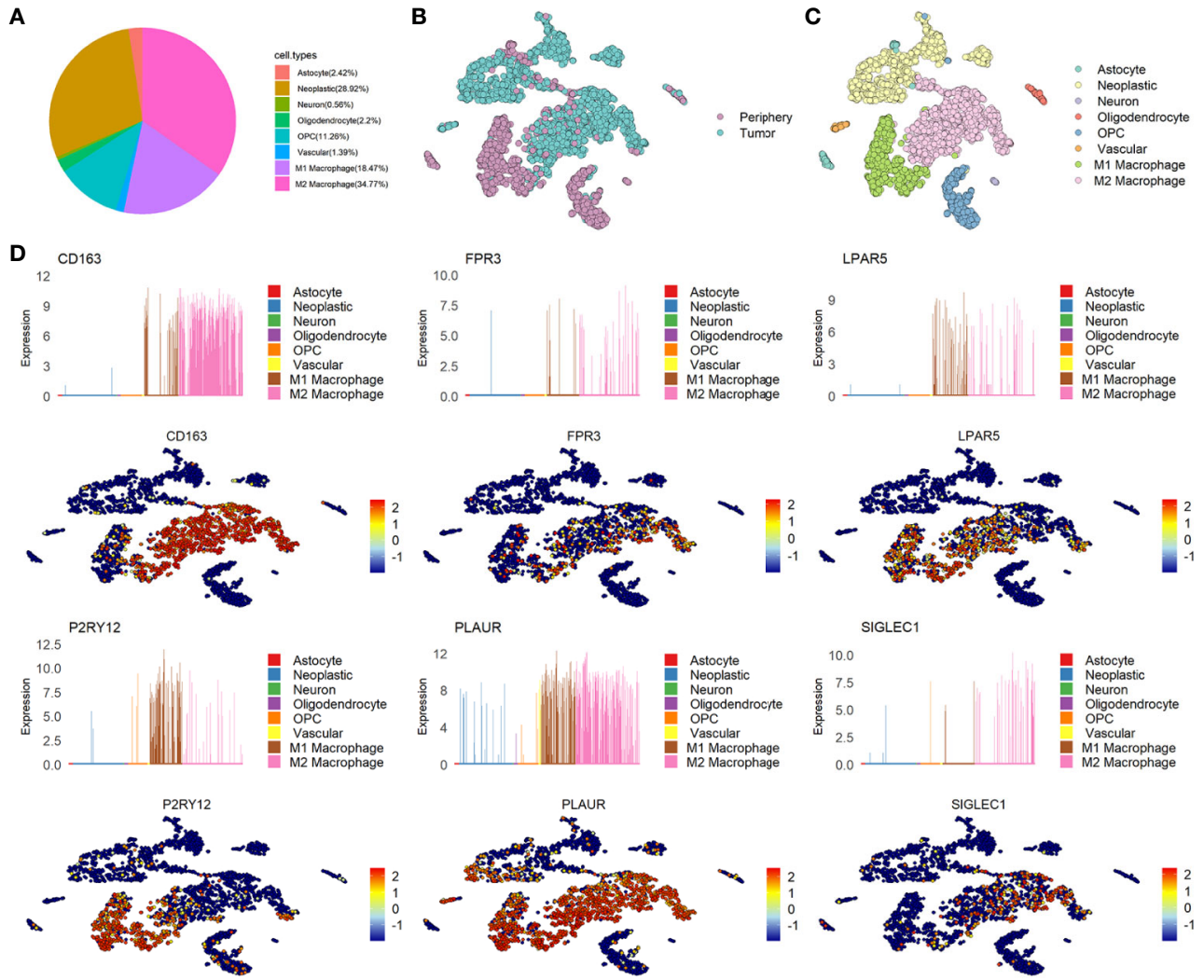

FIGURE 3 | The distribution and expression of six candidate genes in glioma. **(A)** The percentage of each type of cells in glioma. **(B)** The distribution of Tumor and Periphery cells in GSE84465. **(C)** The distribution of each type of cells in glioma in GSE84465. **(D)** The distribution and expression of six candidate genes in GSE84465.

LPAR5 still had no differences between grade II and grade III. However, it can be used to differentiate glioma between grade II and grade IV. The differential expression of other five genes was significant and could be used to well distinguish among different grades and IDH states (**Figures 4A–D**).

Some researches had studied CD163, P2RY12 and PLAUR as biomarkers in glioma. Our TCGA and CGGA analysis results were consistent with the previous studies. However, the role of FPR3 and SIGLEC1 in glioma still not be explored. On account of the six genes were screened from immune cells, we divide gliomas into four groups in line with immune score and stromal score. Unfortunately, only the differential expression of CD163 in TCGA database can distinguish the high or low of immune score and stromal score, while FPR3 just only had a difference between the high score and low score of immune score in CGGA database (**Supplementary Figures 2A–D**).

The protein expression of six genes in glioma and normal brain were acquired from THE HUMAN PROTEIN ATLAS. However, no protein expression information of FPR3 in brain or glioma was found in the database. The expression of PLAUR and SIGLEC1 were not detected, while the expression of P2RY12 protein was high in both normal brain and glioma. The expression of CD163 and LPAR5 protein were lower in normal brain than glioma (**Supplementary Figure 4A**). The number of patients with staining of each protein was shown in **Supplementary Figure 4B**.

Then we performed qPCR to detect the mRNA expression of six genes in normal brain, LGG and GBM. The results revealed that the expression of CD163 and FPR3 were increasing in glioma, especially in GBM, the expression of P2RY12 was high in glioma, but more notable in LGG. SIGLEC1 was higher in GBM but not be detected in LGG. PLAUR was similar to SIGLEC1 and LPAR5 was higher in normal brain (**Supplementary Figure 4C**).

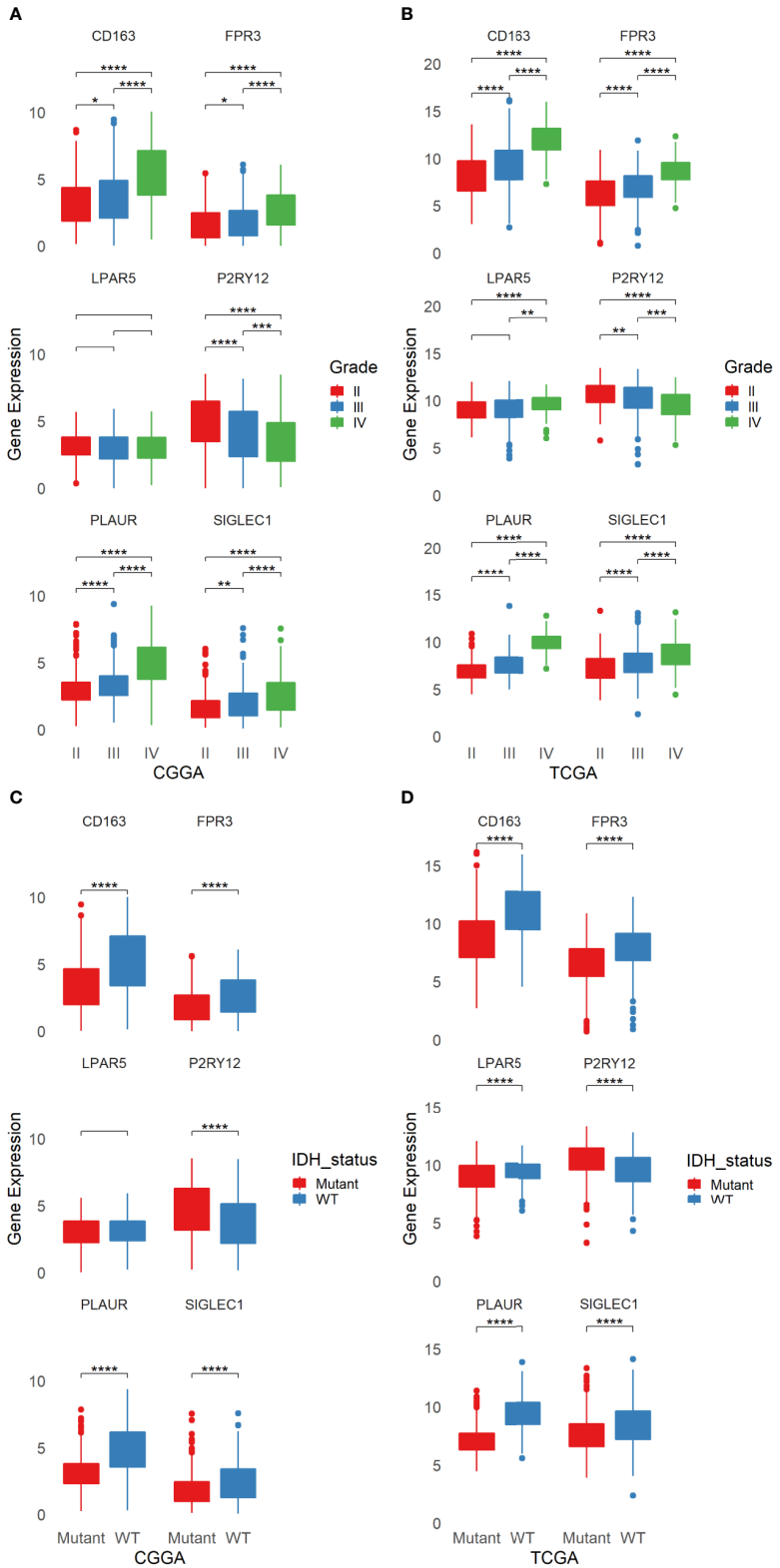

FIGURE 4 | The Expression of six candidate genes in glioma from TCGA and CGGA. **(A)** The expression of six candidate genes in different grades of glioma from CGGA. **(B)** The expression of six candidate genes in different grades of glioma from TCGA. **(C)** The expression of six candidate genes in different status of IDH in glioma from CGGA. **(D)** The expression of six candidate genes in different status of IDH in glioma from TCGA. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p <= 0.0001$.

Prognostic Model Based on Six Candidate Genes Well Evaluate the Prognosis of LGG

In order to analysis the effects of the six genes for prognosis in different grade glioma, We separated patients from TCGA and CGGA into four groups: TCGA LGG, TCGA GBM, CGGA LGG, and CGGA GBM. The analysis of TCGA LGG revealed that patients whose glioma expression high or low of LPAR5 had different outcomes, and consistent with LPAR5, the expression of other five genes all had a relationship with outcomes. The low expression of CD163, LPAR5, PLAUR, FPR3, and SIGLEC1 stand for a better outcomes and survival rates, while P2RY12 had the opposite outcomes (**Figure 5B**). Similarly, CD163, LPAR5, PLAUR, FPR3, and SIGLEC1 had the same effects in CGGA LGG, but P2RY12 had no effects (**Figure 5A**). However, only PLAUR could distinguish the prognosis of TCGA GBM (**Supplementary Figure 3B**) and CD163, PLAUR, and FPR3 could distinguish the prognosis of CGGA GBM (**Supplementary Figure 3A**).

As glioma is a multi-gene disordered disease, we tried to construct a multi-gene model to evaluate the prognosis of LGG. Univariate/multivariate Cox regression analysis were performed to show the prognostic significance of six genes in LGG/GBM patients (**Table 1**). Lasso regression analysis was performed and risk score was calculated by the following formula: risk score = 0.15934970*expression(LPAR5)-0.03816307*expression (CD163)-0.07363766*expression(FPR3)-0.28186165*expression (P2RY12)+0.60211778*expression(PLAUR)+0.09642036* expression(SIGLEC1). The prognostic model was constructed by

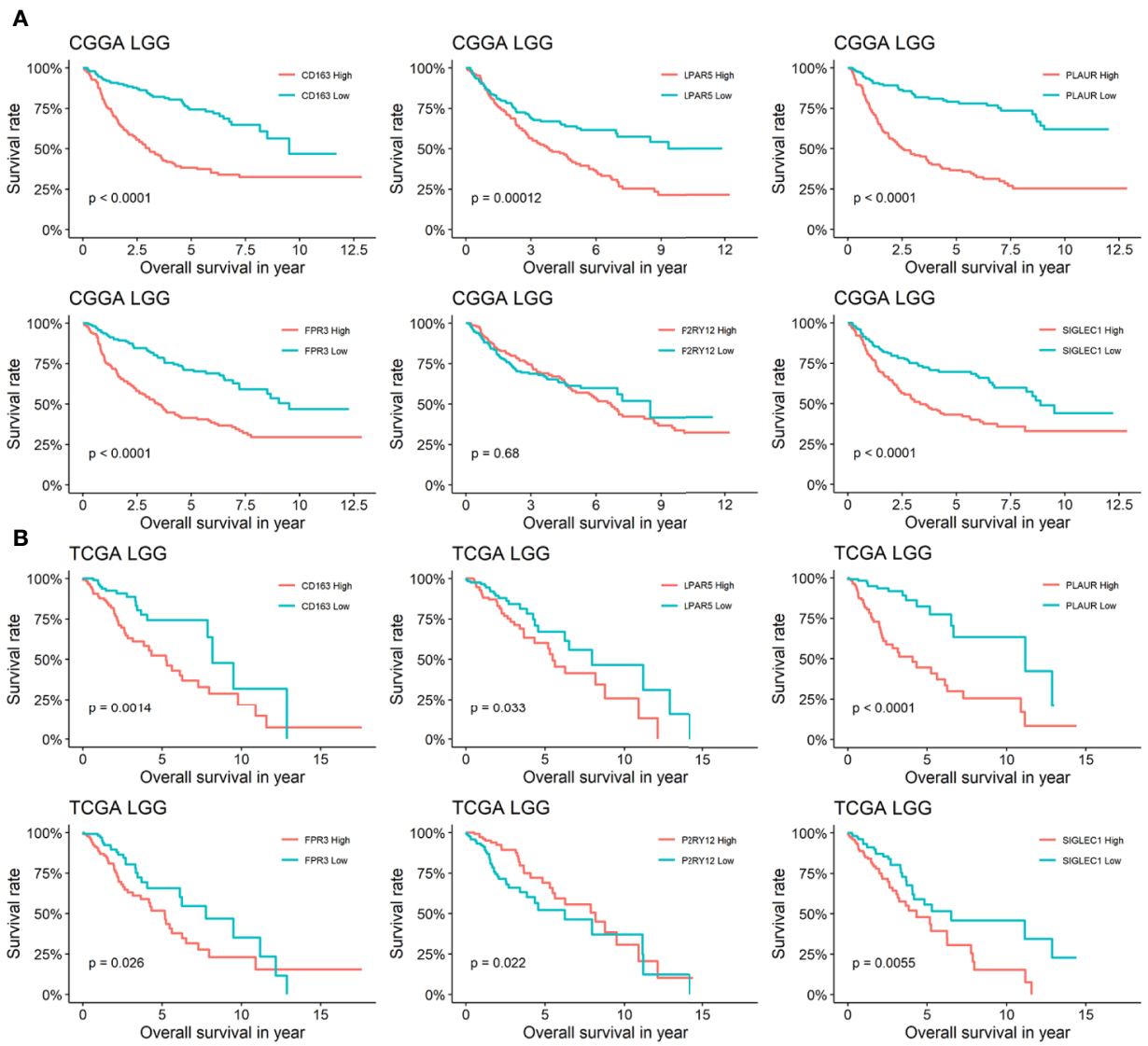

FIGURE 5 | Survival analysis of six genes in LGG. **(A)** Kaplan-Meier curves for CD163, LPAR5, PLAUR, FPR3 P2RY12 and SIGLEC1 of LGG in CGGA. **(B)** Kaplan-Meier curves for CD163, LPAR5, PLAUR, FPR3 P2RY12, and SIGLEC1 of LGG in TCGA.

using TCGA data and verified in CGGA database. The AUC of TCGA and CCGA were 0.784 and 0.736, respectively (**Figure 6C**). K-M curves confirmed that the risk score could well predict the survival of both LGG and HGG patients (**Figures 6A, B**). The AUC of LGG from TCGA and CCGA were 0.666 and 0.683, respectively (**Figure 6D**) and the AUC of GBM from TCGA and

TABLE 1 | Univariate/multivariate Cox regression analysis of six genes in LGG/GBM patients.

Gene	LGG				GBM			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	HR(95%CI)	P-value	HR(95%CI)	P-value	HR(95%CI)	P-value	HR(95%CI)	P-value
CD163	1.172(1.085-1.267)	<0.001*	0.934(0.832–1.049)	0.251	1.089(0.986–1.202)	0.093	0.903(0.765–1.065)	0.225
FPR3	1.166(1.051–1.293)	0.004*	0.889(0.773–1.023)	0.101	1.077(0.941–1.232)	0.281	0.971(0.772–1.220)	0.800
LPAR5	1.166(1.051–1.293)	0.004*	1.456(1.109–1.912)	0.007*	0.950(0.799–1.130)	0.561	0.851(0.572–1.267)	0.428
P2RY12	0.843(0.755–0.941)	0.002*	0.651(0.534–0.794)	<0.001*	0.893(0.794–1.004)	0.058	0.934(0.744–1.173)	0.558
PLAUR	0.843(0.755–0.941)	0.002*	1.576(1.313–1.893)	<0.001*	1.322(1.105–1.581)	0.002*	1.639(1.211–2.219)	0.001*
SIGLEC1	1.275(1.147–1.416)	<0.001*	1.161(1.015–1.329)	0.029*	1.060(0.949–1.183)	0.302	1.098(0.959–1.256)	0.177

CI, confidence interval; LGG, Low-grade glioma; GBM, Glioblastoma; HR, Hazard ratio.
**P < 0.05.*

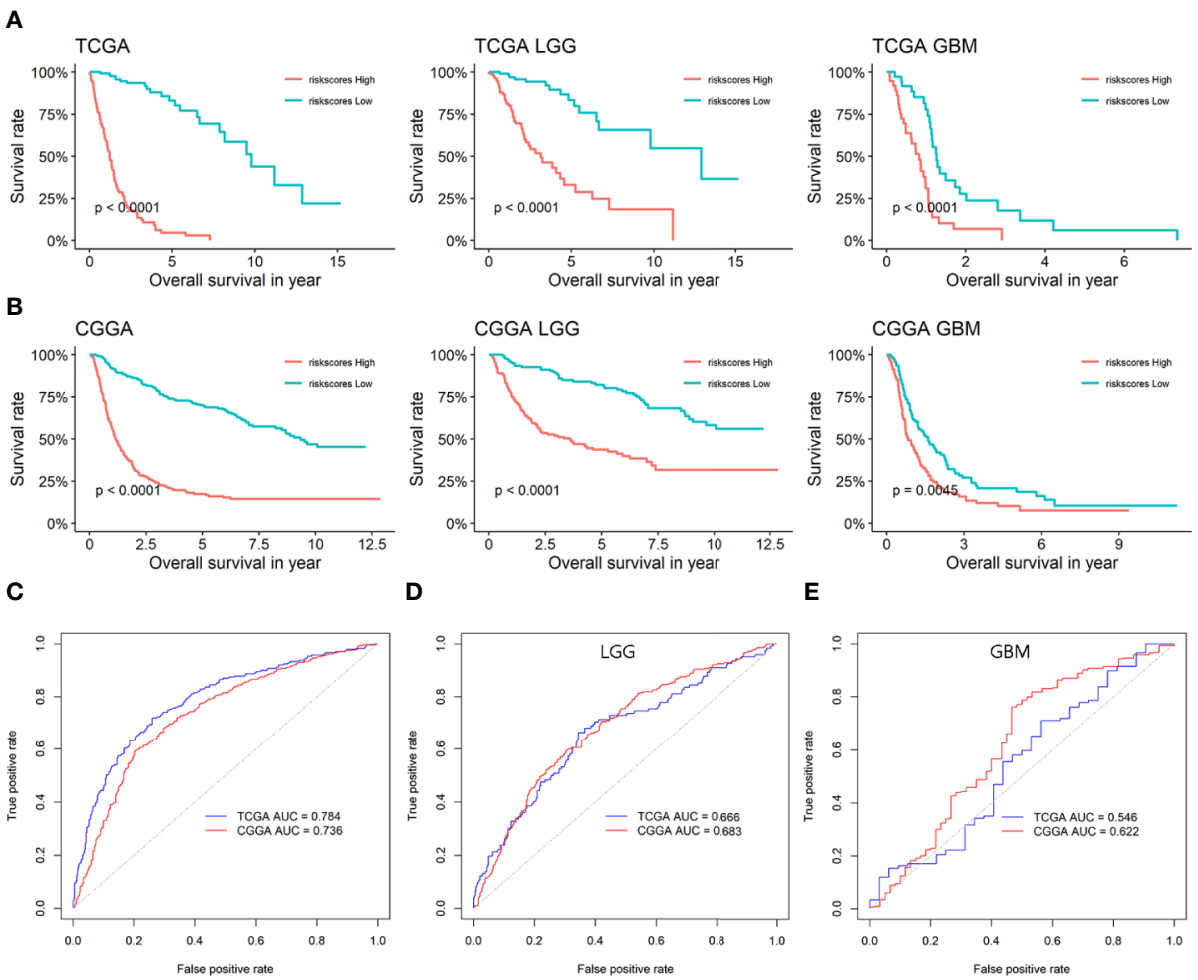

FIGURE 6 | The prognostic efficiency of the six candidate genes and prognostic model. **(A)** Survival analysis of risk score in TCGA. **(B)** Survival analysis of risk score in CGGA. **(C)** ROC curves of the prognostic model based on the six candidate genes. **(D)** ROC curves of the prognostic model in LGG based on the six candidate genes. **(E)** ROC curves of the prognostic model in GBM based on the six candidate genes.

CCGA were 0.546 and 0.622, respectively (**Figure 6E**). The correlation between six genes and immune checkpoint also performed and shown in **Supplementary Figure 5**.

DISCUSSION

In recent years, many studied have highlighted the importance of tumor immune microenvironment in glioma and this has been the subject of intense research (12, 13). Despite the rapid development of tumor immunity research have promoted our understanding of glioma, the immunotherapy for glioma is still far from satisfactory (14). Thus, looking for more immune targets is still needed. Recently, many methods have emerged to predict glioma prognosis based on immune and stromal scores (15–18). Meanwhile, similar methods have been used in many other solid tumor studies to predict prognosis of patients (19–21). In previous studies, bulk RNASeq data downloaded from TCGA and CGGA were used to seek the immune-gene related signatures to evaluate the risk of LGG or GBM. We summarized some researches about immune-related gene to predict prognosis of LGG or GBM listed in **Table S1** (15–18, 22–25). In consideration of the bias of bulk RNASeq data due to mixed cell type in tumor, we performed scRNASeq analysis to target TAMs and found 64 genes that differentially expressed between microglial and TAMs. Although many oncogenes are included in the DEGs, the interaction between TAMs and glioma thought to be taken place in plasma membrane, where cytokines and receptor combined and consequently changes the receptor cells to activate glioma and/or repress immune cell functions (26–28). So we narrowed the DEGs down to 38 genes that are contained in the signal transduction and plasma membrane. In addition, PPI network analysis showed that FPR3 had the most interacting proteins, such as CD163, P2RY12, LPAR5, PLAUR, and SIGLEC1. So we focused on this six genes and made a further research.

Previous studies have shown that CD163 is a biomarker that distinguish between M1 and M2 macrophages and correlated with survival times (29). Similar to our study, Liu (30) analysis a large scale glioma data and revealed that CD163 showed a positive relationship with immune cell populations in glioma and was up-regulated in IDH wild-type glioma. Meanwhile CD163 regulates the stemness of glioma (31), anti-PD-L1 antibody treatment significantly reduced infiltration of CD163 + macrophage in glioma (32). Hence, CD163 might serve as a therapeutic target for glioma. P2RY12 is also relevant to M1 and M2 macrophages according to its location in cells nuclear or cytoplasmic (33) and also differentially expressed between microglia and peripheral monocytes/macrophages in health and glioma (34). Otherwise, P2RY12 is involved in platelet aggregation (35) and is identified as key microglial surface marker (36). LPAR5 is one of the LPA receptor members, of which LPAR1 had been explored in glioma (37), but LPAR5 had been researched in promoting fibrosarcoma (38) and thyroid cancer (39). LPA signaling *via* LPA receptors contributes to the promotion of proliferation, invasion, and metastasis of tumor (40). Otherwise, LPA also regulate immune functions and inflammation (41). In papillary thyroid carcinoma, the LPAR5 is associated with immune infiltration (42). The function of LPAR5 in glioma still unclear, further research is still necessary. PLAUR encodes the urokinase receptor, which is influenced by hypoxia and promotes cell migration in GBM (43, 44). In polyautoimmunity, PLAUR contributes to regulation of apoptotic processes (45). The role of PLAUR is localizing and promoting plasmin formation (46), so the function of PLAUR may related to cell-surface plasminogen activation and localized degradation of the extracellular matrix. SIGLEC1 (also known as CD169), is also abnormal expression in peripheral macrophages of many cancers (47, 48), especially in the lymph node (49–52). The SIGLECs were investigated in glioma (53, 54); however, SIGLEC1 was excluded. The previous study showed Sialoadhesin encoded by SIGLEC1 was undetectable in normal human brain microglia, however was intensely detected in perivascular macrophages (55). This enlightened us that parts of the M2 macrophages of glioma were recruited from periphery. Our ICH images acquired from THE HUMAN PROTEIN ATLAS showed the SIGLEC1 was not detected in both normal brain and glioma. A large sample survey is needed to identify the expression of SIGLEC1 in glioma. FPR3 is Formyl peptide receptor 3, which together with other members of Formyl peptide receptor family been implicated in the regulation of tissue repair and angiogenesis (56). In glioma, the Formyl peptide receptor (FPR, also called FPR1) can regulate the invasion, angiogenesis and growth of tumor (57, 58), however, the function of FPR3 in glioma is still unclear. FPR3 was considered to be a pathogen sensor, due to the up-regulated after stimulation with a bacterial endotoxin (59). Interestingly, the migration of CD4+ T cell can be regulated by FPR3 (60). In consideration of FPR3 expression is mainly in monocytes and relates with the grade, IDH status, and prognosis, it is very promising to be a novel biomarker for glioma.

According to the scRNASeq data, we showed some biological progress enriched in specific cells. The phenotype of macrophages was related to whether the cell is neoplastic or not. The M2 macrophages mainly gathered in neoplastic cells, while the M1 macrophages located in non-neoplastic cells. This phenomenon is consistent with previous research (61).

To analysis the scRNASeq data, we found CD163, SIGLEC1, and FPR3 were mainly located in M2 macrophages, the P2RY12 was both detected in M1 and M2 macrophages, nevertheless a large part of P2RY12 were in M1 macrophages. Previous studies have suggested that the cytoplasmic expression of P2RY12 is associated with the expression of M1 markers and low-grade glioma, while the nuclear expression of P2RY12 is associated with the expression of M2 markers and high-grade glioma (33). The level of mRNA expression of P2RY12 may not be used as an indicator to differentiate M1 and M2 macrophages compared with the location of P2RY12 protein in cell. PLAUR and LAPR5 showed inconsistent results between two scRNASeq data. Heterogeneity of glioma makes it difficult to determine the resource of differential expression, both two scRNASeq data only have four couples of samples. For a better understand all

the six genes, we analyzed the six genes on the basis of grade, IDH statue, immune score, and stromal score. The expression of LPAR5 had no difference, no matter according to grade nor IDH statue; however, the ability of six genes to predict prognosis in LGG was more efficient than them in GBM. Although we screened the six genes from macrophages, only CD163 in TCGA database can distinguish the high or low of immune score and stromal score. The immune score and stromal score are calculated based on 141 stromal signature genes and 141 immune signature genes respectively, and SIGLEC1 is one of the stromal signature genes. The possible explanation is that too many other stromal and immune that not very important diluted the effect of this six genes. A further research in this field may provide the answer someday. Similarly, the relationship between SIGLEC1 and CD163 in glioma also need to be further studied. In view of the types and proportion of immune cells infiltrated in glioma were different between different grades (62), multi-genes may be a suitable method to evaluate statue of glioma, we constructed a prognostic model by the six genes and verified it. The results showed the prognosis of LGG can be predicted more efficient by this prognostic model than GBM. Especially the ability to predict the outcomes of LGG makes the model a more comprehensive evaluation method, addition with the SIGLEC1 and FPR3 could be two novel biomarkers to estimate grade and IDH status of glioma and six genes are correlated with immune checkpoint, the model will be helping for the diagnosis and treatment of glioma, in particular with respect to evaluate LGG.

CONCLUSION

In summary, The six genes construct a prognostic model to predict the outcomes of LGG and are correlated with immune checkpoint which provide a valuable role in diagnosis, prognosis, and immunotherapy of glioma.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Renmin Hospital of Wuhan University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YT contributed to publication search, data extraction, and draft writing. YL, TY, and XY contributed to the quality assessment and editing. BL and JY contributed to the statistical analysis. HZ and QC are correspondence authors and contributed equally to the conception and design. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.606164/full#supplementary-material

SUPPLEMENTARY TABLE 1 | A comparison between the previous studies and our study in immune-related genes in glioma.

SUPPLEMENTARY TABLE 2 | The primer sequence of each gene.

SUPPLEMENTARY FIGURE 1 | (A) The distribution of top-six biological processes in GSE84465. **(B)** The distribution and expression of six candidate genes in GSE135437.

SUPPLEMENTARY FIGURE 2 | (A) The expression of six candidate genes in different immune score group from CGGA. **(B)** The expression of six candidate genes in different immune score group from TCGA. **(C)** The expression of six candidate genes in different stromal score group from CGGA. **(D)** The expression of six candidate genes in different stromal score group from TCGA.

SUPPLEMENTARY FIGURE 3 | Survival analysis of six genes in GBM. **(A)** Kaplan-Meier curves for CD163, LPAR5, PLAUR, FPR3 P2RY12 and SIGLEC1 of GBM in CGGA. **(B)** Kaplan-Meier curves for CD163, LPAR5, PLAUR, FPR3 P2RY12 and SIGLEC1 of GBM in TCGA.

SUPPLEMENTARY FIGURE 4 | (A) The ICH images acquired from THE HUMAN PROTEIN ATLAS. **(B)** The number of patients with staining of each protein. **(C)** The mRNA expression of six genes in normal brain and glioma.

SUPPLEMENTARY FIGURE 5 | (A) The correlation between six genes and immune checkpoint in TCGA. **(B)** The correlation between six genes and immune checkpoint in CGGA.

REFERENCES

1. Goodenberger ML, Jenkins RB. Genetics of adult glioma. *Cancer Genet* (2012) 205:613–21. doi: 10.1016/j.cancergen.2012.10.009

2. Chheda ZS, Kohanbash G, Okada K, Jahan N, Sidney J, Pecoraro M, et al. Novel and shared neoantigen derived from histone 3 variant H3.3K27M mutation for glioma T cell therapy. *J Exp Med* (2018) 215:141–57. doi: 10.1084/jem.20171046

3. Lin L, Cai J, Jiang C. Recent Advances in Targeted Therapy for Glioma. *Curr Med Chem* (2017) 24:1365–81. doi: 10.2174/0929867323666161223150242
4. Anthony P, McArdle S, McHugh M. Tumor Treating Fields: Adjuvant Treatment for High-grade Gliomas. *Semin Oncol Nurs* (2018) 34:454–64. doi: 10.1016/j.soncn.2018.10.007
5. Wang Q, Hu B, Hu X, Kim H, Squatrito M, Scarpace L, et al. Tumor Evolution of Glioma-Intrinsic Gene Expression Subtypes Associates with Immunological Changes in the Microenvironment. *Cancer Cell* (2017) 32:42–56. doi: 10.1016/j.ccell.2017.06.003
6. Hambardzumyan D, Gutmann DH, Kettenmann H. The role of microglia and macrophages in glioma maintenance and progression. *Nat Neurosci* (2016) 19:20–7. doi: 10.1038/nn.4185
7. Bowman RL, Klemm F, Akkari L, Pyonteck SM, Sevenich L, Quail DF, et al. Macrophage Ontogeny Underlies Differences in Tumor-Specific Education in Brain Malignancies. *Cell Rep* (2016) 17:2445–59. doi: 10.1016/j.celrep.2016.10.052
8. Quail DF, Joyce JA. The Microenvironmental Landscape of Brain Tumors. *Cancer Cell* (2017) 31:326–41. doi: 10.1016/j.ccell.2017.02.009
9. Huang X, Li Y, Fu M, Xin HB. Polarizing Macrophages In Vitro. *Methods Mol Biol* (2018) 1784:119–26. doi: 10.1007/978-1-4939-7837-3_12
10. Zhou D, Huang C, Lin Z, Zhan S, Kong L, Fang C, et al. Macrophage polarization and function with emphasis on the evolving roles of coordinated regulation of cellular signaling pathways. *Cell Signal* (2014) 26:192–7. doi: 10.1016/j.cellsig.2013.11.004
11. Dong H, Yang Y, Gao C, Sun H, Wang H, Hong C, et al. Lactoferrin-containing immunocomplex mediates antitumor effects by resetting tumor-associated macrophages to M1 phenotype. *J Immunother Cancer* (2020) 8. doi: 10.1136/jitc-2019-000339
12. Strepkos D, Markouli M, Klonou A, Piperi C, Papavassiliou AG. Insights in the immunobiology of glioblastoma. *J Mol Med (Berl)* (2020) 98:1–10. doi: 10.1007/s00109-019-01835-4
13. Catalano M, D'Alessandro G, Trettel F, Limatola C. Role of Infiltrating Microglia/Macrophages in Glioma. *Adv Exp Med Biol* (2020) 1202:281–98. doi: 10.1007/978-3-030-30651-9_14
14. Sampson JH, Gunn MD, Fecci PE, Ashley DM. Brain immunology and immunotherapy in brain tumours. *Nat Rev Cancer* (2020) 20:12–25. doi: 10.1038/s41568-019-0224-7
15. Yin W, Jiang X, Tan J, Xin Z, Zhou Q, Zhan C, et al. Development and Validation of a Tumor Mutation Burden-Related Immune Prognostic Model for Lower-Grade Glioma. *Front Oncol* (2020) 10:1409. doi: 10.3389/fonc.2020.01409
16. Qin Z, Zhang X, Chen Z, Liu N. Establishment and validation of an immune-based prognostic score model in glioblastoma. *Int Immunopharmacol* (2020) 85:106636. doi: 10.1016/j.intimp.2020.106636
17. Zhang M, Wang X, Chen X, Zhang Q, Hong J. Novel Immune-Related Gene Signature for Risk Stratification and Prognosis of Survival in Lower-Grade Glioma. *Front Genet* (2020) 11:363. doi: 10.3389/fgene.2020.00363
18. Ni J, Liu S, Qi F, Li X, Yu S, Feng J, et al. Screening TCGA database for prognostic genes in lower grade glioma microenvironment. *Ann Transl Med* (2020) 8:209. doi: 10.21037/atm.2020.01.73
19. Pagès F, Galon J, Dieu-Nosjean MC, Tartour E, Sautès-Fridman C, Fridman WH. Immune infiltration in human tumors: a prognostic factor that should not be ignored. *Oncogene* (2010) 29:1093–102. doi: 10.1038/onc.2009.416
20. Yang W, Lai Z, Li Y, Mu J, Yang M, Xie J, et al. Immune signature profiling identified prognostic factors for gastric cancer. *Chin J Cancer Res* (2019) 31:463–70. doi: 10.21147/j.issn.1000-9604.2019.03.08
21. Shen S, Wang G, Zhang R, Zhao Y, Yu H, Wei Y, et al. Development and validation of an immune gene-set based Prognostic signature in ovarian cancer. *EBIOMEDICINE* (2019) 40:318–26. doi: 10.1016/j.ebiom.2018.12.054
22. Xu J, Liu F, Li Y, Shen L. A 1p/19q Codeletion-Associated Immune Signature for Predicting Lower Grade Glioma Prognosis. *Cell Mol Neurobiol* (2020). doi: 10.1007/s10571-020-00959-3
23. Cheng W, Ren X, Zhang C, Cai J, Liu Y, Han S, et al. Bioinformatic profiling identifies an immune-related risk signature for glioblastoma. *Neurology* (2016) 86:2226–34. doi: 10.1212/WNL.0000000000002770
24. Kong Y, Feng ZC, Zhang YL, Liu XF, Ma Y, Zhao ZM, et al. Identification of Immune-Related Genes Contributing to the Development of Glioblastoma Using Weighted Gene Co-expression Network Analysis. *Front Immunol* (2020) 11:1281. doi: 10.3389/fimmu.2020.01281
25. Zhao B, Wang Y, Wang Y, Chen W, Liu PH, Kong Z, et al. Systematic identification, development, and validation of prognostic biomarkers involving the tumor-immune microenvironment for glioblastoma. *J Cell Physiol* (2021) 236:507–22. doi: 10.1002/jcp.29878
26. Glick RP, Lichtor T, Cohen EP. Cytokine immunogene therapy. *Neurosurg Focus* (2000) 9:e2. doi: 10.3171/foc.2000.9.6.3
27. Kan LK, Williams D, Drummond K, O'Brien T, Monif M. The role of microglia and P2X7 receptors in gliomas. *J Neuroimmunol* (2019) 332:138–46. doi: 10.1016/j.jneuroim.2019.04.010
28. Tarassishin L, Casper D, Lee SC. Aberrant expression of interleukin-1β and inflammasome activation in human malignant gliomas. *PloS One* (2014) 9: e103432. doi: 10.1371/journal.pone.0103432
29. Lisi L, Ciotti GM, Braun D, Kalinin S, Currò D, Dello RC, et al. Expression of iNOS, CD163 and ARG-1 taken as M1 and M2 markers of microglial polarization in human glioblastoma and the surrounding normal parenchyma. *Neurosci Lett* (2017) 645:106–12. doi: 10.1016/j.neulet.2017.02.076
30. Liu S, Zhang C, Maimela NR, Yang L, Zhang Z, Ping Y, et al. Molecular and clinical characterization of CD163 expression via large-scale analysis in glioma. *Oncoimmunology* (2019) 8:1601478. doi: 10.1080/2162402X.2019.1601478
31. Chen T, Chen J, Zhu Y, Li Y, Wang Y, Chen H, et al. CD163, a novel therapeutic target, regulates the proliferation and stemness of glioma cells via casein kinase 2. *Oncogene* (2019) 38:1183–99. doi: 10.1038/s41388-018-0515-6
32. Miyazaki T, Ishikawa E, Matsuda M, Sugii N, Kohzuki H, Akutsu H, et al. Infiltration of CD163-positive macrophages in glioma tissues after treatment with anti-PD-L1 antibody and role of PI3Kγ inhibitor as a combination therapy with anti-PD-L1 antibody in in vivo model using temozolomide-resistant murine glioma-initiating cells. *Brain Tumor Pathol* (2020) 37:41–9. doi: 10.1007/s10014-020-00357-z
33. Zhu C, Kros JM, van der Weiden M, Zheng P, Cheng C, Mustafa DA. Expression site of P2RY12 in residential microglial cells in astrocytomas correlates with M1 and M2 marker expression and tumor grade. *Acta Neuropathol Commun* (2017) 5:4. doi: 10.1186/s40478-016-0405-5
34. Haage V, Semtner M, Vidal RO, Hernandez DP, Pong WW, Chen Z, et al. Comprehensive gene expression meta-analysis identifies signature genes that distinguish microglia from peripheral monocytes/macrophages in health and glioma. *Acta Neuropathol Commun* (2019) 7:20. doi: 10.1186/s40478-019-0665-y
35. Schrottmaier WC, Kral JB, Badrnya S, Assinger A. Aspirin and P2Y12 Inhibitors in platelet-mediated activation of neutrophils and monocytes. *Thromb Haemost* (2015) 114:478–89. doi: 10.1160/TH14-11-0943
36. Garcia-Mesa Y, Jay TR, Checkley MA, Luttge B, Dobrowolski C, Valadkhan S, et al. Immortalization of primary microglia: a new platform to study HIV regulation in the central nervous system. *J Neurovirol* (2017) 23:47–66. doi: 10.1007/s13365-016-0499-3
37. Tabuchi S. The autotaxin-lysophosphatidic acid-lysophosphatidic acid receptor cascade: proposal of a novel potential therapeutic target for treating glioblastoma multiforme. *Lipids Health Dis* (2015) 14:56. doi: 10.1186/s12944-015-0059-5
38. Takahashi K, Minami K, Otagaki S, Ishimoto K, Fukushima K, Fukushima N, et al. Lysophosphatidic acid receptor-2 (LPA(2)) and LPA(5) regulate cellular functions during tumor progression in fibrosarcoma HT1080 cells. *Biochem Biophys Res Commun* (2018) 503:2698–703. doi: 10.1016/j.bbrc.2018.08.026
39. Tang J, Kong D, Cui Q, Wang K, Zhang D, Yuan Q, et al. Bioinformatic analysis and identification of potential prognostic microRNAs and mRNAs in thyroid cancer. *PEERJ* (2018) 6:e4674. doi: 10.7717/peerj.4674
40. Lee SC, Dacheux MA, Norman DD, Balázs L, Torres RM, Augelli-Szafran CE, et al. Regulation of Tumor Immunity by Lysophosphatidic Acid. *Cancers (Basel)* (2020) 12. doi: 10.3390/cancers12051202
41. Ray R, Rai V. Lysophosphatidic acid converts monocytes into macrophages in both mice and humans. *Blood* (2017) 129:1177–83. doi: 10.1182/blood-2016-10-743757
42. Xue G, Lin X, Wu JF, Pei D, Wang DM, Zhang J, et al. Identification of key genes of papillary thyroid carcinoma by integrated bioinformatics analysis. *Biosci Rep* (2020) 40. doi: 10.1042/BSR20201555
43. Minchenko OH, Kharkova AP, Kubaichuk KI, Minchenko DO, Hlushchak NA, Kovalevska OV. Effect of hypoxia on the expression of CCN2, PLAU, PLAUR, SLURP1, PLAT and ITGB1 genes in ERN1 knockdown U87 glioma cells. *Ukr Biochem J* (2014) 86:79–89. doi: 10.15407/ubj86.04.079

44. Gilder AS, Natali L, Van Dyk DM, Zalfa C, Banki MA, Pizzo DP, et al. The Urokinase Receptor Induces a Mesenchymal Gene Expression Signature in Glioblastoma Cells and Promotes Tumor Cell Survival in Neurospheres. *Sci Rep* (2018) 8:2982. doi: 10.1038/s41598-018-21358-1
45. Johar A, Sarmiento-Monroy JC, Rojas-Villarraga A, Silva-Lara MF, Patel HR, Mantilla RD, et al. Definition of mutations in polyautoimmunity. *J Autoimmun* (2016) 72:65–72. doi: 10.1016/j.jaut.2016.05.003
46. Stewart CE, Hall IP, Parker SG, Moffat MF, Wardlaw AJ, Connolly MJ, et al. PLAUR polymorphisms and lung function in UK smokers. *BMC Med Genet* (2009) 10:112. doi: 10.1186/1471-2350-10-112
47. Bronte V. Deciphering Macrophage and Monocyte Code to Stratify Human Breast Cancer Patients. *Cancer Cell* (2019) 35:538–9. doi: 10.1016/j.ccell.2019.03.010
48. Zhang Y, Li JQ, Jiang ZZ, Li L, Wu Y, Zheng L. CD169 identifies an anti-tumour macrophage subpopulation in human hepatocellular carcinoma. *J Pathol* (2016) 239:231–41. doi: 10.1002/path.4720
49. Muhsin-Sharafaldine MR, Saunderson SC, Dunn AC, McLellan AD. Melanoma growth and lymph node metastasis is independent of host CD169 expression. *Biochem Biophys Res Commun* (2017) 486:965–70. doi: 10.1016/j.bbrc.2017.03.138
50. Hu J, Xu J, Li M, Zhang Y, Yi H, Chen J, et al. Targeting Lymph Node Sinus Macrophages to Inhibit Lymph Node Metastasis. *Mol Ther Nucleic Acids* (2019) 16:650–62. doi: 10.1016/j.omtn.2019.04.016
51. Junt T, Moseman EA, Iannacone M, Massberg S, Lang PA, Boes M, et al. Subcapsular sinus macrophages in lymph nodes clear lymph-borne viruses and present them to antiviral B cells. *Nature* (2007) 450:110–4. doi: 10.1038/nature06287
52. Strömvall K, Sundkvist K, Ljungberg B, Halin BS, Bergh A. Reduced number of CD169(+) macrophages in pre-metastatic regional lymph nodes is associated with subsequent metastatic disease in an animal model and with poor outcome in prostate cancer patients. *Prostate* (2017) 77:1468–77. doi: 10.1002/pros.23407
53. Santegoets K, Gielen PR, Büll C, Schulte BM, Kers-Rebel ED, Küsters B, et al. Expression profiling of immune inhibitory Siglecs and their ligands in patients with glioma. *Cancer Immunol Immunother* (2019) 68:937–49. doi: 10.1007/s00262-019-02332-w
54. Li GZ, Zhang KN, Wang Z, Hu HM, Wang ZL, Huang RY, et al. Siglecs, Novel Immunotherapy Targets, Potentially Enhance The Effectiveness of Existing Immune Checkpoint Inhibitors in Glioma Immunotherapy. *Onco Targets Ther* (2019) 12:10263–73. doi: 10.2147/OTT.S223406
55. Hartnell A, Steel J, Turley H, Jones M, Jackson DG, Crocker PR. Characterization of human sialoadhesin, a sialic acid binding receptor expressed by resident and inflammatory macrophage populations. *BLOOD* (2001) 97:288–96. doi: 10.1182/blood.V97.1.288
56. Prevete N, Liotti F, Marone G, Melillo RM, de Paulis A. Formyl peptide receptors at the interface of inflammation, angiogenesis and tumor growth. *Pharmacol Res* (2015) 102:184–91. doi: 10.1016/j.phrs.2015.09.017
57. Zhou Y, Bian X, Le Y, Gong W, Hu J, Zhang X, et al. Formylpeptide receptor FPR and the rapid growth of malignant human gliomas. *J Natl Cancer Inst* (2005) 97:823–35. doi: 10.1093/jnci/dji142
58. Chen DL, Ping YF, Yu SC, Chen JH, Yao XH, Jiang XF, et al. Downregulating FPR restrains xenograft tumors by impairing the angiogenic potential and invasive capability of malignant glioma cells. *Biochem Biophys Res Commun* (2009) 381:448–52. doi: 10.1016/j.bbrc.2009.02.065
59. Stempel H, Jung M, Pérez-Gómez A, Leinders-Zufall T, Zufall F, Bufe B. Strain-specific Loss of Formyl Peptide Receptor 3 in the Murine Vomeronasal and Immune Systems. *J Biol Chem* (2016) 291:9762–75. doi: 10.1074/jbc.M116.714493
60. Lee HY, Jeong YS, Lee M, Kweon HS, Huh YH, Park JS, et al. Intracellular formyl peptide receptor regulates naïve CD4 T cell migration. *Biochem Biophys Res Commun* (2018) 497:226–32. doi: 10.1016/j.bbrc.2018.02.060
61. Guo Y, Hong W, Zhang P, Han D, Fang Y, Tu J, et al. Abnormal polarization of macrophage-like cells in the peripheral blood of patients with glioma. *Oncol Lett* (2020) 20:947–54. doi: 10.3892/ol.2020.11602
62. Pinton L, Masetto E, Vettore M, Solito S, Magri S, D'Andolfi M, et al. The immune suppressive microenvironment of human gliomas depends on the accumulation of bone marrow-derived macrophages in the center of the lesion. *J Immunother Cancer* (2019) 7:58. doi: 10.1186/s40425-019-0536-x

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TGF-β Signaling Promotes Glioma Progression Through Stabilizing Sox9

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Gliomas are brain and spinal cord malignancies characterized by high malignancy, high recurrence and poor prognosis, the underlying mechanisms of which remain largely elusive. Here, we found that the Sry-related high mobility group box (Sox) family transcription factor, Sox9, was upregulated and correlated with poor prognosis of clinical gliomas. Sox9 promotes migration and invasion of glioma cells and *in vivo* development of xenograft tumors from inoculated glioma cells. Sox9 functions downstream of the transforming growth factor-β (TGF-β) pathway, in which TGF-β signaling prevent proteasomal degradation of the Sox9 protein in glioma cells. These findings provide novel insight into the wide interplay between TGF-β signaling and oncogenic transcription factors, and have implications for targeted therapy and prognostic assessment of gliomas.

Keywords: glioma, Sox9, transforming growth factor-β, migration, invasion

INTRODUCTION

Glioma is the most common primary central nervous system (CNS) malignant tumor, accounting for about 35–40% of intracranial tumors. Glioma is characterized by high rates of occurrence, invasiveness, and recurrence, with an extremely short overall survival time (OS) and high 5-year mortality rate (1). While the mechanisms underlying their pathogenesis remain largely elusive, gliomas, especially glioblastomas (GBM), often arise from aberrant differentiation of neural cells (2, 3). Genetic mutation is known to drive malignant transformation at least in part by "hijacking" neurodevelopmental programs (4, 5). Increasing evidence has suggested that Sox9, an indispensable transcription factor in the development of the nervous system, plays a pivotal role in the pathogenesis of glioma (6–8).

As a member of the Sry-related high mobility group box (Sox) transcription factors, Sox9 plays various important roles in the development of cartilage, sex organs, and the CNS (9, 10); Sox9 is also crucially involved in the self-renewal and differentiation of neural stem cells (NSCs) (6, 11). Consistent with its critical role in glial differentiation, Sox9 deregulation is closely related to the occurrence and development of glioma. We and others have demonstrated previously that Sox9, which is upregulated *via* various mechanisms, contributes to the occurrence and progression of glioma (12, 13).

Transforming growth factor-β (TGF-β) signaling, a canonical pathway regulating oncogenesis and tissue homeostasis, has been documented to participate in the pathogenesis of divergent malignancies including glioma (14–16). In particular, TGF-β pathway is a key regulator of glioma stem cells (GSCs). Shinojima et al. reported that TGF-β mediates homing of bone marrow-derived human mesenchymal stem cells (BM-hMSCs) to GSCs (17). Bruna et al. found that high TGF-β/Smad activity confers poor prognosis in glioma patients and promotes cell proliferation *via* platelet-derived growth factor B (PDGF-B) (18). Nonetheless, it remains to be fully understood how TGF-β signaling drives the progression of glioma especially considering the divergent genetic context of these clinical malignancies.

In the present study, we investigated the role of Sox9 in regulation of the malignant phenotypes of glioma cells, and explored upstream pathways responsible for Sox9 deregulation. We established that Sox9 overexpression underlies glioma pathogenesis, and TGF-β pathway plays an essential role in upregulating Sox9 and thereby promoting glioma progression.

MATERIALS AND METHODS

Cells and Human Tissue Samples

U87, U373, and U251 cells were purchased from the Chinese Academy of Sciences Cell Bank in 2018. All cell lines were grown in high glucose Dulbecco's Modified Eagle Medium (DMEM) (Hyclone, USA) supplemented with 10% FBS (Gemini, A49F74G) as well as 100 IU/ml penicillin and 100 μg/ml streptomycin (Hyclone, USA), and incubated in 5% CO_2 at 37° C. Eighty-six cases of human gliomas were collected from the neurosurgical specimens of Tangdu Hospital, Fourth Military Medical University, China. All patients were operated for the first time, and all of them were confirmed to be glioma by pathological assays. Normal brain tissues of 14 patients who have encountered with traumatic brain injuries. The study was approved by the Research Ethics Committee of Tangdu Hospital of Fourth Military Medical University, China. All patients involved in this study have signed the informed consent before, and all specimens were handled anonymous processing according to ethical and legal standards. U251, U373, and U87 cell lines, before the western blot, CCK8, wound-healing, transwell, and animal experiments, were treated with TGF-β1 cytokines (Novoprotein, CA59) at the concentration of 5 ng/ml for 2 h, 20 min, and 1 h, respectively. U251 and U373 cell lines, before the western blot, CCK8, wound-healing, and transwell assays, were treated with an inhibitor of TGF-β receptors I/II (Selleck, LY2109761) at the concentration of 5 μM for 12 h. Cells were pretreated with TGF-β receptor inhibitors LY2109761 (Selleck), or DMSO control for 12 h before transfected with Sox9 overexpression. Cells were pretreated with proteasomal inhibitor MG132 (MCE) at the concentration of 25 μg/ml for 6 h. U251 cells were treated with 50 μg/ml of cyclohexamide (Sigma) or DMSO control for 1 h, then treated with TGF-β1 or vehicle control (19).

Gene Knockdown *via* Vector-Based shRNAs

Stable gene knockdown in U251, U373, and U87 cell lines were achieved by infection with recombinant shRNA-expressing lentiviruses and subsequent selection with puromycin at a concentration of 5 μg/ml for about 2 weeks. The shRNA target sequences are as follows: NC, TTCTCCGAACGTGTCACGT; Sox9, GCATCCTTCAA TTTCTGTATA.

CCK8 Assay

Cells were plated into a 96-well plate, and cultured at 37˚C with 5% CO_2 for 12, 24, 48, and 72 h. There are five repeats of each sample. Subsequently, 10 μl CCK8 (5 mg/ml; Life Technologies) was added into 90 μl DMEM (10%FBS). The mixture was transferred into every sample and incubated at 37˚C with 5% CO_2 for 2 h, the cells with CCK8 was detected by determining the optical density (OD) at 450 nm (Thermo Fisher Scientific, Waltham, MA, USA).

Wound Healing Assays

U251 and U373 cells were seeded in six-well plates and cultured 24 h. A wound was then created by manually scraping the cell monolayer with a 200 μl pipette tip. The cultures were washed twice with PBS (Hyclone, USA) to remove floating cells. The cells were then incubated in serum-free DMEM. Cell migration into the wound was observed at three preselected time points (0, 24, and 48 h) in three randomly selected microscopic fields for each condition and time point. Images were acquired with a Nikon DS-5M Camera System mounted on a phase-contrast Leitz microscope and were processed using Image J.

Transwell Assays

Cells were suspended in 100 μl serum-free DMEM and seeded in the top chambers of 24-well transwell plates (Costar, USA) coated with 100 μl Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). The bottom chambers of the transwell plates were filled with 600 μl DMEM containing 10% FBS. Cells were allowed to migrate for 48 h at 37°C. By the time, the cells which invaded to the bottom chambers were fixed in methyl alcohol, and cells in the top chambers were removed using a cotton swab. Then cells were stained with 0.1% crystal violet. The fixed and stained cells were counted in five independent fields under a light microscope. At least three chambers were counted for each experiment. For the migration assays, a similar protocol was followed except for the replacement of the top chamber of the transwell plate with an uncoated chamber. The culture

medium in the bottom chamber was replaced with DMEM containing 10% FBS, and cells were allowed to migrate for 24 h.

Immunohistochemistry Assay

For immunohistochemistry (IHC), 8 μm sections of formalin-fixed and paraffin-embedded brain tissues were first de-waxed and rehydrated before antigen retrieval. The TGF-β1-antibody (1:100 dilution; Proteintech, China) and Sox9-antibody (1:250 dilution; Abcam, ab76997) were used for this study. After incubation with the primary antibodies, the tissues were rinsed and incubated for 1 h with Biotin-labeled secondary antibodies at room temperature (Molecular Probes 1:800). Nuclei were stained by Hematoxylin. Stained sections were examined under a light microscope and the positive cells in five high power fields (1 × 400) were counted for statistic study. The relative expression of TGF-β1 and Sox9 was analyzed by Graphpad *via* Spearman rank correlation test.

Western Blotting

The five cases of peritumor brain tissues and glioma tissues from patients were collected from the neurosurgical specimens of Tangdu Hospital. Peritumor brain tissues were dissected 0.5–1.0 cm away from glioma core regions, which were further histologically confirmed by H&E staining. The study was approved by the Research Ethics Committee of Tangdu Hospital of Fourth Military Medical University, China. All patients involved in this study have signed the informed consent before, and all specimens were handled anonymous processing according to ethical and legal standards. The glioma and peritumor tissues, and total cell lysates were dissolved in middle RIPA Lysis buffer (Beyotime, China) with complete protease inhibitor cocktail (Roche, USA). The protein concentrations were determined by a protein assay kit (Beyotime, China). Twenty micrograms protein was separated with 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto a polyvinylidene difluoride membrane (Roche, USA), which was incubated with TBST containing 5% skim milk 2 h at room temperature; and then with rabbit anti-Sox9 (1:1,000, Abcam, ab185230), rabbit anti-β-actin (1:100,000, Abclonal, China) monoclonal antibodies overnight at 4°C and then with goat anti-rabbit monoclonal IgG (1:10,000; Abclonal, China) secondary antibodies at room temperature for 2 h, followed by chemiluminescence for visualization with an ECL kit (Genshare biological, China).

Animal Experiments

All animal experiments were approved by the Ethics Committee of the Fourth Military Medical University, China. Nude immunocompromised mice were purchased from Fourth Military Medical University, Shanxi, China, and breeding colonies were maintained in SPF conditions. Xenografted transplantation of glioma cells into nude immunocompromised mice was performed as previously described. There are Sox9-NC group and Sox9-KD group for the U87 cell lines. After pre-transplant preparation of the recipient mice and anesthesia with 10% chloral hydrate. Isolated U87 cells of every group (10^7 in 1 ml PBS) and were implanted into the under left axilla of nude mice by subcutaneous injection to establish the xenograft model. The weight change of each animal was measured twice a week. Tumor volumes were determined by measuring the length (a) and the width (b). The tumor volume (V) was calculated according to the formula $V = ab^2/2$.

U87 glioma cells, which were infected by Sox9-NC and Sox9-KD respectively with GFP, were orthotopically implanted in nude mice. U87 cells were pretreated with TGF-β1 (5 ng/ml). Implantation of U87 cells into the brains of nude mice was performed under anesthesia. All procedures re-quiring anesthesia were performed using Chloral hydrate at the concentration of 10% (0.04 ml/10g) i.p. 3 μl of tumor cell suspension (10^5 cells/μl) was stereotactically inoculated in the right forebrain using a 5 μl syringe. On day 21, mice were anesthetized, and the brains were removed under perfusion with sterile 0.9% NaCl and paraformaldehyde. The brains were fixed in paraformaldehyde for 6 h and dehydrated in 10, 20, and 30% sucrose. Brain tissues were Frozen sections of brain tissues were prepared and the fluorescence was detected with the laser confocal microscope. The tissues were confirmed by H&E staining.

Statistical Analysis

Independent samples were analyzed by using two-sided independent Student's t-tests with Graphpad 7.0. Relative expression of TGF-β1 and Sox9 was analyzed *via* Spearman rank correlation test with Graphpad 7.0. Image J was used to cell counts, measurement of migrated distance, relative quantitation of western blot. All statistical results from the quantitative analysis of the *in vitro* experiments are presented as means ± SEM. p values < 0.05 were considered statistically significant.

RESULTS

Sry-Related High Mobility Group Box 9 Expression Correlates With Progression of Clinical Glioma

Sox9 has been documented as an oncogenic transcription factor in various malignancies (20, 21). We examined the expression of Sox9 in clinical glioma. Immunohistochemical staining and western blot of five patients showed that Sox9 was upregulated in glioma tissues when compared with the peritumor tissues (**Figures 1A, B**). Consistently, data from Chinese Glioma Genome Atlas (CGGA) and the Cancer Genome Atlas (TCGA) suggested that high Sox9 expression correlates with short survival of glioma patients (**Figures 1C, D**). Thus, Sox9 is a predictive biomarker for the pathogenesis and prognosis of clinical glioma.

Sry-Related High Mobility Group Box 9 Promotes Malignant Phenotypes of Glioma Cells

To determine the biological function of Sox9 in glioma cells, Sox9 was knocked down in glioma cell lines, U251, U373, and U87, *via* shRNAs expressed from recombinant lentiviral vectors (**Figures 2A, B**). CCK8 assays indicated that Sox9 silencing

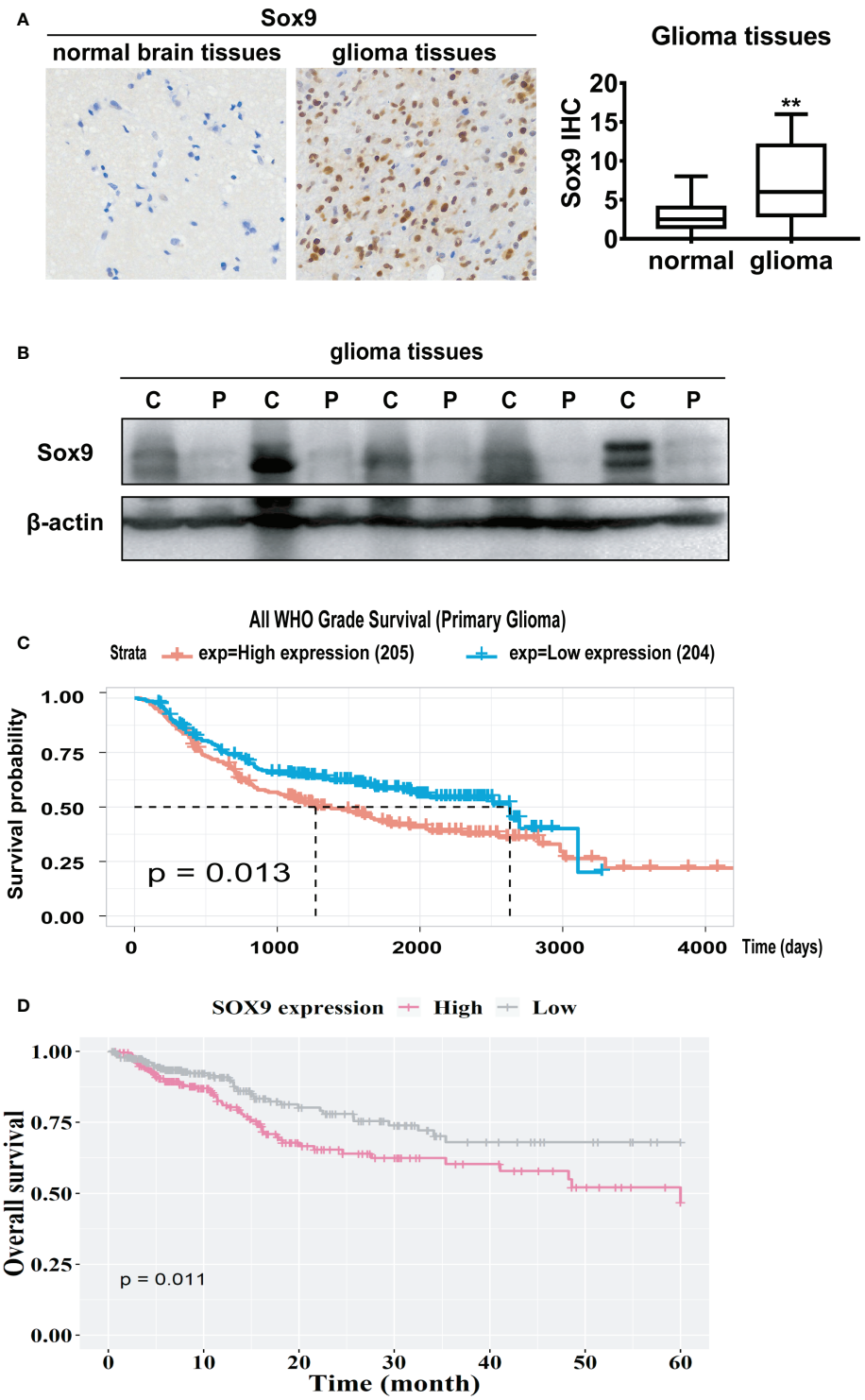

FIGURE 1 | Immunohistological stain of Sox9 in 14 normal brain tissues and 86 glioma tissues ($p < 0.01$). **(A)** Western blot of Sox9 in glioma tissues (C) and paired adjacent tissues (P) of five patients. **(B)** Analysis of the expression of Sox9 and the prognosis of glioma patients from CGGA database **(C)** and TCGA database **(D)**. Value of $p < 0.01$ (**) was considered statistically significant.

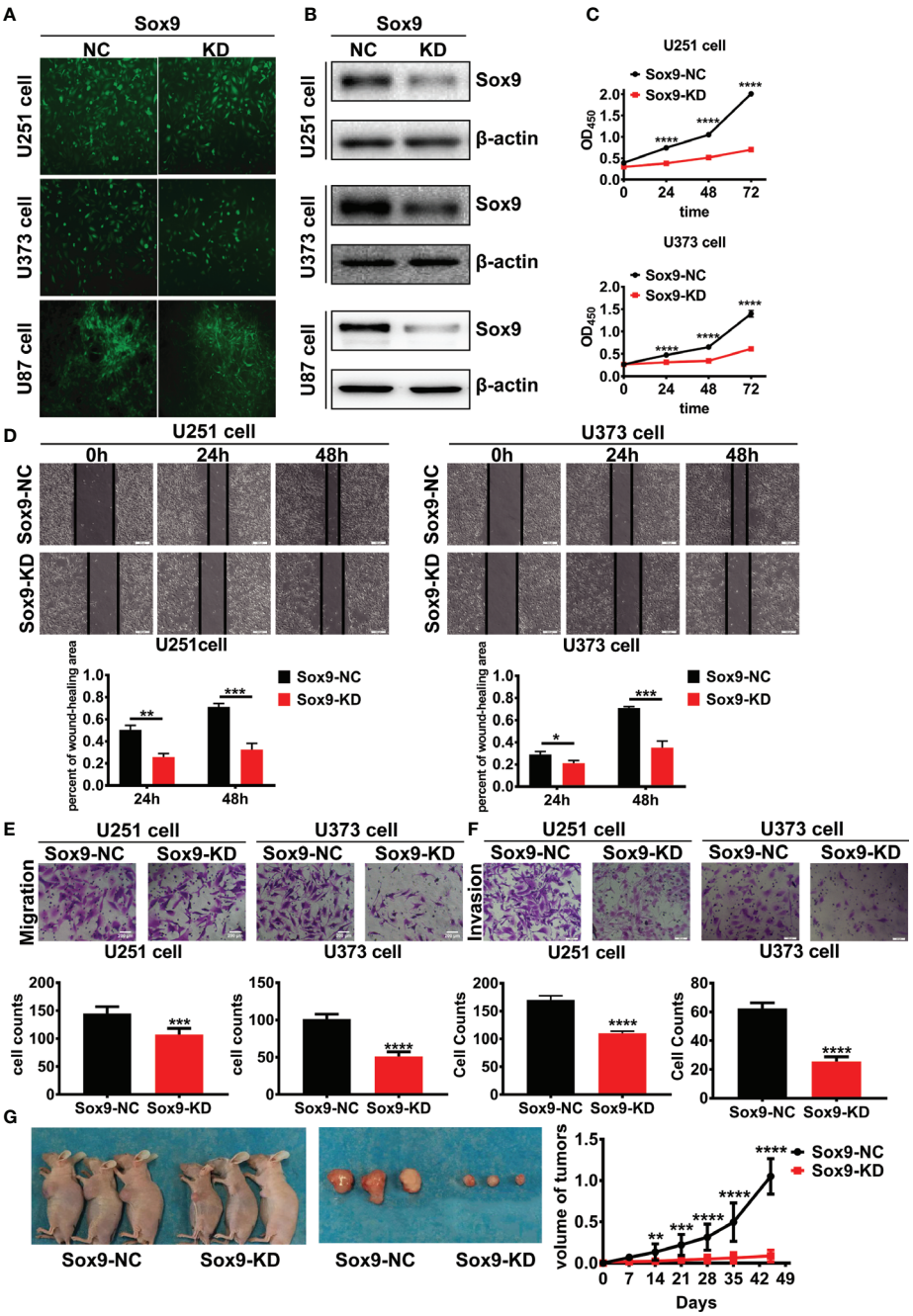

FIGURE 2 | The transfected efficient of Sox9-KD lentivirus in U251, U373, and U87 cells were detected by GFP stain. **(A)** U251, U373, and U87 Sox9-kd stable cell lines were detected by Western blot. **(B)** CCK8 assay of both U251 Sox9-kd cells and U251 Sox9-nc cells, as well as U373 Sox9-kd cells and U373 Sox9-nc cells in 24, 48, and 72 h (n = 3, $p < 0.001$). **(C)** Wound healing assay of both U251 Sox9-kd cells and U251 Sox9-nc cells at 0, 24 ($p < 0.01$), and 48 h ($p < 0.005$), as well as U373 Sox9-kd cells and U373 Sox9-nc cells (24 h: $p < 0.05$; 48 h: $p < 0.005$). **(D)** Transwell (migration) assay in U251 Sox9-kd cells and U251 Sox9-nc cells ($p < 0.005$), as well as in U373 Sox9-kd cells and U373 Sox9-nc cells ($p < 0.001$). **(E)** Transwell (invasion) assay in U251 Sox9-kd cells and U251 Sox9-nc cells ($p < 0.001$), as well as in U373 Sox9-kd cells and U373 Sox9-nc cells ($p < 0.001$). **(F)** Tumorigenesis xenografts with U87 Sox9-nc cells and U87 Sox9- kd cells after 40 days (n = 10). **(G)** Statistical analysis was performed using a two-tailed independent t-test. Values of $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.005$ (***), and $p < 0.001$ (****) were considered statistically significant.

resulted in a moderate growth inhibition of glioma cells (**Figure 2C**). Knockdown of Sox9 also remarkably reduced the migration capability of U251 and U373 cells as shown in wound-healing (**Figure 2D**) and Transwell (**Figure 2E**) assays. Similarly, Sox9 knockdown significantly decreased the invasiveness of glioma cells in a Transwell assay (**Figure 2F**). Sox9 downregulation also inhibited the development of xenograft tumors in nude mice challenged with the U87 glioma cells (**Figure 2G**). Thus, Sox9 plays an essential role in maintenance of the malignant phenotypes of glioma cells.

Sry-Related High Mobility Group Box 9 Functions Downstream of Transforming Growth Factor-β Signaling to Promote Glioma Pathogenesis

We next investigated the oncogenic signal pathways responsible for Sox9 upregulation in glioma cells. TGF-β signaling has been established to promote the progression of various cancers including glioma through substantially affecting the profiles of gene expression in neoplastic cells (22), which is reminiscent the role of oncogenic transcription factors (**Figure 3A**). The expression of Sox9 was positive correlated with TGF-β1 *via* the analysis of TGF-β1 and Sox9 in IHC of glioma tissues (**Figure 3B**). In line with these reports, we found that Sox9 was upregulated by treatment of glioma cells with recombinant TGF-β1 (**Figure 3C**), and Sox9 levels decreased when TGF-β signaling was blocked by a selective inhibitor, LY2109761 (**Figure 3D**). We have clarified that the migration and invasion of glioma cells, treated with recombinant TGF-β1, were significantly increased (23). And inhibition of TGF-β pathway caused remarkably reduced cell proliferation, migration, and invasion in CCK8, wound-healing, and transwell assays (**Figures 3E–H**). However, further overexpression of Sox9 in these cells rescued the capability of migration and invasion (**Figures 4A, C, D, E**), but not the ability of proliferation (**Figure 4B**). Next, to further confirm the relationship between TGF-β and Sox9, we implanted the U87 cells orthotopically to establish xenografts. As we can see, tumors, treated with TGF-β1, were more aggressive. The proliferation of Sox9-NC group and Sox9-KD group, treated with TGF-β1, showed non-significance (which were consistent with the results *in vitro*). The intracranial tumors of Sox9-NC group were more invasive, while the tumors of Sox9-KD groups were limited (**Figure S1A**). These data suggest that Sox9 is a functional target of TGF-β signaling in promoting glioma pathogenesis.

Transforming Growth Factor-β Signaling Represses Proteasomal Degradation of Sry-Related High Mobility Group Box 9 in Glioma Cells

The mechanisms underlying Sox9 regulation by TGF-β pathway in glioma cells were probed. Inhibition of TGF-β signaling decreased the level of Sox9 protein (**Figure 3C**), but not that of the mRNA (**Figure 5A**). Treatment of glioma cells with cycloheximide (CHX), which prevents translocation of elongating ribosomes, revealed that TGF-β pathway protected Sox9 protein from the degradation (**Figure 5B**). In addition, the proteasome inhibitor MG132 counteracted the decrease in Sox9 protein levels induced by the inhibitor of TGF-β signaling (**Figure 5C**). These results suggest that TGF-β signaling represses proteasomal degradation of Sox9 in glioma cells.

DISCUSSION

TGF-β pathway has been well documented to expedite the pathogenesis and recurrence of gliomas by extensively affecting the gene expression profiles of transforming or malignant cells (18, 24). While TGF-β acts *via* the specific heterodimer receptors, TGFBRI/II, to phosphorylate the Smad family proteins, which is subsequently imported into the nucleus and regulate target gene expression (25–27). Alternatively, TGF-β activates Ras/MAPK pathway *via* Smad-independent signaling to orchestrate gene expression and cell behaviors (28, 29). However, little is known how TGF-β signaling regulates transcription factors other than the Smad proteins in the context of glioma cells. We found here that Sox9, a transcription factor commonly overexpressed in various glioma and glioblastoma, is upregulated by TGF-β signaling. Consistent with previous reports in chondrocytes (19, 30), we established that the regulation occurs in the posttranslational level, i.e. TGF-β impairs the degradation of the Sox9 protein. Further study in glioma cells revealed that TGF-β signaling reduces the proteasomal degradation of Sox9. Sox9 is a critical regulatory target of TGF-β since its overexpression rescued the malignant phenotypes of glioma cells caused by inhibition of TGF-β signaling. Nonetheless, additional investigations are needed to determine how TGF-β signaling reduces the proteasmal degradation of Sox9. Sox9 has been reported as targets for proteasomal degradation after ubiquitination by the E3 ligase FBW7 or UBE3A (31, 32). We will further explore that whether TGF-β decreases the ubiquition of Sox9 *via* FBW7 or UBE3A. Carcinogenesis in many tissues has been found to revive a transcriptional network involved in embryonic development. In the central nervous system, Sox9 plays an important role in the differentiation of cranial neural crest cells (8), and was reported as an astrocyte-specific nuclear marker in adult brain outside the neurogenic regions (33). We found here that Sox9 was overexpressed in clinical gliomas, and correlated with a poor prognosis of glioma patients. Sox9 knockdown resulted in significantly suppressed proliferation, migration, and invasion of glioma cells, as well as impaired *in vivo* tumor development in a xenograft model, suggesting that Sox9 facilitates the formation of primary tumors probably *via* improving local invasion. Thus, it is worth additional investigation whether Sox9 overexpression promotes cell transformation through ablating the orchestrated differentiation of neural stem cells or astrocyte progenitors. In terms of the molecular mechanisms downstream of Sox9, Liu et al. found that Sox9 can promote glioma metastasis *via* Wnt/β-Catenin pathway (34); Glasgow et al. demonstrated that Sox9 determines gliogenesis and tumorigenesis of gliomas through differentially regulating the gene of NFIA, which is attributed to

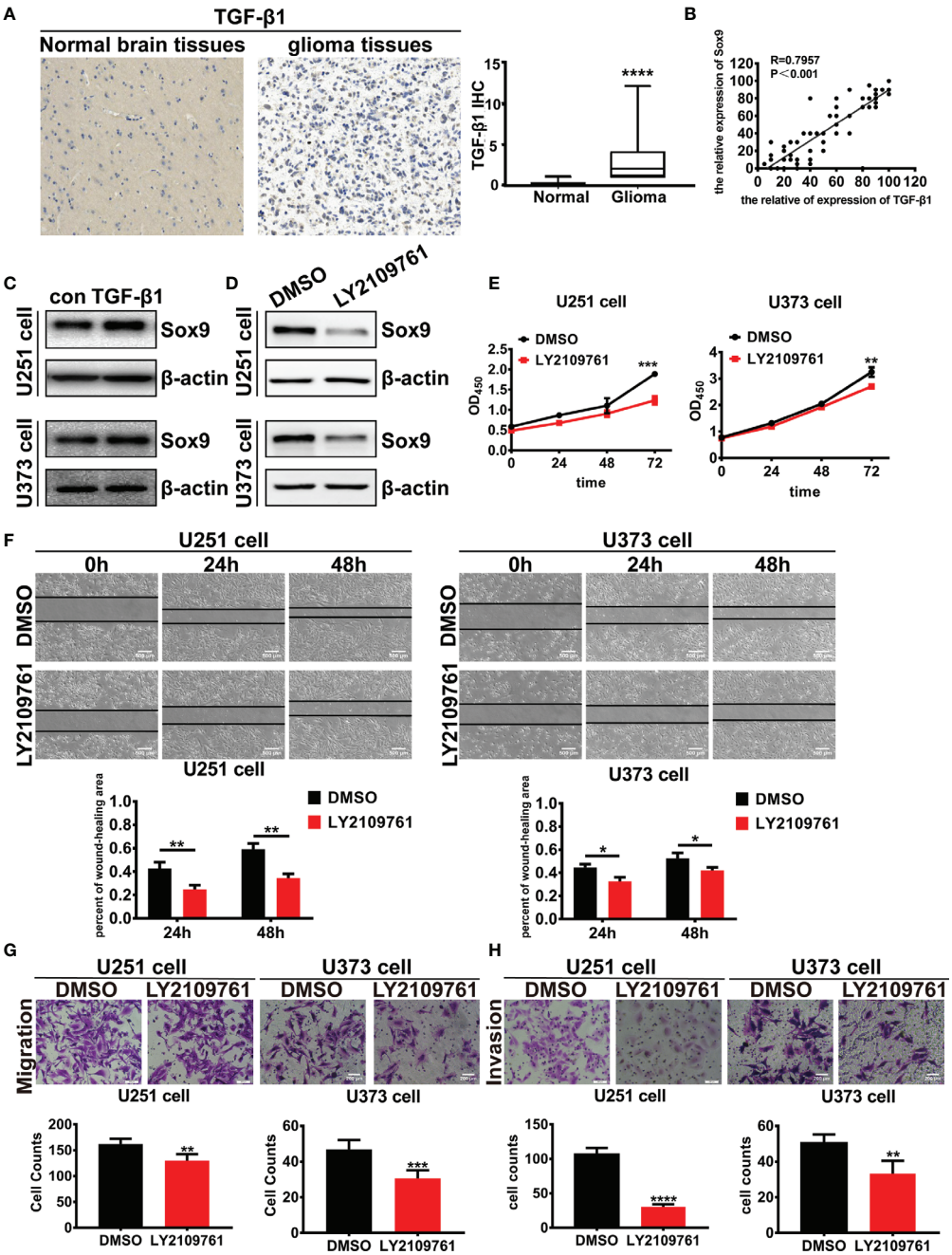

FIGURE 3 | Expression of TGF-β1 in 86 glioma tissues and 14 normal brain tissues were detected by immunohistochemistry ($p < 0.001$). **(A)** Analysis of the correlation of TGF-β1 and Sox9 in IHC assays (R = 0.7957). **(B)** Expression of Sox9 was detected by western blot after that U251 cells were treated with TGF-β1 (5 ng/ml) at 2 h and U373 cells were treated at 20 min. **(C)** Expression of Sox9 was detected by western blot after that U251 cells and U373 cells were treated with LY2109761 (5 μM) at 12 h. **(D)** CCK8 assay of both DMSO-treated U251 cells and LY2109761-treated U251 cells ($p < 0.005$), as well as in U373 cells ($p < 0.001$). **(E)** Wound-healing assay of both DMSO-treated U251 cells and LY2109761-treated U251 cells at 0 h, 24 h ($p < 0.01$), and 48 h ($p < 0.01$), as well as in U373 cells (24 h: $p < 0.05$; 48 h: $p < 0.05$). **(F)** Transwell (migration) assay in DMSO-treated U251 cells and LY2109761-treated U251 cells ($p < 0.01$), as well as in U373 cells ($p < 0.001$). **(G)** Transwell (invasion) assay in DMSO-treated U251 cells and LY2109761-treated U251 cells ($p < 0.001$), as well as in U373 cells ($p < 0.01$). **(H)** Statistical analysis was performed using a two-tailed independent t-test. Values of $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.005$ (***), and $p < 0.001$ (****) were considered statistically significant.

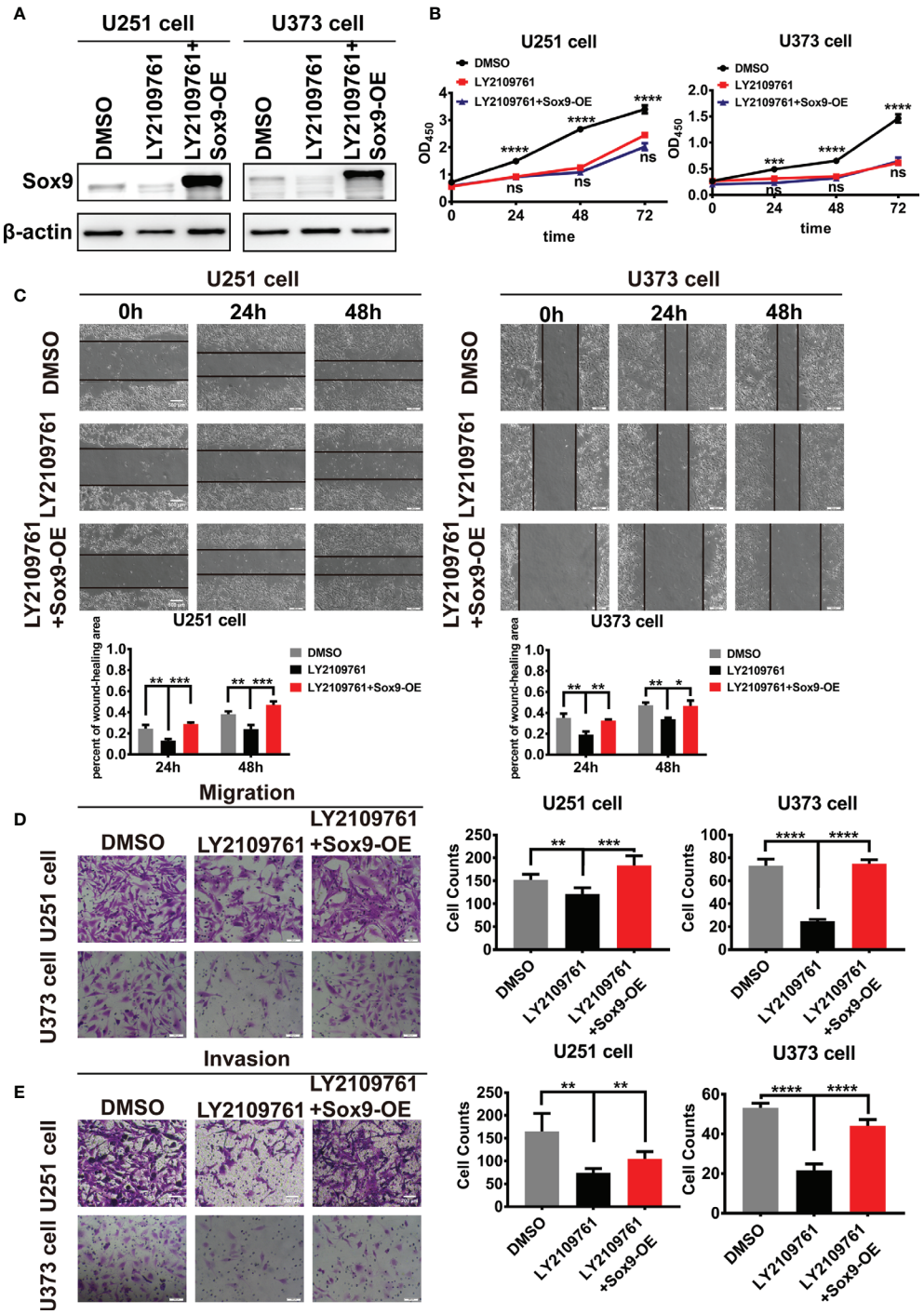

FIGURE 4 | Expression of Sox9 was detected by western blot after that U251 cells and U373 cells were treated with LY2109761 (5 μM) at 12 h, and then while Sox9 was overexpressed (OE). **(A)** CCK8 assay among DMSO-treated U251 cells, LY2109761-treated U251 cells, and LY2109761+Sox9-OE-treated U251 cells, as well as in U373 cells (LY2109763 *vs* LY2109763+Sox9-OE: ns). **(B)** Wound-healing assay of DMSO-treated U251 cells, LY2109761-treated U251 cells, and LY2109761+Sox9-OE-treated U251 cells at 0, 24 (LY2109763 *vs* LY2109763+Sox9-OE: $p < 0.005$), and 48 h (LY2109763 *vs* LY2109763+Sox9-OE: $p < 0.005$), as well as U373 cells (LY2109763 *vs* LY2109763+Sox9-OE: 24 h: $p < 0.0.01$; 48 h: $p < 0.05$). **(C)** Transwell (migration) assay in among DMSO-treated U251 cells, LY2109761-treated U251 cells, and LY2109761+Sox9-OE-treated U251 cells (LY2109763 *vs* LY2109763+Sox9-OE: $p < 0.005$), as well as in U373 cells (LY2109763 *vs* LY2109763+Sox9-OE: $p < 0.001$). **(D)** Transwell (invasion) assay in among DMSO-treated U251 cells, LY2109761-treated U251 cells, and LY2109761+Sox9-OE-treated U251 cells (LY2109763 *vs* LY2109763+Sox9-OE: $p < 0.005$), as well as in U373 cells (LY2109763 *vs* LY2109763+Sox9-OE: $p < 0.001$). **(E)** Statistical analysis was performed using a two-tailed independent t-test. Values of $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.005$ (***), and $p < 0.001$ (****) were considered statistically significant.

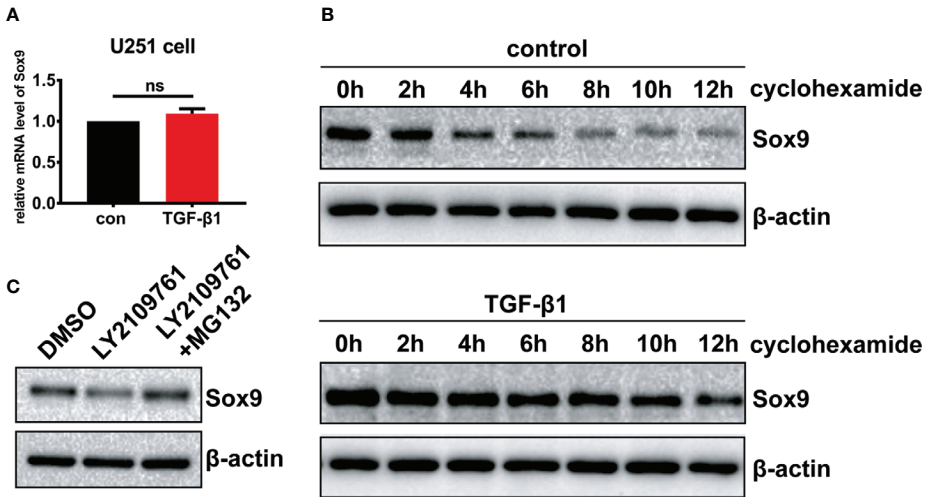

FIGURE 5 | Glioma cells treated with TGF-β1 (5 ng/ml) at 2 h, qRT-PCR showed that the expression of Sox9 mRNA had no changes. **(A)** Treatment of glioma cells with CHX, western blot displayed that TGF-β could stabilize the expression of Sox9 protein. **(B)** Glioma cells treated with LY2109761 showed decrease of Sox9 protein, and MG132 could counteract the effect of LY2109761 to Sox9 protein **(C).**

different modes of long-range enhancer interaction (20). While it is largely unknown whether Sox9 participates in potential cross-talks with other cancer-driving pathways and whether Sox9 play distinct roles dependent upon the molecular subtypes of glioma, our study highlights the function of Sox9 as an oncogenic transcription factor, and has implications for targeted therapy and prognostic assessment of clinical gliomas.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Research Ethics Committee of Tangdu Hospital of Fourth Military Medical University, China. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Ethics Committee of the Fourth Military Medical University, China.

AUTHOR CONTRIBUTIONS

MC and NL performed most experiments, analyzed data, and wrote the manuscript. ZS participated in the animal experiment. YJ and TJ participated in the cell culture assays. MX collected glioma tissue samples. LJ revised the article. YT and LW designed the overall study, supervised the experiments, analyzed the results, and wrote the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.592080/full#supplementary-material

REFERENCES

1. Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJ, Janzer RC, et al. Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. *Lancet Oncol* (2009) 10:459–66. doi: 10.1016/S1470-2045(09)70025-7
2. Trosko JE. Induction of iPS cells and of cancer stem cells: the stem cell or reprogramming hypothesis of cancer. *Anat Rec (Hoboken)* (2014) 297:161–73. doi: 10.1002/ar.22793
3. Schmitt J, Eckardt S, Schlegel PG, Sirén AL, Bruttel VS, McLaughlin KJ, et al. Human Parthenogenetic Embryonic Stem Cell-Derived Neural Stem Cells Express HLA-G and Show Unique Resistance to NK Cell-Mediated Killing. *Mol Med* (2015) 21:185–96. doi: 10.2119/molmed.2014.00188

4. Laug D, Glasgow SM, Deneen B. A glial blueprint for gliomagenesis. *Nat Rev Neurosci* (2018) 19:393–403. doi: 10.1038/s41583-018-0014-3
5. Lek M, Karczewski KJ, Minikel EV, Samocha KE, Banks E, Fennell T, et al. Analysis of protein-coding genetic variation in 60,706 humans. *Nature* (2016) 536:285–91. doi: 10.1038/nature19057
6. Stolt CC, Lommes P, Sock E, Chaboissier MC, Schedl A, Wegner M. The Sox9 transcription factor determines glial fate choice in the developing spinal cord. *Genes Dev* (2003) 17:1677–89. doi: 10.1101/gad.259003
7. Wu S, Wu Y, Capecchi MR. Motoneurons and oligodendrocytes are sequentially generated from neural stem cells but do not appear to share common lineage-restricted progenitors in vivo. *Development* (2006) 133:581–90. doi: 10.1242/dev.02236
8. Sabelström H, Petri R, Shchors K, Jandial R, Schmidt C, Sacheva R, et al. Driving Neuronal Differentiation through Reversal of an ERK1/2-miR-124-SOX9 Axis Abrogates Glioblastoma Aggressiveness. *Cell Rep* (2019) 28:2064–79.e11. doi: 10.1016/j.celrep.2019.07.071
9. Jo A, Denduluri S, Zhang B, Wang Z, Yin L, Yan Z, et al. The versatile functions of Sox9 in development, stem cells, and human diseases. *Genes Dis* (2014) 1:149–61. doi: 10.1016/j.gendis.2014.09.004
10. Ruan H, Hu S, Zhang H, Du G, Li X, Li X, et al. Upregulated SOX9 expression indicates worse prognosis in solid tumors: a systematic review and meta-analysis. *Oncotarget* (2017) 8:113163–73. doi: 10.18632/oncotarget.22635
11. Wang Z, Xu X, Liu N, Cheng Y, Jin W, Zhang P, et al. SOX9-PDK1 axis is essential for glioma stem cell self-renewal and temozolomide resistance. *Oncotarget* (2018) 9:192–204. doi: 10.18632/oncotarget.22773
12. de Bont JM, Kros JM, Passier MM, Reddingius RE, Sillevis Smitt PA, Luider TM, et al. Differential expression and prognostic significance of SOX genes in pediatric medulloblastoma and ependymoma identified by microarray analysis. *Neuro Oncol* (2008) 10:648–60. doi: 10.1215/15228517-2008-032
13. Gao J, Zhang JY, Li YH, Ren F. Decreased expression of SOX9 indicates a better prognosis and inhibits the growth of glioma cells by inducing cell cycle arrest. *Int J Clin Exp Pathol* (2015) 8:10130–8.
14. Massagué J. TGFbeta in Cancer. *Cell* (2008) 134:215–30. doi: 10.1016/j.cell.2008.07.001
15. Kaminska B, Kocyk M, Kijewska M. TGF beta signaling and its role in glioma pathogenesis. *Adv Exp Med Biol* (2013) 986:171–87. doi: 10.1007/978-94-007-4719-7_9
16. Nagaraj NS, Datta PK. Targeting the transforming growth factor-beta signaling pathway in human cancer. *Expert Opin Invest Drugs* (2010) 19:77–91. doi: 10.1517/13543780903382609
17. Shinojima N, Hossain A, Takezaki T, Fueyo J, Gumin J, Gao F, et al. TGF-β mediates homing of bone marrow-derived human mesenchymal stem cells to glioma stem cells. *Cancer Res* (2013) 73:2333–44. doi: 10.1158/0008-5472.CAN-12-3086
18. Bruna A, Darken RS, Rojo F, Ocaña A, Peñuelas S, Arias A, et al. High TGFbeta-Smad activity confers poor prognosis in glioma patients and promotes cell proliferation depending on the methylation of the PDGF-B gene. *Cancer Cell* (2007) 11:147–60. doi: 10.1016/j.ccr.2006.11.023
19. Coricor G, Serra R. TGF-β regulates phosphorylation and stabilization of Sox9 protein in chondrocytes through p38 and Smad dependent mechanisms. *Sci Rep* (2016) 6:38616. doi: 10.1038/srep38616
20. Glasgow SM, Carlson JC, Zhu W, Chaboub LS, Kang P, Lee HK, et al. Glia-specific enhancers and chromatin structure regulate NFIA expression and glioma tumorigenesis. *Nat Neurosci* (2017) 20:1520–8. doi: 10.1038/nn.4638
21. Liu N, Zhang L, Wang Z, Cheng Y, Zhang P, Wang X, et al. MicroRNA-101 inhibits proliferation, migration and invasion of human glioblastoma by targeting SOX9. *Oncotarget* (2017) 8:19244–54. doi: 10.18632/oncotarget.13706
22. Yu Y, Feng XH. TGF-β signaling in cell fate control and cancer. *Curr Opin Cell Biol* (2019) 61:56–63. doi: 10.1016/j.ceb.2019.07.007
23. Chao M, Liu N, Lou M, Liu JH, Zhai YL, Wang L, et al. Mechanism of TGF-β1 Regulation Migration and Invasion of Glioma Cells. *Med Recapitulate* (2020) 26(09):1837–42. doi: 10.3969/j.issn.1006-2084.2020.09.031
24. Platten M, Wick W, Wild-Bode C, Aulwurm S, Dichgans J, Weller M. Transforming growth factors beta(1) (TGF-beta(1)) and TGF-beta(2) promote glioma cell migration via Up-regulation of alpha(V)beta(3) integrin expression. *Biochem Biophys Res Commun* (2000) 268:607–11. doi: 10.1006/bbrc.2000.2176
25. Massagué J. TGF-beta signal transduction. *Annu Rev Biochem* (1998) 67:753–91. doi: 10.1146/annurev.biochem.67.1.753
26. Wrana JL, Attisano L, Wieser R, Ventura F, Massagué J. Mechanism of activation of the TGF-beta receptor. *Nature* (1994) 370:341–7. doi: 10.1038/370341a0
27. Huse M, Muir TW, Xu L, Chen YG, Kuriyan J, Massagué J. The TGF beta receptor activation process: an inhibitor- to substrate-binding switch. *Mol Cell* (2001) 8:671–82. doi: 10.1016/s1097-2765(01)00332-x
28. Mulder KM. Role of Ras and Mapks in TGFbeta signaling. *Cytokine Growth Factor Rev* (2000) 11:23–35. doi: 10.1016/s1359-6101(99)00026-x
29. Lu T, Tian L, Han Y, Vogelbaum M, Stark GR. Dose-dependent cross-talk between the transforming growth factor-beta and interleukin-1 signaling pathways. *Proc Natl Acad Sci U S A* (2007) 104:4365–70. doi: 10.1073/pnas.0700118104
30. Chavez RD, Coricor G, Perez J, Seo HS, Serra R. SOX9 protein is stabilized by TGF-β and regulates PAPSS2 mRNA expression in chondrocytes. *Osteoarthritis Cartilage* (2017) 25:332–40. doi: 10.1016/j.joca.2016.10.007
31. Suryo Rahmanto A, Savov V, Brunner A, Bolin S, Weishaupt H, Malyukova A, et al. FBW7 suppression leads to SOX9 stabilization and increased malignancy in medulloblastoma. *EMBO J* (2016) 35:2192–212. doi: 10.15252/embj.201693889
32. Hattori T, Kishino T, Stephen S, Eberspaecher H, Maki S, Takigawa M, et al. E6-AP/UBE3A protein acts as a ubiquitin ligase toward SOX9 protein. *J Biol Chem* (2013) 288:35138–48. doi: 10.1074/jbc.M113.486795
33. Sun W, Cornwell A, Li J, Peng S, Osorio MJ, Aalling N, et al. SOX9 Is an Astrocyte-Specific Nuclear Marker in the Adult Brain Outside the Neurogenic Regions. *J Neurosci* (2017) 37:4493–507. doi: 10.1523/JNEUROSCI.3199-16.2017
34. Liu H, Liu Z, Jiang B, Peng R, Ma Z, Lu J. SOX9 Overexpression Promotes Glioma Metastasis via Wnt/β-Catenin Signaling. *Cell Biochem Biophys* (2015) 73:205–12. doi: 10.1007/s12013-015-0647-z

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REVIEW
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Immunotherapy: A Potential Approach for High-Grade Spinal Cord Astrocytomas

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Spinal cord astrocytomas (SCAs) account for 6–8% of all primary spinal cord tumors. For high-grade SCAs, the prognosis is often poor with conventional therapy, thus the urgent need for novel treatments to improve patient survival. Immunotherapy is a promising therapeutic strategy and has been used to treat cancer in recent years. Several clinical trials have evaluated immunotherapy for intracranial gliomas, providing evidence for immunotherapy-mediated ability to inhibit tumor growth. Given the unique microenvironment and molecular biology of the spinal cord, this review will offer new perspectives on moving toward the application of successful immunotherapy for SCAs based on the latest studies and literature. Furthermore, we will discuss the challenges associated with immunotherapy in SCAs, propose prospects for future research, and provide a periodic summary of the current state of immunotherapy for SCAs immunotherapy.

Keywords: spinal cord astrocytomas, immunotherapy, immune checkpoint inhibitors, CAR-T therapy, therapeutic vaccines therapy, K27M-mutant histone H3

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INTRODUCTION AND CURRENT MANAGEMENT OF SPINAL CORD ASTROCYTOMAS

Spinal cord astrocytomas (SCAs) comprise approximately 6–8% of primary spinal cord tumors (1) and can be divided into the following categories based on the 2016 World Health Organization (WHO) classification: pilocytic astrocytomas (PAs, Grade I), diffuse astrocytomas (DAs, Grade II), anaplastic astrocytomas (AAs, Grade III), and glioblastomas multiforme (GBMs, Grade IV) (2, 3). In addition, a new histological diagnosis has been proposed to include diffuse midline gliomas (DMGs), with H3K27M mutant, which are found in the spinal cord, brainstem, pineal region, and thalamus (2). Generally, 75% of primary SCAs are low-grade (WHO grade I–II), while the remaining 25% are high-grade (WHO grade III–IV) tumors (4).

The current standard-of-care therapy for SCAs involves maximal safe surgical resection, followed by radiotherapy and chemotherapy. For low-grade primary SCAs, gross total resection is considered the first treatment choice and has an excellent local control rate (5). However, the value of aggressive resection in high-grade SCAs is unknown and cannot be recommended since the infiltrative nature of these SCAs frequently limits the extent of resection. For these tumors, aggressive surgical removal is not associated with any significant survival benefit, with a mortality rate of up to 70% at 6 months (6).

In conclusion, SCAs are rare diseases and are challenging to treat. For high-grade SCAs, the currently available therapies seem to do little to improve the survival of patients. In the future, better therapeutic options are needed to treat high-grade SCAs to prolong the patient's life, and immunotherapy might be a potential treatment for those patients.

SPINAL CORD MICROENVIRONMENT AND ITS IMPACT ON TUMOR BIOLOGY

The spinal cord microenvironment not only plays an important role in the process of tumor occurrence, development, and metastasis but also influences therapeutic effects (7).

In the chapter "Spinal Cord Tumor Microenvironment" in TME in organs, Ellis and colleagues have demonstrated that the TME in the spinal cord and brain are different (8). In their study, the author found that the same source of tumor cells transplanted into the spinal cord tissue showed lower tumor growth than in the brain, which led the researchers to speculate that the occurrence and development of glioma may be due to the different environment present in the brain and spinal cord. In addition, the literature provides evidence that genetic changes in intramedullary astrocytomas are less frequent than in intracranial astrocytomas (8). One possible explanation may be the relatively small spinal canal volume, which makes the tumor more prone to symptoms at an earlier stage of development.

As we all know, the spinal cord is located in the spinal canal, composed of gray and white matter, covered from inside to outside by the pia mater, arachnoid mater, and dural mater. The subarachnoid space is filled with cerebrospinal fluid, which provides mechanical and immune protection for the spinal cord. The existence of the blood-spinal cord barrier (BSCB) makes the spinal cord form a relatively independent microenvironment, which strictly regulates metabolism and immune transport to the spinal cord parenchyma as the same as the function of the blood-brain barrier. Although the spinal cord, like the brain, has long been considered immune exclusion zone, this view has been challenged in recent years.

Glial cells are the most abundant cell types in the spinal cord, including astrocytes, oligodendrocytes, ependymal cells, and microglias. The glia-neuron ratio in the spinal cord is suspected to be much higher than in the brain (9). Among them, astrocytes are the most common cell type that play an important role in the normal functioning of the spinal cord and also participate in the occurrence and development of tumors in many ways. Although relevant studies have been carried out in intracranial gliomas, due to the large regional heterogeneity of astrocytes, intracranial studies cannot be fully applied to SCAs, and relevant studies need to be further carried out.

Lymphoid cells can provide long-term immune monitoring and play an important role in maintaining homeostasis and tumor development. In this context, the concept of checkpoint inhibitor was proposed and received more attention. The discovery of PD-1, PD-L1, CTLA-4, and other molecules and the development of related drugs have been effective in some tumors. In CNS malignancies, tumor molecules can avoid detection by recruiting and coordinating T lymphocytes, and transform the immune system from protective to toxic (10). In spinal cord, the expression rate of PD-L1 is about 20% according to one study (11). Clinical trials targeting immune checkpoints have been carried out in intracranial gliomas, although the results have not been satisfactory and the study of spinal astrocytomas is still in the blank.

In a word, compared to studies on the intracranial glioma microenvironment, there is still a lack of data specific to the spinal cord, which indicates that future research is necessary. With the development of relevant studies and the gradual understanding of the spinal cord microenvironment, the treatment of spinal cord tumor will also change accordingly, making treatment more targeted and efficient.

MOLECULAR BIOLOGY OF SPINAL CORD ASTROCYTOMAS

Characteristic molecular markers in tumor tissues are important for judging tumor pathological grade and treatment prognosis. Therefore, it is of great importance to search for characteristic molecular indicators of SCAs, especially high-grade. Below, we summarize specific biomarkers of SCAs (**Table 1**).

H3K27M

The K27M mutation is one of two mutually exclusive variants of the H3F3A gene. Gliomas harboring this mutation mainly localize to midline structures, such as the thalamus, brainstem, and spinal cord, and are prevalent in adolescents and children with malignant biological characteristics (16, 23). The H3K27M mutation lacks typical cellular genetic abnormalities and is usually found in high-grade gliomas characterized by unusually aggressive tumor progression (24), even if it not classified histologically as low-grade astrocytomas (15).

The K27M mutation in patients with SCAs is associated with grade III–IV tumors. Chai et al. (17) revealed the K27M mutation was detected in 42.1% of cases (n = 83) of SCAs. Nagaishi et al. (23) showed similar data. Thus, this mutation is often associated with grade III–IV SCAs. Another study showed approximately the same mutation frequency rate for grade III–IV astrocytomas in adults and children (52% and 54%; n = 11 and 19, respectively) (14). It should be noted that K27M is absent in other types of malignant tumors, so it may be a pathological feature of primary malignant SCAs and may also serve as an indicator of the worst prognosis (16). Research on H3K27M as an important therapeutic target is under way, which will be described below.

IMMUNOTHERAPY: A POTENTIAL APPROACH FOR HIGH-GRADE SCAS

In recent years, alongside the advancement of research and the continuous improvements in technology, tumor immunotherapy

TABLE 1 | Molecular Markers of Spinal Cord Astrocytomas (SCAs).

Gene/ Mutation	Mutation frequency in SCAs				Comments
	PAs (Grade I)	DAs (Grade II)	AAs (Grade III)	GBMs (Grade IV)	
BRAF KIAA1549	32% (12)	Rare			More common in PAs in the spinal cord and the basilar parts of the brain (13).
BRAF V600E	48% (12)	Rare			Most frequently found in supratentorial PAs.
CDKN2A	+				Crucial to SCAs and, in particular, PAs (13).
H3K27M	–	52% (adults) (14) 54% (children) (14)			SCAs with K27M mutation have malignant biological behavior and are highly invasive (15). Indicated as the pathological basis for high-grade SCAs and is an important indicator of poor prognosis (16).
TERT promoter	–	22% (17)			TERT promoter mutations are associated with shorter survival in SCAs patients (17, 18).
TP53	–	60% (19)			More aggressive disease course (20). Revealed in 60%–67% of grade III-IV SCAs (19, 21).
PTEN	Extremely rare				Only sporadic reports are known in grade III–IV (22).

Green refers to the genes that are related to low-grade (Grade I–II) SCAs; Blue refers to genes that are related to high-grade (Grade III–IV) SCAs. The symbols "+" and "–" stand for the presence or absence of a mutation in SCAs.

has gradually become an important treatment modality in addition to surgical treatment and postoperative radiotherapy and chemotherapy. Immunotherapy has played an increasingly important role in the treatment of hematological malignancies and melanoma and other malignancies. Immunotherapeutic agents have been approved for marketing and have benefited a number of patients. Immunotherapy is also at the experimental stage in glioma. Recent novel advances in immunotherapy include immune checkpoint inhibitors, chimeric antigen receptor (CAR)-T therapy, and vaccine therapy (25). The application of immunotherapy to the treatment of high-grade spinal cord astrocytomas is promising, and the results may be exciting, although at present, there are still considerable challenges. Below, we will summarize the current status of immunotherapy in SCAs and the problems to be resolved in the future.

IMMUNE CHECKPOINT INHIBITORS

Immunocheckpoint inhibitors have played an important role in the treatment of many kinds of tumors. At present, common immune checkpoints include PD-1、PD-L1、CTLA-4. These molecules have been shown to be highly expressed on the surface of intracranial glioma molecules, and their expression level is positively correlated with tumor grade (26). In preclinical studies, relevant studies have shown that anti-PD-1, anti-PD-L1, and anti-CTLA-4 can achieve tumor survival in 50%、20%, and 15% of mice respectively when treated with tumor model alone. Combined use of anti-PD-1 and anti-CTLA-4 can effectively prevent tumor growth (27). However, due to the differences between the animal model and the actual tumor microenvironment, the data of relevant clinical studies were unsatisfactory, and most of them focused on stage I and II. Nivolumab, a monoclonal antibody used to block PD-1, is currently undergoing clinical trials and has shown that its use alone does not extend overall survival (28). In another trial, pembrolizumab, another anti-PD-1, has been used to assess immune response and survival analysis in patients with GBM after surgical resection as a neoadjuvant therapy. The results of this study suggest that the overall survival of patients can be prolonged (29). In general, the effect of immunocheckpoint inhibitors as a single treatment is limited, but combined with other treatment methods, such as targeted drugs, radiotherapy, chemotherapy, and so on, the therapeutic effect may be improved. Although this approach is currently rarely used in high-grade spinal cord astrocytomas, relevant treatment strategies can be used for reference.

CAR-T CELL THERAPY

Chimeric antigen receptor (CAR) T cell therapy is an example of adoptive cell therapy and is a promising immunotherapy approach. CARs are synthetic receptors that alter the specificity, function, and metabolism of T cells (30). A major advantage of CAR-T therapy is that through the short-chain variable fragment (ScFv), T cells can directly recognize tumor surface specific antigens without the need for major histocompatibility complex (MHC) complex antigens, effectively overcoming the limitations of previous TCR-T cells (31).

CAR-T therapy was first described in the mid-1980s (32). However, only in recent years has it been successfully introduced into clinical practice. In 2007, the FDA approved the first application of an anti-CD19-CAR-T clinical trial. The results were encouraging (33), and thus scientists were stimulated to apply this approach to central nervous system tumors. In adult glioblastomas, CAR-T have targeted specific antigens such as human epidermal growth factor receptor 2 (HER2) (34), epidermal growth factor receptor (EGFR) vIII (35, 36), interleukin (IL)-13Rα2 (37), and ephrin type-A receptor 2 (EphA2) (38), and preliminary results are encouraging. Although clinical conversion of these agents is not yet possible, the results have provided scientists with considerable confidence regarding their promising clinical implication.

However, the specific targets identified in glioblastomas of the brain are not suitable for high-grade SCAs. Encouragingly, a recent study (39) has suggested that H3K27M mutation may be a potential target for immunotherapy of high-grade SCAs. The study (39) was conducted in the broad framework of diffuse midline gliomas. The disialoganglioside GD2, a group of galactose-containing complex-lipid structures found on membranes of cells, was identified and confirmed as being specific and highly expressed in the pathological tissues of patient derived diffuse midline glioma, but not on the surface of normal cells. In the H3K27M+DMG orthotopic xenograft model derived from five independent patients (including spinal cord sources), GD2-targeted CAR-T cells could produce the cytokines inteferon(IFN)-γ and interleukin-2 (IL-2) and selectively killed tumor cells. The transplanted tumor cells were significantly reduced in size, which was obviously encouraging. However, the neurotoxicity of CAR-T cell therapy is also of concern. GD2-CAR-T cells in mice showed significant toxicity over the maximum therapeutic period. The death of mice was attributed to local third ventricle compression caused by inflammatory infiltration, rather than the targeted, non-tumor toxicity of specific GD2-CAR-T cells. The author concluded that due to the particular anatomical site of the midline structure, the swelling caused by neuroinflammation is often not tolerated (39); thus, more detailed clinical testing and intensive neuroprotective treatment are needed. Even so, the risk of cerebral hernia may not be effectively reduced. Although the above studies in the mouse xenograft model suggest that GD2-specific CAR T cell therapy has promising therapeutic potential and that most mice can tolerate the inflammatory response associated with the activity of CAR T cells, whether this model is able to predict human outcomes remains to be determined.

THERAPEUTIC VACCINES THERAPY

Therapeutic vaccines are also a promising therapeutic approach, but their exact efficacy is unclear (40). Therapeutic vaccines therapy can be roughly divided into several categories: peptide vaccines, dendritic cell vaccines, tumor cell vaccines, and neoantigen vaccines (41). Importantly, the use of adjuvants is crucial in immunologically privileged environments such as the brain and spinal cord, because the lack of resident immune cells in the CNS may hamper an effective immune response. As a result, vaccines are being developed and refined to adapt to the immunosuppressive tumor microenvironment (TME).

H3K27M is a specific biological marker for primary high-level SCAs and a promising target for immunotherapy. H3K27M-targeted vaccines have shown good therapeutic effects in preclinical models (42, 43). Ochs et al. (42) have done a lot of work in this regard, and their data have provided the basis for the further development of a vaccine against H3K27M. Their results mainly confirmed that H3K27M can be targeted by mutant specific peptide vaccines and can induce specific T cell responses. Besides, the H3K27M peptide vaccine significantly reduced tumor growth in the transplanted tumor mouse model, and induced effective CTL and Th-1 anti-tumor immune responses. The authors' findings provided a solid theoretical basis for vaccine development targeting the H3K27M mutation. It should also be noted, however, that the H3K27M homogenic hypodermic sarcoma model was used in their studies, and that an MHC humanized *in situ* glioma model was lacking to demonstrate the efficacy and reliability of the vaccine.

Limited by the few specific epitopes available, there have been few studies on targeted vaccines for high-grade SCAs. Thus, current treatment of targeted vaccines for SCAs is still in its infancy stage. H3K27M has been shown to be an effective immunogenic epitope for SCAs and represents a promising breakthrough point for the development of targeted vaccines for SCAs in the future. It is also hoped that new immunogenic epitopes will be discovered and confirmed in the future.

DISCUSSION

SCAs, especially high-grade SCAs, tend to occur in adolescents and have a high degree of malignancy and poor prognosis. The existing treatment methods are of little help to improve patient survival. Immunotherapy has shown great anti-tumor potential in other malignant tumors, and it has potential therapeutic significance for spinal cord patients based on the data available. Even so, the current research is limited and mainly focuses on experimental studies in animal models, which is mainly due to the following (44): the incidence of high-grade spinal cord astrocytomas is very low compared with that of intracranial gliomas; thus, it is difficult to obtain enough samples for a full and objective analysis. Furthermore, the spinal cord controls the upper and lower limbs, and because high-grade gliomas are often characterized by invasive growth that is not clearly distinguishable from normal tissue structure, it is difficult to obtain enough tissue for detailed analysis. Therefore, regional or international cooperation is desirable. Since spinal cord astrocytomas are diseases with a low incidence, studies at this stage are more focused on single centers, with a limited number of cases, and the conclusions drawn are often incomplete. Therefore, in the future, it is expected to enrich the pathological sample size of spinal cord astrocytomas through the cooperation of all parties, so as to conduct a systematic and comprehensive analysis and continuously deepen the understanding of this disease. Only with a deeper understanding of the disease can we better diagnose and treat it.

There are still many difficulties and challenges that need to be addressed in the future, including (i) antigen escape and paucity of tumor specific antigens; (ii) drug delivery crossing the blood spinal cord barrier (BSCB); (iii) neurotoxicity on the spinal cord; and (iv) the unique immune cohort in SCAs.

The number of specific antigens identified in SCAs, especially high-grade SCAs, is too small at present, which seriously restricts the development of relevant immunotherapy drugs in SCAs. Recent studies have shown that the genomic landscape of SCAs is significantly different from that of intracranial astrocytomas,

with unique and highly recurrent mutations in the genes encoding the H3 variant of the histone (45, 46). Other genes identified contain TP53 and the TERT promoter. With this knowledge, a new approach for the discovery of tumor-specific targets for SCAs is necessary, instead of treating and testing intracranial astrocytomas as in the past. Scientists will need to determine how to identify new specific molecular markers expressed in SCAs tissue samples that can more effectively guide the immune system toward cancer eradication.

Antigenic escape is a thorny issue for scientists and clinic doctors. Antigen escape has been identified as an important cause of drug resistance and tumor relapse in acquired leukemia (33). This problem may also be encountered in the immunotherapy of SCAs. In view of the limited number of targeted antigens available at present, only single antigens can be used for CAR-T preparation, which further increases the probability of antigen escape and reduces the anti-tumor effectiveness of drugs, while the existence of tumor heterogeneity makes this problem more prominent. It is no exaggeration to say that the task of finding more specific targets is urgent so as to include effective and safe immunotherapy.

How to improve the targeted drug's ability to cross the BSCB, so as to better reach the lesion to improve efficacy, is a question that future research should consider. Methods to improve the penetration of drugs through the blood-brain barrier (BBB) can be used for reference. A potential approach would the use of nanoparticle systems that can co-opt existing signaling pathways (47). Physically breaching the BBB may be another approach, for example, by reversibly doing so using pulsed ultrasonic sound waves; this is already an option clinically available for glioma treatment (48). The BSCB exerts roughly similar functions as BBB, but there is evidence that the interface between the blood circulation and CNS is not evenly distributed along the entire neural axis (49, 50); thus, whether these methods can be directly applied to BSCB, or modified according to the specific characteristics of the BSCB, using biological engineering technology so as to improve the bioavailability of effective drugs to penetrate BSCB required investigation.

It remains to be seen whether the drugs used for immunotherapy cause potential damage to the CNS. Unlike other organs, the CNS cannot tolerate even minimal autoimmune responses (51). There is a lack of data and analysis on the long-term effects of immunotherapy, and the results remain unknown. In the current model, no significant CNS adverse events have been reported for CAR and therapeutic vaccine therapy. However, the potential damage to patients from immunotherapy is inestimable due to differences between the mouse model and humans. At the same time, given that patients with primary CNS tumors may have a different disease burden than patients with leukemia secondary CNS diseases, the risk of potential neurotoxicity in the SCA population may be higher (25). The question of how to monitor the response of SCAs also needs to be addressed.

Lastly, how to obtain enough peptides to antigen-presenting cells (APCs) and the subsequent immune response cascade will be a major challenge for peptide vaccines in the future (52). The CNS had been considered an immunologically privileged site for a long time (52). However, according to existing studies, a series of unique immune characteristics exist in CNS, including the natural expression of immunosuppressant factors such as transforming growth factor (TGF) and IL-10, low expression of MHCs, lack of effective APCs, and the presence of the BBB and the BSCB. Although T cells and antibodies can be exposed to CNS antigens in gliomas, the lack of adequate APCs in the spinal TME may affect the effectiveness of some immunotherapies, including vaccines (52). Increasing APCs recruitment at injection sites has been explored through a variety of adjuvants; in addition, designing continuous APCs recruitment before and during vaccination may have good therapeutic potential.

The application of immunotherapy to SCAs presents many unique challenges, in particular how to monitor responses and the effects of treatment on the spinal cord. We hope that future development in immunotherapy will allow improved anti-tumor efficacy of highly malignant SCAs. As a result, the question of how to combine this new treatment with traditional therapies will become increasingly important.

CONCLUSION

High-grade SCAs is an aggressive tumor with malignant biological behavior; it is more common in adolescents and children and has a very poor prognosis. Due to its lower incidence than brain glioma and invasive growth, the survival rates of these patients have not been significantly improved even with currently available standard treatment. Immunotherapy is considered as a promising approach due to the cytotoxic potential of the immune system and the precision of molecular guidance (53). However, while immunotherapy has shown promising results in other cancers, little progress has been made in tumors of the CNS, including brain gliomas and SCAs. In the future, immunotherapy of SCAs will need to take into consideration the penetration of the BSCB, the escape mechanism of immune antigens, the lack of known specific targets, the neurotoxicity of the drug to the normal spinal cord structures, and the ability to enhance the local immune response. Researchers need to combine more advanced technology with multi-center collaborations to further expand the sample size to better understand the microenvironmental and biological characteristics of high-grade SCAs, and to use this information to develop combinations of multiple immunotherapies as a meaningful therapy, able to overcome poor clinical results in this subgroup (25).

AUTHOR CONTRIBUTIONS

JH and TL: manuscript preparation and wrote the main part of the manuscript. ST: wrote parts of the manuscript and manuscript editing. BH and GH: helped to perform the analysis with constructive discussions. YX: critically reviewed the manuscript and gave many professional suggestions. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Tobin MK, Geraghty JR, Engelhard HH, Linninger AA, Mehta AI. Intramedullary spinal cord tumors: a review of current and future treatment strategies. *Neurosurg Focus* (2015) 39(2):E14. doi: 10.3171/2015.5.FOCUS15158
2. Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-Branger D, Cavenee WK, et al. The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. *Acta Neuropathol* (2016) 131(6):803–20. doi: 10.1007/s00401-016-1545-1
3. Banan R, Hartmann C. The new WHO 2016 classification of brain tumors-what neurosurgeons need to know. *Acta Neurochir (Wien)* (2017) 159 (3):403–18. doi: 10.1007/s00701-016-3062-3
4. Raco A, Esposito V, Lenzi J, Piccirilli M, Delfini R, Cantore G. Long-term follow-up of intramedullary spinal cord tumors: a series of 202 cases. *Neurosurgery* (2005) 56(5):972–81.
5. Klekamp J. Treatment of intramedullary tumors: analysis of surgical morbidity and long-term results. *J Neurosurg Spine* (2013) 19(1):12–26. doi: 10.3171/2013.3.SPINE121063
6. Chamberlain MC, Tredway TL. Adult primary intradural spinal cord tumors: a review. *Curr Neurol Neurosci Rep* (2011) 11(3):320–8. doi: 10.1007/s11910-011-0190-2
7. Wu T, Dai Y. Tumor microenvironment and therapeutic response. *Cancer Lett* (2017) 387:61–8. doi: 10.1016/j.canlet.2016.01.043
8. Ellis JA, Castelli M, Assanah M, Bruce JN, Canoll P, Ogden AT. Unique microenvironmental responses to PDGF stimulation in brain and spinal cord gliomas determine tumor phenotype. *J Neurooncol* (2015) 123(1):27–33. doi: 10.1007/s11060-015-1769-2
9. Bahney J, von Bartheld CS. The Cellular Composition and Glia-Neuron Ratio in the Spinal Cord of a Human and a Nonhuman Primate: Comparison With Other Species and Brain Regions. *Anat Rec (Hoboken)* (2018) 301 (4):697–710. doi: 10.1002/ar.23728
10. Chen Z, Hambardzumyan D. Immune Microenvironment in Glioblastoma Subtypes. *Front Immunol* (2018) 9:1004. doi: 10.3389/fimmu.2018.01004
11. Jha P, Manjunath N, Singh J, Mani K, Garg A, Kaur K, et al. Analysis of PD-L1 expression and T cell infiltration in different molecular subgroups of diffuse midline gliomas. *Neuropathology* (2019) 39(6):413–24. doi: 10.1111/neup.12594
12. Shankar GM, Lelic N, Gill CM, Thorner AR, Van Hummelen P, Wisoff JH, et al. BRAF alteration status and the histone H3F3A gene K27M mutation segregate spinal cord astrocytoma histology. *Acta Neuropathol* (2016) 131 (1):147–50. doi: 10.1007/s00401-015-1492-2
13. Schindler G, Capper D, Meyer J, Janzarik W, Omran H, Herold-Mende C, et al. Analysis of BRAF V600E mutation in 1,320 nervous system tumors reveals high mutation frequencies in pleomorphic xanthoastrocytoma, ganglioglioma and extra-cerebellar pilocytic astrocytoma. *Acta Neuropathol* (2011) 121(3):397–405. doi: 10.1007/s00401-011-0802-6
14. Gessi M, Gielen GH, Dreschmann V, Waha A, Pietsch T. High frequency of H3F3A (K27M) mutations characterizes pediatric and adult high-grade gliomas of the spinal cord. *Acta Neuropathol* (2015) 130(3):435–7. doi: 10.1007/s00401-015-1463-7
15. Aihara K, Mukasa A, Gotoh K, Saito K, Nagae G, Tsuji S, et al. H3F3A K27M mutations in thalamic gliomas from young adult patients. *Neuro Oncol* (2014) 16(1):140–6. doi: 10.1093/neuonc/not144
16. Solomon DA, Wood MD, Tihan T, Bollen AW, Gupta N, Phillips JJJ, et al. Diffuse Midline Gliomas with Histone H3-K27M Mutation: A Series of 47 Cases Assessing the Spectrum of Morphologic Variation and Associated Genetic Alterations. *Brain Pathol* (2016) 26(5):569–80. doi: 10.1111/bpa.12336
17. Chai R, Zhang Y, Liu Y, Chang Y, Pang B, Jiang T, et al. The molecular characteristics of spinal cord gliomas with or without H3 K27M mutation. *Acta Neuropathol Commun* (2020) 8(1):40. doi: 10.1186/s40478-020-00913-w
18. Alvi MA, Ida CM, Paolini MA, Kerezoudis P, Meyer J, Barr Fritcher EG, et al. Spinal cord high-grade infiltrating gliomas in adults: clinico-pathological and molecular evaluation. *Mod Pathol* (2019) 32(9):1236–43. doi: 10.1038/s41379-019-0271-3
19. Walker C, Baborie A, Crooks D, Wilkins S, Jenkinson MD. Biology, genetics and imaging of glial cell tumours. *Br J Radiol* (2011) 84 Spec No 2(Spec Iss 2): S90–S106. doi: 10.1259/bjr/23430927
20. England B, Huang T, Karsy M. Current understanding of the role and targeting of tumor suppressor p53 in glioblastoma multiforme. *Tumour Biol* (2013) 34(4):2063–74. doi: 10.1007/s13277-013-0871-3
21. Johnson A, Severson E, Gay L, Vergilio J, Elvin J, Suh J, et al. Comprehensive Genomic Profiling of 282 Pediatric Low- and High-Grade Gliomas Reveals Genomic Drivers, Tumor Mutational Burden, and Hypermutation Signatures. *Oncologist* (2017) 22(12):1478–90. doi: 10.1634/theoncologist.2017-0242
22. Shows J, Marshall C, Perry A, Kleinschmidt-DeMasters BK. Genetics of Glioblastomas in Rare Anatomical Locations: Spinal Cord and Optic Nerve. *Brain Pathol* (2016) 26(1):120–3. doi: 10.1111/bpa.12327
23. Nagaishi M, Nobusawa S, Yokoo H, Sugiura Y, Tsuda K, Tanaka Y, et al. Genetic mutations in high grade gliomas of the adult spinal cord. *Brain Tumor Pathol* (2016) 33(4):267–9. doi: 10.1007/s10014-016-0263-7
24. Sturm D, Witt H, Hovestadt V, Khuong-Quang D, Jones DTW, Konermann C, et al. Hotspot mutations in H3F3A and IDH1 define distinct epigenetic and biological subgroups of glioblastoma. *Cancer Cell* (2012) 22(4):425–37. doi: 10.1016/j.ccr.2012.08.024
25. Wang SS, Bandopadhayay P, Jenkins MR. Towards Immunotherapy for Pediatric Brain Tumors. *Trends Immunol* (2019) 40(8):748–61. doi: 10.1016/j.it.2019.05.009
26. Garber ST, Hashimoto Y, Weathers S, Xiu J, Gatalica Z, Verhaak RGW, et al. Immune checkpoint blockade as a potential therapeutic target: surveying CNS malignancies. *Neuro Oncol* (2016) 18(10):1357–66. doi: 10.1093/neuonc/now132
27. Reardon DA, Gokhale PC, Klein SR, Ligon KL, Rodig SJ, Ramkissoon SH, et al. Glioblastoma Eradication Following Immune Checkpoint Blockade in an Orthotopic, Immunocompetent Model. *Cancer Immunol Res* (2016) 4(2):124–35. doi: 10.1158/2326-6066.CIR-15-0151
28. Weller M, Cloughesy T, Perry JR, Wick W. Standards of care for treatment of recurrent glioblastoma–are we there yet? *Neuro Oncol* (2013) 15(1):4–27. doi: 10.1093/neuonc/nos273
29. Cloughesy TF, Mochizuki AY, Orpilla JR, Hugo W, Lee AH, Davidson TB, et al. Neoadjuvant anti-PD-1 immunotherapy promotes a survival benefit with intratumoral and systemic immune responses in recurrent glioblastoma. *Nat Med* (2019) 25(3):477–86. doi: 10.1038/s41591-018-0337-7
30. June CH, Sadelain M. Chimeric Antigen Receptor Therapy. *N Engl J Med* (2018) 379(1):64–73. doi: 10.1056/NEJMra1706169
31. Kwok D, Okada H. T-Cell based therapies for overcoming neuroanatomical and immunosuppressive challenges within the glioma microenvironment. *J Neurooncol* (2020) 147:281–95. doi: 10.1007/s11060-020-03450-7
32. Gross G, Waks T, Eshhar Z. Expression of immunoglobulin-T-cell receptor chimeric molecules as functional receptors with antibody-type specificity. *Proc Natl Acad Sci USA* (1989) 86(24):10024–8. doi: 10.1073/pnas.86.24.10024
33. Maude SL, Frey N, Shaw PA, Aplenc R, Barrett DM, Bunin NJ, et al. Chimeric antigen receptor T cells for sustained remissions in leukemia. *N Engl J Med* (2014) 371(16):1507–17. doi: 10.1056/NEJMoa1407222; Erratum in: *N Engl J Med* (2016) 374(10):998.
34. Ahmed N, Brawley V, Hegde M, Bielamowicz K, Kalra M, Landi D, et al. HER2-Specific Chimeric Antigen Receptor-Modified Virus-Specific T Cells for Progressive Glioblastoma: A Phase 1 Dose-Escalation Trial. *JAMA Oncol* (2017) 3(8):1094–101. doi: 10.1001/jamaoncol.2017.0184
35. O'Rourke DM, Nasrallah MP, Desai A, Melenhorst JJ, Mansfield K, Morrissette JJD, et al. A single dose of peripherally infused EGFRvIII-directed CAR T cells mediates antigen loss and induces adaptive resistance in patients with recurrent glioblastoma. *Sci Transl Med* (2017) 9(399): eaaa0984. doi: 10.1126/scitranslmed.aaa0984
36. Goff SL, Morgan RA, Yang JC, Sherry RM, Robbins PF, Restifo NP, et al. Pilot Trial of Adoptive Transfer of Chimeric Antigen Receptor-transduced T Cells Targeting EGFRvIII in Patients With Glioblastoma. *J Immunother* (2019) 42 (4):126–35. doi: 10.1097/CJI.0000000000000260
37. Brown CE, Alizadeh D, Starr R, Weng L, Wagner JR, Naranjo A, et al. Regression of Glioblastoma after Chimeric Antigen Receptor T-Cell Therapy. (2016) 2561–9. doi: 10.1056/NEJMoa1610497
38. Chow KKH, Naik S, Kakarla S, Brawley VS, Shaffer DR, Yi Z, et al. T cells redirected to EphA2 for the immunotherapy of glioblastoma. *Mol Ther* (2013) 21(3):629–37. doi: 10.1038/mt.2012.210

39. Mount CW, Majzner RG, Sundaresh S, Arnold EP, Kadapakkam M, Haile S, et al. Potent antitumor efficacy of anti-GD2 CAR T cells in H3-K27M diffuse midline gliomas. *Nat Med* (2018) 24(5):572–9. doi: 10.1038/s41591-018-0006-x
40. Sampson JH, Gunn MD, Fecci PE, Ashley DM. Brain immunology and immunotherapy in brain tumours. *Nat Rev Cancer* (2020) 20(1):12–25. doi: 10.1038/s41568-019-0224-7
41. Wong KK, Li WA, Mooney DJ, Dranoff G. Advances in Therapeutic Cancer Vaccines. *Adv Immunol* (2016) 130:191–249. doi: 10.1016/bs.ai.2015.12.001
42. Ochs K, Ott M, Bunse T, Sahm F, Bunse L, Deumelandt K, et al. K27M-mutant histone-3 as a novel target for glioma immunotherapy. *Oncoimmunology* (2017) 6(7):e1328340. doi: 10.1080/2162402X.2017.1328340
43. Chheda ZS, Kohanbash G, Okada K, Jahan N, Sidney J, Pecoraro M, et al. Novel and shared neoantigen derived from histone 3 variant H3.3K27M mutation for glioma T cell therapy. *J Exp Med* (2018) 215(1):141–57. doi: 10.1084/jem.20171046
44. Abd-El-Barr MM, Huang KT, Moses ZB, Iorgulescu JB, Chi JH. Recent advances in intradural spinal tumors. *Neuro Oncol* (2018) 20(6):729–42. doi: 10.1093/neuonc/nox230
45. Khuong-Quang D, Buczkowicz P, Rakopoulos P, Liu X, Fontebasso AM, Bouffet E, et al. K27M mutation in histone H3.3 defines clinically and biologically distinct subgroups of pediatric diffuse intrinsic pontine gliomas. *Acta Neuropathol* (2012) 124(3):439–47. doi: 10.1007/s00401-012-0998-0
46. Vinci M, Burford A, Molinari V, Kessler K, Popov S, Clarke M, et al. Functional diversity and cooperativity between subclonal populations of pediatric glioblastoma and diffuse intrinsic pontine glioma cells. *Nat Med* (2018) 24(8):1204–15. doi: 10.1038/s41591-018-0086-7
47. von Maltzahn G, Park J, Lin KY, Singh N, Schwöppe C, Mesters R, et al. Nanoparticles that communicate in vivo to amplify tumour targeting. *Nat Mater* (2011) 10(7):545–52. doi: 10.1038/nmat3049
48. Carpentier A, Canney M, Vignot A, Reina V, Beccaria K, Horodyckid C, et al. Clinical trial of blood-brain barrier disruption by pulsed ultrasound. *Sci Transl Med* (2016) 8(343):342r–3r. doi: 10.1186/2050-5736-3-S1-O14
49. Bartanusz V, Jezova D, Alajajian B, Digicaylioglu M. The blood-spinal cord barrier: morphology and clinical implications. *Ann Neurol* (2011) 70(2):194–206. doi: 10.1002/ana.22421
50. Wilhelm I, Nyúl-Tóth Á, Suciu M, Hermenean A, Krizbai IA. Heterogeneity of the blood-brain barrier. *Tissue Barriers* (2016) 4(1):e1143544. doi: 10.1080/21688370.2016.1143544
51. Lyon JG, Mokarram N, Saxena T, Carroll SL, Bellamkonda RV. Engineering challenges for brain tumor immunotherapy. *Adv Drug Deliv Rev* (2017) 114:19–32. doi: 10.1016/j.addr.2017.06.006
52. Weathers S, Gilbert MR. Current challenges in designing GBM trials for immunotherapy. *J Neurooncol* (2015) 123(3):331–7. doi: 10.1007/s11060-015-1716-2
53. Swartz AM, Shen SH, Salgado MA, Congdon KL, Sanchez-Perez L. Promising vaccines for treating glioblastoma. *Expert Opin Biol Ther* (2018) 18(11):1159–70. doi: 10.1080/14712598.2018.1531846

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REVIEW
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Mitochondria's Role in the Maintenance of Cancer Stem Cells in Glioblastoma

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Glioblastoma (GBM), one of the deadliest primary brain malignancies, is characterized by a high recurrence rate due to its limited response to existing therapeutic strategies such as chemotherapy, radiation therapy, and surgery. Several mechanisms and pathways have been identified to be responsible for GBM therapeutic resistance. Glioblastoma stem cells (GSCs) are known culprits of GBM resistance to therapy. GSCs are characterized by their unique self-renewal, differentiating capacity, and proliferative potential. They form a heterogeneous population of cancer stem cells within the tumor and are further divided into different subpopulations. Their distinct molecular, genetic, dynamic, and metabolic features distinguish them from neural stem cells (NSCs) and differentiated GBM cells. Novel therapeutic strategies targeting GSCs could effectively reduce the tumor-initiating potential, hence, a thorough understanding of mechanisms involved in maintaining GSCs' stemness cannot be overemphasized. The mitochondrion, a regulator of cellular physiological processes such as autophagy, cellular respiration, reactive oxygen species (ROS) generation, apoptosis, DNA repair, and cell cycle control, has been implicated in various malignancies (for instance, breast, lung, and prostate cancer). Besides, the role of mitochondria in GBM has been extensively studied. For example, when stressors, such as irradiation and hypoxia are present, GSCs utilize specific cytoprotective mechanisms like the activation of mitochondrial stress pathways to survive the harsh environment. Proliferating GBM cells exhibit increased cytoplasmic glycolysis in comparison to terminally differentiated GBM cells and quiescent GSCs that rely more on oxidative phosphorylation (OXPHOS). Furthermore, the Warburg effect, which is characterized by increased tumor cell glycolysis and decreased mitochondrial metabolism in the presence of oxygen, has been observed in GBM. Herein, we highlight the importance of mitochondria in the maintenance of GSCs.

Keywords: glioblastoma, GSC, stem cell, mitochondria, quiescence, metabolism, stemness

INTRODUCTION

Glioblastoma (GBM) is the most common primary brain malignancy and is characterized by a variable survival time ranging from 4 to 16 months, depending on the status and the type of therapy the patients receive. Unlike most other types of malignancies, distant or extraneural metastasis of GBM is rare (1). However, GBM remains one of the incurable primary brain malignancies due to several factors. For instance, the absence of a single targetable oncogenic pathway is one of the contributing factors that further complicate the course of GBM treatment and research. GBM resistance to temozolomide (TMZ), a principal first-line chemotherapeutic agent, is mediated through several pathways and mechanisms. These include, methylguanine-DNA-methyltransferase (MGMT) (2, 3), long non-coding RNAs such as lncRNA TP73-AS1 (4), increased angiogenesis (5), resistance to apoptosis and apoptosis-inducing agents (6), mitochondrial DNA mutation, and most importantly, the presence of GBM initiating cells (GICs). According to Gimple et al., GICs are a heterogeneous population of GBM cells formed by the mutation of neural progenitor cells, immature neural stem cells (NSCs), or mature cells such as neurons. GICs give rise to glioblastoma stem-like tumor-initiating cells (GSLTICs) and their smaller subpopulation, GSCs, which are known to be the leading cause of GBM therapy resistance (7, 8). Interestingly, not only GSCs but also other subpopulations of GBM cells (such as GSLTICs) are capable of displaying stem cell properties (7). In response to microenvironmental changes such as hypoxia, these cells undergo a "state" transition and display phenotypic adaptation resulting from intrinsic tumor plasticity. In summary, plasticity imposed by microenvironment will determine the fate of the original GSC. Plasticity may also be responsible for reprogramming committed GBM progenitor cells and differentiated GBM cells to dedifferentiate into GSCs (8). It is noteworthy that the terms glioblastoma stem-like cells (GSLCs) and GSCs are vaguely described and used interchangeably in various reports. However, in our report, we introduce a three compartment model comprising; a) GSCs that are quiescent, self-renew slowly or infrequently and have the potential to proliferate, whereas GSLCs are proliferating GSCs that can self-renew under certain conditions, b) glioblastoma progenitor cells that proliferate rapidly and are committed to differentiate, and c) differentiated GBM cells. GSLCs are similar to progenitor cells in that, they are dedicated to differentiate and proliferate. Regarding metabolism, GSCs exhibit flexibility compared to neural stem cells due to the presence of certain enzymes [like pyruvate kinase isozyme 1 (PKM1) and pyruvate kinase isozyme 2 (PKM 2)] that enable GSCs to switch between glycolysis and oxidative phosphorylation (9). Both mitochondrial function and dysfunction play a significant role in GBM tumorigenesis, as mitochondria modulate the maintenance of GBM stemness, quiescence, and differentiation, whereas mitochondrial impairment is essential in arbitrating GSCs' resistance to treatment. Previous studies during the last decades have not been successful in resolving this issue. That said, understanding the involvement of mitochondria in GSC quiescence might shed some light on GBM pathophysiology. This review emphasizes the importance of mitochondria in maintaining GSC stemness, quiescence, and metabolism. Also, we highlight the general features of GSCs, GBM progenitor, and differentiated GBM cells.

Research History on Cancer Stem Cells

The history of cancer stem cell (CSC) research goes back to 1994 when leukemia initiating cells were identified (10). Identification of CSCs was a major breakthrough that could explain highly recurrent malignancies, such as GBM. Primarily, the extent to which oncogenesis and metastasis involve CSCs is unknown; however, as we learned more about CSCs in different types of malignancies such as liver, colorectal, ovarian, and brain cancers (for example, GBM), we realized how important these cells could be for an effective targeted cancer therapy. CSCs, characterized by their unique self-renewal and differentiating capacity, generate various tumor cells with different genetic constitutions, such as new GSCs and GBM neural progenitor cells that, in turn, give rise to the differentiated cells. The ability to stay in the quiescent state (during the G0 phase of the cell cycle) allows them to survive during the intensive cancer treatment. Recent discoveries have attributed glioblastoma resistance to the presence of cancer stem cells or so-called glioblastoma stem cells (GSC). GSCs, which originate from malignant transformation of neural stem cells (NSCs) of the subventricular zone (SVZ) tissues and differentiated neural cells such as astrocytes, maintain GBM tumor heterogeneity (11, 12).

General Features of GSCs

GSCs are distinguished from neural stem cells by their molecular, genetic, metabolic, and dynamic features. Cancer stem-like cells have fragmented mitochondria compared to differentiated GBM cells, which possess tubular-shaped mitochondria (13, 14). Stem cells are said to have fewer and less mature mitochondria that are relatively inactive compared to those of differentiated cells, resulting in decreased ROS generation and, thus, low ROS levels required for the maintenance of stem cell quiescence and self-renewal potential (15, 16). Previously, it was said that CSCs might favor glycolysis as it regulates stemness and minimizes ROS generation (17). However, recent studies suggest quiescent CSCs depend largely on OXPHOS. This is also true for differentiated non-proliferating GBM cells that cannot further differentiate. On the other hand, proliferating CSCs utilize both glycolytic and oxidative pathways. Depending on oxygenation, nutrient availability and tumor microenvironment, proliferating GSCs can transition between glycolytic and oxidative pathways (7, 18, 19). CSCs utilize both glycolysis and OXPHOS since they switch between quiescent and proliferation "states." A study on human TS1 GSLCs, upon acidic pH shift-induced quiescence, demonstrated the remodeling of mitochondria from tubular to donut shape to corroborate this. Similarly, placing the quiescent cells in a less acidic environment induced the alteration of mitochondria from donut to tubular shape (20). This study not only implies that donut-shaped mitochondria might be a feature of quiescent GSLCs but also suggests that mitochondria shape and function is dependent on GSLCs microenvironment. The influence of tumor microenvironment on CSCs has been extensively discussed elsewhere (14). Other features of GSCs and differentiated glioblastoma cells are shown in **Figure 1**.

Molecular and Genetic Features of GSCs

GSCs' surface molecular biomarkers include $CD49f^+$, $CD90^+$, $CD44^+$, $CD36^+$, $EGFR^+$, $A2B5^+$, $L1CAM^+$, and $CD133^+$ (21). Glycerol-3-phosphate dehydrogenase 1 (GPD1) is another important marker that distinguishes GSCs from normal neural stem cells and can be used as a prognostic factor. Following chemotherapy, dormant GSCs, expressing GPD1 and mainly located at the GBM tumor borders, can be activated (22). Neural stem cells (NSCs) or transformed astrocytes might give rise to GSCs following gaining access to stem-specific transcriptional programs. GSCs are maintained through epigenetic regulators and modify the gene expression in response to external cues (7). Radiation enhances tumor recurrence due to tumor cell DNA mutations conferred by radiation, thus, rendering the tumor cells resistant to treatment. However, GSCs are not only able to survive the extensive course of chemoradiotherapy but can also promote radiotherapy resistance through the preferential activation of DNA damage checkpoint response that, in turn, promotes their DNA repair capacity. As shown in **Figure 2**, cell cycle checkpoints are critical regulators of cell proliferation and development. Quiescent GSCs express a higher amount of G0/G1-phase regulatory molecules such as cyclin D1, cyclin D2, and cyclin E) at the transcriptional and translational levels (23). Certain genes, such as ectonucleotidase ENPP1 (ectonucleotide pyrophosphatase/phosphodiesterase 1), are overexpressed in GSCs. Their function is usually related to stem cell feature maintenance, cell cycle control, cell death, and potential to proliferate (24).

Transcriptomic analyses of samples of recurrent and newly diagnosed GBM have shown that GSCs, locating in different regions of the tumor, are characterized by different degrees of stemness and gene expression pattern; however, this intratumoral heterogeneity is not random and depends on the intratumoral architecture. Studies have shown that harvesting four samples from a single tumor is sufficient to predict and optimize therapy outcomes. It is important to note that post-operative radiochemotherapy can further induce longitudinal changes in gene expression of GSCs. On the other hand, the limitation of performing biopsy after each round of therapy is another challenge for studying these longitudinal mutational alterations. These result in an increased resistance rate after each therapy session (25–27).

Metabolic Features of GSCs

Metabolic alterations are evident in GSCs. Though rapidly proliferating cells from GBM patients are glycolytic, only a small fraction of these are GSCs which are quiescent and capable of self-renewal (28). Self-renewing GSCs, similar to most other types of

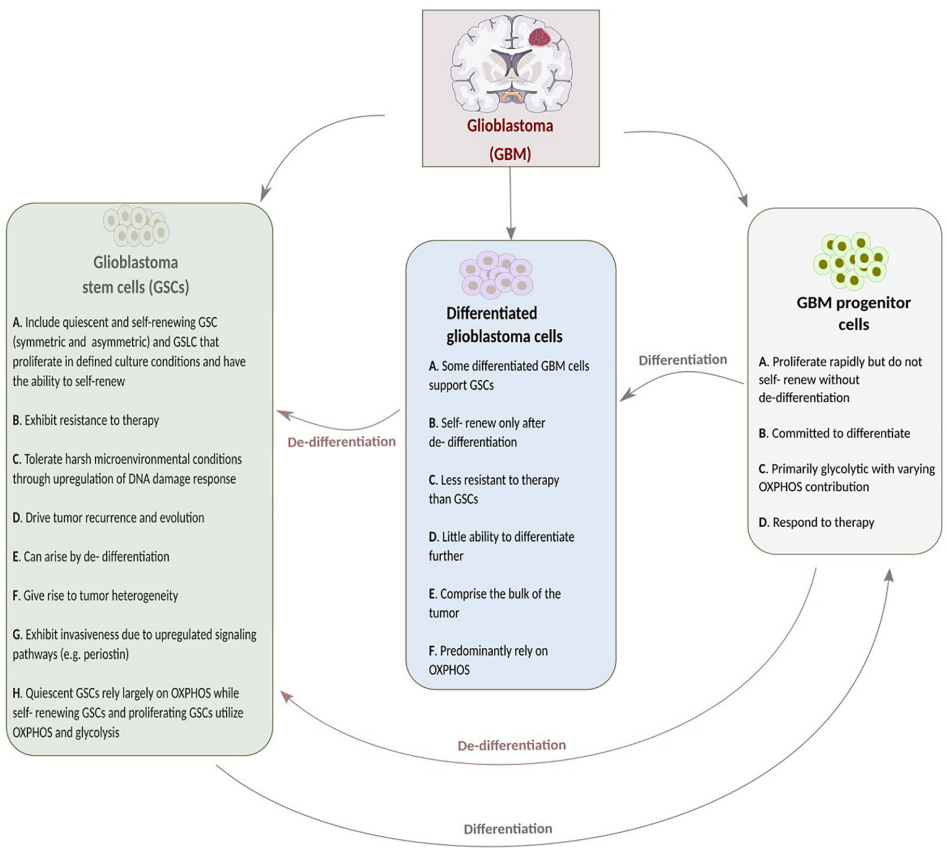

FIGURE 1 | A summary of the features of glioblastoma stem cells (GSCs), glioblastoma (GBM) progenitor cells and differentiated glioblastoma stem cells.

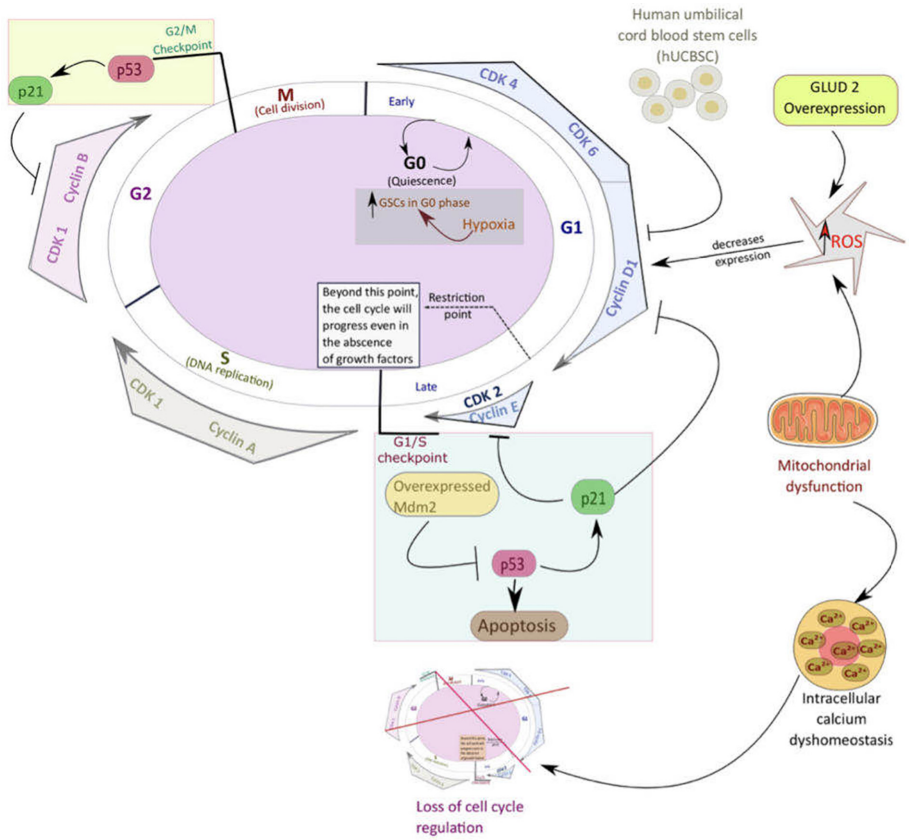

FIGURE 2 | An overview of the pathways that mediate quiescence in glioblastoma stem cells (GSCs). Mitochondria play a significant role in critical cellular processes such as cell cycle control, cell metabolism, regulation of calcium homeostasis, and reactive oxygen species (ROS) generation. Like most other types of cancer stem cells, GSCs utilize mitochondria oxidative phosphorylation (OXPHOS) to keep up their increased proliferation, resistance, and stemness. Mitochondrial dysfunction enhances tumorigenesis through different pathways such as loss of cell cycle control, intracellular calcium dyshomeostasis, increased transition of GSCs into the quiescent state, and decreased apoptosis.

cancer stem cells, utilize both glycolytic and OXPHOS. To keep up their increased proliferation, rapidly proliferating GBM cells utilize glycolysis while quiescent GSCs depend on OXPHOS to maintain their stemness. Unlike previous speculation, GSCs can switch between different energy pathways and exhibit intermediate metabolic features to adapt their metabolism according to the different conditions such as environmental stressors such as radiation. Moreover, quiescent GSCs exhibit lower glycolysis and oxygen consumption and a much acidic extracellular space compared to the differentiated GBM cells (18, 19).

Specific features of GSCs such as GSCs' self-renewal and decreased apoptosis are the hallmark of GBM resistance. Several factors regulate GSC proliferation and survival. One of the significant factors contributing to the increased tumorigenicity of GSCs is their high capacity for self-renewal. Early studies of mechanisms responsible for sustaining GSCs' self-renewal property highlighted the importance of SRY-box transcription factor 2 (SOX2) gene expression. Commonly, SOX2 expression is upregulated during neural development and is essential in inducing pluripotency (29). However, its overexpression in GSCs is associated with increased tumorigenicity and resistance. Further experiments on tumor-initiating cells (TICs) showed that SOX2 knockdown leads to decreased proliferation and self-renewal capacity. Moreover, these studies showed that GSCs share a similar mechanism with normal neural stem cells to sustain their stemness (30).

GBM Tumor Constitution and the Surrounding Tumor Microenvironment

The hierarchical model proposed for GBM involves the progression from stem cell populations to more differentiated progeny (31). Single-cell RNA-seq (scRNAseq) studies of IDH mutant gliomas have shown glioblastoma trilineage hierarchy, including progenitor, neuronal, and astro-mesenchymal cancer cells, among which the progenitor cancer cells have the highest proliferative and lowest differentiated properties (32). Furthermore, in IDH wild type GBM cells, proliferating GSCs, referred to as "progenitor GSCs" display a more rapid growth rate and a higher chemoresistance property. Previously, several pathways, such as EZH2, FOXM1, and Wnt, associated with GSC self-renewal and tumorigenicity, have been

identified. Recently, another critical pathway, the E2F4 pathway, has been identified by Couturier and colleagues. E2F gene family plays a key role as a cell cycle regulator and is critical for GSC progenitor cells. The inhibition of E2F4 is negatively correlated with GSC progenitor proliferation (33). In addition to the previously mentioned pathways, mitochondrial dynamics is crucial in regulating postmitotic cell fate. Iwata and colleagues showed that shortly after mitosis of neural stem cells, daughter cells that undergo and displays mitochondrial fusion maintain their self-renewal property, and those with mitochondrial fission differentiate into neurons (34). However, further studies are required to determine whether a similar mechanism exists in different GSC lineages.

As in normal tissues, quiescent and active CSCs coexist in the tumor bulk (35).GSCs consist of a small subpopulation of stem-like cells conferring tumor recurrence (36). Normal neural stem cells (NSCs) of the brain are located in the subventricular zone and hippocampus (37). Since GSCs' surrounding microenvironment has to fit their need to maintain their stemness, intratumoral GSCs reside in specific locations such as perivascular, hypoxic, and necrotic niches. The perivascular niches can provide essential signals (such as Wnts) necessary for GSC maintenance, growth, and invasion (38–40).

Along with GSLCs and differentiated glioblastoma cells, other types of cells such as neural precursor cells (NPCS), astrocytes, neurons, macrophages, microglia, and endothelial cells as well as vascular components and extracellular matrix (ECM) are contributing to the intratumoral heterogeneity (41). Cellular components of the tumor communicate with each other and distant cells through extracellular vesicles (EVs). These EVs can also alter tumor growth, resistance, and death (42).

Role of Mitochondria in GBM Tumorigenesis and Metastasis

Mitochondria, known to be responsible for cellular respiration, generation of oxidative radicals and their central role in apoptosis, DNA repair, autophagy, and cell cycle control, have recently been the focus of attention for the role of their genome in cancer development. The proposed role of mitochondria in tumorigenesis and metastasis has been studied in several types of malignancies, such as breast, lung, and prostate cancer. Mitochondrial dysfunction is associated with altered metabolism and can lead to enhanced tumorigenesis and metastasis. A broad study on mitochondrial cancer genome has shown that hypermutation, variations in structure and copy- number, and somatic transfer of mtDNA into the nuclear genome are associated with increased risk of cancer development and growth, and metastasis (43). Studies have shown that autophagy plays a critical role in the process of tumor cell survival, growth, and resistance. Different cancer therapeutic agents exert different regulatory effects on autophagy, leading to activation or inhibition of cytoprotective or cytotoxic autophagy. Moreover, in some types of malignancies such as GBM, chemoresistance to the first-line therapy agents such as TMZ can be mediated *via* ROS induced- activation of cytoprotective autophagy. Therefore, understanding the interplay between mitochondria, autophagy, tumor growth, resistance, and metastasis will provide us with better clues to new treatment strategies (44).

Mitochondria are responsible for maintaining the oxidant-antioxidant system in a cell. Oxidative damage, which has been implicated in tumorigenesis, usually follows mitochondria dysfunction. Mutations in genes encoding components of mitochondrial protein complexes such as NADH-ubiquinone oxidoreductase chain 4 (ND4) subunit can lead to elevated superoxide radical ($O_2^{\bullet-}$) production, thus resulting in sustained ROS-dependent oncogenic pathways and induction of mitochondrial DNA (mtDNA). These changes are associated with an increased risk of tumorigenesis and metastasis in GBM (45).

GLUD2, which encodes for glutamate dehydrogenase (GDH), plays a critical role in regulating GBM tumorigenesis and is involved in normal cellular processes such as Krebs cycle and energy production as well as ammonia homeostasis (46). GDH is a mitochondrial enzyme, and its primary function is the reversible catabolization of glutamate to α-KG and ammonia. Typically, GDH exhibits high activity levels in specific mammalian organs such as the brain, liver, pancreas and kidney (47). Overexpression of GLUD2 is associated with the modification of mitochondrial function and metabolic profile of human GBM cells. GLUD2 overexpression is associated with increased ROS production due to increased mitochondrial oxidative metabolism and increased oxygen consumption levels (48). An increase in ROS levels causes cell cycle arrest in G0/G1 due to the decreased cyclin D1 and E expression (49). Also depicted in **Figure 2**, increased ROS levels inhibit the cell cycle's progression, hence, causing cells to remain in their quiescent stage.

The Warburg effect, which is characterized by increased tumor cell glycolysis and decreased mitochondrial energy metabolism even in the presence of oxygen, can be seen in various malignancies such as GBM (50). Furthermore, malignant cells raise the mitochondrial apoptotic threshold by activating mitochondrial maintenance programs, which is important for enhancing cancer cell survival, proliferation, and metastasis. Other organelles such as the nucleus and endoplasmic reticulum and their crosstalk with mitochondria are essential components of cancer cell physiology such as survival, proliferation, metastasis, and stemness (51). In extreme environmental conditions such as hypoxia and acidic shift of the environment, nutritional deficiency and radiation, GSCs use specific protective mechanisms such as activation of stress response pathways to counteract the anti-cancer effects of endogenous stressors such as increased ROS production and exogenous stressors such as chemotherapy agents. These pathways, such as cytosolic heat shock response (HSR), the integrated stress response (ISR), and unfolded protein response (UPR), are either mediated by mitochondria or endoplasmic reticulum (ER) or cooperation of both organelles (52, 53).

Glioblastoma Stem Cell Maintenance, Differentiation, and Quiescence

Stem Cell Maintenance

Stem cell maintenance is critical for GBM tumor recurrence, tumorigenicity, and metastasis. This stem cell feature is mediated through different mechanisms. It is noteworthy that differentiated GBM cells demonstrate lower therapy resistance

compared to GSCs. The more we learn about these novel pathways, the better we can develop anti-cancer agents effectively targeting GSCs and induce their differentiation into the less resistant GBM cell types. GSCs employ specific mechanisms to maintain their stem cell features. One of these mechanisms is to counteract factors that can induce cell differentiation, such as bone morphogenetic proteins (BMPs). In response to anti-GSCs effects of BMP, GSCs secrete gremlin1, a BMP antagonist that inhibits BMP signaling, resulting in maintenance of stem cell features such as self-renewal capacity (54).

Hypoxia is another crucial factor that maintains and regulates stemness features and undifferentiated state in neural, hematopoietic stem cells, and GSCs (55, 56). Under hypoxic conditions, the number of GSCs in the G0 phase increases and more differentiated glioblastoma cells are induced into the undifferentiated form. Hypoxia maintains GSCs through the activation of NOTCH pathway, which is mediated by hypoxia-inducible factor-1α (HIF-1α) and 2α (HIF-2α) (56–58). Moreover, hypoxia can induce mixed-lineage leukemia 1 (MLL1), a histone methyltransferase, to increase the sensitivity and response of GSCs to hypoxia-induced regulation of stemness features (55).

An important tumor suppressor, p53 regulates different cellular functions such as cell differentiation, DNA repair, and angiogenesis. Mouse double minute 2 homolog (MDM2) gene is a negative regulator of p53. Within cells, p53 is usually present in low levels albeit, in certain types of malignancies, due to disrupted MDM2 and p53 interaction, p53 is upregulated to prevent cells' malignant transformation in response to oncogenic stress (59). Conversely, in some malignancies such as GBM, MDM2 is overexpressed, and as a result, the activity of p53 is inhibited (60). In addition, Oliner et al. demonstrated the importance of MDM2 in maintaining GSC stemness, inhibition of which can cause further inhibition of factors related to GSCs stemness (61). Intriguingly, cholesterol might be involved in GSC stemness. RNA sequencing comparison of patient-derived GSCs and differentiated GBM cells showed the importance of cholesterol biosynthesis pathway in maintaining GSC stemness. More studies revealed that farnesyl diphosphate synthase (FDPS), which serves as an important enzyme in isoprenoid biosynthesis, has a vital role in GSC stemness maintenance (62). It is of note that GSCs highly express ectonucleotidase ENPP1 (ectonucleotide pyrophosphatase/phosphodiesterase 1) compared to other types of cells such as NSCs. Ectonucleotidase ENPP1 is involved in maintaining GSCs, and its knockdown induces GSCs to differentiate into GBM cells, lowers cellular proliferation rate, induces cell death, and decreases chemotherapy resistance (24).

Long non-coding RNAs (long ncRNA, lncRNA) are other essential mediators of GBM resistance, involved in various diseases and act as critical biological regulators. Follow-up of patients with GBM showed that overexpression of TP73-AS1, a GBM-associated lncRNA, maintains stemness of GSCs through interactions involving multiple pathways, thus leading to increased resistance of GBM cells to TMZ therapy (4). That said, lncRNA are good targets for potential therapeutic options.

Transformation of GSCs Into Differentiated Cells and Dedifferentiation of GBM Cells Into Stem-Like Cells

Early studies have established the importance of c-Jun N-terminal kinase (JNK) signaling pathway in GSC maintenance, self-renewal, and differentiation. Activation of the JNK pathway is necessary for self-renewal and inhibition of GSC from differentiation. Therefore, JNK pathway inhibition promotes GSC differentiation and diminishes tumor-initiating potential, making them more prone to cancer therapy strategies (63). Nutritional stress, acidic environment, and hypoxia induce dedifferentiation of GBM cells into GSCs. However, eliminating any of these conditions permit GSCs proliferation and transition into differentiated GBM cells, with increased sensitivity to the anti-cancer therapy (23).

Dedifferentiation of GBM cells into stem-like cells, possible through various mechanisms, is required for tumor continuity and is usually associated with a low survival rate. As we previously mentioned, hypoxia can induce transformation of differentiated GBM tumor cells into an undifferentiated state that exhibits stem-cell-like features. The tumor microenvironment plays a critical role in the stemness and differentiation state of different tumor cells. Cancer therapy, such as irradiation, can alter the tumor microenvironment and promote stem-like cell features, angiogenesis, recruitment of inflammatory cells such as $Ly6G^+$ inflammatory cells like tumor-associated neutrophils (TANs) and granulocytic myeloid-derived suppressor cells (G-MDSCs) (64). Following radiation therapy, GBM tumor cells are driven to dedifferentiation. Besides, $Ly6G^+$ inflammatory cells further promote the secretory feature of senescent GBM cells and alteration of tumor microenvironment, which are mediated through NFκB signaling pathway. $Ly6G^+$ inflammatory cells promote GBM tumor cells dedifferentiation through the NO-ID4 axis. Inhibitors of differentiation (ID) family members are important regulators of GSCs with stem-like features and GBM cells' transformation into GSCs (65–68).

Nutritional stress or nutritional deprivation instigates dedifferentiation of GBM cell into GSCs and is associated with an increased expression of GBM stem-like cell features, including biomarkers such as CD133, therapy resistance, and angiogenesis. Moreover, nutritional stress activates Wnt and Hedgehog signaling pathways and causes overexpression and nuclear localization of stemness markers such as Sox2, Oct 4, and Nanog at the transcriptional and translational levels (23).

Transition of GSCs Into Quiescence and Mechanisms Involved in Quiescent State Maintenance

The transition of GSCs into quiescent state (G0–G1 phase arrest) is a tumor protective response following chemoradiotherapy. Proteins such as Cdk 4, Cdk 6, cyclin B1, and cyclin D1 regulating the cell cycle are down-regulated upon entry into the quiescent state. Inhibition of Cyclin D1, which regulates cell cycle progression through the G1 phase in human umbilical cord blood stem cells (hUCBSC), can induce glioblastoma cell lines to enter cell cycle arrest (69). A decrease in intracellular pH is associated with GSCs induction into the quiescent state, increased stemness and increased expression of stemness

markers (20). Though it was proposed that the simultaneous treatment with TMZ and glucose starvation could promote GBM tumor cell death, a recent study by Wang et al. suggested that glucose starvation can induce resistant GBM tumor cells to enter quiescence, thus leading to their increased resistance to chemotherapy (70).

Quiescent GSCs stay in a functional reversible G0 phase, vigorously maintained by several pathways until reactivation and reentry into the cell cycle. In **Figure 2**, various pathways involved in GBM quiescence are illustrated. BMP signaling, found to be the mediator of GSCs quiescence, is further regulated by its downstream targets, ID1 and p21, and is also associated with increased chemoradiotherapy resistance. A series of experiments by Sachdeva and colleagues showed that BMP4 not only modulates GSC phenotype but also causes an inhibition of GSC self-renewal capacity and tumorigenicity (71). In recent years, mitochondria have been recognized as a crucial regulator of GSC quiescent state maintenance, potentially serving as an important target against GBM resistance.

As aforementioned, mitochondria can counteract the destructive effects of endogenous and exogenous stressors in GSCs. One of these mechanisms is the activation of mitochondrial stress pathways such as mitochondrial unfolded protein response (UPR^{mt}). Chaperones and proteases of the UPR^{mt} pathway maintain cellular homeostasis through proteotoxic stress elimination. Intracellular calcium ion (Ca^{2+}) homeostasis, regulated by mitochondria, is necessary as intracellular Ca^{2+} modulates cell-cycle progression (72). Mitochondrial Ca^{2+} uptake and regulation of store-operated Ca^{2+} entry (SOCE) activity controls Ca^{2+} levels through store-operated channels (73, 74).

Reactivation of Quiescent GSCs

Quiescent GSCs reside mainly in pre-necrotic areas of the tumor. Upon removal of exogenous and endogenous stressors, GSCs reactivate and migrate into the oxygen and nutrient-rich areas such as perivascular zones for proliferation and differentiation. Nevertheless, how these cells get reactivated and enter the proliferative phase is yet to be clarified.

GINS complex, a heterotetrameric complex which consists of four subunits including Sld5, Psf1, Psf2, and Psf3, is important in initiating DNA replication and progression by serving as a DNA helicase in association with CDC45 and MCM2-7 (75, 76). Recent studies have shown that induction of GINS expression is not only required for the reactivation of quiescent GBM cells residing in peri-necrotic niches, but also determines the proliferative phenotypes of quiescent GBM cells. Quiescent GSCs show decreased GINS protein subunit levels, which positively correlate with the results stating that GINS is involved in the reactivation of quiescent GSCs (77).

Therapeutic Implications of Proliferative and Quiescent GSCs

A significant hurdle in GBM treatment is the presence of resistant intratumoral GSCs. Most current treatment strategies show little to no efficacy due to the evasiveness of GSCs. However, engineered oncolytic viruses are a promising treatment strategy for some malignancies, such as GBM. Recent discoveries have shown that the Zika virus (ZIKV; the primary cause of newborn microcephaly outbreak in 2015) could treat resistant GBM. The Zika virus primarily kills different brain cells, such as neural precursor cells (NPC), leading to microcephaly. Further studies have shown that ZIKV displays higher oncolytic activity toward GSCs than NPCs and differentiated glioblastoma cells, and at the same time, causes no harm to normal brain tissue. ZIKV confers its oncolytic property by inhibiting the self-renewal capacity of GSCs (78). Earlier studies demonstrated that the upregulated expression of SOX2 in GSCs is associated with GBM's higher tumorigenicity due to an increased self-renewal capacity (30). SOX2 acts by modulating GSCs ZIKV infection and regulating their expression of the Integrin α_v subunit. Integrin α_v plays a major role in cellular migration, proliferation, and intracellular signaling by the formation of a heterodimer with one of the distinct β subunits including β_1, β_3, β_5, β_6, and β_8 (79). Further experiments showed that $\alpha_v\beta_5$ plays a critical role in ZIKV infection of GSCs by maintaining the GSCs (80).

Manipulation of GSC differentiation and proliferation can serve as an important target for effective treatment of resistant GBM. Theoretically, each GSC has three choices: self-renewal to produce two GSCs, asymmetric division to produce one GSC and one cell that proliferates but cannot self-renew, and commitment to differentiate to produce two cells that proliferate but cannot self-renew. Generally, GSC are present in two different niches: quiescent and active in cell division. Differentiated GBM cells exhibit a lower resistance to chemoradiotherapy compared to undifferentiated and quiescent cancer stem cells. GSCs exhibit low expression levels of MKP1, which is a dual-specificity phosphatase and negatively regulates ERK1/2 and p38 MAPK. The role of MKP1 is significant since the high expression level of MKP1 is associated with the differentiation of GSCs and their increased sensitivity to TMZ (81). What makes these findings significant is that a group of glioblastoma patients with a higher expression level of MKP1 showed improved prognosis and overall survival rate. Studies on histone deacetylase inhibitors (HDACIs) showed that these agents could cause an upregulation of glioma cell MKP1; thus, MKP1 is a promising treatment strategy targeting resistant GSCs (82).

Another critical target of resistant GBM therapy is mitochondria. As aforementioned, mitochondria play a critical role in tumor biology by regulating cell cycle, metabolism, apoptosis, DNA repair, and maintenance of stemness in cancer stem cells. Mitochondria enable cancer cells to be more tolerant against hypoxia, radiation, and cytotoxic agents by activating stress response pathways and altering cell metabolism. A small synthetic molecule named KHS101 was discovered to effectively impair mitochondrial heat shock protein family D member 1 (HSPD1) and its dependent metabolic pathway. KHS101 is a good anti-tumor agent since it can effectively exert its anti-tumor effect on different subtypes of cancer cells, including GSCs, without negatively impacting intact cells. KHS101 interrupts GBM cell aerobic glycolysis and mitochondria respiration-dependent pathways and causes aggregation of HSPD1 and metabolic enzymes in GBM cells,

thereby promoting their metabolic exhaustion. Induction of acute metabolic stress, which leads to alteration of the cell cycle, metabolic, and stemness pathways, causes different subtypes of GBM cells to undergo autophagy and apoptosis after KSH101 treatment. All of this could lead to the loss of stem cell-like features of GBM cells and an increase in cell death. Further experiments on patient-derived tumor xenografts in mice showed that KHS101 treatment could successfully diminish tumor growth and increase the survival rate (83).

Induction of reactive oxygen species (ROS), by-products of mitochondrial metabolism, can be used as another effective treatment strategy. In GSCs, ROS is present at low levels due to the free radical scavenging system. Moreover, low levels of ROS are associated with a higher malignant potential. Further, studies have shown that high ROS levels can prevent the cancer progression (84, 85). Curcumin, the main component of turmeric, has previously shown its antioxidant effects on the prevention and progression of different types of cancers. However, what makes curcumin an even more valuable anti-cancer agent is that it can target non-GSCs (GBM cell that do not have GSCs) and effectively target GSCs through different mechanisms such as the induction of mitochondrial ROS, leading to MAPK activation, STAT3 inactivation, and downregulation of STAT3 targets. Together, these mechanisms could decrease the self-renewal and survival of GSCs and non-GSCs (86).

Previously, we stated that p53 inactivation due to MDM2 overexpression could lead to GBM tumor recurrence *via* the absence of inhibition of stemness-related factors in GSCs. Experiments on patient-derived GSCs have shown that GBM stemness can be inhibited by MDM2 inhibitor, AMG232. However, p53 reactivation is required to increase the sensitivity of GBM tumor cells to MDM2 inhibitors (61). Besides, targeting the cholesterol biosynthesis pathway has shown to be a promising treatment strategy against resistant GSCs. Alendronate, a popular anti-osteoporotic agent, is effective in GBM treatment as it inhibits farnesyl diphosphate synthase (an enzyme involved in isoprenoid biosynthesis and GSCs' maintenance) that in turn, reduces embryonic stem-cell features and activation of pathways related to necrosis and development in GBM cells (62).

Lack of selectivity of specific agents to target GSCs is another obstacle in managing GBM. Recently, an RNA aptamer (a shortened form of aptamer 40L) known as A40s, was developed to bind to $CD133^{+}$-GSCs selectively. Moreover, GSCs can internalize these aptamers, which could be used as a means of drug delivery such as microRNAs targeting and inhibiting GSCs (87). Induction of apoptosis through mitochondria ROS formation is an important mechanism employed by certain agents such as sulforaphane, which is an isothiocyanate found in cruciferous vegetables that exhibits anti-cancer properties (88).

The few aforementioned therapeutic strategies could potentially be used in the management of GBM. However, none of these therapeutic agents can achieve effective GBM treatment. An effective therapeutic strategy would be one that prevents GBM progression, recurrence, and reduces the possibility of GBM resistance development. An ideal therapeutic agent should possess specific characteristics such as high affinity to its target cells (GSCs), reasonable price, public availability and, most importantly, effective against GSLCs and quiescent GSCs.

DISCUSSION

GSCs are a distinct subpopulation of GBM cells with unique self-renewal properties, the potential to proliferate and differentiate. In the presence of environmental stressors (such as chemoradiotherapy, nutritional deprivation, hypoxia, and acidic shift of the environment), these cells undergo cell cycle arrest and become quiescent. The quiescent state is phase G0 of the cell cycle, where cell inactivity is observed. Chemoradiotherapy mostly affects rapidly dividing cells, which explains why the quiescent state protects GSCs during chemoradiotherapy. GSCs are dormant until an activating signal causes them to reactivate, and migrate to the perivascular regions, which provides them with enough nutrition and oxygen for proliferation. Currently, we are faced with several obstacles in the effective treatment of GBM such as the presence of quiescent GSCs. Conventional therapies lack specificity for quiescent GSCs, making it difficult to eradicate these tumor-driving cells. Although extensive research has identified most of the pathways and mechanisms involved in quiescent state activation and reactivation in other kinds of malignancies and neural stem cells, understanding of quiescent GSCs is not well elucidated, therefore, more studies are warranted.

For effective GBM treatment, future therapeutic strategies focusing on reducing GSC transition into the quiescent state and reactivation of existing quiescent GSCs, might be beneficial. Another focus could be induction of GSCs to proliferate and become committed to differentiate. This way, the tumor-initiating cell population could be significantly reduced. More research on the specific role of the brain lymphatic and immune system in GBM and the interaction between these systems is also warranted. It is necessary to understand how proliferative GBM cells and quiescent GSCs behave in different microenvironments, including in an inflammatory setting.

Methods such as High-throughput Automated Single Cell Imaging Analysis (HASCIA) facilitate the assessment of heterogeneity and state transition in GSCs at the single-cell level, which is vital for future GBM research and discovery of new anti-cancer drugs that can target state transitions, for instance, inhibition of quiescent state transformation or activation of differentiated state (89). A thorough understanding of GSC transition between quiescent, self-renewing and proliferative progenitor states that cannot self-renew could help develop targeted therapy to these specific populations with little influence on the normal neural stem cells. We believe that this ideal therapy will most likely be a combinational therapy due to the complexity of the GBM hierarchy.

AUTHOR CONTRIBUTIONS

YI, BJ, and FO contributed equally and thus share the first authorship. CS, JL, JW, and ZD made sure this article was up to

standard. CS is the corresponding author and JL is the co-corresponding author. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Kim W, Yoo H, Shin SH, Gwak HS, Lee SH. Extraneural Metastases of Glioblastoma without Simultaneous Central Nervous System Recurrence. *Brain Tumor Res Treat* (2014) 2(2):124–7. doi: 10.14791/btrt.2014.2.2.124
2. Wu Q, Berglund AE, Wang D, MacAulay RJ, Mulé JJ, Etame AB. Paradoxical epigenetic regulation of XAF1 mediates plasticity toward adaptive resistance evolution in MGMT-methylated glioblastoma. *Sci Rep* (2019) 9(1):14072. doi: 10.1038/s41598-019-50489-2
3. Nie E, Miao F, Jin X, Wu W, Zhou X, Zeng A, et al. Fstl1/DIP2A/MGMT signaling pathway plays important roles in temozolomide resistance in glioblastoma. *Oncogene* (2019) 38(15):2706–21. doi: 10.1038/s41388-018-0596-2
4. Mazor G, Levin L, Picard D, Ahmadov U, Carén H, Borkhardt A, et al. The lncRNA *TP73-AS1* is linked to aggressiveness in glioblastoma and promotes temozolomide resistance in glioblastoma cancer stem cells. *Cell Death Dis* (2019) 10:246. doi: 10.1038/s41419-019-1477-5
5. Soni H, Bode J, Nguyen CDL, Puccio L, Neßling M, Piro RM, et al. PERK-mediated expression of peptidylglycine α-amidating monooxygenase supports angiogenesis in glioblastoma. *Oncogenesis* (2020) 9(2):18. doi: 10.1038/s41389-020-0201-8
6. Yang X, Cao W, Wang X, Zhang X, Zhang W, Li Z, et al. Down-regulation of 14-3-3zeta reduces proliferation and increases apoptosis in human glioblastoma. *Cancer Gene Ther* (2020) 27:399–411. doi: 10.1038/s41417-019-0097-7
7. Gimple RC, Bhargava S, Dixit D, Rich JN. Glioblastoma stem cells: lessons from the tumor hierarchy in a lethal cancer. *Genes Dev* (2019) 33(11-12):591–609. doi: 10.1101/gad.324301.119
8. Dirkse A, Golebiewska A, Buder T, Nazarov PV, Muller A, Poovathingal S, et al. Stem cell-associated heterogeneity in Glioblastoma results from intrinsic tumor plasticity shaped by the microenvironment. *Nat Commun* (2019) 10 (1):1787. doi: 10.1038/s41467-019-09853-z
9. Vlashi E, Lagadec C, Vergnes L, Matsutani T, Masui K, Poulou M, et al. Metabolic state of glioma stem cells and nontumorigenic cells. *Proc Natl Acad Sci U S A* (2011) 108(38):16062–7. doi: 10.1073/pnas.1106704108
10. Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, et al. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* (1994) 367(6464):645–8. doi: 10.1038/367645a0
11. Matarredona ER, Pastor AM. Neural Stem Cells of the Subventricular Zone as the Origin of Human Glioblastoma Stem Cells. Therapeutic Implications. *Front Oncol* (2019) 9:779:779. doi: 10.3389/fonc.2019.00779
12. Lee JH, Lee JE, Kahng JY, Kim SH, Park JS, Yoon SJ, et al. Human glioblastoma arises from subventricular zone cells with low-level driver mutations. *Nature* (2018) 560(7717):243–7. doi: 10.1038/s41586-018-0389-3
13. Xie Q, Wu Q, Horbinski C, Flavahan WA, Yang K, Zhou W, et al. Mitochondrial control by DRP1 in brain tumor initiating cells. *Nat Neurosci* (2015) 18:501–10. doi: 10.1038/nn.3960
14. Peiris-Pagès M, Martinez-Outschoorn UE, Pestell RG, Sotgia F, Lisanti MP. Cancer stem cell metabolism. *Breast Cancer Res* (2016) 18(1):55. doi: 10.1186/s13058-016-0712-6
15. Prigione A, Fauler B, Lurz R, Lehrach H, Adjaye J. The senescence-related mitochondrial/oxidative stress pathway is repressed in human induced pluripotent stem cells. *Stem Cells* (2010) 28(4):721–33. doi: 10.1002/stem.404
16. Jang YY, Sharkis SJ. A low level of reactive oxygen species selects for primitive hematopoietic stem cells that may reside in the low-oxygenic niche. *Blood* (2007) 110(8):3056–63. doi: 10.1182/blood-2007-05-087759
17. Jang H, Yang J, Lee E, Cheong JH. Metabolism in embryonic and cancer stemness. *Arch Pharm Res* (2015) 38(3):381–8. doi: 10.1007/s12272-015-0558-y
18. Michelakis ED, Sutendra G, Dromparis P, Webster L, Haromy A, Niven E, et al. Metabolic modulation of glioblastoma with dichloroacetate. *Sci Transl Med* (2010) 2(31):31ra34. doi: 10.1126/scitranslmed.3000677
19. Weinberg SE, Chandel NS. Targeting mitochondria metabolism for cancer therapy. *Nat Chem Biol* (2015) 11(1):9–15. doi: 10.1038/nchembio.1712
20. Aulestia FJ, Néant I, Dong J, Haiech J, Kilhoffer MC, Moreau M, et al. Quiescence status of glioblastoma stem-like cells involves remodelling of Ca^{2+} signalling and mitochondrial shape. *Sci Rep* (2018) 8:9731. doi: 10.1038/s41598-018-28157-8
21. Yang L, Shi P, Zhao G, Xu J, Peng W, Zhang J, et al. Targeting cancer stem cell pathways for cancer therapy. *Sig Transduct Target Ther* (2020) 5:8. doi: 10.1038/s41392-020-0110-5
22. Rusu P, Shao C, Neuerburg A, Acikgöz AA, Wu Y, Zou P, et al. GPD1 Specifically Marks Dormant Glioma Stem Cells with a Distinct Metabolic Profile. *Cell Stem Cell* (2019) 25(2):241–257.e8. doi: 10.1016/j.stem.2019.06.004
23. Mondal S, Bhattacharya K, Mandal C. Nutritional stress reprograms dedifferention in glioblastoma multiforme driven by PTEN/Wnt/Hedgehog axis: a stochastic model of cancer stem cells. *Cell Death Discovery* (2018) 4:110. doi: 10.1038/s41420-018-0126-6
24. Bageritz J, Puccio L, Piro RM, Hovestadt V, Phillips E, Pankert T, et al. Stem cell characteristics in glioblastoma are maintained by the ecto-nucleotidase E-NPP1. *Cell Death Differ* (2014) 21(6):929–40. doi: 10.1038/cdd.2014.12
25. Kim EL, Sorokin M, Kantelhardt SR, Kalasauskas D, Sprang B, Fauss J, et al. Intratumoral Heterogeneity and Longitudinal Changes in Gene Expression Predict Differential Drug Sensitivity in Newly Diagnosed and Recurrent Glioblastoma. *Cancers (Basel)* (2020) 12(2):520. doi: 10.3390/cancers12020520
26. Prabhu A, Kesarwani P, Kant S, Graham SF, Chinnaiyan P. Histologically defined intratumoral sequencing uncovers evolutionary cues into conserved molecular events driving gliomagenesis. *Neuro Oncol* (2017) 19(12):1599–606. doi: 10.1093/neuonc/nox100
27. Morrissy AS, Cavalli FMG, Remke M, Ramaswamy V, Shih DJH, Holdago BL, et al. Spatial heterogeneity in medulloblastoma. *Nat Genet* (2017) 49(5):780–8. doi: 10.1038/ng.3838
28. Spehalski EI, Lee JA, Peters C, Tofilon P, Camphausen K. The Quiescent Metabolic Phenotype of Glioma Stem Cells. *J Proteomics Bioinform* (2019) 12 (6):96–103. doi: 10.35248/0974-276x.19.12.502
29. Sarkar A, Hochedlinger K. The sox family of transcription factors: versatile regulators of stem and progenitor cell fate. *Cell Stem Cell* (2013) 12(1):15–30. doi: 10.1016/j.stem.2012.12.007
30. Gangemi RM, Griffero F, Marubbi D, Perera M, Capra MC, Malatesta P, et al. SOX2 silencing in glioblastoma tumor-initiating cells causes stop of proliferation and loss of tumorigenicity. *Stem Cells* (2009) 27(1):40–8. doi: 10.1634/stemcells.2008-0493
31. Lan X, Jörg DJ, Cavalli FMG, Richards LM, Nguyen LV, Vanner RJ, et al. Fate mapping of human glioblastoma reveals an invariant stem cell hierarchy. *Nature* (2017) 549(7671):227–32. doi: 10.1038/nature23666
32. Venteicher AS, Tirosh I, Hebert C, Yizhak K, Neftel C, Filbin MG, et al. Decoupling genetics, lineages, and microenvironment in IDH-mutant gliomas by single-cell RNA-seq. *Science* (2017) 355(6332):eaai8478. doi: 10.1126/science.aai8478
33. Couturier CP, Ayyadhury S, Le PU, Nadaf J, Monlong J, Riva G, et al. Single-cell RNA-seq reveals that glioblastoma recapitulates a normal

neurodevelopmental hierarchy. *Nat Commun* (2020) 11:3406. doi: 10.1038/s41467-020-17186-5
34. Iwata R, Casimir P, Vanderhaeghen P. Mitochondrial dynamics in postmitotic cells regulate neurogenesis. *Science* (2020) 369(6505):858–62. doi: 10.1126/science.aba9760
35. Li L, Clevers H. Coexistence of quiescent and active adult stem cells in mammals. *Science* (2010) 327(5965):542–5. doi: 10.1126/science.1180794
36. Zhou D, Alver BM, Li S, Hlady RA, Thompson JJ, Schroeder MA, et al. Distinctive epigenomes characterize glioma stem cells and their response to differentiation cues. *Genome Biol* (2018) 19(1):43. doi: 10.1186/s13059-018-1420-6
37. Li SC, Vu LT, Ho HW, Yin HZ, Keschrumrus V, Lu Q, et al. Cancer stem cells from a rare form of glioblastoma multiforme involving the neurogenic ventricular wall. *Cancer Cell Int* (2012) 12(1):41. doi: 10.1186/1475-2867-12-41
38. Griveau A, Seano G, Shelton SJ, Kupp R, Jahangiri A, Obernier K, et al. A Glial Signature and Wnt7 Signaling Regulate Glioma-Vascular Interactions and Tumor Microenvironment. *Cancer Cell* (2018) 33(5):874–889.e7. doi: 10.1016/j.ccell.2018.03.020
39. Kim JK, Jeon HY, Kim H. The molecular mechanisms underlying the therapeutic resistance of cancer stem cells. *Arch Pharm Res* (2015) 38 (3):389–401. doi: 10.1007/s12272-014-0531-1
40. Cabarcas SM, Mathews LA, Farrar WL. The cancer stem cell niche–there goes the neighborhood? *Int J Cancer* (2011) 129(10):2315–27. doi: 10.1002/ijc.26312
41. Tang M, Xie Q, Gimple RC, Zhong Z, Tam T, Tian J, et al. Three-dimensional bioprinted glioblastoma microenvironments model cellular dependencies and immune interactions. *Cell Res* (2020) 30(10):833–53. doi: 10.1038/s41422-020-0338-1
42. Walker S, Busatto S, Pham A, Tian M, Suh A, Carson K, et al. Extracellular vesicle-based drug delivery systems for cancer treatment. *Theranostics* (2019) 9(26):8001–17. doi: 10.7150/thno.37097
43. Yuan Y, Ju YS, Kim Y, Li J, Wang Y, Yoon CJ, et al. Comprehensive molecular characterization of mitochondrial genomes in human cancers. *Nat Genet* (2020) 52(3):342–52. doi: 10.1038/s41588-019-0557-x
44. Ulasov I, Fares J, Timashev P, Lesniak MS. Editing Cytoprotective Autophagy in Glioma: An Unfulfilled Potential for Therapy. *Trends Mol Med* (2020) 26 (3):252–62. doi: 10.1016/j.molmed.2019.11.001
45. Raimondi V, Ciccarese F, Ciminale V. Oncogenic pathways and the electron transport chain: a dangeROS liaison. *Br J Cancer* (2020) 122(2):168–81. doi: 10.1038/s41416-019-0651-y
46. Comelli M, Pretis I, Buso A, Mavelli I. Mitochondrial energy metabolism and signalling in human glioblastoma cell lines with different PTEN gene status. *J Bioenerg Biomembr* (2018) 50(1):33–52. doi: 10.1007/s10863-017-9737-5
47. Spanaki C, Plaitakis A. The role of glutamate dehydrogenase in mammalian ammonia metabolism. *Neurotox Res* (2012) 21(1):117–27. doi: 10.1007/s12640-011-9285-4
48. Shashidharan P, Plaitakis A. The discovery of human of GLUD2 glutamate dehydrogenase and its implications for cell function in health and disease. *Neurochem Res* (2014) 39(3):460–70. doi: 10.1007/s11064-013-1227-5
49. Wang X, Liu J, Jiang L, Wei X, Niu C, Wang R, et al. Bach1 Induces Endothelial Cell Apoptosis and Cell-Cycle Arrest through ROS Generation. *Oxid Med Cell Longev* (2016) 2016:6234043. doi: 10.1155/2016/6234043
50. Nie H, Ju H, Fan J, Shi X, Cheng Y, Cang X, et al. O-GlcNAcylation of PGK1 coordinates glycolysis and TCA cycle to promote tumor growth. *Nat Commun* (2020) 11(1):36. doi: 10.1038/s41467-019-13601-8
51. O'Malley J, Kumar R, Inigo J, Yadava N, Chandra D. Mitochondrial Stress Response and Cancer. *Trends Cancer* (2020) 6(8):688–701. doi: 10.1016/j.trecan.2020.04.009
52. Pakos-Zebrucka K, Koryga I, Mnich K, Ljujic M, Samali A, Gorman AM. The integrated stress response. *EMBO Rep* (2016) 17(10):1374–95. doi: 10.15252/embr.201642195
53. Hetz C. The unfolded protein response: controlling cell fate decisions under ER stress and beyond. *Nat Rev Mol Cell Biol* (2012) 13(2):89–102. doi: 10.1038/nrm3270
54. Yan K, Wu Q, Yan DH, Lee CH, Rahim N, Tritschler I, et al. Glioma cancer stem cells secrete Gremlin1 to promote their maintenance within the tumor hierarchy. *Genes Dev* (2014) 28(10):1085–100. doi: 10.1101/gad.235515.113
55. Heddleston JM, Wu Q, Rivera M, Minhas S, Lathia JD, Sloan AE, et al. Hypoxia-induced mixed-lineage leukemia 1 regulates glioma stem cell tumorigenic potential. *Cell Death Differ* (2012) 19(3):428–39. doi: 10.1038/cdd.2011.109
56. Li P, Zhou C, Xu L, Xiao H. Hypoxia enhances stemness of cancer stem cells in glioblastoma: an in vitro study. *Int J Med Sci* (2013) 10(4):399–407. doi: 10.7150/ijms.5407
57. Qiang L, Wu T, Zhang HW, Lu N, Hu R, Wang YJ, et al. HIF-1α is critical for hypoxia-mediated maintenance of glioblastoma stem cells by activating Notch signaling pathway. *Cell Death Differ* (2012) 19(2):284–94. doi: 10.1038/cdd.2011.95
58. Lee G, Auffinger B, Guo D, Hasan T, Deheeger M, Tobias AL, et al. Dedifferentiation of Glioma Cells to Glioma Stem-like Cells By Therapeutic Stress-induced HIF Signaling in the Recurrent GBM Model. *Mol Cancer Ther* (2016) 15(12):3064–76. doi: 10.1158/1535-7163.MCT-15-0675
59. Mello SS, Attardi LD. Deciphering p53 signaling in tumor suppression. *Curr Opin Cell Biol* (2018) 51:65–72. doi: 10.1016/j.ceb.2017.11.005
60. Oliner JD, Saiki AY, Caenepeel S. The Role of MDM2 Amplification and Overexpression in Tumorigenesis. *Cold Spring Harb Perspect Med* (2016) 6(6): a026336. doi: 10.1101/cshperspect.a026336
61. Her N, Oh J, Oh YJ, Han S, Cho HJ, Lee Y, et al. Potent effect of the MDM2 inhibitor AMG232 on suppression of glioblastoma stem cells. *Cell Death Dis* (2018) 9:792. doi: 10.1038/s41419-018-0825-1
62. Kim HY, Kim DK, Bae S, Gwak H, Jeon JH, Kim JK, et al. Farnesyl diphosphate synthase is important for the maintenance of glioblastoma stemness. *Exp Mol Med* (2018) 50:1–12. doi: 10.1038/s12276-018-0166-2
63. Matsuda K, Sato A, Okada M, Shibuya K, Seino S, Suzuki K, et al. Targeting JNK for therapeutic depletion of stem-like glioblastoma cells. *Sci Rep* (2012) 2:516. doi: 10.1038/srep00516
64. Coffelt SB, Kersten K, Doornebal CW, Weiden J, Vrijland K, Hau CS, et al. IL-17-producing γδ T cells and neutrophils conspire to promote breast cancer metastasis. *Nature* (2015) 522(7556):345–8. doi: 10.1038/nature14282
65. Jin X, Jeon HM, Jin X, Kim EJ, Yin J, Jeon HY, et al. The ID1-CULLIN3 Axis Regulates Intracellular SHH and WNT Signaling in Glioblastoma Stem Cells. *Cell Rep* (2016) 16(6):1629–41. doi: 10.1016/j.celrep.2016.06.092
66. Jin X, Yin J, Kim SH, Sohn YW, Beck S, Lim YC, et al. EGFR-AKT-Smad signaling promotes formation of glioma stem-like cells and tumor angiogenesis by ID3-driven cytokine induction. *Cancer Res* (2011) 71(22):7125–34. doi: 10.1158/0008-5472.CAN-11-1330
67. Jeon HM, Jin X, Lee JS, Oh SY, Sohn YW, Park HJ, et al. Inhibitor of differentiation 4 drives brain tumor-initiating cell genesis through cyclin E and notch signaling. *Genes Dev* (2008) 22(15):2028–33. doi: 10.1101/gad.1668708
68. Jeon H, Ham SW, Kim JK, Jin X, Lee SY, Shin YJ, et al. $Ly6G^+$ inflammatory cells enable the conversion of cancer cells to cancer stem cells in an irradiated glioblastoma model. *Cell Death Differ* (2019) 26:2139–56. doi: 10.1038/s41418-019-0282-0
69. Velpula KK, Dasari VR, Tsung AJ, Gondi CS, Klopfenstein JD, Mohanam S, et al. Regulation of glioblastoma progression by cord blood stem cells is mediated by downregulation of cyclin D1. *PloS One* (2011) 6(3):e18017. doi: 10.1371/journal.pone.0018017
70. Wang L, Shang Z, Zhou Y, Hu X, Chen Y, Fan Y, et al. Autophagy mediates glucose starvation-induced glioblastoma cell quiescence and chemoresistance through coordinating cell metabolism, cell cycle, and survival. *Cell Death Dis* (2018) 9(2):213. doi: 10.1038/s41419-017-0242-x
71. Sachdeva R, Wu M, Johnson K, Kim H, Celebre A, Shahzad U, et al. BMP signaling mediates glioma stem cell quiescence and confers treatment resistance in glioblastoma. *Sci Rep* (2019) 9(1):14569. doi: 10.1038/s41598-019-51270-1
72. Humeau J, Bravo-San Pedro JM, Vitale I, Nuñez L, Villalobos C, Kroemer G, et al. Calcium signaling and cell cycle: Progression or death. *Cell Calcium* (2018) 70:3–15. doi: 10.1016/j.ceca.2017.07.006
73. Li G, Zhang Z, Wang R, Ma W, Yang Y, Wei J, et al. Suppression of STIM1 inhibits human glioblastoma cell proliferation and induces G0/G1 phase arrest. *J Exp Clin Cancer Res* (2013) 32(1):20. doi: 10.1186/1756-9966-32-20
74. El Boustany C, Katsogiannou M, Delcourt P, Dewailly E, Prevarskaya N, Borowiec AS, et al. Differential roles of STIM1, STIM2 and Orai1 in the control of cell proliferation and SOCE amplitude in HEK293 cells. *Cell Calcium* (2010) 47 (4):350–9. doi: 10.1016/j.ceca.2010.01.006

75. Chang YP, Wang G, Bermudez V, Hurwitz J, Chen XS. Crystal structure of the GINS complex and functional insights into its role in DNA replication. *Proc Natl Acad Sci U S A* (2007) 104(31):12685–90. doi: 10.1073/pnas.0705558104
76. Aparicio T, Guillou E, Coloma J, Montoya G, Méndez J. The human GINS complex associates with Cdc45 and MCM and is essential for DNA replication. *Nucleic Acids Res* (2009) 37(7):2087–95. doi: 10.1093/nar/gkp065
77. Kimura T, Cui D, Kawano H, Yoshitomi-Sakamoto C, Takakura N, Ikeda E . Induced expression of GINS complex is an essential step for reactivation of quiescent stem-like tumor cells within the peri-necrotic niche in human glioblastoma. *J Cancer Res Clin Oncol* (2019) 145(2):363–71. doi: 10.1007/s00432-018-2797-z
78. Zhu Z, Gorman MJ, McKenzie LD, Chai JN, Hubert CG, Prager BC, et al. Zika virus has oncolytic activity against glioblastoma stem cells. *J Exp Med* (2017) 214(10):2843–57. doi: 10.1084/jem.20171093
79. Desgrosellier JS, Cheresh DA. Integrins in cancer: biological implications and therapeutic opportunities. *Nat Rev Cancer* (2010) 10(1):9–22. doi: 10.1038/nrc2748
80. Zhu Z, Mesci P, Bernatchez JA, Gimple RC, Wang X, Schafer ST, et al. Zika Virus Targets Glioblastoma Stem Cells through a SOX2-Integrin $\alpha_v\beta_5$ Axis. *Cell Stem Cell* (2020) 26(2):187–204.e10. doi: 10.1016/j.stem.2019.11.016
81. Tejero R, Huang Y, Katsyv I, Kluge M, Lin JY, Tome-Garcia J, et al. Gene signatures of quiescent glioblastoma cells reveal mesenchymal shift and interactions with niche microenvironment. *EBioMedicine* (2019) 42:252–69. doi: 10.1016/j.ebiom.2019.03.064
82. Arrizabalaga O, Moreno-Cugnon L, Auzmendi-Iriarte J, Aldaz P, Ibáñez-de-Caceres I, Garros-Regulez L, et al. High expression of MKP1/DUSP1 counteracts glioma stem cell activity and mediates HDAC inhibitor response. *Oncogenesis* (2017) 6:401. doi: 10.1038/s41389-017-0003-9
83. Polson ES, Kuchler VB, Abbosh C, Ross EM, Mathew RK, Beard HA, et al. KHS101 disrupts energy metabolism in human glioblastoma cells and reduces tumor growth in mice. *Sci Transl Med* (2018) 10(454):eaar2718. doi: 10.1126/scitranslmed.aar2718
84. Diehn M, Cho RW, Lobo NA, Kalisky T, Dorie MJ, Kulp AN, et al. Association of reactive oxygen species levels and radioresistance in cancer stem cells. *Nature* (2009) 458(7239):780–3. doi: 10.1038/nature07733
85. Burdon RH, Gill V, Rice-Evans C. Oxidative stress and tumour cell proliferation. *Free Radic Res Commun* (1990) 11(1-3):65–76. doi: 10.3109/10715769009109669
86. Gersey ZC, Rodriguez GA, Barbarite E, Sanchez A, Walters WM, Ohaeto KC, et al. Curcumin decreases malignant characteristics of glioblastoma stem cells via induction of reactive oxygen species. *BMC Cancer* (2017) 17(1):99. doi: 10.1186/s12885-017-3058-2
87. Affinito A, Quintavalle C, Esposito CL, Roscigno G, Vilardo C, Nuzzo S, et al. The Discovery of RNA Aptamers that Selectively Bind Glioblastoma Stem Cells. *Mol Ther - Nucleic Acids* (2019) 18:99–109. doi: 10.1016/j.omtn.2019.08.015
88. Jin CY, Moon DO, Lee JD, Heo MS, Choi YH, Lee CM, et al. Sulforaphane sensitizes tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis through downregulation of ERK and Akt in lung adenocarcinoma A549 cells. *Carcinogenesis* (2007) 28(5):1058–66. doi: 10.1093/carcin/bgl251
89. Chumakova AP, Hitomi M, Sulman EP, Lathia JD. High-Throughput Automated Single-Cell Imaging Analysis Reveals Dynamics of Glioblastoma Stem Cell Population During State Transition. *Cytom A* (2019) 95(3):290–301. doi: 10.1002/cyto.a.23728

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Putting Proteomics Into Immunotherapy for Glioblastoma

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In glioblastoma, the most aggressive brain cancer, a complex microenvironment of heterogeneity and immunosuppression, are considerable hurdles to classify the subtypes and promote treatment progression. Treatments for glioblastoma are similar to standard therapies for many other cancers and do not effectively prolong the survival of patients, due to the unique location and heterogeneous characteristics of glioblastoma. Immunotherapy has shown a promising effect for many other tumors, but its application for glioma still has some challenges. The recent breakthrough of high-throughput liquid chromatography–mass spectrometry (LC-MS/MS) systems has allowed researchers to update their strategy for identifying and quantifying thousands of proteins in a much shorter time with lesser effort. The protein maps can contribute to generating a complete map of regulatory systems to elucidate tumor mechanisms. In particular, newly developed unicellular proteomics could be used to determine the microenvironment and heterogeneity. In addition, a large scale of differentiated proteins provides more ways to precisely classify tumor subtypes and construct a larger library for biomarkers and biotargets, especially for immunotherapy. A series of advanced proteomic studies have been devoted to the different aspects of immunotherapy for glioma, including monoclonal antibodies, oncolytic viruses, dendritic cell (DC) vaccines, and chimeric antigen receptor (CAR) T cells. Thus, the application of proteomics in immunotherapy may accelerate research on the treatment of glioblastoma. In this review, we evaluate the frontline applications of proteomics strategies for immunotherapy in glioblastoma research.

Keywords: glioblastoma, proteomics, heterogeneous microenvironment, immunotherapy, biotarget

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INTRODUCTION

Glioblastoma is one of the top malignant brain cancers. Standard therapies only result in poor prognosis and low survival rates. Novel treatment approaches are desperately needed. Subtype classification is very important for precision medicine of cancer treatment to achieve a better prognosis. Even though advanced nucleic acid technology together with other clinical features have made considerable progress in this step for glioblastoma, the heterogeneous characteristics still cannot be overcome.

The standard care for glioblastoma is similar to that of other cancers, but due to the special location of glioblastoma and its heterogeneity, standard therapies do not turn out the ideal prognosis for glioblastoma. The appearance of immunotherapy provided a more specific and efficient approach to prolong the survival of patients with cancer. Several different strategies have been proposed to target different parts of the tumor. However, heterogeneity again makes

it difficult to apply single or several existing immunotherapy methods to yield better consequences in glioblastoma. There are several challenges facing immunotherapy for glioblastoma. A more complicated mechanism needs to be elucidated to identify more useful biomarkers and biotargets, which has been almost beyond the ability of many prime research methods. Efficient evaluation methods are also necessary for immunotherapy.

Proteomics, which has been developing rapidly in the last decade, is important for whole-tumor research. Compared with whole-genome sequencing or transcriptome sequencing, which can only indicate the origin of tumors, proteomics can reveal the actual state of tumor cells by quantifying functional proteins, the cell function operators. High-throughput mass spectrometry (MS) technology can be used to evaluate tumors with higher dimensions. The ability to quantify thousands of proteins at the same time simplifies the process of studying the mechanisms of tumor development and can filter certain biomarkers and target candidates. Thus, the application of proteomics can enhance the efficiency of glioblastoma research. In particular, single-cell proteomics has also provided an even more specific tool to investigate the heterogeneous microenvironment of glioblastoma. In this review, we discuss immunotherapy for glioblastoma and its challenges, and proteomics methods are presented and shown as applications for solving these challenges.

GLIOBLASTOMA

Glioma is responsible for 27% of all central nervous system (CNS) tumors and 80% of malignant tumors (1) occurring among people aged from 15 to 34 years around the world. About 2.5% of cancer-related death is caused by malignant gliomas (2). In 2016, the WHO classified gliomas into three main types based on histological methods: astrocytoma, oligodendroglioma, and oligoastrocytoma (3, 4). Later, the newly published World Health Organization Classification of Tumors of the Central Nervous System (WHO CNS 2016) further classified tumors as WHO I–IV based on the combination of both histological and molecular information (5). Patients categorized under WHO IV had the most malignant degree of tumors, which were called high-grade colloid tumors or glioblastoma (6).

Glioblastoma is mostly diagnosed as primary glioblastoma (*de novo*) and is more common in elderly patients. Astrocytomas would transform to a malignant tumor to become the source of some secondary glioblastomas (7). From a microscopic view, glioblastoma is characterized by growth and morphology, including cell number, anaplasia, mitotic activity, and microvascular condition (8). Besides the histological information, the mutations of genes, IDH, ATRX, TERT, EGFR, MGMT, etc., have all been included to further diagnose the subtypes of glioma or predict progress benefit. Specifically, for glioblastoma, EGFR, TERT, and +7/−10 cytogenetic signature are the molecular markers, and MGMT is a predictive biomarker of the benefit from alkylating chemotherapy (9).

ADVANCES IN PROTEOMICS FOR GLIOMA

"Proteome" is a word combining "protein" and "genome" that was proposed by Wilkins in 1994. Proteome refers to all the proteins in cells, tissues, or even in creatures and is extraordinarily complicated. Proteomics is a new technology to identify and analyze all the proteins present in biological samples from a holistic perspective. Proteomics can study the expression of proteins and the interaction between proteins. With the fast development of equipment and software, the most advanced proteomics techniques are based on MS and can be generally put into two categories: bottom-up proteomics (BUP) and top-down proteomics (TDP) (10). BUP differs from TDP in the prior steps of enzyme digestion of proteins to peptides and the liquid chromatography–mass spectrometry (LC-MS) separation and analysis. High-throughput MS systems make it possible to identify thousands of proteins at one time. Consequently, proteomics has become a more important technology to study the omics of different creatures and a powerful tool to research the mechanisms of tumor development to locate treatment markers. The proteomic strategy can be easily applied to the research of natural production mechanism in plants (11) or microorganism (12). This strategy has also been successfully applied to different types of diseases, such as Alzheimer's (13), periodontal disease (14), or thyroid-related diseases (15) and various kinds of tumors.

Since 2016, glioblastomas have mainly been classified based on the molecular genetic properties accompanied by other features (16). The ideal marker should not only be 100% sensitive, specific, and efficient for detection but should also be easily accessible for analysis and provide a simple analytical method and accurate information (17). Various biomarkers have been applied for different types of tumors. For glioblastoma, microRNAs (miRNAs), small molecules, circulating tumor cells (CTCs), extracellular vesicles, tumor tissues, and biological fluids are the most widely used besides nucleic acid and proteins. Unlike gene markers, which only indicate the possibility of having a type of tumor, identified proteins would confirm what is ongoing in the tissue and further divide tumors into more specific subtypes.

Proteins are becoming diagnostic and prognostic markers in different tumors including glioblastoma. Proteins are widely located in cancer tissues (18) and liquid matrices such as blood (19) and cerebrospinal fluid (CSF) (20). Though studies have verified that certain nucleic acids are more specific than other features including proteins, the breakthrough of MS technology has made proteins a strong assistant method. As nucleic acids cannot be used to evaluate the specific situation of tumors in cancer development, the combination of gene expression and proteomics is still necessary (20). Full proteomics tumor profiles would compare both natural and posttranslational changes during cancer development so that the mechanism of tumor development would be more specifically elucidated (2). For instance, the proteomic has been integrated with other methods to research on Pediatric Brain Cancer to explore novel biomarkers in recently published research (21). The study of protein posttranslational modifications could lead to the discovery of novel biomarkers and novel strategies for treatment (22).

In view of the possible lack of specificity of protein markers, a multiparameter comprehensive evaluation method was proposed, which is a combination of qualitative and quantitative analysis of several different protein markers to simultaneously filter the misleading false results in the identification of proteins (20, 23). This multiparametric evaluation can not only distinguish healthy or ill patients but also allow the diagnosis of specific tumor subtypes (20, 24, 25). A low concentration of proteins in biological fluid samples might be the most important problem. Moreover, extensive validation is still required when using proteins as biomarkers due to their heterogeneous nature (26). For instance, different glioblastoma cells with different microenvironments exhibit different *in vitro* invasion and cell migration abilities (27).

Proteomics Strategy

Bottom-Up Strategy for Proteomics

The prime procedure and most widely used strategy are BUP, which is performed from peptides (bottom) to proteins (up) (**Figure 1**). Generally, the proteins would be extracted from samples and then digested into peptides, and then the peptides would be purified and detected by LC-MS system to acquire peptide ion information, which is assembled and analyzed using specific software. The majority of researches on microorganisms, plants, or animals have regarded BUP as a prime option. Typically, BUP applies enzymes to cleave extracted mixed proteins from collected samples, including formalin-fixed and paraffin-embedded (FFPE), tissues or cultured cells, to small peptides of ∼6–50 amino acids, which are optimum for MS detection and computational analysis (10). Trypsin is one of the most commonly used enzymes for an average output length of ∼14 amino acids (28). The advantages of small peptide fragments are that they increase the separation efficiency, avoid the inability to detect isotopic peaks of proteins, and lighten the burden of searching through a database and assigning them to certain proteins. However, there is a key limitation of BUP that when the proteins are turned into fragments, the information regarding the proteoform, including the location and number of posttranslational modifications (PTMs) and endogenous proteolysis is lost (29). Furthermore, due to the increased complexity of mixtures of peptides, only some peptides can be detected, and the coverage of the assembled protein sequences is normally under 20%. To compensate for the shortcomings of BUP, the middle-down strategy was proposed such that the proteins could be digested into longer peptides and then sequenced.

Higher resolution and throughput would cover more peptides and identify more proteins. The newly applied orbitrap technology developed by Thermo Fisher (30) boosts the coverage and efficiency of BUP. Considering the large quantities of MS data, a powerful software and a complete database are necessary. The major companies involved in the proteomics business have developed their own systems to assist with their equipment. Other platforms such as Spectronaut, Peaks, MaxQuant (31), and Skyline (32) might be widely chosen by many researchers. However, as open databases are quite limited and the MS data from different types of machines might differ from each other, this is a great obstacle for research exchange between different labs.

Top-Down Strategy for Proteomics

On the other hand, a novel developed strategy, TDP, is becoming available. TDP aims to separate protein mixtures first and then sequence the intact proteins. Thus, the protein sequences from the TDP strategy would mostly be 100% complete, and even the PTMs of proteins with the same sequences could be distinguished. This could provide a deeper understanding of proteoforms *in vivo* (33). The three typical steps are as follows: separation of the protein mixture; detection of the molecular weight by MS; and data processing and database searching/scoring (34).

Multiple methods have been proposed to improve the sample condition before MS in the first step. Hydrophilic interaction liquid chromatography (HILIC) (35), weak cation exchange (WCX) (36), capillary reversed-phase liquid chromatography (RPLC) (37), and capillary isoelectric focusing (CIEF) (38) are typical representative on-line technologies that are used before MS detection. High sensitivity, high resolution, and high throughput are necessary for the sequencing of mixed proteins with a large m/z. Thus, Fourier transform ion cyclotron resonance (FTICR) MS and orbitrap MS are among the top choices (39). In addition to the separation and detection methods, the key bottleneck is the identification software. There are several welcomed free software and databases. ProSightP^{TM} (40) and TDPortal (41) might be the most widely used for TDP and proteoform identification, and TopPIC, TopMG, and Proteoform Suite might also be worthy of implementation (42). MetaMorpheus is an integrated software program for both BUP and TDP to identify peptides and proteoforms (43).

In addition to the mentioned whole-proteome strategy, the target-proteome strategy is sometimes preferred. An antiproteomics approach for the selection of nanobodies specific for overexpressed glioblastoma proteins was proposed recently (44). This straightforward antiproteomics approach led to the identification of seven novel candidate biomarkers for glioma formation, progression, and prognosis.

Quantitative Proteomics

Quantitative proteomics can identify and accurately quantify proteins in biological samples and has become an effective research tool in the field of life sciences. Compared with the various oncogenes and tumor suppressors identified by genomics and transcriptomics research, the research objective of proteomics is the protein synthesized during translation. Proteins are the executors of most physiological processes; thus, through proteomics research, we can visually analyze physiological processes.

The isotopic-labeling strategies, in particular the isotope-coded affinity tag (ICAT), and stable isotope labeling by amino acids in cell culture (SILAC) were applied to evaluate metabolic marking proteins by using the principle of the dependence of mammalian cell proliferation on essential amino acids (45). Later, chemical labeling by isobaric tags for relative and absolute quantification (iTRAQ) (46), tandem mass tags (TMTs)

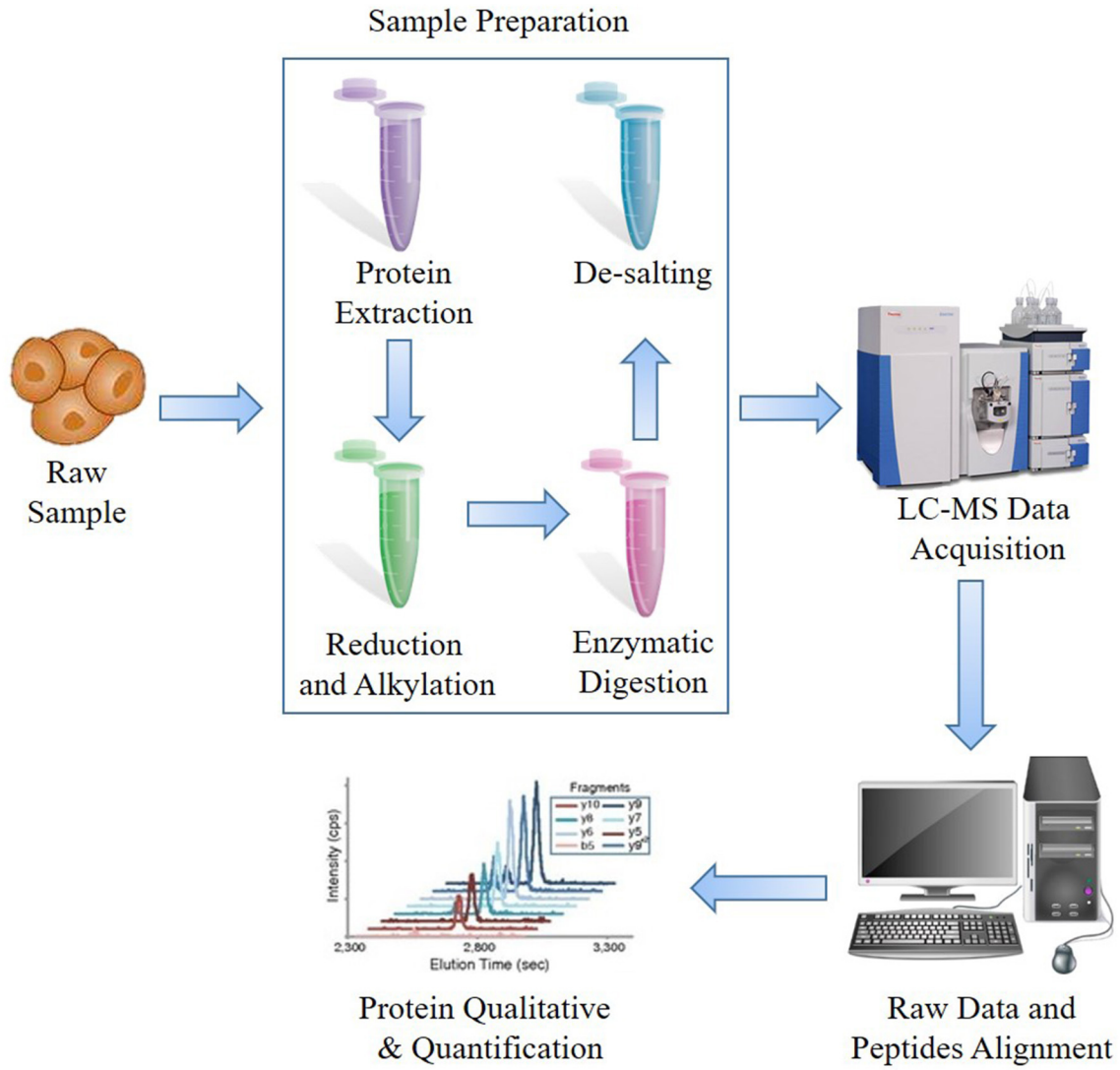

FIGURE 1 | General procedure of bottom-up proteomics (BUP) mass spectrometry (MS)-based proteomics.

(47), and dimethyl labeling (48) protocols were developed to improve quantification accuracy. These techniques use multiple stable isotope labels and amino groups of specific labeled peptides for tandem MS analysis. Although the quantitative information provided by SILAC and iTRAQ is considerable, the labeling reagent is relatively expensive, and the cost of each sample is large; therefore, these strategies are more suitable for quantitative analysis of protein expression changes at the whole proteomics level.

With the advancements in the field of proteomics technologies, it is now possible to measure an accurate amount of proteins in different biological specimens with label-free quantitative methods (49). Data-independent acquisition (DIA), is a remarkably developed label-free quantitative method in the past 5 years, which does not require expensive stable isotope labels as internal standards, but only needs to analyze the MS data generated with large-scale protein identification and compare the signal strength of corresponding peptides in different products to carry out relative quantitative analysis for proteins corresponding to peptide segments. DIA/SWATH-type techniques have been applied successfully in a variety of studies and are becoming increasingly prevalent in the quantitative proteomics field, especially in studies requiring consistent analysis of large sample cohorts, like the continuous collection of tumor samples for a long period.

Multiple reaction monitoring (MRM) is a targeted quantitative proteomics method to study target protein molecules, based on the information of target molecules. MRM MS is a high-precision protein quantitative identification technology, which is an excellent method for a one-time accurate quantitative study of multiple target proteins in complex samples.

Single-Cell Proteomics

Analysis of single-cell transcriptomes using next-generation sequencing (NGS) has been intensively developed for decades. The methods to study glioblastoma multiforme (GBM) heterogeneity are mainly genetic and transcriptomic profiling, which cannot reflect instant functional changes (50, 51). Moreover, non-uniform results between genetic/transcriptome and protein levels have been shown, particularly for epidermal growth factor receptor (EGFR) (52, 53).

Recently, mass cytometry (MC) has become a more widely accepted platform for accurate proteomic analysis of single-cell dimension. MC is a technique proposed for the analysis of individual proteins in single cells. In this method, target proteins are quantified using antibodies conjugated with ions of isotopically pure transition metals. Protein complexes with the antibodies are sent through the inductively coupled plasma, which ionizes the metal conjugated antibodies, and their mass spectra are analyzed with a time-of-flight MS. MC has demonstrated the possibility of quantitative profiling of the immune response or evaluating the functional response of signaling at the single-cell level (54). This method has been increasingly used to analyze single cells when the research interest is focused on a limited group of proteins. Thus, MC was recently applied for quantitative analysis of transcription factors responsible for differentiation of hematopoietic cells (55).

Single-cell measurements, such as qFlow cytometry, provide a powerful tool to elucidate GBM heterogeneity. The fluorescent calibration is applied in qFlow to convert signal to accurate protein concentrations (56). Research on anti-VEGF efficacy based on qFlow cytometry and systems biology revealed that this efficacy is related to the concentrations of endothelial VEGFR1 in plasma membrane (57).

IMMUNOTHERAPY AND ITS CHALLENGES

For malignant glioma, neuroimaging, surgical resection, radiotherapy, and chemotherapy are still standard care (58). In all grades of gliomas, surgical resection is necessary, and the maximal safe resection is still worthy to protect patients' neurological function (59). However, if the tumor is located in an important/non-resectable position of the brain and the tumor grows into the adjacent normal brain tissue, it is still difficult to completely remove the whole tumor. The highly specific and efficient method of immunotherapy is considered a promising therapy.

The immune system of patients with tumors is generally suppressed; thus, for the tumors with strong invasive ability, this feature makes it easier for them to become targets of treatment. Cancer immunotherapy (CIT) has developed fast in recent years and is increasingly playing an important role in cancer treatment. Tumor immunotherapy has shown a significant therapeutic effect in a variety of cancer types; thus, more and more research has focused on glioma immunotherapy. Immunotherapy can achieve a sustained response from the immune system without many side effects (60).

Immunotherapy methods are currently under research and mainly including the following methods: peptide vaccines, oncolytic viruses (OVs), DC vaccines, CAR T cells, and immune checkpoint inhibitors (**Figure 2**) (58). However, there are still many challenges before these technologies can be applied. Although there are many successful applications of CIT on various human cancers, only a small number of patients benefit from these therapies. Specifically for glioma, the two important immune pathways have not shown many benefits (61). The main hurdles for immunotherapy in glioma include the low tumor mutational burden (TMB), heterogenetic microenvironment, restricted immune access, and sequestration of systemic T cells (58).

Among the challenges facing CIT, 10 top challenges were highlighted by Priti S. Hegde and Daniel S. Chen to promote cooperation (62). Here, based on the advantages of proteomics, we focus on the challenges in glioma related to heterogeneity and personalized biomarkers, driver mechanism elucidation, blocking points, combination of multiple immunotherapies, and prognostic evaluation methods (62). With the advantage of proteomics, the biotargets for immunotherapy and the mechanisms could be much more direct and easier to be discovered than the nucleic method. Like research in melanoma, the proteomic strategy has been a valuable platform for discovering novel biomarkers (63). Besides, the proteomics from different cells would be quite different which is even suitable for the research of heterogenetic characteristics. For now, the proteomics has been tried for the evaluation of therapeutic effects on glioblastoma (64).

Monoclonal Antibodies Targeting Glioma Stem Cells

In immunotherapy research of glioma, the scheme of glioma-specific antibodies is also popular (65–71). It has been more than 30 years since monoclonal antibodies (mAbs) were used to target tumor antigens in immunotherapy. This scheme mainly depends on the antigens specifically expressed in glioma or the molecules overexpressed in tumor cells. mAbs have played an important role in tumor immunotherapy due to their direct cytotoxic and immunomodulatory effects (72).

Immunotherapy has been proved to be able to activate the antitumor response in the brain, which lays a solid foundation for treatment strategies for malignant glioma. The mAbs against PD-1 (nivolumab and pembrolizumab), PD-L1 (atezolizumab and durvalumab), CTLA-4 (ipilimumab) (58), EGFR (cetuximab) (73), and vascular endothelial growth factor (VEGF; Bevacizumab) (74) have been revealed with significant potential. A series of clinical trials of the mentioned targets are also underway (NCT02974883, NCT020177717, NCT01952769, etc.), but many challenges still need to be solved, such as the non-effect of anti-TGF-β antibody GC1008 on tumor progression in the late stage of treatment (75) and the non-survival benefit of bevacizumab (76). Additionally, methods to overcome the brain–blood barrier to deliver the mAbs to tumor sites should be developed, such as in the study of nimotuzumab, which targets the EGFR (77).

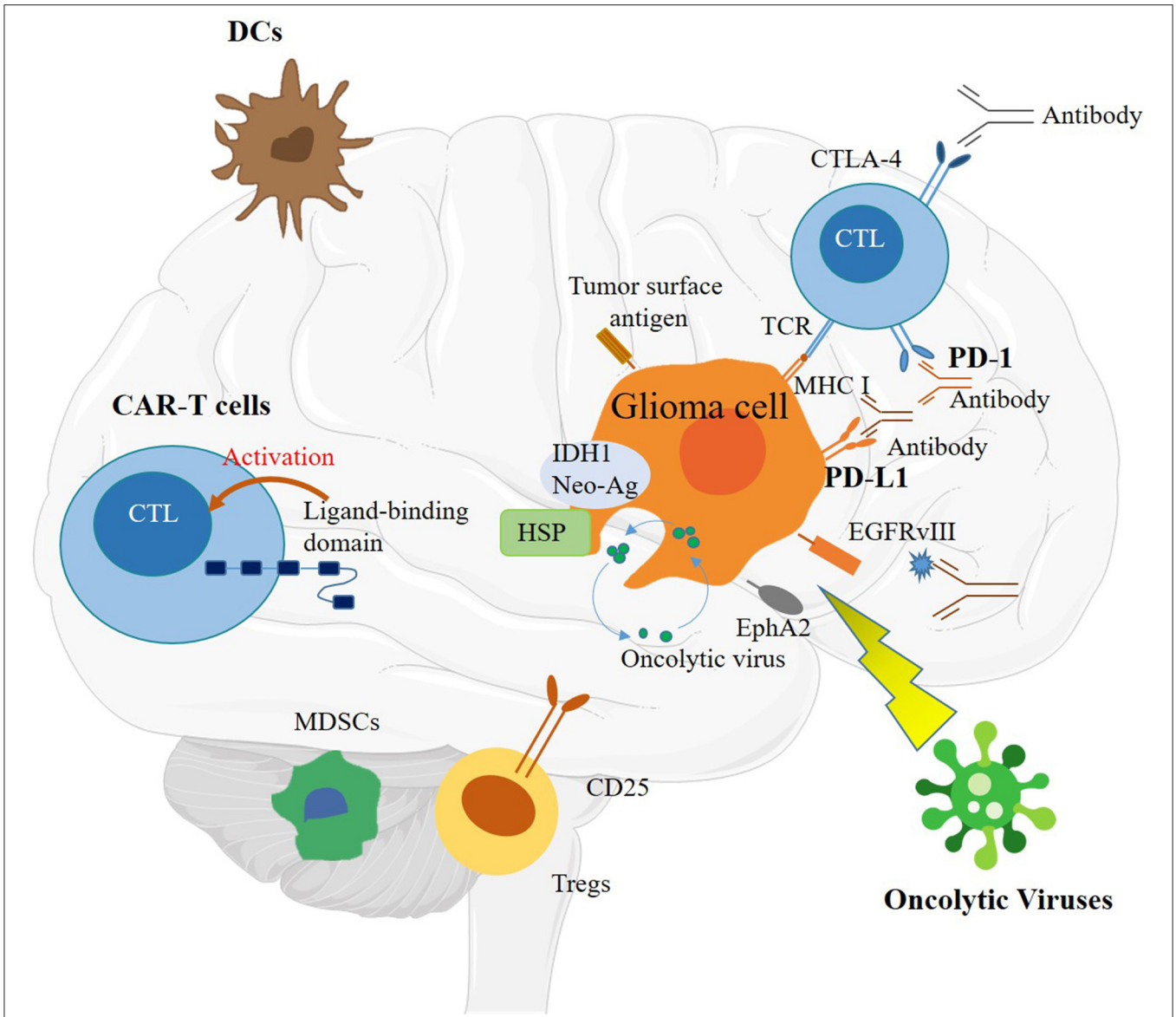

FIGURE 2 | Schematic of immunotherapy, including immune checkpoint inhibitors, dendritic cell (DC) vaccines, chimeric antigen receptor (CAR) T cells, and oncolytic viruses (OVs).

The recent cases have proven that proteomics studies could make some challenges easier to solve. Using proteomics, it is actually easier and more direct to detect and locate biotargets, namely, proteins. Differential expression brain-derived proteins, such as the EGFR, MMP9, TIMP, and fibulin-2 and−5, were validated to be released at the same time in one study of high-grade glioblastomas (19). The circulating biomarkers from the serum have been regarded as important sources for targeted therapy in brain cancers (78). A small panel of three proteins S100A8, S100A9, and CXCL4 were identified by proteomic strategy and validated by ELISA in early research (79). Another study to identify blood biomarkers also suggested another eight potential valuable ones, and three of them, LRG1, CRP, and C9, are closely related to the size of tumor (24). Different grades of glioma are analyzed by iTRAQ-based quantitative method, and it is found that nucleophosmin, glucose-regulated protein 78 kDa, nucleolin, and heat shock protein 90 kDa are highly expressed, and Raf kinase inhibitor protein is lowly expressed in glioblastoma. The expression levels of the RNA-binding protein nova 1 (NOVA1) in different subtypes of glioma were different (80). For all these proteins with altered expression, potential novel biotargets might be inside them. A series of proteins have been reported to have changes in their qualitative or quantitative composition during cancer development as determined with conventional methods (81, 82).

Bone marrow-derived human mesenchymal stem cells (BM-hMSCs) are expected to become cell vectors for glioma therapy due to their inherent glioma characteristics. Some GSCs are called attractors for they can attract the injected BM-hMSCs. The proteomics strategy could extend methodologies to further study various pathways related to inflammation-related cues for BM-hMSC homing (83). The results of the study present the first proof to link nutrition metabolism to N-glycosylation.

Oncolytic Viruses

The abnormal expression of proteins in tumor cells caused by engineered oncolytic adenoviruses could be utilized to increase their anti-tumor efficacy. Oncolytic virus therapy is also a strategy for glioma immunotherapy. In addition to inducing cell death, virus infection can also cause endogenous and acquired immune responses, which are also promising immunotherapies. OVs are designed drugs that can selectively reproduce and kill tumor cells and then destroy the microenvironment of the tumor; thus, the innate immune system could be activated to adapt the immune response to tumor. It is an important design principle to weaken or delete viral virulence factors, making OVs safe for normal tissues, but still able to kill tumor cells in tumors (84). Delta-24-RGD (DNX-2401) and PSVRIPO are two promising engineered OVs resulting in better progress in clinical trials (85, 86).

For OVs, the current challenge is to understand the response mechanism of glioma cells to OVs, which will aid in the development of novel vectors with the stronger release of virus progeny to gain more effective oncolysis. DNX-2401, the E1A mutant of adenovirus, has shown proper toxicity and significant efficacy. Thus, the proteomics strategy and other techniques were conducted on cytosolic, nuclear, and secreted glioma proteomes to elucidate the interaction mechanism. The Delta-24-RGD can inhibit signal transducer and activator of transcription 3 (STAT3) and c-JUN (transcription factor AP-1), or increase nuclear factor kappa B (NF-κB) and protein kinase C (PKC), extracellular signal–regulated kinase 1/2 (ERK1/2) and p38 mitogen-activated protein kinase (p38 MAPK) pathways (87). Herpes simplex virus type 1 (HSV-1) is a vector with a great potential for application on solid tumors. The release of proteins is validated to be associated with metabolites, transportation, stress responses, apoptosis, proteolysis, the extracellular matrix, and cell adhesion by the proteomics analysis of HSV-1 infected human macrophages (88). In addition, filamin, tubulin, t-complex protein 1, and heat shock proteins are found to be upregulated, and extracellular matrix proteins are found to be downregulated by analyzing the secreted proteins and secretomes from tumor cells infected by oncolytic HSV-1 (89). These changes caused by HSV-1 RH2 infection indicated the potential to change the tumor microenvironment to improve the effect of immunotherapy.

Dendritic Cell Vaccines

Tumor vaccines are an active form of immunotherapy, which can trigger the immune system to defend against tumors. The best way to activate the immune system is to stimulate dendritic cells (DCs), which are one kind of multifunctional antigen-presenting cells (APCs). The granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-4 (IL-4), IL-1b, IL-6, prostaglandin E2 (PGE2), and tumor necrosis factor-α (TNF-α) mixture are utilized to stimulate peripheral blood mononuclear cells (PBMCs) to obtain mature DCs, which is the isolation source of DCs (90). The main purpose of DC vaccines to treat tumors is to generate specific T helper cells (Th) to activate the antitumor effect of cytotoxic T cells (91).

Though multiple glioma-related antigens, such as IL-13Rα2, HER2, EphA2, gp100, and aim-2, are targets of peptide vaccines and related clinical trials have shown that peptide vaccine treatment can also significantly prolong the survival period (NCT00643097, NCT00458601), the dominant drivers are still unclear, and the evaluation of prognosis is difficult. Even the driver scheme is complex, and proteomics could provide direct evidence to assist in discovery. The proteomics profile of tumor-associated macrophages (TAM) indicated that Cat Eye Syndrome Critical Region Protein 1 (CECR1) can promote differentiation of M2 TAMs and affects the proliferation and migration of glioma cells, and 67 proteins are upregulated by CECR1 siRNA transfection in THP-1-derived macrophages (MQs) (92). There have been studies based on proteomics to develop DC vaccines for solid tumors such as melanoma (93). The proteomics technique was applied to uncover the mechanism of how an original melanoma cell-derived lysate (TRIMEL) induced the immune responses mediated by T cell and DC maturation. Similarly, such an induced mechanism study could be applied in future glioma DC vaccine research.

Chimeric Antigen Receptor T Cells

Cell adoptive T-cell therapy (ACT) has direct antitumor activity and could be developed as a personalized treatment. CAR T-cell therapy requires isolated T cells infiltrated by tumor from the patient's body. After stimulation with IL-2, it can be cultured *in vitro* to have the ability to specifically recognize the tumor and then returned to the patient (94). Unlike active immunity that stimulates the innate immune system with tumor-associated antigens, adoptively transferred CAR T cells can directly target tumor-associated antigens without relying on the antigen presentation process. CAR T cells have been successfully used in the treatment of hematological malignancies. This therapy targets EGFRvIII to clear tumor cells expressing EGFRvIII in tumor-bearing mouse models and phase I clinical trials (Trial No. NCT02209376) (95). However, due to the lack of specific antigens on the surface of solid tumors, the application of this therapy in solid tumors remains to be explored in depth (96). On the other hand, considering the adverse effects of CAR T-cell therapy on the CNS, such as cognitive dysfunction and hydrocephalus, there have been few reports on its use in gliomas (97).

In this immunotherapy area, advanced single-cell proteomics provides a more powerful method to evaluate the heterogenetic microenvironment. A study targeting on GBM39 indicated that over 70% target cells have more than 6,000 VEGFR2 (∼five-fold higher) or PDGFRα/cell (∼four-fold higher) plasma membrane proteins with higher expression levels (98). Within a 33-marker panel proteomics research, the complex immune microenvironments of single cell were illustrated, and the presence of various immune cells was confirmed. The increase

of T cells with PD-1&CD8 or TIM-3&CD4 will induce the immunosuppressive effects in the microenvironments (99). A cohort analysis of 259 patients with primary and metastatic brain tumors ranging from benign to malignant by flow cytometry found that the myeloid-derived suppressor cells (MDSCs) of patients with GBM were significantly increased, which indicates a poor prognosis and provides a theoretical basis for formulating strategies for MDSCs (100).

As mentioned before, locating more biotargets for T cells to activate is also urgently need to apply CAR T-cell therapy. The reported 17 antigens with 41 different HLA ligands were identified through an MS analysis of HLA-presenting peptides in GSCs and glioblastoma patient specimens. Importantly, these become the best option for antigen-specific immunotherapy of glioblastoma for they are proved to be functional CD8+ T-cell epitopes in the tests of *in vitro* immunogenicity and killing antigen-specific target cells (101). In addition, comprehensive methods based on proteomics revealed that stable expression of GSC-specific antigen is related to higher T-cell infiltration and positive immunomodulator expression, indicating that the antigens are at reduced risk and suitable for further clinical application (102). The laboratory team of Sidi Chen applied membrane proteomics to update T cell–based immunotherapies (103). The detailed information validated that the edition of Pdia3, Mgat5, Emp1, or Lag3 genes in adoptively transferred CD8+ T cells can improve the survival rate of mice with syngeneic and T-cell receptor transgenic modification.

Proteomics-Related Mechanisms to Assist With Immunotherapy

Mass spectrometry-based proteomics technology has not only started to contribute directly to immunotherapy but has also already aided to elucidate the signal and protein interaction mechanisms to improve the understanding of glioma diagnosis and molecular mechanism to assist the application of immunotherapy.

For glioma, the induction mechanism still needs to be clearly explained. Proteomics can currently cover a larger number of proteins and subsequently solidify the final drivers of glioma. Whole-genome sequencing and transcriptome sequencing only provide a hint of what is leading to the occurrence of the tumor; thus, the identification and quantification of specific proteins by proteomics could finally verify what is arising in the tumor to transfer the cells. Many large-scale proteomics studies have revealed that there are more possible candidate proteins to elucidate these mechanisms. A label-free quantitative proteomic study of low-grade astrocytoma (LGA) or GBM revealed 136 regulated proteins (86 up and 50 down) with at least a five-fold change in GBM (104). An unbiased quantitative proteomics analysis of human glioma biopsies revealed that up- or downregulation could be observed in multiple pathways. For instance, both clathrin-dependent and -independent endocytosis would be affected by a large reduction in various mechanical components related to the initiation, formation, and rupture of endocytic vectors, such as clathrin, AP-2 adaptins, and endophilins (105).

Beyond the whole proteomics comparison and filtering, more researchers have also applied proteomics to investigate specific pathways. The study of phosphorylated OLIG2 applies proteomics methods to reveal that glioma cells will have a stronger invasive mesenchymal character with the induction of non-phosphorylated OLIG2 to activate TGF-b2, providing a mechanistic insight for the transformation of cells from proliferation to invasion (22). The glioma cell line GL261 cultured with the 3T3-L1 adipocyte line verified that angiogenesis is necessary for adipose tissue expansion and is an important factor in the formation of malignant tumors (106) as well as in cancer progression and metastasis. Some identified factors from adipocyte cells are found underexpressed, such as STI1, hnRNPs, and PGK1 in conditioned glioma cells, and some are found upregulated in contrast, such as RFC1, KIF5C, ANXA2, N-RAP, and RACK1 in GL261 cell. In addition, pro-inflammatory and angiogenic factors are also with different regulations (107). A proteomics research on mouse glial culture indicated that glial cells will activate the MAPK/ERK pathway and upregulate a variety of proteins participating in inflammation, cell adhesion, and extracellular structural organization after exposure to GBM cells (108).

CONCLUSION

For now, glioblastoma is one of the most lethal tumors due to its heterogeneity, which causes poor diagnosis and treatment. Even with multiple molecular markers, these complications make it hard to diagnose and classify cancer development and subtypes, even with the most advanced nucleic acid detection technology. Many novel technologies have been applied to mine more biomarkers to distinguish subtypes, and a series of new genes or proteins seem to be worthy of deeper research for both mechanism elucidation and identification of target therapies.

The standard treatments for tumors have not yielded much hope for patients. Significant progress has been made in developing immunotherapeutic regimens, and these may soon be included in the SOC. The development of immunotherapy is a valuable method to extend the lives of patients, but several challenges still need to be overcome. The tumors that do not respond to immunotherapy are often referred to as "cold tumors." GBM is considered to be a cold tumor, and immunotherapy fails for many reasons, including the highly immunosuppressive tumor microenvironment, special physical microenvironment of glioma, and decreased tumor antigen presentation. The interaction of these factors together with the difficulty of T-cell activation recruitment and administration lead to the dilemma of immunotherapy for glioma. Thus, due to its complexity, the requirement to better elucidate the induction mechanism is urgently needed. Furthermore, the heterogenetic characteristics are a key challenge to identify more available biomarkers to activate the immune system. Additionally, similar to mechanism studies, the efficient observations of the therapeutic effect are another hurdle before clinical researchers.

Many drugs and vaccines mentioned above might contribute considerably to other cancers but are still problematic for the treatment of glioblastoma due to the intratumor heterogeneity (109). The different combinations of multiple immunotherapies and standard care have also been evaluated by a series of clinical trials. However, locating more target positions might be more urgent and requires more devotion and better methodology.

Proteins can be used more feasibly than nucleic acids to assess the immediate situation of tumor development. Furthermore, most drugs have their final effect on proteins, which are the main life executors in the body compared with nucleic acids and metabolites. Thus, proteomics is a promising direction to mine more targets for diagnosis and treatment. The large-scale screening of quantity changes in proteomes makes it a more efficient filter for biomarker candidates. On the other hand, the heterogeneity of glioblastoma makes it more important to study several pathways simultaneously to unveil the mechanisms of occurrence, development, and immunosuppression.

FUTURE PROSPECTS

With the enormous increase in the availability of gene expression, epigenetic and molecular pathway analyses, a personalized therapeutic approach tailored to the tumor would be ideal, especially for glioblastoma with intratumoral heterogeneity. With the rapid development of detection equipment and software, proteomics would be another promising and powerful tool to facilitate personalized therapeutics. The combination of multiple high-throughput technologies would enhance the progression rate of identifying more unique biomarkers.

With increasing research attention devoted to the application of proteomics for glioblastoma, more specific diagnostic procedures can be proposed based on MS detection. There might be four directions for the improvement of quantitative MS techniques to accelerate the application in biology and medicine: (1) updating and innovation of instrumentations, (2) optimizing sample preparation or fraction separation strategy, (3) developing more sensitive single-cell proteomics technology, and (4) developing more automated software tools. Though it is very exciting to be able to study proteomes, the next stage would be research on highly abundant proteoforms with large-scale analysis. Multiplexed proteomics technologies such as the reverse-phase protein arrays (RPPA) would also allow us to apply multi-omics, including genomic, transcriptomic, and metabonomic, to gain deeper understanding of tumor biology in the future.

AUTHOR CONTRIBUTIONS

LC, DQ, XG, and QW participated in writing the paper and figure preparation. JL guided the writing and editing of the article. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Ostrom QT, Gittleman H, Fulop J, Liu M, Blanda R, Kromer C et al. CBTRUS statistical report: primary brain and central nervous system tumors diagnosed in the United States in 2008-2012. *Neuro Oncol.* (2015) 17(Suppl. 4):v1–v62. doi: 10.1093/neuonc/nov189
2. Silantyev AS, Falzone L, Libra M, Gurina OI, Kardashova KS, Nikolouzakis TK et al. Current and future frends on diagnosis and prognosis of glioblastoma: from molecular biology to proteomics. *Cells-Basel.* (2019) 8:863. doi: 10.3390/cells8080863
3. Kamran N, Alghamri MS, Nunez FJ, Shah D, Asad AS, Candolfi M et al. Current state and future prospects of immunotherapy for glioma. *Immunotherapy-Uk.* (2018) 10:317–39. doi: 10.2217/imt-2017-0122
4. Wen PY, Huse JT. 2016 world health organization classification of central nervous system tumors. *Continuum (Minneap Minn).* (2017) 23:1531–47. doi: 10.1212/CON.0000000000000536
5. Sonoda Y. Clinical impact of revisions to the WHO classification of diffuse gliomas and associated future problems. *Int J Clin Oncol.* (2020) 25:1004–9. doi: 10.1007/s10147-020-01628-7
6. Reifenberger G, Wirsching HG, Knobbe-Thomsen CB, Weller M. Advances in the molecular genetics of gliomas - implications for classification and therapy. *Nat Rev Clin Oncol.* (2017) 14:434–52. doi: 10.1038/nrclinonc.2016.204
7. Yang P, Wang Y, Peng X, You G, Zhang W, Yan W et al. Management and survival rates in patients with glioma in China (2004-2010): a retrospective study from a single-institution. *J Neurooncol.* (2013) 113:259–66. doi: 10.1007/s11060-013-1103-9
8. Jiang T, Mao Y, Ma W, Mao Q, You Y, Yang X et al. CGCG clinical practice guidelines for the management of adult diffuse gliomas. *Cancer Lett.* (2016) 375:263–73. doi: 10.1016/j.canlet.2016.01.024
9. Weller M, van den Bent M, Preusser M, Le Rhun E, Tonn JC, Minniti G et al. EANO guidelines on the diagnosis and treatment of diffuse gliomas of adulthood. *Nat Rev Clin Oncol.* (2020) 1–17. doi: 10.1038/s41571-020-00447-z
10. Zhang Y, Fonslow BR, Shan B, Baek MC, Yates JR. Protein analysis by shotgun/bottom-up proteomics. *Chem Rev.* (2013) 113:2343–94. doi: 10.1021/cr3003533
11. Tocmo R, Veenstra JP, Huang Y, Johnson JJ. Covalent modification of proteins by plant-derived natural products: proteomic approaches and biological impacts. *Proteomics (Weinheim).* (2020) 21:e1900386. doi: 10.1002/pmic.201900386
12. Gouveia D, Grenga L, Pible O, Armengaud J. Quick microbial molecular phenotyping by differential shotgun proteomics. *Environ Microbiol.* (2020) 22:2996–3004. doi: 10.1111/1462-2920.14975
13. Wesenhagen KEJ, Teunissen CE, Visser PJ, Tijms BM. Cerebrospinal fluid proteomics and biological heterogeneity in Alzheimer's disease: a literature review. *Crit Rev Cl Lab Sci.* (2019) 57:1–13. doi: 10.1080/10408363.2019.1670613
14. Binti Badlishah Sham NI, Lewin SD, Grant MM. Proteomic investigations of *in vitro* and *in vivo* models of periodontal disease. *Proteomics.* (2019) 14:1900043. doi: 10.1002/prca.201900043
15. Ucal Y, Ozpinar A. Proteomics in thyroid cancer and other thyroid-related diseases: a review of the literature. *Biochim Biophys Acta.* (2020) 1868:140510. doi: 10.1016/j.bbapap.2020.140510
16. Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-Branger D, Cavenee WK et al. The 2016 world health organization classification of tumors of the central nervous system: a summary. *Acta Neuropathol.* (2016) 131:803–20. doi: 10.1007/s00401-016-1545-1
17. Cheng L, Davison DD, Adams J, Lopez-Beltran A, Wang L, Montironi R et al. Biomarkers in bladder cancer: translational and clinical implications.

Crit Rev Oncol Hematol. (2014) 89:73–111. doi: 10.1016/j.critrevonc.2013.08.008
18. Kros JM, Mustafa DM, Dekker LJ, Sillevis SP, Luider TM, Zheng PP. Circulating glioma biomarkers. *Neuro Oncol.* (2015) 17:343–60. doi: 10.1093/neuonc/nou207
19. Lange RP, Everett A, Dulloor P, Korley FK, Bettegowda C, Blair C et al. Evaluation of eight plasma proteins as candidate blood-based biomarkers for malignant gliomas. *Cancer Invest.* (2014) 32:423–9. doi: 10.3109/07357907.2014.933237
20. Liu J, Yu J, Shen H, Zhang J, Liu W, Chen Z et al. Mass spectrometric analysis of cerebrospinal fluid protein for glioma and its clinical application. *Contemp Oncol (Pozn).* (2014) 18:100–5. doi: 10.5114/wo.2014.40455
21. Petralia F, Tignor N, Reva B, Koptyra M, Chowdhury S, Rykunov D et al. Integrated proteogenomic characterization across major histological types of pediatric brain cancer. *Cell.* (2020) 183:1962–85. doi: 10.1016/j.cell.2020.10.044
22. Petushkova NA, Zgoda VG, Pyatnitskiy MA, Larina OV, Teryaeva NB, Potapov AA et al. Post-translational modifications of FDA-approved plasma biomarkers in glioblastoma samples. *PLoS ONE.* (2017) 12:e177427. doi: 10.1371/journal.pone.0177427
23. Furnari FB, Fenton T, Bachoo RM, Mukasa A, Stommel JM, Stegh A et al. Malignant astrocytic glioma: genetics, biology, and paths to treatment. *Genes Dev.* (2007) 21:2683–710. doi: 10.1101/gad.1596707
24. Miyauchi E, Furuta T, Ohtsuki S, Tachikawa M, Uchida Y, Sabit H et al. Identification of blood biomarkers in glioblastoma by SWATH mass spectrometry and quantitative targeted absolute proteomics. *PLoS ONE.* (2018) 13:e193799. doi: 10.1371/journal.pone.0193799
25. Heroux MS, Chesnik MA, Halligan BD, Al-Gizawiy M, Connelly JM, Mueller WM et al. Comprehensive characterization of glioblastoma tumor tissues for biomarker identification using mass spectrometry-based label-free quantitative proteomics. *Physiol Genomics.* (2014) 46:467–81. doi: 10.1152/physiolgenomics.00034.2014
26. Stetson LC, Dazard JE, Barnholtz-Sloan JS. Protein markers predict survival in glioma patients. *Mol Cell Proteomics.* (2016) 15:2356–65. doi: 10.1074/mcp.M116.060657
27. Koh I, Cha J, Park J, Choi J, Kang SG, Kim P. The mode and dynamics of glioblastoma cell invasion into a decellularized tissue-derived extracellular matrix-based three-dimensional tumor model. *Sci Rep.* (2018) 8:4608. doi: 10.1038/s41598-018-22681-3
28. Burkhart JM, Schumbrutzki C, Wortelkamp S, Sickmann A, Zahedi RP. Systematic and quantitative comparison of digest efficiency and specificity reveals the impact of trypsin quality on MS-based proteomics. *J Proteomics.* (2012) 75:1454–62. doi: 10.1016/j.jprot.2011.11.016
29. Smith LM, Kelleher NL. Proteoform: a single term describing protein complexity. *Nat Methods.* (2013) 10:186–7. doi: 10.1038/nmeth.2369
30. Danielson SR, Markowski T, Higgins L, Jagtap P, Griffiin T, Chen Y. *Rapid profiling of proteomes and sub-proteomes on the Orbitrap Fusion Tribrid Mass Spectrometer.* Thermo Scientific.
31. Tyanova S, Temu T, Cox J. The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. *Nat Protoc.* (2016) 11:2301–19. doi: 10.1038/nprot.2016.136
32. MacLean B, Tomazela DM, Shulman N, Chambers M, Finney GL, Frewen B et al. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics.* (2010) 26:966–8. doi: 10.1093/bioinformatics/btq054
33. Cupp-Sutton KA, Wu S. High-throughput quantitative top-down proteomics. *Mol Omics.* (2020) 16:91–9. doi: 10.1039/C9MO00154A
34. Siuti N, Kelleher NL. Decoding protein modifications using top-down mass spectrometry. *Nat Methods.* (2007) 4:817–21. doi: 10.1038/nmeth1097
35. Tian Z, Tolic N, Zhao R, Moore RJ, Hengel SM, Robinson EW et al. Enhanced top-down characterization of histone post-translational modifications. *Genome Biol.* (2012) 13:R86. doi: 10.1186/gb-2012-13-10-r86
36. Tian Z, Zhao R, Tolic N, Moore RJ, Stenoien DL, Robinson EW et al. Two-dimensional liquid chromatography system for online top-down mass spectrometry. *Proteomics.* (2010) 10:3610–20. doi: 10.1002/pmic.201000367
37. Lee SW, Berger SJ, Martinovic S, Pasa-Tolic L, Anderson GA, Shen Y et al. Direct mass spectrometric analysis of intact proteins of the yeast large ribosomal subunit using capillary LC/FTICR. *Proc Natl Acad Sci USA.* (2002) 99:5942–7. doi: 10.1073/pnas.082119899
38. Martinovic S, Berger SJ, Pasa-Tolic L, Smith RD. Separation and detection of intact noncovalent protein complexes from mixtures by on-line capillary isoelectric focusing-mass spectrometry. *Anal Chem.* (2000) 72:5356–60. doi: 10.1021/ac0004557
39. Loo JA, Quinn JP, Ryu SI, Henry KD, Senko MW, McLafferty FW. High-resolution tandem mass spectrometry of large biomolecules. *Proc Natl Acad Sci USA.* (1992) 89:286–9. doi: 10.1073/pnas.89.1.286
40. Zamdborg L, LeDuc RD, Glowacz KJ, Kim YB, Viswanathan V, Spaulding IT et al. ProSight PTM 2.0: improved protein identification and characterization for top down mass spectrometry. *Nucleic Acids Res.* (2007) 35:W701–6. doi: 10.1093/nar/gkm371
41. Blankenberg D, Von Kuster G, Coraor N, Ananda G, Lazarus R, Mangan M et al. Galaxy: a web-based genome analysis tool for experimentalists. *Curr Protoc Mol Biol.* (2010) 19:10–9. doi: 10.1002/0471142727.mb1910s89
42. Schaffer LV, Millikin RJ, Miller RM, Anderson LC, Fellers RT, Ge Y et al. Identification and quantification of proteoforms by mass spectrometry. *Proteomics.* (2019) 19:e1800361. doi: 10.1002/pmic.201970085
43. Solntsev SK, Shortreed MR, Frey BL, Smith LM. Enhanced global post-translational modification discovery with MetaMorpheus. *J Proteome Res.* (2018) 17:1844–51. doi: 10.1021/acs.jproteome.7b00873
44. Jovčevska I, Zupanec N, Urlep }, Vranič A, Matos B, Stokin CL et al. Differentially expressed proteins in glioblastoma multiforme identified with a nanobody-based anti-proteome approach and confirmed by OncoFinder as possible tumor-class predictive biomarker candidates. *Oncotarget.* (2017) 8:44141. doi: 10.18632/oncotarget.17390
45. Vidova V, Spacil Z. A review on mass spectrometry-based quantitative proteomics: targeted and data independent acquisition. *Anal Chim Acta.* (2017) 964:7–23. doi: 10.1016/j.aca.2017.01.059
46. Evans C, Noirel J, Ow SY, Salim M, Pereira-Medrano AG, Couto N et al. An insight into iTRAQ: where do we stand now? *Anal Bioanal Chem.* (2012) 404:1011–27. doi: 10.1007/s00216-012-5918-6
47. Thompson A, Schäfer J, Kuhn K, Kienle S, Schwarz J, Schmidt G et al. Tandem mass tags: a novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. *Anal Chem.* (2003) 75:1895–904. doi: 10.1021/ac0262560
48. Lemeer S, Mohammed S, Raijmakers R, Boersema PJ, Heck AJR. Multiplex peptide stable isotope dimethyl labeling for quantitative proteomics. *Nat Protoc.* (2009) 4:484–94. doi: 10.1038/nprot.2009.21
49. Samuel N, Remke M, Rutka JT, Raught B, Malkin D. Proteomic analyses of CSF aimed at biomarker development for pediatric brain tumors. *J Neurooncol.* (2014) 118:225–38. doi: 10.1007/s11060-014-1432-3
50. Ellis HP, Greenslade M, Powell B, Spiteri I, Sottoriva A, Kurian KM. Current challenges in glioblastoma: intratumour heterogeneity, residual disease, and models to predict disease recurrence. *Front Oncol.* (2015) 5:251. doi: 10.3389/fonc.2015.00251
51. Taniguchi Y, Choi PJ, Li GW, Chen H, Babu M, Hearn J et al. Quantifying *E. coli* proteome and transcriptome with single-molecule sensitivity in single cells. *Science.* (2010) 329:533–8. doi: 10.1126/science.1188308
52. Brennan C, Momota H, Hambardzumyan D, Ozawa T, Tandon A, Pedraza A et al. Glioblastoma subclasses can be defined by activity among signal transduction pathways and associated genomic alterations. *PLoS ONE.* (2009) 4:e7752. doi: 10.1371/journal.pone.0007752
53. Lemée J, Clavreul A, Aubry M, Com E, de Tayrac M, Mosser J et al. Integration of transcriptome and proteome profiles in glioblastoma: looking for the missing link. *Bmc Mol Biol.* (2018) 19:13. doi: 10.1186/s12867-018-0115-6
54. Bendall SC, Simonds EF, Qiu P, Amir E, Krutzik PO, Finck R et al. Single-cell mass cytometry of differential immune and drug responses across a human hematopoietic continuum. *Science.* (2011) 332:687–96. doi: 10.1126/science.1198704
55. Palii CG, Cheng Q, Gillespie MA, Shannon P, Mazurczyk M, Napolitani G et al. Single-cell proteomics reveal that quantitative changes in co-expressed lineage-specific transcription factors determine cell fate. *Cell Stem Cell.* (2019) 24:812–20. doi: 10.1016/j.stem.2019.02.006
56. Chen S, Weddell J, Gupta P, Conard G, Parkin J, Imoukhuede PI. qFlow cytometry-based receptoromic screening: a high-throughput quantification

approach informing biomarker selection and nanosensor development. *Methods Mol Biol.* (2017) 1570:117–38. doi: 10.1007/978-1-4939-6840-4_8
57. Weddell JC, Imoukhuede PI. Quantitative characterization of cellular membrane-receptor heterogeneity through statistical and computational modeling. *PLoS ONE.* (2014) 9:e97271. doi: 10.1371/journal.pone.0097271
58. Wang H, Xu T, Huang Q, Jin W, Chen J. Immunotherapy for malignant glioma: current status and future directions. *Trends Pharmacol Sci.* (2020) 41:123–38. doi: 10.1016/j.tips.2019.12.003
59. Lacroix M, Abi-Said D, Fourney DR, Gokaslan ZL, Shi W, DeMonte F et al. A multivariate analysis of 416 patients with glioblastoma multiforme: prognosis, extent of resection, and survival. *J Neurosurg.* (2001) 95:190–8. doi: 10.3171/jns.2001.95.2.0190
60. Sanmamed MF, Chen L. A paradigm shift in cancer immunotherapy: from enhancement to normalization. *Cell.* (2019) 176:677. doi: 10.1016/j.cell.2019.01.008
61. Topalian SL, Drake CG, Pardoll DM. Immune checkpoint blockade: a common denominator approach to cancer therapy. *Cancer Cell.* (2015) 27:450–61. doi: 10.1016/j.ccell.2015.03.001
62. Hegde PS, Chen DS. Top 10 challenges in cancer immunotherapy. *Immunity.* (2020) 52:17–35. doi: 10.1016/j.immuni.2019.12.011
63. Babacic H, Lehtio J, Pico DCY, Pernemalm M, Eriksson H. In-depth plasma proteomics reveals increase in circulating PD-1 during anti-PD-1 immunotherapy in patients with metastatic cutaneous melanoma. *J Immunother Cancer.* (2020) 8:e000204. doi: 10.1136/jitc-2019-000204
64. Erhart F, Hackl M, Hahne H, Buchroithner J, Meng C, Klingenbrunner S et al. Combined proteomics/miRNomics of dendritic cell immunotherapy-treated glioblastoma patients as a screening for survival-associated factors. *Npj Vaccines.* (2020) 5:5. doi: 10.1038/s41541-019-0149-x
65. Chinot OL, Wick W, Mason W, Henriksson R, Saran F, Nishikawa R et al. Bevacizumab plus radiotherapy-temozolomide for newly diagnosed glioblastoma. *N Engl J Med.* (2014) 370:709–22. doi: 10.1056/NEJMoa1308345
66. Gilbert MR, Dignam JJ, Armstrong TS, Wefel JS, Blumenthal DT, Vogelbaum MA et al. A randomized trial of bevacizumab for newly diagnosed glioblastoma. *N Engl J Med.* (2014) 370:699–708. doi: 10.1056/NEJMoa1308573
67. Hasselbalch B, Lassen U, Hansen S, Holmberg M, Sorensen M, Kosteljanetz M et al. Cetuximab, bevacizumab, and irinotecan for patients with primary glioblastoma and progression after radiation therapy and temozolomide: a phase II trial. *Neuro Oncol.* (2010) 12:508–16. doi: 10.1093/neuonc/nop063
68. Massimino M, Biassoni V, Miceli R, Schiavello E, Warmuth-Metz M, Modena P et al. Results of nimotuzumab and vinorelbine, radiation and re-irradiation for diffuse pontine glioma in childhood. *J Neurooncol.* (2014) 118:305–12. doi: 10.1007/s11060-014-1428-z
69. Westphal M, Heese O, Steinbach JP, Schnell O, Schackert G, Mehdorn M et al. A randomised, open label phase III trial with nimotuzumab, an anti-epidermal growth factor receptor monoclonal antibody in the treatment of newly diagnosed adult glioblastoma. *Eur J Cancer.* (2015) 51:522–32. doi: 10.1016/j.ejca.2014.12.019
70. Phillips AC, Boghaert ER, Vaidya KS, Mitten MJ, Norvell S, Falls HD et al. ABT-414, an antibody-drug conjugate targeting a tumor-selective EGFR epitope. *Mol Cancer Ther.* (2016) 15:661–9. doi: 10.1158/1535-7163.MCT-15-0901
71. Reardon DA, Lassman AB, van den Bent M, Kumthekar P, Merrell R, Scott AM et al. Efficacy and safety results of ABT-414 in combination with radiation and temozolomide in newly diagnosed glioblastoma. *Neuro Oncol.* (2017) 19:965–75. doi: 10.1093/neuonc/now257
72. Scott AM, Allison JP, Wolchok JD. Monoclonal antibodies in cancer therapy. *Cancer Immun.* (2012) 12:14.
73. Taylor TE, Furnari FB, Cavenee WK. Targeting EGFR for treatment of glioblastoma: molecular basis to overcome resistance. *Curr Cancer Drug Targets.* (2012) 12:197–209. doi: 10.2174/156800912799277557
74. Esparza R, Azad TD, Feroze AH, Mitra SS, Cheshier SH. Glioblastoma stem cells and stem cell-targeting immunotherapies. *J Neurooncol.* (2015) 123:449–57. doi: 10.1007/s11060-015-1729-x
75. Han J, Alvarez-Breckenridge CA, Wang QE, Yu J. TGF-beta signaling and its targeting for glioma treatment. *Am J Cancer Res.* (2015) 5:945–55.
76. Zhuang H, Yuan X, Zheng Y, Li X, Chang JY, Wang J et al. A study on the evaluation method and recent clinical efficacy of bevacizumab on the treatment of radiation cerebral necrosis. *Sci Rep.* (2016) 6:24364. doi: 10.1038/srep24364
77. Han L, Liu C, Qi H, Zhou J, Wen J, Wu D et al. Systemic delivery of monoclonal antibodies to the central nervous system for brain tumor therapy. *Adv Mater.* (2019) 31:1805697. doi: 10.1002/adma.201805697
78. Tanase C, Albulescu R, Codrici E, Popescu ID, Mihai S, Enciu AM et al. Circulating biomarker panels for targeted therapy in brain tumors. *Future Oncol.* (2015) 11:511–24. doi: 10.2217/fon.14.238
79. Popescu ID, Codrici E, Albulescu L, Mihai S, Enciu AM, Albulescu R et al. Potential serum biomarkers for glioblastoma diagnostic assessed by proteomic approaches. *Proteome Sci.* (2014) 12:47. doi: 10.1186/s12953-014-0047-0
80. Gimenez M, Marie SKN, Oba-Shinjo S, Uno M, Izumi C, Oliveira JB et al. Quantitative proteomic analysis shows differentially expressed HSPB1 in glioblastoma as a discriminating short from long survival factor and NOVA1 as a differentiation factor between low-grade astrocytoma and oligodendroglioma. *BMC Cancer.* (2015) 15:481. doi: 10.1186/s12885-015-1473-9
81. Schuhmann MU, Zucht HD, Nassimi R, Heine G, Schneekloth CG, Stuerenburg HJ et al. Peptide screening of cerebrospinal fluid in patients with glioblastoma multiforme. *Eur J Surg Oncol.* (2010) 36:201–7. doi: 10.1016/j.ejso.2009.07.010
82. Bernardi D, Padoan A, Ballin A, Sartori M, Manara R, Scienza R et al. Serum YKL-40 following resection for cerebral glioblastoma. *J Neurooncol.* (2012) 107:299–305. doi: 10.1007/s11060-011-0762-7
83. Wildburger NC, Lichti CF, LeDuc RD, Schmidt M, Kroes RA, Moskal JR et al. Quantitative proteomics and transcriptomics reveals metabolic differences in attracting and non-attracting human-in-mouse glioma stem cell xenografts and stromal cells. *EuPA Open Proteomics.* (2015) 8:94–103. doi: 10.1016/j.euprot.2015.06.006
84. Muik A, Stubbert LJ, Jahedi RZ, Geibeta Y, Kimpel J, Dold C et al. Re-engineering vesicular stomatitis virus to abrogate neurotoxicity, circumvent humoral immunity, and enhance oncolytic potency. *Cancer Res.* (2014) 74:3567–78. doi: 10.1158/0008-5472.CAN-13-3306
85. Jiang H, Gomez-Manzano C, Aoki H, Alonso MM, Kondo S, McCormick F et al. Examination of the therapeutic potential of Delta-24-RGD in brain tumor stem cells: role of autophagic cell death. *J Natl Cancer Inst.* (2007) 99:1410–4. doi: 10.1093/jnci/djm102
86. Lang FF, Conrad C, Gomez-Manzano C, Yung W, Sawaya R, Weinberg JS et al. Phase I study of DNX-2401 (Delta-24-RGD) oncolytic adenovirus: replication and immunotherapeutic effects in recurrent malignant glioma. *J Clin Oncol.* (2018) 36:1419–27. doi: 10.1200/JCO.2017.75.8219
87. González-Morales A, Zabaleta A, Guruceaga E, Alonso MM, García-Moure M, Fernández-Irigoyen J et al. Spatial and temporal proteome dynamics of glioma cells during oncolytic adenovirus Delta-24-RGD infection. *Oncotarget.* (2018) 9:31045. doi: 10.18632/oncotarget.25774
88. Miettinen JJ, Matikainen S, Nyman TA. Global secretome characterization of herpes simplex virus 1-infected human primary macrophages. *J Virol.* (2012) 86:12770–8. doi: 10.1128/JVI.01545-12
89. Tada S, Hamada M, Yura Y. Proteomic analysis of secretomes of oncolytic herpes simplex virus-infected squamous cell carcinoma cells. *Cancers (Basel).* (2018) 10:28. doi: 10.3390/cancers10020028
90. Datta J, Terhune JH, Lowenfeld L, Cintolo JA, Xu S, Roses RE et al. Optimizing dendritic cell-based approaches for cancer immunotherapy. *Yale J Biol Med.* (2014) 87:491–518.
91. Cho DY, Lin SZ, Yang WK, Hsu DM, Lee HC, Lee WY et al. Recent advances of dendritic cells (DCs)-based immunotherapy for malignant gliomas. *Cell Transplant.* (2009) 18:977–83. doi: 10.3727/096368909X12483162196962
92. Zhu C, Mustafa D, Krebber MM, Chrifi I, Leenen P, Duncker DJ et al. Comparative proteomic analysis of cat eye syndrome critical region protein 1- function in tumor-associated macrophages and immune response regulation of glial tumors. *Oncotarget.* (2018) 9:33500–14. doi: 10.18632/oncotarget.26063
93. Gonzalez FE, Chernobrovkin A, Pereda C, Garcia-Salum T, Tittarelli A, Lopez MN et al. Proteomic identification of heat shock-induced danger signals in a melanoma cell lysate used in

dendritic cell-based cancer immunotherapy. *J Immunol Res.* (2018) 2018:3982942. doi: 10.1155/2018/3982942
94. Rosenberg SA, Restifo NP. Adoptive cell transfer as personalized immunotherapy for human cancer. *Science.* (2015) 348:62–8. doi: 10.1126/science.aaa4967
95. Choi BD, O'Rourke DM, Maus MV. Engineering chimeric antigen receptor T cells to treat glioblastoma. *J Target Ther Cancer.* (2017) 6:22–5.
96. Kosti P, Maher J, Arnold JN. Perspectives on chimeric antigen receptor T-cell immunotherapy for solid tumors. *Front Immunol.* (2018) 9:1104. doi: 10.3389/fimmu.2018.01104
97. Santomasso BD, Park JH, Salloum D, Riviere I, Flynn J, Mead E et al. Clinical and biological correlates of neurotoxicity associated with CAR T-cell therapy in patients with B-cell acute lymphoblastic leukemia. *Cancer Discov.* (2018) 8:958–71. doi: 10.1158/2159-8290.CD-17-1319
98. Imoukhuede PI, Popel AS. Quantitative fluorescent profiling of VEGFRs reveals tumor cell and endothelial cell heterogeneity in breast cancer xenografts. *Cancer Med.* (2014) 3:225–44. doi: 10.1002/cam4.188
99. Fu W, Wang W, Li H, Jiao Y, Weng J, Huo R et al. High dimensional mass cytometry analysis reveals characteristics of the immunosuppressive microenvironment in diffuse astrocytomas. *Front Oncol.* (2020) 10:78. doi: 10.3389/fonc.2020.00078
100. Alban TJ, Alvarado AG, Sorensen MD, Bayik D, Volovetz J, Serbinowski E et al. Global immune fingerprinting in glioblastoma patient peripheral blood reveals immune-suppression signatures associated with prognosis. *JCI Insight.* (2018) 3:e122264. doi: 10.1172/jci.insight.122264
101. Neidert MC, Kowalewski DJ, Silginer M, Kapolou K, Backert L, Freudenmann LK et al. The natural HLA ligandome of glioblastoma stem-like cells: antigen discovery for T cell-based immunotherapy. *Acta Neuropathol.* (2018) 135:923–38. doi: 10.1007/s00401-018-1836-9
102. Rapp C, Warta R, Stamova S, Nowrouzi A, Geisenberger C, Gal Z et al. Identification of T cell target antigens in glioblastoma stem-like cells using an integrated proteomics-based approach in patient specimens. *Acta Neuropathol.* (2017) 134:297–316. doi: 10.1007/s00401-017-1702-1
103. Ye L, Park JJ, Dong MB, Yang Q, Chow RD, Peng L et al. In vivo CRISPR screening in CD8 T cells with AAV–Sleeping Beauty hybrid vectors identifies membrane targets for improving immunotherapy for glioblastoma. *Nat Biotechnol.* (2019) 37:1302–13. doi: 10.1038/s41587-019-0246-4
104. Bi B, Li F, Guo J, Li C, Jing R, Lv X et al. Label-free quantitative proteomics unravels the importance of RNA processing in glioma malignancy. *Neuroscience.* (2017) 351:84–95. doi: 10.1016/j.neuroscience.2017.03.023
105. Buser DP, Ritz M, Moes S, Tostado C, Frank S, Spiess M et al. Quantitative proteomics reveals reduction of endocytic machinery components in gliomas. *Ebiomedicine.* (2019) 46:32–41. doi: 10.1016/j.ebiom.2019.07.039
106. Carmeliet P. Angiogenesis in health and disease. *Nat Med.* (2003) 9:653–60. doi: 10.1038/nm0603-653
107. Almeida J, Costa J, Coelho P, Cea V, Galesio M, Noronha JP et al. Adipocyte proteome and secretome influence inflammatory and hormone pathways in glioma. *Metab Brain Dis.* (2019) 34:141–52. doi: 10.1007/s11011-018-0327-y
108. Oliveira AI, Anjo SI, Vieira De Castro J, Serra SC, Salgado AJ, Manadas B et al. Crosstalk between glial and glioblastoma cells triggers the "go-or-grow" phenotype of tumor cells. *Cell Commun Signal.* (2017) 15:37. doi: 10.1186/s12964-017-0194-x
109. Wang Q, Tan Y, Fang C, Zhou J, Wang Y, Zhao K et al. Single-cell RNA-seq reveals RAD51AP1 as a potent mediator of EGFRvIII in human glioblastomas. *Aging.* (2019) 11:7707. doi: 10.18632/aging.102282

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REVIEW
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Current Immunotherapies for Glioblastoma Multiforme

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Glioblastoma multiforme (GBM) is the most common and aggressive malignant tumor found in the central nervous system. Currently, standard treatments in the clinic include maximal safe surgical resection, radiation, and chemotherapy and are mostly limited by low therapeutic efficiency correlated with poor prognosis. Immunotherapy, which predominantly focuses on peptide vaccines, dendritic cell vaccines, chimeric antigen receptor T cells, checkpoint inhibitor therapy, and oncolytic virotherapy, have achieved some promising results in both preclinical and clinical trials. The future of immune therapy for GBM requires an integrated effort with rational combinations of vaccine therapy, cell therapy, and radio- and chemotherapy as well as molecule therapy targeting the tumor microenvironment.

Keywords: immunotherapy, glioblastoma multiforme, glioma, vaccines, checkpoint inhibitors

INTRODUCTION

Glioblastoma multiforme (GBM) is the most common and aggressive primary malignant tumor in the central nervous system (CNS) in adults (1). It is mainly classified into two groups: isocitrate dehydrogenase (IDH)-wildtype GBM, which has been previously referred to as primary GBM and represents about 90% of cases, and IDH-mutant GBM, which is developed from a lower-grade diffuse glioma and represents about 10% of cases. The current standard treatments for GBM include a combination of surgical resection, radiation, and chemotherapy. At present, there are only two drugs approved by the FDA to treat GBM *via* systematical administration: temozolomide (TMZ) for the treatment of newly diagnosed GBM (ndGBM) and bevacizumab for the treatment of recurrent GBM (rGBM) (2, 3). Unfortunately, current therapeutic approaches have very limited impact on improving the prognosis of GBM patients, showing 15 months of median survival and less than 5% of a 5-year survival rate (1). Thus, opportunities and challenges remain in finding more efficient treatments against GBM.

Immunotherapy, which manipulates the immune system to attack tumor cells with minimal adverse effects and prevents tumor remission, has drawn extensive attention (4). However, there are still challenges that need to be overcome in the development of immunotherapy for GBM. The CNS is considered to be an "immune-privileged" organ, attributed to the lack of lymphatic involvement and the selectivity of the blood–brain barrier (BBB) to immune cells (5). Antigens in the brain can

still drain into the cervical lymph nodes through lymphatic vessels in the dura and meninges (6). Moreover, microglia, as the brain's resident immune cells, can function as potential antigen presentation cells (APCs), and T cells are activated in the cervical lymph nodes entering the brain parenchyma through the cerebrospinal fluid (CSF) (7). These observations suggest that the brain is immune privileged to a certain degree, and blood-derived immune cells are not completely precluded from the brain (8, 9). Moreover, GBM cells can exert local immunosuppressive effects in many ways. On the one hand, GBM cells themselves can secrete various protumor cytokines and/or chemokines, which can influence macrophage polarization, promote regulatory T cell (Treg) recruitment, and inhibit dendritic cell (DC) maturation and natural killer (NK) cell function. On the other hand, GBM cells can express immunosuppressive molecules, such as programmed cell death protein 1 ligand (PD-L1), which can prevent T cell proliferation and activation (10). In spite of these challenges, immunotherapy for GBM still obtains considerable achievements, which have given rise to a number of clinical trial investigations. Increasing immunotherapeutic approaches for GBM treatments have also been established. In this review, we present an overview of the current immunotherapy for GBM, including peptide vaccines, DC vaccines, chimeric T-cell receptors, checkpoint inhibitors, and oncolytic virotherapy.

PEPTIDE VACCINES

Peptide vaccines are about 8–30 amino acids in length. They are designed to encompass tumor-specific antigens (TSA), which derive from mutations only expressed in tumor cells but are absent in normal cells, or tumor-associated antigens (TAA), which derive from overexpressed normal proteins that are present in both tumoral and normal tissue. Unlike other solid tumors, GBM is notorious for possessing a relatively low level of mutation, resulting in only a minority of mutations used as TSA (11). At present, the peptide vaccines under investigation in GBM include rindopepimut (12), IMA950, and isocitrate dehydrogenase 1 (IDH1). The epidermal growth factor receptor variant III (EGFRvIII), with a mutated deletion in 20%–30% of tumors, is the most relevant and uncontroversial TSA for GBM. Thus, targeting EGFRvIII as a primary example of TSA-based peptide vaccines has been extensively investigated in the immunotherapy against GBM. In a phase II clinical trial, 65 patients with EGFRvIII-positive GBM were administrated with rindopepimut as well as with standard adjuvant TMZ (13) (**Table 1**). As a result, a progression-free survival (PFS) at 5.5 months of 66% and a median overall survival (OS) of 21.8 months were observed (13). In another phase II clinical trial, bevacizumab plus rindopepimut or a placebo were tested in rGBM patients, indicating that PFS at 6 months was 27%, and the median OS

TABLE 1 | Completed representative clinical trials of immunotherapy.

Immunotherapy approach	Phase	Sample size	PFS(m)	OS(m)	Characteristics
Vaccine					
Rindopepimut (15)	III	745	8	20.1	First clinical trial of an EGFRvIII-targeted therapy for newly diagnosed GBM
IMA950 (18)	I	45	NR	15.3	Evaluated the most biologically effective and clinically feasible
DCs vaccine (110)	II	26	12.7	23.4	Vaccine schedule design to deliver vaccine before radiation therapy
CMV pp65 DCs vaccine (32)	I	11	25.3	41.1	Provides evidence for targeting the association between CMV and GBM
SurVaxM peptide vaccine (111)	I	9	17.6	86.6	First study of SurVaxM in recurrent malignant gliomas
CDX-110 (13)	II	65	5.5	21.8	Multi-center phase II trials of CDX-110 with TMZ and radiation in GBM
HSPPC- 96 vaccine (112)	I/II	41	4.5	9.5	Establishes HSPPC-96 vaccine for recurrent malignant gliomas
GSCs derived mRNA transfected DCs vaccine (113)	I	20	23.1	25.5	First study targeting GSCs demonstrating feasibility, safety of an active immunotherapy targeting GSCs
Adaptive T cells					
IL13Rα2-CAR-T cells (57)	I	3	NR	11	First-in-human pilot safety and feasibility trial evaluating CAR-T cell targeting IL13Rα2 for recurrent GBM
INNOCELL Immuncell-LC (114)	III	180	8.1	22.5	First prospective, multicenter, randomized,controlled study of cytokine-induced killer cells therapy for newly diagnosed GBM
CMV-specific T cells (115)	I	19	8.2	13.3	First clinical trial of adoptive CMV-specific T cells for recurrent GBM
HER2-CAR-CMV-T cells (61)	I	16	3.5	24.5	First phase I trial of autologous HER2-CAR-CMV-T cells in GBM
Checkpoint Inhibitor					
Pembrolizumab (83)	II	80	4.1	8.8	First trial of pembrolizumab with Bevacizumab in recurrent GBM
Ipilimumab (116)	II	72	NR	7vs 4	First open label study of ipilimumab in melanoma patients with brain metastases
Nivolumab	III	369	1.5	9.8	First large randomized clinical trial of PD-1 inhibition in GBM

GBM, glioblastoma; CAR-T, chimeric antigen receptor T cells; OS, overall survival; PFS, progression-free survival; HSPPC-96, heat shock protein peptide complexes 96; CMV, cytomegalovirus; EGFR vIII. epidermal growth factor receptor variant III; HER-2, human epidermal-growth-factor receptor 2; GSCs, glioma stem cells; CMV pp65, cytomegalovirus phosphoprotein 65 RNA; DCs dendritic cells.

was 12 months, which is significantly improved compared with the control group of a PSF at 6 months of 11% and a median OS of 8.8 months (14). Following these achievements, a large, randomized, double-blind, placebo-controlled phase III clinical trial, enrolling 745 patients with ndGBM was terminated early after showing no significant improvement in the median OS. However, the data demonstrate patients with decent humoral immune responses (15) (**Table 1**). Notably, lost expression of EGFRvIII (antigen escape) was observed in the control arm to a similar degree as that of the treatment arm, which challenges the notion that therapies targeting EGFRvIII should be responsible for the outgrowth of EGFRvIII-deficient GBM cells (16, 17). In addition, this study also highlights that targeting a single tumor antigen may not be sufficient enough to induce durable antitumor responses.

IMA950 is a novel therapeutic vaccine that includes nine synthetic tumor-associated HLA-A2-restricted peptides (TUMAP), two MHC class II–binding peptides, and one HLA-A2-restricted HBV-derived peptide, and the last one was also used as a marker of vaccine immunogenicity. IMA950 can trigger the stimulation of TUMAP-specific cytotoxic T cells, leading to the destruction of malignant tumor cells. In a phase I trial, patients diagnosed with ndGBM after tumor resection were injected intradermally with IMA950 either prior to or just after the initiation of chemoradiotherapy. The majority of patients were found to respond well with a PFS at 6 months of 74% and a median OS of 15.3 months (18) (**Table 1**). In a recently completed phase I/II trial, IMA950 with vaccine adjuvant poly-ICLC in combination with TMZ were tested in 19 patients (16 with GBM and three with anaplastic astrocytoma). Patients from the overall cohort showed a median OS of 21 months from the date of surgery, compared with the GBM-only cohort of 19 months. PFS of patients from the overall cohort were 93% and 56% at 6 and 9 months, respectively (19). As for rGBM, however, IMA950 has no benefit in any preclinical trial. In a previous clinic trial, patients with recurrent high-grade gliomas who were administrated bevacizumab with the IMA950/poly-ICLC peptide vaccine did not show improved OS and PFS compared to nonvaccinated patients (20).

IDH1 mutations can be found in nearly 90% of low-grade gliomas, and more than 90% of IDH1 mutations contain an arginine-to-histidine switch at position 132 ($IDH1^{R132H}$). In GBM, IDH1 mutations can predict whether the tumors are secondarily developed from lower-grade gliomas because IDH1 mutations are rarely found in primary GBM. This high-frequency neoantigen is expressed in more than 70% of rGBMs, which can induce the formation of the oncometabolite 2-hydroxyglutarate and the inhibition of NADPH production (21, 22). Preclinical studies suggest peptide vaccines spanning the IDH1 mutation, may elicit $IDH1^{R132H}$-reactive $CD4^+$ and $CD8^+$ responses for antitumor (23). A phase I clinical trial at Duke University is ongoing in which the intradermal IDH1 peptide vaccine is tested in IDH1-positive grade II primary brain tumors (ClinicalTrials.gov identifier: NCT02193347). In another phase I trial, the safety of the IDH1 peptide vaccine for high-grade gliomas was also being evaluated. This study was completed in 2019 (ClinicalTrials.gov identifier: NCT02454634). Data collection is ongoing, and the therapeutic efficiency of IDH1 vaccines will be further estimated.

To date, several peptide vaccine strategies are shown to have safe and efficient profiles in phase I and II clinical trials, and some vaccines have significantly improved patient survival compared with historical controls. However, supportive data from phase III trials are still lacking. Although a phase III clinical trial on the EGFRvIII-based vaccine has failed in ndGBM patients, this vaccine could still induce decent humoral immune responses (15). Accordingly, more phase III trials on the peptide vaccine are required to support the therapeutic potential of peptide vaccines in GBM treatment. In addition, the single-antigen targeted strategy may lead to antigen escape due to high heterogeneity in the tumor. Therefore, alternative vaccine approaches are needed to target multiple tumor neoantigens. Heat shock protein (HSP) peptide complexes 96 (HSPPC-96) is one solution to handling this problem. HSPPC-96 is a primary resident chaperone of the endoplasmic reticulum, which can be internalized into APCs for efficient class I and II MHC-mediated presentation of tumor peptides (24). In a phase I clinical trial, an HSPPC-96 vaccination induced a tumor-specific peripheral immune response in 11 of 12 high-grade glioma patients (25). A subsequent open-label phase II multicenter clinical trial in surgically resectable rGBM patients treated with HSPPC-96-loaded antigens, which were extracted from patient-derived glioma tissue, showed an impressive median OS of 42.6 weeks and a 6-month OS of 29.3%, respectively (26). These results have sparked multiple ongoing clinical trials: NCT00905060, a completed phase II trial exploring the application of autologous HSPPC-96 following tumor resection and adjuvant RT and TMZ in ndGBM, and NCT01814813, a multi-institutional trial investigating the safety, tolerability, and efficacy of HSPPC-96 combined with bevacizumab in rGBM patients.

DC VACCINES

DCs are able to present tumor antigens to $CD4^+$ and $CD8^+$ T cells to stimulate an immune response. Therefore, vaccines based on DCs represent another immunotherapeutic approach. This type of vaccine is typically produced through the *ex vivo* generation of DCs harvested from patients. The isolated DCs are stimulated by either tumor antigens or mRNA-expressing MHC molecules before administration (27, 28). Currently, there are strategies for DC vaccines exposed to either single specific antigens or multiple tumor antigens. In a phase I trial, seven patients with high-grade gliomas were administered Wilms' tumor 1 (WT1)-pulsed autologous DCs. Five patients showed stable clinical responses, and the OS was 12.3 months in the cohort after the first DC vaccination (29). Cytomegalovirus phosphoprotein 65 RNA (CMV pp65) is also incorporated into DC vaccines because CMV nucleic acids and proteins are found in both primary and recurrent GBM (30). In another phase I trial, patients with ndGBM were administered pp65-specific DCs in combination with preconditioning using tetanus-diphtheria toxoid (Td). It achieved a promising PFS of 15.4–47.3 months and OS of 20.6–

47.3 months (31). Batich et al. applied dose-intensified TMZ followed by a CMV pp65 DC vaccine to treat 11 ndGBM patients in a phase I trial. Both median PFS and OS are longer than predicted ones (32) (**Table 1**). Currently, a randomized phase II trial involving a CMV pp65 DC vaccine is recruiting ndGBM patients (ClinicalTrials.gov identifier: NCT02465268) (**Table 2**). Another similar clinical trial on IDH1 DC vaccine for glioma treatments is also under investigation in China (ClinicalTrials.gov identifier: NCT02771301).

In addition, there are also DC vaccines exposed to multiple tumor antigens to induce a more robust immune response. In a phase I clinical study, an autologous DC vaccine pulsed with class I peptides from TAA highly expressed on gliomas and a cancer stem cell population (ICT-107) were administered to 15 ndGBM patients. Median PFS was 16.9 months and median OS was 38.4 months. It is also worth noting that six patients showed no evidence of tumor recurrence in a follow-up of 40.1 months (33). In another phase I/II trial, patients with recurrent glioma were administered α-type 1 polarized DCs loaded with EphA2, IL13Rα2, YKL-40, and gp100 and combined with poly-ICLC. It was observed that nine of 22 patients achieved PFS lasting at least 12 months, and one rGBM patient exhibited a sustained complete response (34). Recently, a novel DC vaccine, called DCVax-L, has been prepared from tumor lysate. In a phase I/II clinical trial, a DC vaccine was prepared with the patient's own tumor cells prior to administration to the patients. Sixteen GBM

TABLE 2 | Ongoing clinical trials involving DC vaccine, checkpoint inhibitor and CAR-T.

NCT number	Phase	Name of trial	Status	Patient enrolled
DC Vaccine				
NCT02649582	I/II	Adjuvant DC-immunotherapy Plus TMZ in GBM Patients	Recruiting	20
NCT02709616	I/II	Personalized Cellular Vaccine for Glioblastoma	Recruiting	20
NCT01567202	II	Study of DC Vaccination Against Glioblastoma	Recruiting	100
NCT02772094	II	Dendritic Cell-Based Tumor Vaccine Adjuvant Immunotherapy Human GBM	Ongoing	50
NCT02366728	II	DC Migration Study for Newly-Diagnosed GBM	Recruiting	100
NCT02465268	II	Vaccine Therapy for the Treatment of Newly Diagnosed Glioblastoma Multiforme	Recruiting	150
NCT01204684	II	Dendritic Cell Vaccine for Patients With Brain Tumors	Ongoing	60
NCT02754362	II	A Toll-like Receptor Agonist as an Adjuvant to TAA Mixed With Montanide ISA-51 VG With Bevacizumab for Patients With Recurrent GBM	Recruiting	30
NCT03395587	II	Efficiency of Vaccination With Lysate-loaded Dendritic Cells in Patients With Newly Diagnosed Glioblastoma	Recruiting	136
NCT03400917	II	Autologous Dendritic Cells Loaded With Autologous TAA for Treatment of Newly Diagnosed GBM	Recruiting	55
Checkpoint Inhibitor				
NCT02530502	I/II	Radiation Therapy With TMZ and Pembrolizumab in Treating Patients With Newly Diagnosed GBM	Ongoing	4
NCT02337686	II	Pharmacodynamic Study of Pembrolizumab in Patients With Recurrent GBM	Ongoing	18
NCT02337491	II	Pembrolizumab +/- Bevacizumab for Recurrent GBM	Ongoing	80
CAR-T				
NCT01454596	I/II	CAR-T Cell Receptor Immunotherapy Targeting EGFRvIII for Patients With Malignant Gliomas Expressing EGFRvIII	Recruiting	107
NCT02617134	I/II	CAR-T Cell Immunotherapy in MUC1 Positive Solid Tumor	Recruiting	20
NCT02839954	I/II	CAR-pNK Cell Immunotherapy in MUC1 Positive Relapsed or Refractory Solid Tumor	Recruiting	10
NCT02208362	I	Genetically Modified T-cells in Treating Patients With Recurrent or Refractory Malignant Glioma	Recruiting	135
NCT02713984	I/II	A Clinical Research of CAR T Cells Targeting HER2 Positive Cancer	Recruiting	60
NCT02209376	I	Autologous T Cells Redirected to EGFRVIII-With a CAR in Patients With EGFRVIII^{+} Glioblastoma	Ongoing	12
NCT02664363	I	EGFRvIII CAR T Cells for Newly Diagnosed GBM	Recruiting	48
NCT02844062	I	Pilot Study of Autologous Anti-EGFRvIII CAR T Cells in Recurrent Glioblastoma Multiforme	Recruiting	20
NCT01109095	I	CMV-specific Cytotoxic T Lymphocytes Expressing CAR Targeting HER2 in Patients With GBM	Ongoing	16
NCT02442297	I	T Cells Expressing HER2-specific CAR for Patients With Glioblastoma	Recruiting	14
NCT02937844	I	Pilot Study of Autologous Chimeric Switch Receptor Modified T Cells in Recurrent GBM	Recruiting	20

GBM, glioblastoma; CAR-T, chimeric antigen receptor T cells; TMZ, temozolomide; HSPPC-96, heat shock protein peptide complexes 96; CMV, cytomegalovirus; TAA, tumor-associated antigen; EGFR vIII, epidermal growth factor receptor variant III; HER-2, human epidermal-growth-factor receptor 2; CMV pp65, cytomegalovirus phosphoprotein 65 RNA.

patients were enrolled in this trial. The data show that median and 5-year survival were 525 days and 18.8%, respectively (35). A randomized phase II trial on DCVax-L and nivolumab in rGBM patients is ongoing (ClinicalTrials.gov identifier: NCT03014804). Another randomized phase III trial on DCVax-L is currently underway in 348 GBM patients (ClinicalTrials.gov identifier: NCT00045968). Given that DC cocultured with tumor lysate for the generation of DCVax-L, this kind of vaccine should be more efficient in the elimination of tumor cells because it is able to target more tumor-related antigens. However, theoretically there is also a high risk that it may cause an autoimmune response. Therefore, it still remains a challenge for researchers to choose a suitable tumor lysate for the generation of DC vaccines regarding the high heterogeneity of GBM. There is still much work that needs to be done to understand the influences of tumor genotypes and microenvironments on DC vaccine production to prevent the undesired autoimmune response during administration.

ADOPTIVE T CELL THERAPY

The functional advantage of adoptive T cell therapy lies in its ability to harvest, train, and expand autologous T cells which are then transfered back into patients (36). The primary forms of adoptive T cell therapy can be generally classified as tumor-infiltrating lymphocytes (TILs), T-cell receptor (TCR) treatment, and chimeric antigen receptor T (CAR-T) cells. The application of TILs requires highly accessible and immunogenic tumor cells; however, only melanoma can meet sufficient expansion of TILs from their respective tumor samples (37). In a prospective pilot study including six rGBM, locally infused autologous TILs did not show powerful cytotoxicity against the autologous tumor (38). Apart from the desire for improvement in expansion of brain tumor–derived TILs, this study also implied the significance of maintaining autologous TIL activation within the brain TME. TCR treatment was the first successful application of adoptive T cell therapy that utilized autologous T cells transduced with human TCR recognizing a melanoma antigen recognized by T cells 1 (MART-1) to treat patients with metastatic melanoma (39). As far as gliomas are concerned, however, no clinical trials based on TCR-T cell therapies have been initiated. The little progress made in TILs and TCR against gliomas force researchers to seek other ways, and the efforts to overcome MHC restriction result in the development of CAR-T cell therapy.

Recently, genetically engineered T cells expressing chimeric antigen receptors (CARs) to recognize specific tumor antigens have brought in a new era of cancer immunotherapy. CARs are artificial fusion proteins that incorporate an intracellular T-cell signaling domain that consists of one or more single-chain variable fragments (scFv) and an extracellular antigen-recognition domain to target specific neoplastic cells. These complex domains include CD28, CD3ζ, 4-1BB, or OX40 derived from the same part of CD28/CD8 or a corresponding domain of T-cell receptors (TCRs) (40, 41) (**Figure 1**). In addition to being endowed with a specific affinity to TSAs or targets of interest, CAR-T cells can be stimulated without MHC involvement and prevent the challenges associated with adoptive T-cell transfer (42, 43). Currently, CD19-specific CAR-T cells have induced sustained and durable antitumor immune responses in patients with multiple myeloma, acute and chronic lymphocytic leukemia, and refractory diffuse large B-cell lymphoma (DLBCL) (44–49). These encouraging results have prompted FDA approvals of two therapies: CTL019, a treatment for patients younger than 25 with relapsed or refractory B-cell precursor acute lymphoblastic leukemia, and another CD19-targeted CAR T-cell treatment, axicabtagene ciloleucel, for patients with failed DLBCL for at least two prior therapies (50, 51). Inspired by the success in blood tumors, increasing interest has focused on the treatments of CAR-T cells against GBM. These CAR-T cells mainly target the following antigens: EGFRvIII, IL-13Rα2, and HER2. EGFRvIII is abundantly expressed in approximately 30% of GBM to enhance glioma cell proliferation, angiogenesis, and invasiveness (52). In preclinical studies, CAR-T cells targeting

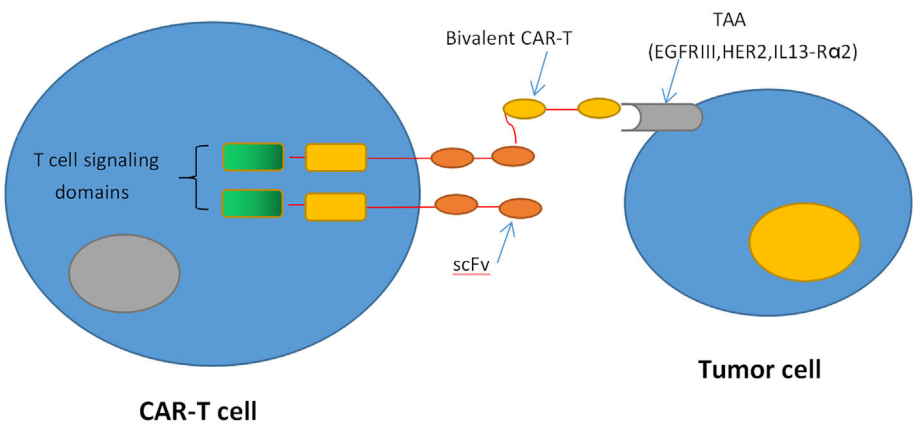

FIGURE 1 | CAR T-cell therapy. CAR-T cells produce an artificial T cell receptor that has big tumor-specific surface antigens.

EGFRvIII could effectively infiltrate to tumor sites and suppress the growth of glioma xenografts in murine models (53). In a human clinical trial, EGFRvIII-targeting CAR-T cells showed feasibility and safety in the treatment for 10 rGBM patients without toxicity or cytokine release syndromes (54). It demonstrates that transient expansion of EGFRvIII-targeting CAR-T cells could be detected in peripheral blood of all patients. The median OS was approximately 8 months, and one patient experienced residual stable disease at 18 months (54). The promising clinical trials are still ongoing to assess the efficiency of this approach (**Figure 1**).

Another target of CAR-T cell treatment for GBM patients is IL-13Rα2, which presents in more than 75% of GBM tumors associated with tumor invasiveness and poor prognosis (55, 56). As the first CAR-T targeting IL-13Rα2 therapy, the feasibility and safety of IL13-zetakine $CD8^+$ CTL against rGBM have been evaluated by Brown et al. In this trial, intracranial delivery of the IL13-zetakine$^+$ cytotoxic T lymphocytes (CTL) into the resection cavity was well tolerated in three patients. A transient antiglioma response was observed in two patients (57) (**Table 1**). In a following report, CAR T-cells targeting IL-13Rα2 incorporated with costimulatory immunoreceptor CD137 were initially delivered into the resection cavity of grade 3 or higher GBM. Consequently, regression of all intracranial and spinal tumors was observed without any toxic effects. Moreover, a robust increase of inflammatory cytokines and chemokines in the CSF with limited CAR T-cell accumulation and expansion was also found. Eventually, this clinical response lasted for 7.5 months after the initiation of CAR T-cell therapy (58).

Human epidermal growth factor receptor 2 (HER2) is a transmembrane tyrosine kinase receptor overexpressed in 80% of GBM. It is identified as an independent unfavorable prognostic indicator for GBM patients (59, 60). Considering that HER2 is also expressed in normal tissues, there is a theoretical high risk of off-target toxicity resulting from HER2-targeting CAR-T cells. Intriguingly, a phase I clinical trial demonstrated the feasibility and safety of HER2-targeting CAR-T cells, which were well-tolerated in 17 patients with progressive HER2-positive GBM without any dose-limiting toxic effects (61) (**Table 1**). The median OS was 11.1 months (95% CI, 4.1-27.2 months) from the first T-cell infusion and 24.5 months (95% CI, 17.2-34.6 months) from diagnosis. Three patients had no progression between 24 to 29 months (61).

Although the results from these studies are encouraging, CAR-T cells targeting a single antigen may still inevitably lead to antigen escape. To deal with this intractable dilemma, CAR-T cells targeting multiple tumor antigens have been established to overcome the heterogeneity of GBM. Hegde et al. created CAR-T cells expressing a HER2-binding scFv and an IL-13Rα2-binding IL-13 mutein, which could efficiently recognize and kill either HER2 or IL-13Rα2 positive tumor cells (62). These bispecific CAR-T cells are more sustainable and capable of improving the survival in GBM murine models and mitigating antigen escape (62). Taking this approach one step further, the same research group generated trivalent CAR-T cells targeting HER2, IL-13Rα2, and EphA2, which could overcome the interpatient variability and capture nearly 100% of tumor cells. In a murine model, the trivalent CAR-T cells exhibited superior antitumor efficacy. It significantly inhibited tumor growth and improved animal survival compared with biCAR-T cells or single CAR T-cells (63).

CAR T-cell therapy in GBM has just begun. Preliminary results demonstrate its feasibility and safety, and bi- or tri-CAR-T cells may be a promising strategy for the intractable dilemma of antigen loss. However, several problems and challenges in solving CAR-T treatment still exist.. First, T-cell proliferation and persistence is still a limitation for solid tumor treatment because the peripheral blood is not the therapeutic site. It also raises a related issue regarding whether precondition of lymphodepleting, which has been approved as a standard in CAR-T treatment of hematologic malignancies, is able to improve CAR T-cell expansion and efficacy in GBM (64, 65). Although it has not been reported to use lymphodepleting preconditioning in ndGBM (54, 57, 58), rGBM patients often accept "lymphodepletion" before CAR-T treatment due to the effects of standard radiation and TMZ (66). Another issue that needs to be addressed is the immunosuppressive TME. The TME of GBM can present many obstacles to CAR-T cells, including immunosuppressive immune cells, tumor-derived soluble factors and cytokines, and physical and metabolic barriers (67, 68). Therefore, intensive investigations are urgently needed to improve the efficacy of CAR-T treatment in GBM patients.

CHECKPOINT INHIBITORS

Immune checkpoints are the coinhibitory molecules that could attenuate the intensity and duration of T-cell-mediated immune responses to maintain self-tolerance and prevent uncontrolled inflammatory responses. Currently, the most well-studied coinhibitory molecules in hematologic and solid tumors include cytotoxic T-lymphocyte antigen 4 (CTLA-4), programmed cell death protein 1 (PD-1) and its ligand PD-L1, T-cell immunoglobulin and mucin domain 3 (TIM-3), and indoleamine 2,3-dioxygenase-1 (IDO1).

CTLA-4 is one of the most extensively studied immune checkpoint inhibitors, and it suppresses T-cell stimulation by competing with the costimulatory molecule CD28 for binding its ligands CD80 and CD86 (69, 70) (**Figure 2**). Ipilimumab (trade name Yervoy) was the first FDA-approved checkpoint for immunotherapy targeting CTLA-4 applied in metastatic melanoma and now approved for several solid tumors. In murine glioma models, blockade of CTLA-4 could induce tumor regression and promote long-term survival without eliciting experimental allergic encephalomyelitis (71). For GBM, combinatorial blockade of CTLA-4 and PD-1 were demonstrated to cure 75% of immunocompetent murine GBM models even against advanced, later-stage tumors (72). Until now, blockade of CTLA-4 could lead to robust antitumor immunity only at the preclinical stage. Although there has been no published data on CTLA-4 inhibitors solely treating GBM yet, some clinical trials are currently ongoing to evaluate

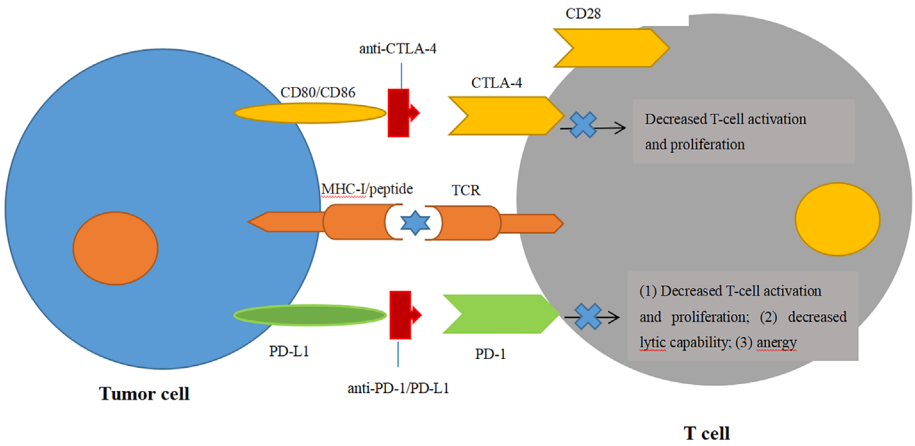

FIGURE 2 | Blockade of immune checkpoint inhibitors. Engagement of CTLA-4 with its ligands CD80/CD86 can prevent the ligands binding to the T-cell activation and proliferation. Engagement of PD-1 with one of its ligands, PD-L1, can decrease the T-cell tumor lytic capacity and induces T-cell anergy.

CTLA-4 inhibitors in GBM combined with other therapeutic agents, such as VEGF inhibitors, checkpoint inhibitors, tumor treating fields, and radiation therapy (73).

PD-1, an immunoglobulin receptor belonging to the extended CD28/CTLA-4 family of T-cell regulators, is expressed on activated T, B, myeloid, and NK cells. It binds to the ligands PD-L1 and PD-L2 (74). The PD-1/PD-L1 axis is proven to be the major negative regulation of CTL in the TME, whose protumor function, including suppression of T-cell activation and infiltration, is inhibiting the secretion of pro-inflammatory factors and inactivation of TCR signaling (74, 75) (**Figure 2**). Owing to the success of antibodies targeting the PD-1/PD-L1 axis in the clinical trials against advanced melanoma, monoclonal PD-1 antibodies (Pembrolizumab and Nivolumb) were approved by the FDA for the treatment of melanoma, non-small cell lung cancer (NSLC), and other solid tumors (76–80). For GBM, the therapeutic effects of PD-1/PD-L1 antibodies remain largely elusive. In a preclinical study, the combination of PD-1 antibody and radiotherapy achieved a twofold increase in median survival in GL261 glioma mouse models, and 15%–40% of mice gained long-term survival compared with a single treatment (81). In another preclinical trial, the combination of a DC vaccine and PD-1 antibody achieved long-term survival in intracranial glioma tumor-bearing mice that were solely dependent on CD8+ T cells (82). Moreover, this combination of a DC vaccine and PD-1 antibody also resulted in the upregulation of homing integrin and immunologic memory markers on TILs (82). These encouraging preclinical studies prompted the first large phase III clinical trial of PD-1 checkpoint blockade in rGBM through the comparison of nivolumab monotherapy with standard care using bevacizumab (NCT02017717). Although the median OS was comparable between nivolumab and bevacizumab among the overall enrolled patients, this trial was still closed in 2017 on account of failing to meet the primary OS endpoint (83) (**Table 1**). Another phase III randomized trial, CheckMate 548, was processed to evaluate the effects of nivolumab with or without radiation therapy and TMZ in O6-methylguanine-DNA methyltransferase (MGMT)-methylated ndGBM patients. This study has also failed to achieve the endpoint for the inability of nivolumab concomitant with radiation therapy and TMZ to improve the median OS (84). Another similar phase III trial, CheckMate 498, for patients with MGMT-unmethylated tumors also declared that nivolumab combined with TMZ failed to improve patients' median OS. Although nivolumab has not yet shown efficiency in clinical trials, other antibody therapies targeting the PD-1/PD-L1 axis have emerged in clinical trials. Pembrolizumab, another PD-1 antibody, was tested as neoadjuvant or adjuvant-only therapy in 35 surgically resectable rGBM patients in a single-arm phase II clinical trial. Patients accepting pembrolizumab showed a statistically significant increase in OS with a median value of 417 days compared with those in the adjuvant group with 228.5 days. PFS in the neoadjuvant group was also significantly increased over the adjuvant group (99.5 days vs. 72.5 days). The study also found that neoadjuvant anti-PD-1 blockade was related to an upregulation of the IFN-γ responsive gene signature and a declined cell cycle–related gene signature in the tumor (85). In a single-arm phase I trial, pembrolizumab accompanied by hypofractionated stereotactic irradiation and bevacizumab were well tolerated in 23 rGBM patients. More than half of the patients achieved durable objective responses, and 64% of the patients were still alive within 12 months (86). Another phase I trial on combinatorial pembrolizumab with bevacizumab (NCT02337491) in rGBM patients showed a median OS of 8.8 months and PFS of 4.1 months (https://clinicaltrials.gov/ct2/show/results/NCT02337491) (**Table 2**). Additionally, durvalumab (MEDI4736), a humanized PD-Ll monoclonal antibody, is currently being tested in a multicenter phase II trial combined with radiotherapy and bevacizumab in GBM patients (NCT02336165) (87). It is striking that one patient obtained a long-period OS of 86 weeks (87). In contrast to the monotherapy by PD-1/PD-L1 inhibitors with few successes, combinatorial therapy

of PD-1/PD-L1 antibodies with radiation therapy and/or chemotherapy seem more promising in the clinical trials against GBM.

In addition to CTLA-4 and PD-1/PD-L1 therapy, another two checkpoint targets have received researcher interest. TIM-3, an immunosuppressive receptor expressed on T cells, Tregs, DCs, NK cells, and macrophages, can promote T-cell exhaustion similar to PD-1 (88, 89). There are ongoing clinical trials testing TIM-3-targeted antibodies in solid tumors (NCT02608268, NCT02817633) and hematological malignancies (NCT03066648). IDO1 is a cytoplasmic enzyme promoting tryptophan catabolism through the kynurenine pathway. It is demonstrated that depletion of IDO1 can suppress T-cell function and elevate expression of IDO1 in a tumor, which is correlated with poor prognosis in GBM patients (90, 91). So far, there are some clinical trials evaluating IDO1 inhibitors in melanoma (92) and breast cancer (NCT01792050) but none showing a survival benefit. There is a phase I clinical trial including various treatments, such as IDO1 inhibitor therapy, chemotherapy, and radiation therapy in pediatric brain tumors. Twenty-nine patients enrolled in this trial showing a median PFS of 6.2 months, and the median time to regimen failure is 11.7 months (NCT02502708). There are also other ongoing clinical trials testing the IDO1 inhibitor combined with other therapies in malignant brain tumors (93) and rGBM (NCT03707457). Results from these trials are still pending. Furthermore, the efficacy and safety of these agents need to be evaluated in GBM patients.

Despite the great advances in treating hematological malignancies and solid tumors as well as promising results from preclinical and early-phase trials in GBM, immune checkpoint inhibitors have not yet demonstrated efficacy in GBM through large phase III clinical trials as a monotherapy or combination therapy with other treatments. The BBB should first be taken into account as it may block the antibody penetration into the CNS. Moreover, a tumor mutational burden that predicts the efficacy of immune checkpoint inhibitors across multiple solid tumors is actually associated with poor prognosis in glioma patients (94). Last but not least, the immunosuppressive TME and dynamic responses to tumorigenesis of GBM may also contribute to the obstacles faced by the immune checkpoint inhibitors. Thus, further investigations on the optimal combinations of multiple therapies as well as tumor genomic and immune characteristics are urgently required to clarify the role of checkpoint inhibitors in GBM in the future.

ONCOLYTIC VIROTHERAPY

Oncolytic virotherapy (OV) employs naturally occurring or artificially engineered viruses, which are typically delivered intratumorally or postsurgically into the resection cavity to infect and lyse tumor cells, simultaneously triggering inflammation and immune responses to tumor cells and the virus (95). Multifarious virus species have been studied as oncolytic virus platforms for cancer therapy, such as herpes simplex virus (HSV), adenovirus, vaccinia virus, measles virus, poliovirus, and reovirus. In 2015, talimogene laherparepvec (T-VEC), a genetically modified HSV, was approved by the FDA for advanced melanoma as the first OV therapeutic in the United States (96). GBM virotherapy clinical trials started in 1991; Martuza et al. first reported engineered HSV for their capability of selective replication and killing of GBM cells (97). Since then, multifarious OVs have been tested in gliomas; however, they seldomly demonstrate efficacy in improving median OS in randomized trials (98). Here, we present evidence that OVs have recently been advanced to phase I/II trials in glioma patients, demonstrating remarkable efficacy in subsets of patients.

DNX-2401 (Ad5-Delta-24-RGD;tasadenoturev) is a replication-competent adenovirus with enhanced infectivity, high tumor selectivity, and a specific mutation to restrict viral replication. This virus can target integrins on GBM cells with a glycine/aspartic/arginine acid motif, which can increase infective specificity for tumor cells (99, 100). In a phase I trial of DNX-2401 (NCT00805376), 37 rGBM patients received a single intratumoral injection of DNX-2401 through the biopsy needle (cohort 1) or a permanently implanted catheter followed by tumor resection (cohort 2). In cohort 1, 20% of patients survived more than 3 years after treatment, and 3 patients showed more than 3 years of PFS with dramatic tumor reduction (95% or more, CR). Immunohistochemical analysis of post-treatment surgical specimens from cohort 2 revealed that DNX-2401 replicated and spread within the tumor and induced $CD8^+$ and T-bet^+ cell infiltration. No dose-limiting toxicities were observed, and adverse effects were reported in 15% of patients with no serious virus-related events of grade 3 or higher noted (101). Thus, this clinical trial, for the first time, showed direct oncolytic effects in GBM and provided evidence for elicitation of anti-GBM immune responses. In another phase I/II clinical trial that was initiated in 2010 for rGBM patients (NCT01582516), DNX-2401 was administered by catheters targeting the tumor mass and the surrounding infiltrated brain. Analysis of CSF from patients showed an elevated level of some cytokines that can increase the levels of CD64, a marker of M1-polarization, implying that DNX-2401 therapy can promote a macrophage phenotype shift from M2 to M1 (102). Currently, the combination of DNX-2401 treatment with pembrolizumab is under investigation in a phase II trial for rGBM patients (CAPTIVE/KEYNOTE-192, NCT02798406). Interim results were reported at the SNO 2018 annual meeting, including that the combinatorial therapy was well tolerated, and 100% 9-month survival for the first seven patients treated was noted (103). Publication of longer follow-up data is eagerly awaited.

The polio-rhinovirus chimera (PVS-RIPO) is a replication-competent, live attenuated poliovirus vaccine/human rhinovirus chimera that is engineered with a foreign (rhinovirus) ribosome entry site to ablate neurovirulence. PVS-RIPO can target the poliovirus receptor CD155 that is expressed on APC or overexpressed on tumor cells. In a phase I trial (NCT01491893), 61 patients with recurrent supratentorial grade IV malignant glioma received PVS-RIPO intratumorally by convection-enhanced delivery *via* a catheter. The patients

who received PVS-RIPO had an OS rate of 21% at 24 and 36 months with two patients obtaining complete response and remaining alive for more than 70.4 months (104). A randomized phase II trial of PVS-RIPO solely or combined with lomustine in patients with recurrent grade IV malignant glioma (NCT02986178) is ongoing.

Other OVs, such as ParvOryx (oncolytic H-1 parvovirus), Toca 511 (a retroviral replication-competent vector), Reovirus, and HSV type 1 have also been tested in a phase I/II trial for GBM patients and obtained promising results (105–108). Although these early phase clinical trials demonstrate a survival benefit that OV has brought, these benefits were only appreciated by some subsets of patients with glioma. Recently, a comprehensive analysis of virotherapy trials for rGBM revealed that virotherapy can improve the 2- and 3-year survival rates compared with non-virotherapy clinical trials (2-year survival: 15% vs. 12%; 3-year survival rate: 9% vs. 6%) (109). Thus, further investigations and more large randomized controlled phase II/III trials need to be done to evaluate the benefit of OV.

CONCLUSION

Current clinical trials of immunotherapy predominantly focus on the investigation of peptide vaccines, DC vaccines, CAR-T cells, checkpoint inhibitors, and OV. Many promising clinical outcomes have been achieved (110–116) however, immunotherapeutic successes in GBM are still lacking. Multiple factors challenge immunotherapy in GBM, including the immunosuppressive TME, tumor heterogeneity, tumor genomic characteristics, persistence and delivery of the vaccines, and efficiency of drug penetration through the BBB. Moreover, there remains a need for appropriate pre- and post-therapeutic biomarkers that may facilitate the establishment of a valid and standardized assessment for clinical efficacy in GBM. Immunotherapy for GBM requires integrated efforts with rational combinations of vaccine therapy, cell therapy, and radio- and chemotherapy, as well as molecule therapy targeting TME. These contributions promote the development of an optimal personalized therapeutic strategy for GBM patients.

AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by BH, XL, YL, ZZ, and HZ. The first draft of the manuscript was written by BH and JZ, and all authors commented on previous versions of the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Davis ME. Epidemiology and Overview of Gliomas. *Semin Oncol Nurs* (2018) 34(5):420–9. doi: 10.1016/j.soncn.2018.10.001
2. Stupp R, Mason WP , van den Bent, MJ, Weller M, Fisher B, Taphoorn MJB, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *New Engl J Med* (2005) 352:987–96. doi: 10.1056/NEJMoa043330
3. Cohen MH, Shen YL, Keegan P, Pazdur R. FDA Drug Approval Summary: Bevacizumab (Avastin®) as Treatment of Recurrent Glioblastoma Multiforme. *Oncologist* (2012) 17:1482–2. doi: 10.1634/theoncologist.2009-0121erratum
4. Sanmamed MF, Chen L. A paradigm shift in cancer immunotherapy: from enhancement to normalization. *Cell* (2019) 176:677. doi: 10.1016/j.cell.2019.01.008
5. Sampson JH, Maus MV, June CH. Immunotherapy for brain tumors. *J Clin Oncol* (2017) 35:2450–6. doi: 10.1200/JCO.2017.72.8089
6. McGranahan T, Li G, Nagpal S. History and current state of immunotherapy in glioma and brain metastasis. *Ther Adv Med Oncol* (2017) 9:347–68. doi: 10.1177/1758834017693750
7. Sampson JH, Maus MV, June CH. Immunotherapy for brain tumors. *J Clin Oncol* (2017) 35:2450–6. doi: 10.1200/JCO.2017.72.8089
8. Mrdjen D, Pavlovic A, Hartmann FJ, Schreiner B, Utz SG, Leung BP, et al. High-dimensional single-cell mapping of central nervous system immune cells reveals distinct myeloid subsets in health, aging, and disease. *Immunity* (2018) 48:380–395.e6. doi: 10.1016/j.immuni.2018.01.011
9. Thion MS, Low D, Silvin A, Chen J, Grisel P, Schulte-Schrepping J, et al. Microbiome infuences prenatal and adult microglia in a sex-specific manner. *Cell* (2018) 172:500–16.e16. doi: 10.1016/j.cell.2017.11.042
10. Nduom EK, Weller M, Heimberger AB. Immunosuppressive mechanisms in glioblastoma. *Neuro Oncol* (2015) 17 Suppl 7:vii9–vii14. doi: 10.1093/neuonc/nov151
11. Hodges TR, Ott M, Xiu J, Gatalica Z, Swensen J, Zhou S, et al. Mutational burden, immune checkpoint expression, and mismatch repair in glioma: implications for immune checkpoint immunotherapy. *Neuro Oncol* (2017) 19:1047–57. doi: 10.1093/neuonc/nox026
12. Heimberger AB, Crotty LE, Archer GE, Hess KR, Wikstrand CJ, Friedman AH, et al. Epidermal growth factor receptor VIII peptide vaccination is efficacious against established intracerebral tumors. *Clin Cancer Res* (2003) 9(11):4247–54.
13. Schuster J, Lai RK, Recht LD, Reardon DA, Paleologos NA, Groves MD, et al. A phase II, multicenter trial of rindopepimut (CDX-110) in newly diagnosed glioblastoma: the ACT III study. *Neuro Oncol* (2015) 17:854–61. doi:10.1093/neuonc/nou348
14. Reardon DA, Schuster J, Tran DD, Fink KL, Nabors LB, Li G, et al. ReACT: overall survival from a randomized phase II study of rindopepimut (CDX-110) plus bevacizumab in relapsed glioblastoma. *J Clin Oncol* (2015) 33 (suppl):2009. doi:10.1200/jco.2015.33.15_suppl.2009
15. Weller M, Butowski N, Tran DD, Recht LD, Lim M, Hirte H, et al. Rindopepimut with temozolomide for patients with newly diagnosed, EGFRvIII expressing glioblastoma (ACT IV): a randomized, double-blind, international phase 3 trial. *Lancet Oncol* (2017) 18:1373–785. doi: 10.1016/S1470-2045(17)30517-X
16. Sampson JH, Heimberger AB, Archer GE, Aldape KD, Friedman AH, Friedman HS, et al. Immunologic escape after prolonged progression-free survival with epidermal growth factor receptor variant III peptide vaccination in patients with newly diagnosed glioblastoma. *J Clin Oncol* (2010) 28(31):4722–9. doi: 10.1200/JCO.2010.28.6963

17. Sampson JH, Aldape KD, Archer GE, Coan A, Desjardins A, Friedman AH, et al. Greater chemotherapy-induced lymphopenia enhances tumor-specific immune responses that eliminate EGFRvIII-expressing tumor cells in patients with glioblastoma. *Neuro Oncol* (2011) 13(3):324–33. doi: 10.1093/neuonc/noq157
18. Rampling R, Peoples S, Mulholland PJ, James A, Al-Salihi O, Twelves CJ, et al. A Cancer Research UK First Time in Human Phase I Trial of IMA950 (Novel Multipeptide Therapeutic Vaccine) in Patients with Newly Diagnosed Glioblastoma. *Clin Cancer Res* (2016) 22(19):4776–85. doi: 10.1158/1078-0432.CCR-16-050
19. Migliorini D, Dutoit V, Allard M, Grandjean Hallez N, Marinari E, Widmer V, et al. Phase I/II trial testing safety and immunogenicity of the multipeptide IMA950/poly-ICLC vaccine in newly diagnosed adult malignant astrocytoma patients. *Neuro Oncol* (2019) 21(7):923–33. doi: 10.1093/neuonc/noz040
20. Boydell E, Marinari E, Migliorini D, Dietrich PY, Patrikidou A, Dutoit V. Exploratory Study of the Effect of IMA950/Poly-ICLC Vaccination on Response to Bevacizumab in Relapsing High-Grade Glioma Patients. *Cancers (Basel)* (2019) 11(4):E464. doi: 10.3390/cancers11040464
21. Nobusawa S, Watanabe T, Kleihues P, Ohgaki H. IDH1 mutations as molecular signature and predictive factor of secondary glioblastomas. *Clin Cancer Res* (2009) 15(19):6002–7. doi: 10.1158/1078-0432.CCR-09-0715
22. Cohen A, Holmen S, Colman H. IDH1 and IDH2 mutations in gliomas. *Curr Neuro Neurosci Rep* (2013) 13:345. doi: 10.1007/s11910-013-0345-4
23. Schumacher T, Bunse L, Pusch S, Sahm F, Wielster B, Quandt J, et al. A vaccine targeting mutant IDH1 induces antitumour immunity. *Nature* (2014) 512(7514):324–7. doi: 10.1038/nature13387
24. Amato RJ. Heat-shock protein-peptide complex-96 for the treatment of cancer. *Expert Opin Biol Ther* (2007) 7(8):1267–73. doi: 10.1517/14712598.7.8.1267
25. Crane CA, Han SJ, Ahn B, Oehlke J, Kivett V, Fedoroff A, et al. Individual patient-specifc immunity against high-grade glioma after vaccination with autologous tumor derived peptides bound to the 96 KD chaperone protein. *Clin Cancer Res* (2013) 19:205–14. doi: 10.1158/1078-0432.CCR-11-3358
26. Bloch O, Crane CA, Fuks Y, Kaur R, Aghi MK, Berger MS, et al. Heat-shock protein peptide complex-96 vaccination for recurrent glioblastoma: a phase II, single-arm trial. *Neuro-Oncology* (2014) 16:274–9. doi: 10.1093/neuonc/not203
27. Filley AC, Dey M. Dendritic cell based vaccination strategy: an evolving paradigm. *J Neurooncol* (2017) 133:223–35. doi: 10.1007/s11060-017-2446-4
28. Lynes J, Sanchez V, Dominah G, Nwankwo A, Nduom E. Current Options and Future Directions in Immune Therapy for Glioblastoma. *Front Oncol* (2018) 8:578. doi: 10.3389/fonc.2018.00578
29. Sakai K, Shimodaira S, Maejima S, Udagawa N, Sano K, Higuchi Y, et al. Dendritic cell-based immunotherapy targeting Wilms' tumor 1 in patients with recurrent malignant glioma. *J Neurosurg* (2015) 123(4):989–97. doi: 10.3171/2015.1.JNS141554
30. Nair SK, Sampson JH, Mitchell DA. Immunological targeting of cytomegalovirus for glioblastoma therapy. *OncoImmunology* (2014) 3: e29289. doi: 10.4161/onci.29289
31. Mitchell DA, Batich KA, Gunn MD, Huang MN, Sanchez-Perez L, Nair SK, et al. Tetanus toxoid and CCL3 improve dendritic cell vaccines in mice and glioblastoma patients. *Nature* (2015) 519(7543):366–9. doi: 10.1038/nature14320
32. Batich KA, Reap EA, Archer GE, Sanchez-Perez L, Nair SK, Schmittling RJ, et al. Long-term survival in glioblastoma with cytomegalovirus pp65-targeted vaccination. *Clin Cancer Res* (2017) 23(8):1898–909. doi: 10.1158/1078-0432.CCR-16-2057
33. Phuphanich S, Wheeler CJ, Rudnick JD, Mazer M, Wang H, Nuño MA, et al. Phase I trial of a multi-epitope-pulsed dendritic cell vaccine for patients with newly diagnosed glioblastoma. *Cancer Immunol Immunother* (2013) 62 (1):125–35. doi: 10.1007/s00262-012-1319-0
34. Okada H, Kalinski P, Ueda R, Hoji A, Kohanbash G, Donegan TE, et al. Induction of CD8+ T-cell responses against novel glioma-associated antigen peptides and clinical activity by vaccinations with {alpha}-type 1 polarized dendritic cells and polyinosinic-polycytidylic acid stabilized by lysine and carboxymethylcellulose in patients with recurrent malignant glioma. *J Clin Oncol* (2011) 29(3):330–6. doi: 10.1200/JCO.2010.30.7744
35. Chang CN, Huang YC, Yang DM, Kikuta K, Wei KJ, Kubota T, et al. A phase I/II clinical trial investigating the adverse and therapeutic effects of a postoperative autologous dendritic cell tumor vaccine in patients with malignant glioma. *J Clin Neurosci* (2011) 18(8):1048–54. doi: 10.1016/j.jocn.2010.11.034
36. Rosenberg SA, Restifo NP. Adoptive cell transfer as personalized immunotherapy for human cancer. *Science* (2015) 348:62–8. doi: 10.1126/science.aaa4967
37. Reinhard K, Rengstl B, Oehm P, Michel K, Billmeier A, Hayduk N, et al. An RNA vaccine drives expansion and efficacy of claudin-CAR-T cells against solid tumors. *Science* (2020) 367:446–53. doi: 10.1126/science.aay5967
38. Quattrocchi KB, Miller CH, Cush S, Bernard SA, Dull ST, Smith M, et al. Pilot study of local autologous tumor infiltrating lymphocytes for the treatment of recurrent malignant gliomas. *Clin Trial J Neurooncol* (1999) 45(2):141–57. doi: 10.1023/A:1006293606710
39. Morgan RA, Dudley ME, Wunderlich JR, Hughes MS, Yang JC, Sherry RM, et al. Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science* (2006) 314:5. doi: 10.1126/science.1129003
40. Rabinovich GA, Gabrilovich D, Sotomayor EM. Immunosuppressive strategies that are mediated by tumor cells. *Annu Rev Immunol* (2007) 25:267–96. doi: 10.1146/annurev.immunol.25.022106.141609
41. Sadelain M, Brentjens R, Rivière I. The basic principles of chimeric antigen receptor design. *Cancer Discovery* (2013) 3(4):388–98. doi: 10.1158/2159-8290.CD-12-0548
42. Srivastava S, Riddell SR. Engineering CAR-T cells: design concepts. *Trends Immunol* (2015) 36(8):494–502. doi: 10.1016/j.it.2015.06.004
43. van der Stegen SJ, Hamieh M, Sadelain M. The pharmacology of second-generation chimeric antigen receptors. *Nat Rev Drug Discovery* (2015) 14 (7):499–509. doi: 10.1038/nrd4597
44. Garfall AL, Maus MV, Hwang WT, Lacey SF, Mahnke YD, Melenhorst JJ, et al. Chimeric antigen receptor T cells against CD19 for multiple myeloma. *N Engl J Med* (2015) 373:1040–7. doi: 10.1056/NEJMoa1504542
45. Maude SL, Frey N, Shaw PA, Aplenc R, Barrett DM, Bunin NJ, et al. Chimeric antigen receptor T cells for sustained remissions in leukemia. *N Engl J Med* (2014) 371:1507–17. doi: 10.1056/NEJMoa1407222
46. Turtle CJ, Hanafi LA, Berger C, Gooley TA, Cherian S, Hudecek M, et al. CD19 CAR-T cells of defined CD4+:CD8+ composition in adult B cell ALL patients. *J Clin Invest* (2016) 126:2123–38. doi: 10.1172/JCI85309
47. Porter DL, Hwang WT, Frey NV, Lacey SF, Shaw PA, Loren AW, et al. Chimeric antigen receptor T cells persist and induce sustained remissions in relapsed refractory chronic lymphocytic leukemia. *Sci Transl Med* (2015) 7:303ra139. doi: 10.1126/scitranslmed.aac5415
48. Locke FL, Neelapu SS, Bartlett NL, Siddiqi T, Chavez JC, Hosing CM, et al. Phase 1 results of ZUMA-1: a multicenter study of KTE-C19 anti-CD19 CAR t cell therapy in refractory aggressive lymphoma. *Mol Ther* (2017) 25:285–95. doi: 10.1016/j.ymthe.2016.10.020
49. Kochenderfer JN, Dudley ME, Kassim SH, Somerville RP, Carpenter RO, Stetler-Stevenson M, et al. Chemotherapy-refractory diffuse large B-cell lymphoma and indolent B-cell malignancies can be effectively treated with autologous T cells expressing an anti-CD19 chimeric antigen receptor. *J Clin Oncol* (2015) 33:540–9. doi: 10.1200/JCO.2014.56.2025
50. Buechner J, Grupp SA, Maude SL, Boyer M, Bittencourt H, Laetsch TW, et al. Global reigstration trial of effcacy and safety of CTL019 in pediatric and young adult patients with relapsed/refractory (R/R) acute lymphoblastic leukemia (ALL): update to the interim analysis. *Haematologica* (2017) 102 (2):178. doi: 10.1016/j.clml.2017.07.030
51. Neelapu SS, Locke FL, Bartlett NL, Lekakis LG, Miklos D, Jacobson CA, et al. Axicabtagene ciloleucel (axicel; kte-c19) in patients with refractory aggressive non-Hodgkin lymphomas (NHL): primary results of the pivotal trial ZUMA-1. *Hematol Oncol* (2017) 35(Suppl 2):28. doi: 10.1002/hon.2437_7
52. Yang J, Yan J, Liu B. Targeting EGFRvIII for glioblastoma multiforme. *Cancer Lett* (2017) 403:224–30. doi: 10.1016/j.canlet.2017.06.024
53. Miao H, Choi BD, Suryadevara CM, Sanchez-Perez L, Yang S, De Leon G, et al. EGFRvIII-specific chimeric antigen receptor T cells migrate to and kill tumor deposits infiltrating the brain parenchyma in an invasive xenograft model of glioblastoma. *PloS One* (2014) 9:e94281. doi: 10.1371/journal.pone.0094281

54. O'Rourke DM, Nasrallah MP, Desai A, Melenhorst JJ, Mansfield K, Morrissette JJD, et al. A single dose of peripherally infused EGFRvIII-directed CAR T cells mediates antigen loss and induces adaptive resistance in patients with recurrent glioblastoma. *Sci Transl Med* (2017) 9(399): eaaa0984. doi: 10.1126/scitranslmed.aaa0984
55. Debinski W, Gibo DM, Hulet SW, Connor JR, Gillespie GY. Receptor for interleukin 13 is a marker and therapeutic target for human high-grade gliomas. *Clin Cancer Res* (1999) 5:985–90.
56. Brown CE, Warden CD, Starr R, Deng X, Badie B, Yuan YC, et al. Glioma IL13Rα2 is associated with mesenchymal signature gene expression and poor patient prognosis. *PloS One* (2013) 8(10):e77769. doi: 10.1371/journal.pone.0077769
57. Brown CE, Badie B, Barish ME, Weng L, Ostberg JR, Chang WC, et al. Bioactivity and Safety of IL13Rα2-Redirected Chimeric Antigen Receptor CD8+ T Cells in Patients with Recurrent Glioblastoma. *Clin Cancer Res* (2015) 21(18):4062–72. doi: 10.1158/1078-0432.CCR-15-0428
58. Brown CE, Alizadeh D, Starr R, Weng L, Wagner JR, Naranjo A, et al. Regression of glioblastoma after chimeric antigen receptor T-cell therapy. *N Engl J Med* (2016) 375:2561–9. doi: 10.1056/NEJMoa1610497
59. Mineo JF, Bordron A, Baroncini M, Maurage CA, Ramirez C, Siminski RM, et al. Low HER2-expressing glioblastomas are more often secondary to anaplastic transformation of low-grade glioma. *J Neurooncol* (2007) 85:281–7. doi: 10.1007/s11060-007-9424-1
60. Koka V, Potti A, Forseen SE, Pervez H, Fraiman GN, Koch M, et al. Role of Her-2/neu overexpression and clinical determinants of early mortality in glioblastoma multiforme. *Am J Clin Oncol* (2003) 26:332–5. doi: 10.1097/01.COC.0000020922.66984.E7
61. Ahmed N, Brawley V, Hegde M, Bielamowicz K, Kalra M, Landi D, et al. HER2-specifc chimeric antigen receptormodifed virus-specifc T cells for progressive glioblastoma: a phase 1 dose-escalation trial. *JAMA Oncol* (2017) 3:1094–101. doi: 10.1001/jamaoncol.2017.0184
62. Hegde M, Mukherjee M, Grada Z, Pignata A, Landi D, Navai SA, et al. Tandem CAR T cells targeting HER2 and IL13Ra2 mitigate tumor antigen escape. *J Clin Invest* (2016) 126:3036–52. doi: 10.1172/JCI83416
63. Bielamowicz K, Fousek K, Byrd TT, Samaha H, Mukherjee M, Aware N, et al. Trivalent CAR T cells overcome interpatient antigenic variability in glioblastoma. *Neuro Oncol* (2018) 20:506–18. doi: 10.1093/neuonc/nox182
64. Newick K, Moon E, Albelda SM. Chimeric antigen receptor T-cell therapy for solid tumors. *Mol Ther Oncolytics* (2016) 3:16006. doi: 10.1038/mto.2016.6
65. Porter DL, Hwang WT, Frey NV, Lacey SF, Shaw PA, Loren AW, et al. Chimeric antigen receptor T cells persist and induce sustained remissions in relapsed refractory chronic lymphocytic leukemia. *Sci Transl Med* (2015) 7 (303):303ra139. doi: 10.1126/scitranslmed.aac5415
66. Yovino S, Kleinberg L, Grossman SA, Narayanan M, Ford E. The etiology of treatment-related lymphopenia in patients with malignant gliomas: modeling radiation dose to circulating lymphocytes explains clinical observations and suggests methods of modifying the impact of radiation on immune cells. *Cancer Invest* (2013) 31(2):140–4. doi: 10.3109/07357907.2012.762780
67. Newick K, O'Brien S, Moon E, Albelda SM. CAR T cell therapy for solid tumors. *Annu Rev Med* (2017) 68:139–52. doi: 10.1146/annurev-med-062315-120245
68. Mirzaei R, Sarkar S, Yong VW. T cell exhaustion in glioblastoma: intricacies of immune checkpoints. *Trends Immunol* (2017) 38(2):104–15. doi: 10.1016/j.it.2016.11.005
69. Engels B, Engelhard VH, Sidney J, Sette A, Binder DC, Liu RB, et al. Relapse or eradication of cancer is predicted by peptide-major histocompatibility complex afnity. *Cancer Cell* (2013) 23(4):516–26. doi: 10.1016/j.ccr.2013.03.018
70. Pardoll DM. The Blockade of Immune Checkpoints in Cancer Immunotherapy. *Nat Rev Cancer* (2012) 12(4):252–64. doi: 10.1038/nrc3239
71. Fecci PE, Ochiai H, Mitchell DA, Grossi PM, Sweeney AE, Archer GE, et al. Systemic CTLA-4 Blockade Ameliorates Glioma-Induced Changes to the CD4 + T Cell Compartment Without Affecting Regulatory T-cell Function. *Clin Cancer Res* (2007) 13(7):2158–67. doi: 10.1158/1078-0432.CCR-06-2070
72. Reardon DA, Gokhale PC, Klein SR, Ligon KL, Rodig SJ, Ramkissoon SH, et al. Glioblastoma Eradication Following Immune Checkpoint Blockade in an Orthotopic, Immunocompetent Model. *Cancer Immunol Res* (2016) 4 (2):124–35. doi: 10.1158/2326-6066.CIR-15-0151
73. Desai K, Hubben A, Ahluwalia M. The Role of Checkpoint Inhibitors in Glioblastoma. *Target Oncol* (2019) 14(4):375–94. doi: 10.1007/s11523-019-00655-3
74. Sharpe AH, Pauken KE. The diverse functions of the PD1 inhibitory pathway. *Nat Rev Immunol* (2018) 18:153–67. doi: 10.1038/nri.2017.108
75. Wintterle S, Schreiner B, Mitsdoerfer M, Schneider D, Chen L, Meyermann R, et al. Expression of the B7-related molecule B7–H1 by glioma cells: a potential mechanism of immune paralysis. *Cancer Res* (2003) 63(21):7462–7.
76. Ansell SM, Lesokhin AM, Borrello I, Halwani A, Scott EC, Gutierrez M, et al. PD-1 blockade with nivolumab in relapsed or refractory Hodgkin's Lymphoma. *N Engl J Med* (2015) 372(4):311–9. doi: 10.1056/NEJMoa1411087
77. Bellmunt J, de Wit R, Vaughn DJ, Fradet Y, Lee J-L, Fong L, et al. Pembrolizumab as Second-Line Therapy for Advanced Urothelial Carcinoma. *N Engl J Med* (2017) 376(11):1015–26. doi: 10.1056/NEJMoa1613683
78. Ferris RL, Blumenschein G Jr, Fayette J, Guigay J, Colevas AD, Licitra L, et al. Nivolumab for Recurrent Squamous-Cell Carcinoma of the Head and Neck. *N Engl J Med* (2016) 375(19):1856–67. doi: 10.1056/NEJMoa1602252
79. Motzer RJ, Tannir NM, McDermott DF, Frontera OA, Melichar B, Choueiri TK, et al. Nivolumab Plus Ipilimumab Versus Sunitinib in Advanced Renal-Cell Carcinoma. *N Engl J Med* (2018) 378(14):1277–90. doi: 10.1056/NEJMoa1712126
80. Overman MJ, Lonardi S, Wong KYM, Lenz H-J, Gelsomino F, Aglietta M, et al. Durable Clinical Benefit With Nivolumab Plus Ipilimumab in DNA Mismatch Repair-Deficient/Microsatellite Instability-High Metastatic Colorectal Cancer. *J Clin Oncol* (2018) 36(8):773–9. doi: 10.1200/JCO.2017.76.9901
81. Zeng J, See AP, Phallen J, Jackson CM, Belcaid Z, Ruzevick J, et al. Anti-PD-1 Blockade and Stereotactic Radiation Produce Long-Term Survival in Mice With Intracranial Gliomas. *Int J Radiat Oncol Biol Phys* (2013) 86(2):343–9. doi: 10.1016/j.ijrobp.2012.12.025
82. Antonios JP, Soto H, Everson RG, Orpilla J, Moughon D, Shin N, et al. PD-1 Blockade Enhances the Vaccination-Induced Immune Response in Glioma. *JCI Insight* (2016) 1(10):e87059. doi: 10.1172/jci.insight.87059
83. Reardon DA, Brandes AA, Omuro A, Mulholland P, Lim M, Wick A, et al. Effect of Nivolumab vs Bevacizumab in Patients With Recurrent Glioblastoma: The CheckMate 143 Phase 3 Randomized Clinical Trial. *JAMA Oncol* (2020). doi: 10.1001/jamaoncol.2020.1024
84. Bristol-Myers Squibb Provides Update on Phase 3 Opdivo. *(nivolumab) CheckMate -548 Trial in Patients with Newly Diagnosed MGMT-Methylated Glioblastoma Multiforme*. Available at: https://news.bms.com/category/press-release-category/%5Bcatpath-raw%5D.
85. Schalper KA, Rodriguez-Ruiz ME, Diez-Valle R, López-Janeiro A, Porciuncula A, Idoate MA, et al. Neoadjuvant Nivolumab Modifies the Tumor Immune Microenvironment in Resectable Glioblastoma. *Nat Med* (2019) 25(3):470–6. doi: 10.1038/s41591-018-0339-5
86. Reardon DA, Kaley TJ, Dietrich J, Clarke JL, Dunn GP, Lim M, et al. Phase 2 study to evaluate safety and efcacy of MEDI4736 (durvalumab [DUR]) in glioblastoma (GBM) patients: an update. *J Clin Oncol* (2017) 35 (15_Suppl):2042. doi: 10.1200/JCO.2017.35.15_suppl.2042
87. Reardon DA, Kaley TJ, Dietrich J, Clarke JL, Dunn GP, Lim M, et al. Phase 2 study to evaluate safety and efcacy of MEDI4736 (durvalumab [DUR]) in glioblastoma (GBM) patients: an update. *J Clin Oncol* (2017) 35 (15_Suppl):2042. doi: 10.1200/JCO.2017.35.15_suppl.2042
88. Monney L, Sabatos CA, Gaglia JL, Ryu A, Waldner H, Chernova T, et al. Th1-specifc cell surface protein TIM-3 regulates macrophage activation and severity of an autoimmune disease. *Nature* (2002) 415(6871):536–41. doi: 10.1038/415536a
89. Kanzaki M, Wada J, Sugiyama K, Nakatsuka A, Teshigawara S, Murakami K, et al. Galectin-9 and T cell immunoglobulin mucin-3 pathway is a therapeutic target for type 1 diabetes. *Endocrinology* (2012) 153(2):612–20. doi: 10.1210/en.2011-1579
90. Prendergast GC, Malachowski WP, DuHadaway JB, Muller AJ. Discovery of IDO1 inhibitors: from bench to bedside. *Cancer Res* (2017) 77(24):6795–811. doi: 10.1158/0008-5472.CAN-17-2285
91. Zhai L, Ladomersky E, Lauing KL, Wu M, Genet M, Gritsina G, et al. Infltrating T cells increase IDO1 expression in glioblastoma and contribute

to decreased patient survival. *Clin Cancer Res* (2017) 23(21):6650–60. doi: 10.1158/1078-0432.CCR-17-0120
92. Soliman HH, Minton SE, Ismail-Khan R, Han HS, Vahanian NN, Ramsey WJ, et al. A phase 2 study of docetaxel in combination with indoximod in metastatic breast cancer. *J Clin Oncol* (2014) 32(15_Suppl):TPS3124. doi: 10.1200/jco.2014.32.15_suppl.tps3124
93. Zakharia Y, Johnson TS, Colman H, Vahanian NN, Link CJ, Kennedy E, et al. A phase I/II study of the combination of indoximod and temozolomide for adult patients with temozolomide-refractory primary malignant brain tumors. *J Clin Oncol* (2014) 32(15_Suppl):TPS2107. doi: 10.1200/jco.2014.32.15_suppl.tps2107
94. Samstein RM, Lee CH, Shoushtari AN, Hellmann MD, Shen R, Janjigian YY, et al. Tumor mutational load predicts survival after immunotherapy across multiple cancer types. *Nat Genet* (2019) 51(2):202–6. doi: 10.1038/s41588-018-0312-8
95. Russell SJ, Peng KW, Bell JC. Oncolytic virotherapy. *Nat Biotechnol* (2012) 30(7):658–70. doi: 10.1038/nbt.2287
96. Andtbacka RHII, Kaufman HL, Collichio F, Amatruda T, Senzer N, Chesney J, et al. Talimogene Laherparepvec Improves Durable Response Rate in Patients With Advanced Melanoma. *J Clin Oncol* (2015) 33(25):2780–8. doi: 10.1200/JCO.2014.58.3377
97. Martuza RL, Malick A, Markert JM, Ruffner KL, Coen DM. Experimental therapy of human glioma by means of a genetically engineered virus mutant. *Science* (1991) 252(5007):854–6. doi: 10.1126/science.1851332
98. Eissa IR, ustos-Villalobos I, Ichinose T, Matsumura S, Naoe Y, Miyajima N, et al. The Current Status and Future Prospects of Oncolytic Viruses in Clinical Trials against Melanoma, Glioma, ancreatic, and Breast Cancers. *Cancers (Basel)* (2018) 10(10):356. doi: 10.3390/cancers10100356
99. Lang FF, Conrad C, Gomez-Manzano C, Yung WKA, Sawaya R, Weinberg JS, et al. Phase I study of DNX-2401 (Delta-24-RGD) oncolytic adenovirus: replication and immunotherapeutic effects in recurrent malignant glioma. *J Clin Oncol* (2018) 36:1419–27. doi: 10.1200/JCO.2017.75.8219
100. Fueyo J, Alemany R, Gomez-Manzano C, Fuller GN, Khan A, Conrad CA, et al. Preclinical characterization of the antiglioma activity of a tropism-enhanced adenovirus targeted to the retinoblastoma pathway. *J Natl Cancer Inst* (2003) 95:652–60. doi: 10.1093/jnci/95.9.652
101. Lang FF, Conrad C, Gomez-Manzano C, Yung WKA, Sawaya R, Weinberg JS, et al. Phase I Study of DNX-2401 (Delta-24-RGD) Oncolytic Adenovirus: Replication and Immunotherapeutic Effects in Recurrent Malignant Glioma. *J Clin Oncol* (2018) 36(14):1419–27. doi: 10.1200/JCO.2017.75.8219
102. van Den Bossche WBL, Kleijn A, Teunissen CE, Voerman JSA, Teodosio C, Noske DP, et al. Oncolytic virotherapy in glioblastoma patients induces a tumor macrophage phenotypic shift leading to an altered glioblastoma microenvironment. *Neuro Oncol* (2018) Oct 920(11):1494–504. doi: 10.1093/neuonc/noy082
103. Gea Z. Abstract – interim results of a phase II multicenter study of the conditionally replicative oncolytic adenovirus DNX-2401 with pembrolizumab (Keytruda) for recurrent glioblastoma; CAPTIVE STUDY (LEYNOTE-192). *Neuro-Oncology* (2018) 20(suppl_6):vi6. doi: 10.1093/neuonc/noy148.019
104. Desjardins A, Gromeier M, Herndon JE2, Beaubier N, Bolognesi DP, Friedman AH, et al. Recurrent Glioblastoma Treated with Recombinant Poliovirus. *N Engl J Med* (2018) 379(2):150–61. doi: 10.1056/NEJMoa1716435
105. Geletneky K, Hajda J, Angelova AL, Leuchs B, Capper D, Bartsch AJ, et al. Oncolytic H-1 Parvovirus Shows Safety and Signs of Immunogenic Activity in a First Phase I/IIa Glioblastoma Trial. *Mol Ther* (2017) 25(12):2620–34. doi: 10.1016/j.ymthe.2017.08.016
106. Markert JM, Liechty PG, Wang W, Gaston S, Braz E, Karrasch M, et al. Phase Ib trial of mutant herpes simplex virus G207 inoculated pre-and post-tumor resection for recurrent GBM. *Mol Ther* (2009) 17(1):199–207. doi: 10.1038/mt.2008.228
107. Cloughesy TF, Landolfi J, Hogan DJ, Bloomfield S, Carter B, Chen CC, et al. Phase 1 trial of vocimagene amiretrorepvec and 5-fluorocytosine for recurrent high-grade glioma. *Sci Transl Med* (2016) 8(341):341ra75. doi: 10.1126/scitranslmed.aad9784
108. Kicielinski KP, Chiocca EA, Yu JS, Gill GM, Coffey M, Markert JM. Phase 1 clinical trial of intratumoral reovirus infusion for the treatment of recurrent malignant gliomas in adults. *Mol Ther* (2014) 22(5):1056–62. doi: 10.1038/mt.2014.21
109. Antonio Chiocca E, Nassiri F, Wang J, Peruzzi P, Zadeh G. Viral and other therapies for recurrent glioblastoma: is a 24-month durable response unusual? *Neuro Oncol* (2019) 21(1):14–25. doi: 10.1093/neuonc/noy170
110. Inogés S, Tejada S, de Cerio ALD, Pérez–Larraya 6 JG, Espinós J, Idoate MG, et al. A phase II trial of autologous dendritic cell vaccination and radiochemotherapy following fluorescence-guided surgery in newly diagnosed glioblastoma patients. *J Transl Med* (2017). doi: 10.1186/s12967-017-1202-z
111. Fenstermaker RA, Ciesielski MJ, Qiu J, Yang N, Frank CL, Lee KP, et al. Clinical study of a survivin long peptide vaccine (SurVaxM) in patients with recurrent malignant glioma. *Cancer Immunol Immunother* (2016). doi: 10.1007/s00262-016-1890-x
112. Bloch O, Crane CA, Fuks Y, Kaur R, Aghi MK, Berger MS, et al. Heat-shock protein peptide complex-96 vaccination for recurrent glioblastoma: A phase II, single-arm trial. *Neuro Oncol* (2014) 16(2):274–9. doi: 10.1093/neuonc/not203
113. Vik-Mo EO, Nyakas M, Mikkelsen BV, Moe MC, Due–Tønnesen P, Suso EMI, et al. Therapeutic vaccination against autologous cancer stem cells with mRNA-transfected dendritic cells in patients with glioblastoma. *Cancer Immunol Immunother* (2013). doi: 10.1007/s00262-013-1453-3
114. Kong D-S, Nam D-H, Kang S-H, Lee JW, Chang J-H, Kim J-H, et al. Phase III randomized trial of autologous cytokine-induced killer cell immunotherapy for newly diagnosed glioblastoma in korea. *Oncotarget* (2015). doi: 10.18632/oncotarget.12273
115. Schuessler A, Smith C, Beagley L, Boyle GM, Rehan S, Matthews K, et al. Autologous t-cell therapy for cytomegalovirus as a consolidative treatment for recurrent glioblastoma. *Cancer Res* (2014) 74(13):3466–76. doi: 10.1158/0008-5472.CAN-14-0296
116. Margolin K, Ernstoff MS, Hamid O, Lawrence D, McDermott D, Puzanov I, et al. Ipilimumab in patients with melanoma and brain metastases: An open-label, phase 2 trial. *Lancet Oncol* (2012) 13(5):459–65. doi: 10.1016/S1470-2045(12)70090-6

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Immune Checkpoint-Associated Locations of Diffuse Gliomas Comparing Pediatric With Adult Patients Based on Voxel-Wise Analysis

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Objective: Pediatric diffuse gliomas (pDGs) are relatively rare and molecularly distinct from pediatric pilocytic astrocytoma and adult DGs. Immunotherapy is a promising therapeutic strategy, requiring a deep understanding of tumor immune profiles. The spatial locations of brain tumors might be related to the molecular profiles. We aimed to analyze the relationship between the immune checkpoint molecules with the locations of DGs comparing pediatric with adult patients.

Method: We studied 20 pDGs patients (age $\leq$ 21 years old), and 20 paired adult patients according to gender and histological types selected from 641 adult patients with DGs. Immune checkpoint molecules including B7-H3, CD47, and PD-L1, as well as tumor-infiltrating lymphocytes (TILs) and tumor-associated macrophages (TAMs), were manifested by immunohistochemical staining. Expression difference analyses and Spearman's correlation were performed. MRI data were voxel-wise normalized, segmented, and analyzed by Fisher's exact test to construct the tumor frequency and p value heatmaps. Survival analyses were conducted by Log-rank tests.

Result: The median age of pediatric patients was 16 years. 55% and 30% of patients were WHO II and III grades, respectively. The left frontal lobe and right cerebellum were the statistically significant locations for pDGs, while the anterior horn of ventricles for adult DGs. A potential association between the expression of PD-L1 and TAMs was found in pDGs ($p = 0.002$, $R = 0.670$). The right posterior external capsule and the lateral side of the anterior horn of the left ventricle were predominant locations for the adult patients with high expression of B7-H3 and low expression of PD-L1 compared to pediatric ones, respectively. Pediatric patients showed significantly improved overall survival compared with adults. The prognostic roles of immune checkpoint molecules and TILs/TAMs were not significantly different between the two groups.

Conclusion: Immune checkpoint-associated locations of diffuse gliomas comparing pediatric with adult patients could be helpful for the immunotherapy decisions and design of clinical trials.

Keywords: pediatric diffuse gliomas, immune checkpoint molecules, spatial locations, B7-H3, CD47, PD-L1, immunotherapy

INTRODUCTION

The incidence of CNS tumors in children and adolescents in the United States is 6.06 per 100,000 according to the latest CBTRUS statistical report (1). High childhood cancer-related mortality is observed in pediatric patients with CNS tumors, which is the second most malignancy after leukemia (2). Pediatric gliomas are the most common type therein, and the low-grade gliomas (WHO grades I and II) constitute a majority of pediatric gliomas, such as pilocytic astrocytoma and subependymal giant cell astrocytoma (3). The high-grade gliomas (WHO grades III and IV) are relatively rare but extremely fatal (4). Pediatric diffuse gliomas (pDGs) are a subgroup of pediatric gliomas that histologically including anaplastic/non-anaplastic astrocytoma, oligodendroglioma, oligoastrocytoma, and glioblastoma multiform (5). Patients with pDGs are highly heterogeneous and differ from the adult counterparts and the common pediatric gliomas such as pilocytic astrocytoma molecularly, clinically, and prognostically (4, 6, 7). However, the distinctive characteristics of pDGs remain largely unknown.

Neuroimaging including CT and MRI is a pivotal method to detect the pDGs. Imaging information include tumor location, volume, and edema which are associated with clinical symptoms. It was reported that less neurologic impairments of pediatric gliomas were observed when tumors located at cerebral hemispheres compared with ones at midline, optic pathway, posterior fossa, and brainstem (8). Besides the advantages in the determination of symptom and diagnosis, the tumor location in radiology promotes the accuracy of surgical resection and the efficacy of outcome evaluation (9–11).

Immunotherapy is an emerging approach treating the refractory gliomas in addition to surgery and chemoradiotherapy. Since the chemoradiotherapy may cause developmental disorders and other side effects in pediatric patients, immunotherapy becomes the novel alternative for glioma management (12). Checkpoint inhibitors work by promoting the antitumor immune response. The effect of PD-1 blockage Nivolumab was investigated in patients with recurrent glioblastoma but the overall survival was not improved, probably due to the low permeability of blood-brain barrier and suppressive immune microenvironment (13). Notably, immunotherapy is facing more challenges in pediatric gliomas, a unique group differing from adult patients (14). For instance, the expression of immune checkpoint molecules may be highly distinct between pediatric and adult gliomas.

In the present study, using voxel-wise analysis, we aim to investigate the association between the spatial locations of pDGs and the expression of immune checkpoint molecules including B7-H3, CD47, and PD-L1, as well as the tumor-infiltrating lymphocytes (TILs) and tumor-associated macrophages (TAMs), when compared to the adult DGs. The location-associated immune characteristics may be valuable for the design of the immunotherapy regimen.

MATERIALS AND METHODS

Patient Cohort

Patients diagnosed with brain tumors from March 2012 to December 2017 were initially searched in our institutional database and 2,048 patients were reviewed. The histopathological data and preoperative craniocerebral contrast-enhanced MRI were collected. A total of 661 patients with DGs including anaplastic/non-anaplastic astrocytoma, oligodendroglioma, oligoastrocytoma, and glioblastoma multiform were confirmed. Pediatric (aged $\leq$ 21 years) patients were grouped into one cohort, adults into the other (15). Among the adult patients, 20 were randomly selected and paired according to the gender and histological types of every pediatric patient for further analyses.

Patient Consent

This study was approved by the ethical committee on clinical human research in the institution (No. 2020-876). The informed consent of the pathological examination of surgical specimens was signed by every patient as soon as the admission to hospital. Because of no clinical intervention, the committee had waived the specific informed consent agreement for the review of clinical information and imaging data in the current study.

Magnetic Resonance Imaging

The contrast-enhanced MRI was acquired in patients with intravenous injection of gadodiamide (0.2 ml/kg body weight, up to a maximum of 20 ml, Omniscan, GE Healthcare) followed by the use of 1.5 (Signa Excite, GE Healthcare, Milwaukee, Wisconsin) or 3.0 Tesla (Discovery 750, GE Healthcare, Milwaukee, Wisconsin) MRI.

Imaging Data

The imaging data in the standard Digital Imaging and Communications in Medicine (DICOM) format were converted to the Neuroimaging Informatics Technology Initiative (NIfTI) format using dcm2nii converter software (University of Nottingham School of Psychology, Nottingham, UK). The data were registered to the standard brain template (MNI152; Montreal Neurological Institute, McGill University, Montreal, Quebec, Canada) using Statistical Parametric Mapping Software version 12 (SPM12, Institute of Neurology, University College London, London, UK) in MATLAB (version R2012a, The MathWorks, Natick, MA, USA). The regions of interest

(ROIs) were obtained after the semiautomatic segmentation of normalized data using 3D Slicer (version 4.10.0; http://www.slicer.org/) (16). The processes were conducted by trained authors (LZ and ZD), and were reviewed by two experienced neurosurgeons and a neuroradiologist (CS, JZ, and BJ).

Construction of Frequency and *P*-Value Heatmaps

To visualize the spatial distribution of DGs, the ROIs were overlapped on the MNI152 by MRIcron (University of Nottingham School of Psychology, Nottingham, UK) to create frequency heatmaps. The *p*-value heatmaps comparing two different phenotypes (e.g., pediatric patients and adult ones) were constructed using the analysis of differential involvement (ADIFFI) as previously described by Ellingson et al. (17, 18). Briefly, a 2×2 contingency table was used to perform a two-tailed Fisher's exact test for the significance calculation of a particular voxel:

$$p = \frac{(a+b)!\,(c+d)!\,(a+c)!\,(b+d)!}{a!b!c!d!n}$$

In the formula, "a" is the frequency of tumor occurrence under phenotype A, "b" is the frequency of tumor occurrence under phenotype B, "c" is the frequency of tumor-free patients under phenotype A, "d" is the frequency of tumor-free patients under phenotype B, and "n" is the total number of patients. The exclamation mark refers to the factorial operation.

Immunohistochemical Staining

The DGs tissues were fixed, dehydrated, and paraffin-embedded. The 4 μm sections were deparaffinized and rehydrated in 100, 95, and 75% ethanol. The antigen retrieval solution with EDTA (pH 9.0) was used for PD-L1 staining, while the solution with sodium citrate (pH 6.0) for B7-H3 and CD47 staining. After endogenous peroxidase activity blocking, the sections were rinsed and incubated with the primary antibodies including anti-human PD-L1 (1:1,000, Abcam, ab228462, Cambridge, MA, USA), CD47 (1:2000, Abcam, ab218810) and B7-H3 (1:2,000, Abcam, ab219648) overnight at 4°C. TILs were stained by the CD45 (1:1,000, Abcam, ab40763) antibody, and TAMs were stained by the CD68 antibody (1:1,000, Abcam, ab213363). Images were acquired after the incubation with secondary antibodies and DAB, and counterstained with Hematoxylin. The expression level of three immune checkpoint molecules was determined by the percentage of positive cells and the staining intensity: low (negative intensity and intensity 1, and intensity 2 with positive cells $< 10\%$) and high (intensity 2 with positive cells $\geq 10\%$ and intensity 3) expression (19). The expression of TILs/TAMs was similarly evaluated as previously described (20, 21).

Statistical Analysis

The data were presented as the mean ± standard error of mean (SEM) by nonparametric paired *t*-test. The paired four-fold table was statistically analyzed by McNemar's test. Spearman's correlation analysis was performed between immune checkpoints and TILs/TAMs. The two-tailed Fisher's exact test was mentioned above. Overall survival (OS) was defined as the time of imaging detection until death or the last follow-up. The Kaplan-Meier analysis with the Log-rank test was conducted to evaluate the OS. We used GraphPad Prism (version 8.0.2; GraphPad Software, San Diego, CA, USA) and SPSS (version 22.0; IBM SPSS Statistics, Armonk, NY, USA) for all statistical analyses. $P < 0.05$ was considered significant.

TABLE 1 | Demographics of all patients with diffuse gliomas and 20 pediatric patients with diffuse gliomas, as well as 20 paired adult patients based on the gender and histological types of pediatric patients.

Characteristics	All patients with diffuse gliomas (*n* = 661)	Pediatric patients with diffuse gliomas (*n* = 20)	Paired adult patients with diffuse gliomas (*n* = 20)
Age (years)			
Range	6–87	6–21	27–68
Median	51	16	43
Gender (%)			
Male	385 (58.2)	16 (80)	16 (80)
Female	276 (41.8)	4 (20)	4 (20)
Histological type (%)			
Diffuse astrocytoma	57 (8.6)	4 (20)	4 (20)
Oligodendroglioma	32 (4.8)	5 (25)	5 (25)
Oligoastrocytoma	22 (3.3)	2 (10)	2 (10)
Anaplastic astrocytoma	89 (13.5)	3 (15)	3 (15)
Anaplastic oligodendroglioma	58 (8.8)	1 (5)	1 (5)
Anaplastic oligoastrocytoma	47 (7.1)	2 (10)	2 (10)
Glioblastoma	356 (53.9)	3 (15)	3 (15)

RESULTS

Demographics

A total of 20 pediatric patients (age ≤ 21 years old) with DGs were analyzed. The demographics including age, gender and histological types were listed in **Table 1**. The median age of pediatric patients was 16 years. There were 55% of WHO grade II DGs. According to the gender and histological types of pediatric patients, 20 adult patients (age > 21 years old) from the adult cohort (641 patients) were randomly paired. In the entire cohort (661 patients), the percentage of patients with glioblastoma was 53.9% (**Table 1**).

Left Frontal Lobe and Right Cerebellum Were the Preferred Locations for pDGs

The frequency heatmaps of DGs were constructed by ROIs overlapping to display the spatial distribution of DGs. The color ranging from dark blue to red suggested the tumor frequency from 5 to 20% and above. For pediatric patients, left frontal lobe showed relatively high incidence of pDGs (**Figure 1A**). For the remaining 641 patients with DGs, the paraventricular and subventricular regions, especially the anterior horn of lateral ventricles, were frequently affected (**Figure 1B**). By using

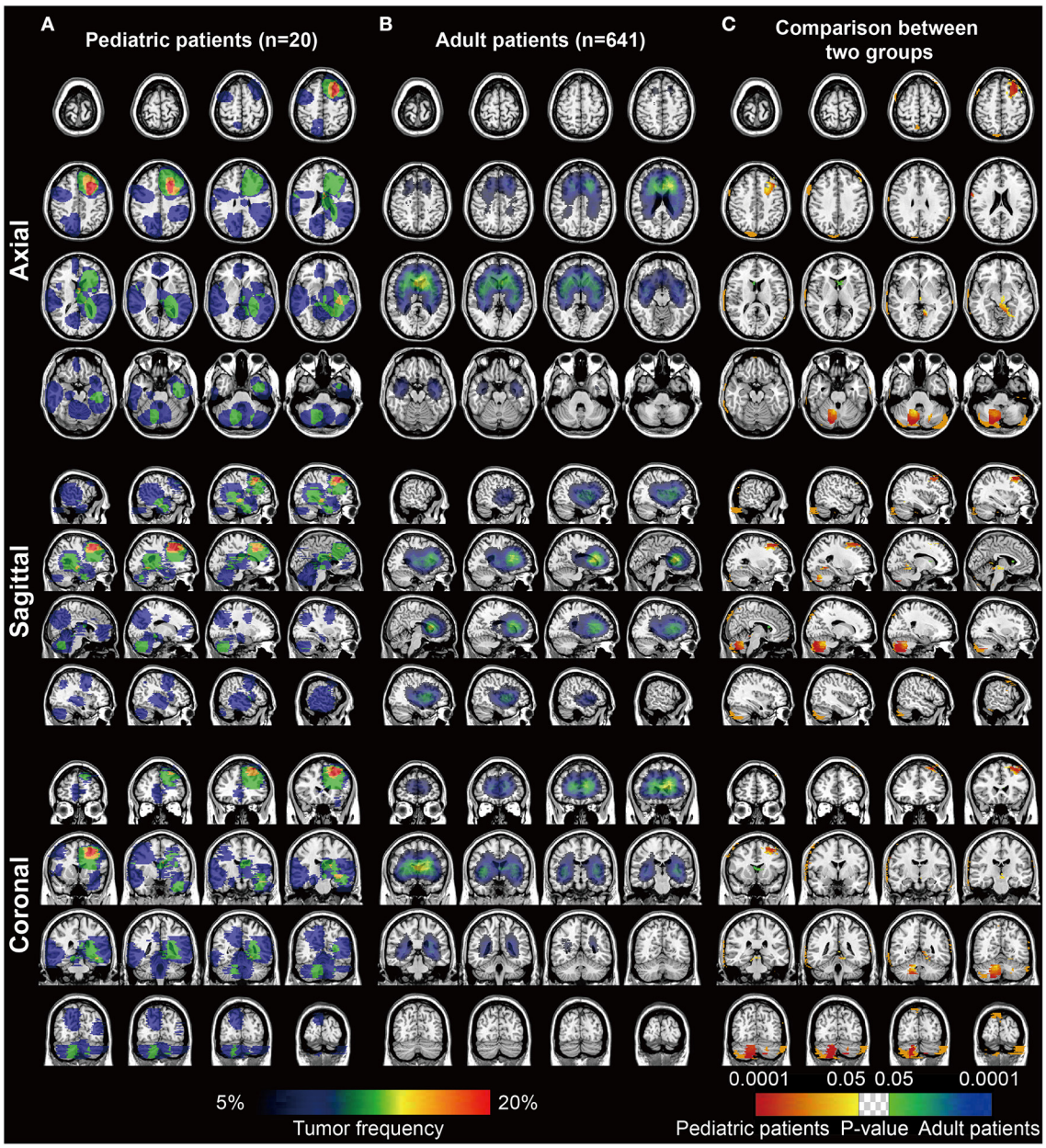

FIGURE 1 | The frequency and p value heatmaps in axial, sagittal, and coronal positions comparing pediatric and adult patients with DGs. **(A)** The frequency heatmap of pDGs. **(B)** The frequency heatmap of adult DGs. The color ranging from dark blue to red suggested the tumor frequency from 5 to 20% and above. **(C)** The p value heatmap comparing the two groups after the Fisher's exact test. The color ranging from dark blue to green, and red to bright yellow, both suggested the *p*-value from 0.0001 to 0.05.

Fisher's exact test, significant clusters for pDGs were identified in the left frontal lobe and right cerebellum, and clusters for adult DGs in the anterior horn of ventricles ($p < 0.05$, **Figure 1C**).

IHC Results of B7-H3, CD47, PD-L1 and TILs/TAMs

Three immune checkpoints molecules, B7-H3, CD47 and PD-L1 were immunohistochemically stained in pediatric and paired adult DGs, as shown in **Figure 2A**. To reveal the difference of checkpoints expression between pediatric and paired adult DGs, the positive cells, and patient quantity with high or low expression (determined by positive cells and staining intensity), were respectively compared. However, no significance was found (**Table 2** and **Figures 2B–D**). Furthermore, the expression level of TILs (CD45 staining) and TAMs (CD68 staining) were determined by IHC, and no significance was either found comparing patient quantity (**Table 2**).

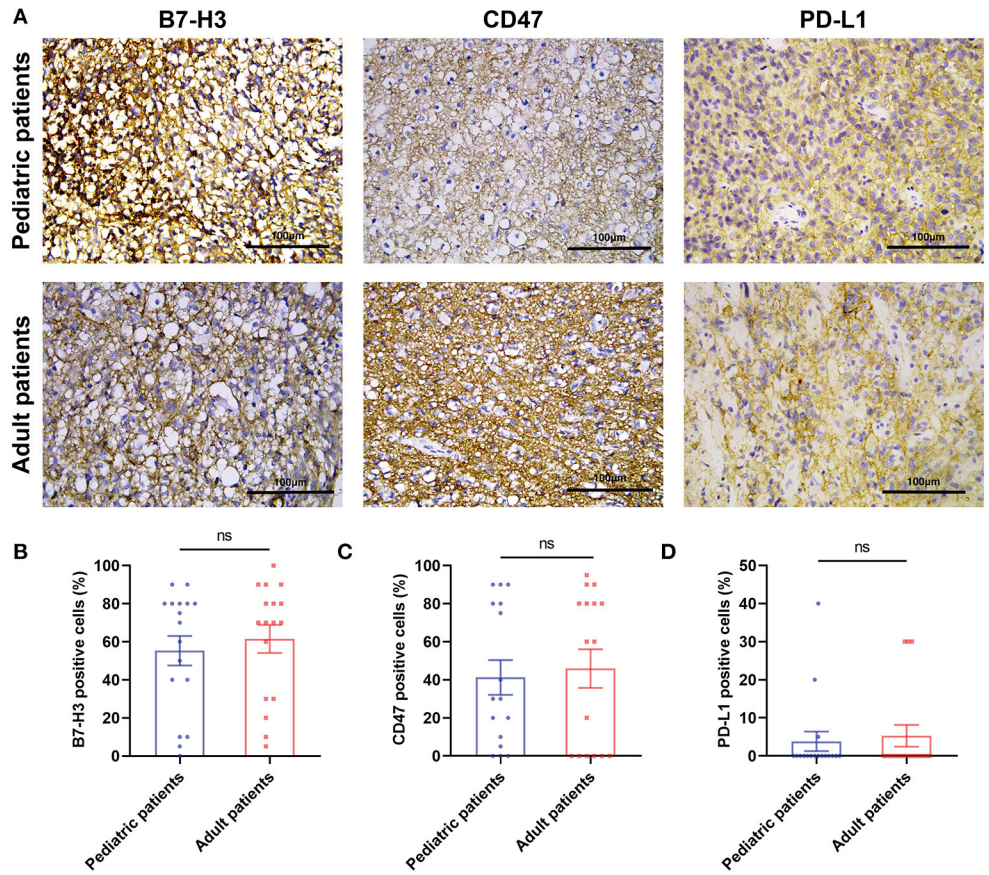

FIGURE 2 | The IHC results of three immune checkpoint molecules in pediatric and adult patients with DGs. **(A)** Representative images of IHC staining of B7-H3, CD47, and PD-L1 in pediatric and paired adult patients with DGs. The scale bar is 100 μm. **(B)** Paired *t*-test analyzing the percentage of B7-H3 positive cells in the two groups. **(C)** Paired *t*-test analyzing the percentage of CD47 positive cells in the two groups. **(D)** Paired *t*-test analyzing the percentage of PD-L1 positive cells in the two groups. The ns refers to "not significant".

TABLE 2 | Patient quantity in terms of three immune checkpoints, TILs and TAMs.

	Pediatric patients*		Paired adult patients*		*P*-value#
	Patient quantity with high expression	Patient quantity with low expression	Patient quantity with high expression	Patient quantity with low expression	
B7-H3	12	7	12	6	0.359
CD47	7	12	6	12	0.238
PD-L1	1	18	1	17	NA
TILs	7	12	8	10	0.503
TAMs	7	12	4	14	0.077

**Because of the loss of specimens, the total number was not 20.*
#The p-value was calculated by McNemar's test.
TILs refer to the tumor infiltrating-lymphocytes, and TAMs refer to the tumor-associated macrophages. NA refers to "not available".

The relationship between immune checkpoints molecules and immune cells such as lymphocytes and macrophages was profoundly studied. Therefore, the Spearman's correlation was performed to analyze the potential relationship in the current study (**Table 3**). For pediatric patients, the percentage of positive cells of PD-L1 was significantly correlated to the expression level of TAMs ($p = 0.002$, $R = 0.670$). For the group of pediatric and adult patients, the expression level of TAMs was significantly correlated to the B7-H3 ($p = 0.009$, $R = 0.428$) and PD-L1 ($p = 0.005$, $R = 0.458$). No significant correlation was found in the paired adult group. The results may indicate the distinct role of macrophages in pDGs.

Immune Checkpoint-Associated Locations of DGs

We assumed that the location of DGs may be associated with the immune characteristics. Thus, voxel-wise Fisher's exact test was applied to visualize the significant clusters by comparing the pediatric and the paired adult groups based on the expression level of immune checkpoints. Paired adult patients with high expression of B7-H3 displayed a remarkable location in the right posterior external capsule compared to the pediatric group ($p < 0.05$, **Figure 3A**). The lateral side of the anterior horn of the left ventricle was identified as a distinct location in

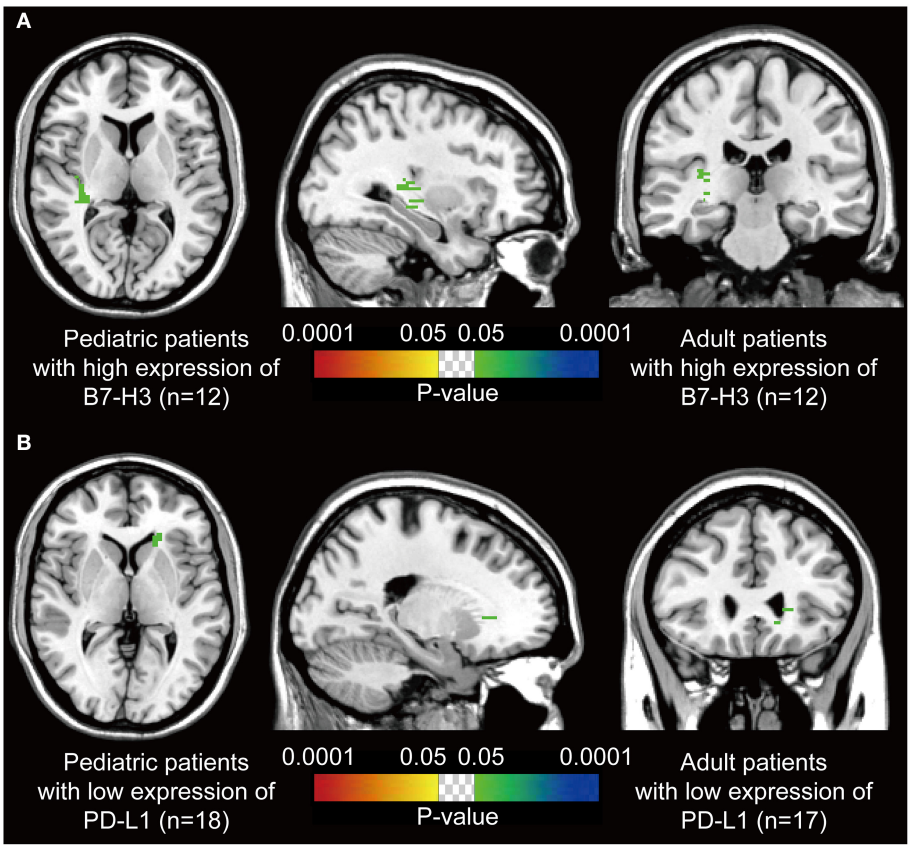

FIGURE 3 | *P*-value heatmaps visualize the immune checkpoint-associated locations of DGs. **(A)** The *p*-value heatmap showed the right posterior external capsule was the predominant location for the adult DGs with high expression of B7-H3 compared to pediatric ones. **(B)** The *p*-value heatmap showed the lateral side of the anterior horn of the left ventricle was the predominant location for the adult DGs with low expression of PD-L1 compared to pediatric ones. The color ranging from dark blue to green, and red to bright yellow, both suggested the *p*-value from 0.0001 to 0.05.

TABLE 3 | Spearman's correlation of TILs/TAMs with the immune checkpoints.

Spearman's correlation		TILs			TAMs		
		Pediatric patients	Paired adult patients	Pediatric and paired adult patients	Pediatric patients	Paired adult patients	Pediatric and paired adult patients
B7-H3	*P*-value	0.537	0.053	0.080	0.087	0.226	**0.009**
	R	0.151	0.477	0.296	0.403	0.300	**0.428**
CD47	*P*-value	0.651	0.439	0.420	0.298	0.086	0.070
	R	−0.111	−0.201	−0.139	−0.252	−0.429	−0.306
PD-L1	*P*-value	0.569	0.426	0.308	**0.002**	0.637	**0.005**
	R	0.140	0.200	0.175	**0.670**	0.120	**0.458**

P-values with statistical significance and the corresponding R are boldface.
TILs refer to the tumor-infiltrating lymphocytes, and TAMs refer to the tumor-associated macrophages.

paired adult patients with low expression of PD-L1 compared to the pediatric group ($p < 0.05$, **Figure 3B**). The checkpoint-associated locations of DGs may provide the potential value for immune therapeutic strategies according to the location of the tumor.

Survival Differences According to the Expression Level of Checkpoints and TILs/TAMS

The clinical immunotherapy strategies largely depend on the expression status of immune checkpoint molecules, which

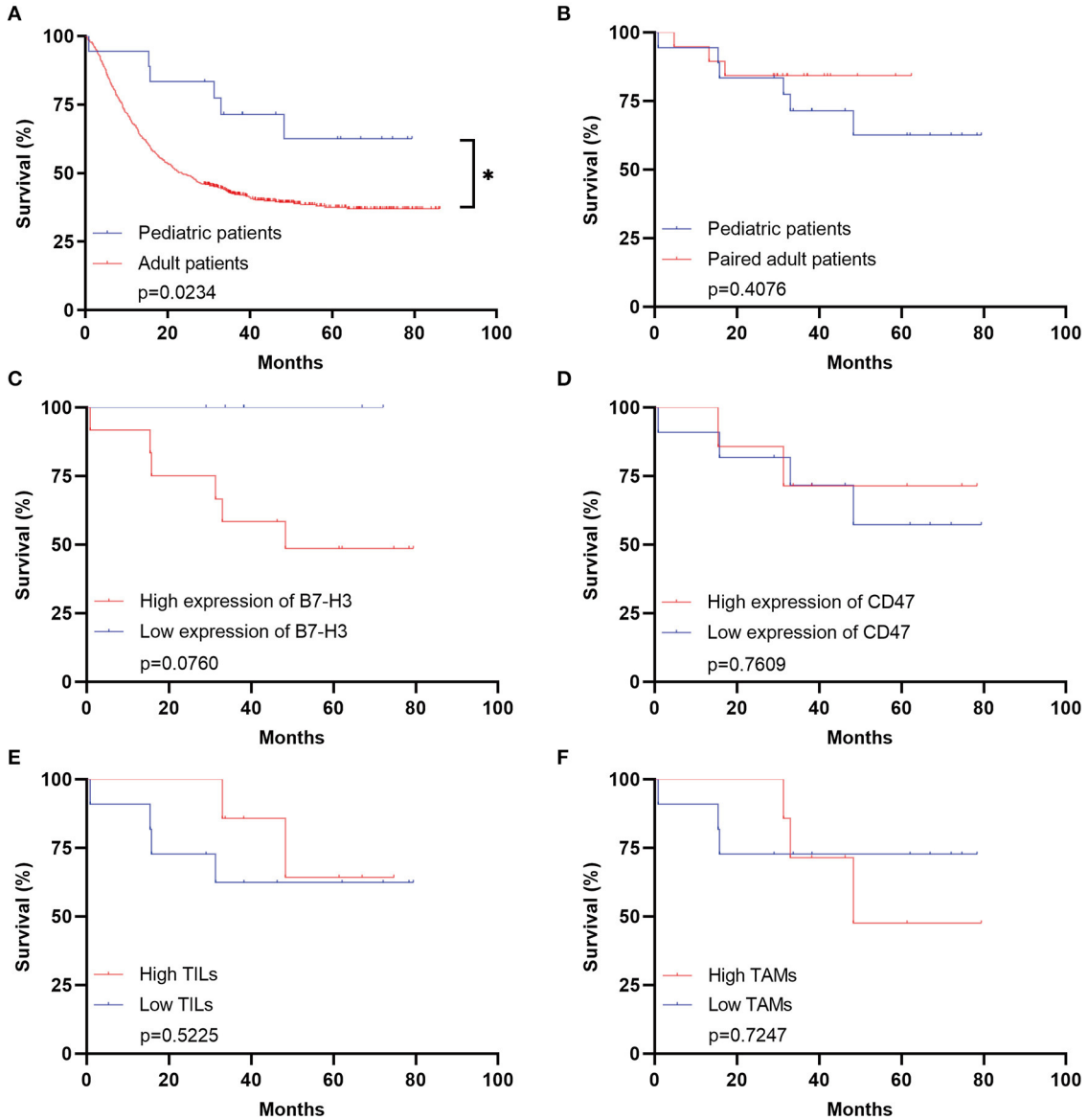

FIGURE 4 | Survival analyses for pediatric patients with DGs. **(A)** Survival analysis comparing the pediatric group with the adult group ($n = 641$, $p = 0.0234$). **(B)** Survival analysis comparing the pediatric group with the paired adult group ($n = 20$, $p = 0.4076$). **(C)** Survival analysis comparing the high expression of B7-H3 with the low expression in the pediatric group ($p = 0.0760$). **(D)** Survival analysis comparing the high expression of CD47 with the low expression in the pediatric group ($p = 0.7609$). **(E)** Survival analysis comparing the high TILs with the low TILs in the pediatric group ($p = 0.5225$). **(F)** Survival analysis comparing the high TAMs with the low TAMs in the pediatric group ($p = 0.7247$). TILs refer to the tumor-infiltrating lymphocytes; TAMs refer to the tumor-associated macrophages; * refers to the $p < 0.05$.

leads to different prognosis. We first performed the survival analysis comparing the 20 pediatric patients and 641 adult patients with DGs. Expectedly, the OS of adult patients was statistically shorter than the pediatric ones (**Figure 4A**). The result comparing 20 pediatric and 20 paired adult patients was not significant (**Figure 4B**). No significance was found analyzing the prognosis in pediatric patients according to the expression level of B7-H3, CD47, or TILs/TAMs, though an unfavorable trend was observed in the group with high expression of B7-H3 compared to the one with low expression (**Figures 4C–F**). For paired adult patients, the expression level of B7-H3 and CD47 had no statistical impact on OS (**Figures 5A,B**). Though the OS was not significantly affected by the expression of TILs, high TAMs remarkably shortened the survival time (**Figures 5C,D**). Moreover, the survival difference between the pediatric and paired adult patients under a similar expression level of checkpoints or TILs/TAMs was analyzed. However, no significance was found regarding the OS difference between the two groups (**Figures 6A–F**).

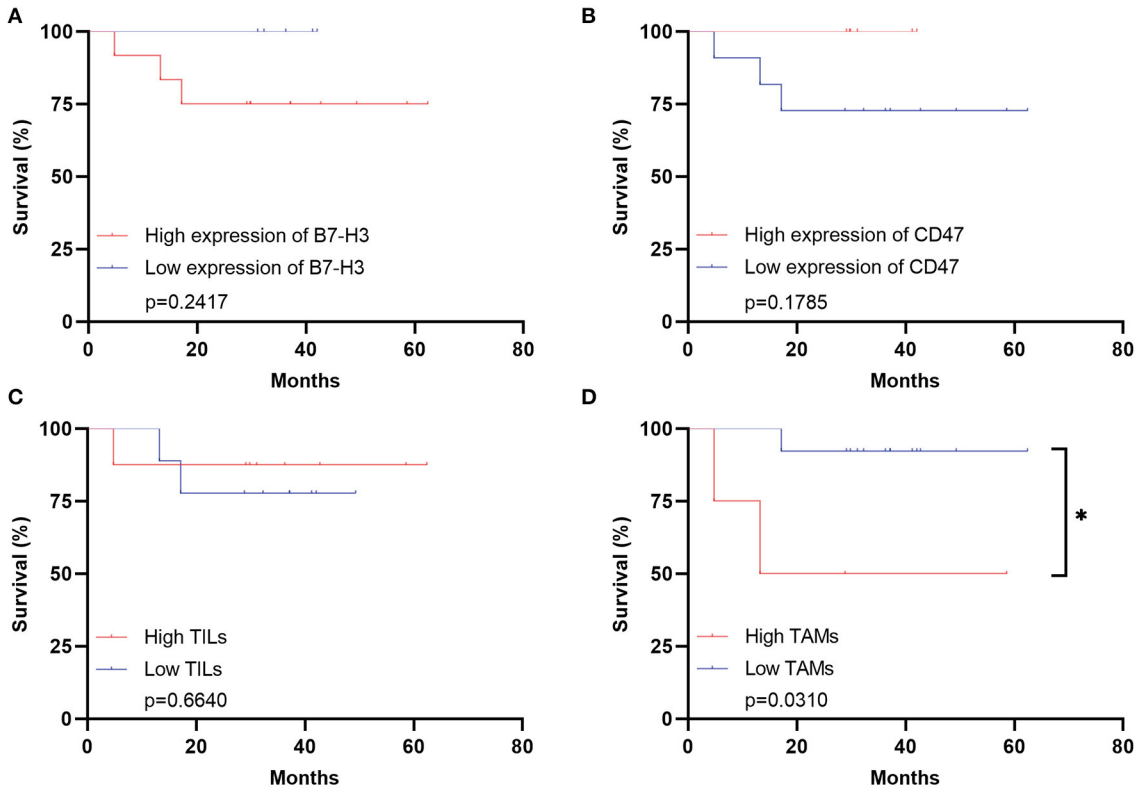

FIGURE 5 | Survival analyses for adult patients with DGs. **(A)** Survival analysis comparing the high expression of B7-H3 with the low expression in the adult group ($p = 0.2417$). **(B)** Survival analysis comparing the high expression of CD47 with the low expression in the adult group ($p = 0.1785$). **(C)** Survival analysis comparing the high TILs with the low TILs in the adult group ($p = 0.6640$). **(D)** Survival analysis comparing the high TAMs with the low TAMs in the adult group ($p = 0.0310$). TILs refer to the tumor-infiltrating lymphocytes; TAMs refer to the tumor-associated macrophages; * refers to the $p < 0.05$.

DISCUSSION

This is the first study analyzing the relationship between the expression of immune checkpoint molecules and the intracranial locations of pDGs after comparing to the adult counterparts.

DGs commonly affected adults, especially the middleaged and elderly patients. The WHO grade I gliomas were not included in the DGs, therefore the number of pediatric patients with DGs was limited and much lower than the adult counterparts. Glioblastoma, the most aggressive DGs, accounts for 53.9% in the cohort of 661 patients but only 15% in the pediatric cohort. We considered the difference of DGs incidence between pediatric and adult patients might be related to the intracranial location difference. By applying the ADIFFI method proposed by Ellingson et al., we found the left frontal lobe and right cerebellum were the preferred locations for pDGs. It was described that the cerebellum was the most vulnerable site affected by pilocytic astrocytoma (36.22%), which was the most common pediatric CNS tumors (22). The cerebellum might be a distinctive area to develop pilocytic astrocytoma and DGs in pediatric patients compared to the adults. The cerebellar location of pediatric high-grade glioma was reported to have a worse survival (23). A differential diagnosis for pilocytic astrocytoma and DGs in the pediatric cerebellum is necessary. Additionally, the anterior horn of ventricles is a significant location for adult DGs, which could be explained by the glioma origin from the subventricular zone (SVZ) (24).

Three immune checkpoints were typically selected to perform IHC staining in pediatric patients and paired adult patients who were chosen according to gender and histological types. B7-H3 (B7 homolog 3 protein), also known as CD276, belongs to the B7 superfamily (25). The roles of co-stimulator and co-inhibitor of B7-H3 during T-cell activation were reported, and the inhibition of B7-H3 checkpoint suppressed tumor growth by enhancing cytotoxic lymphocyte function (26). B7-H3 was highly expressed in gliomas and meningiomas, which could be treated by B7-H3-targeted CAR-T (27, 28). CD47 is overexpressed in hematologic and solid tumors, presenting the "don't eat me" signal against phagocytosis of macrophages after binding and activating signal regulatory protein–α (SIRPα) (29). It was demonstrated disrupting the CD47-SIRPα axis could exert antitumor effects on gliomas, and malignant pediatric brain tumors (30, 31). PD-L1 is the cognate ligand for PD-1, which is upregulated on tumor cells, and targeting the PD-1-PD-L1 axis is the robust immunotherapy (32). The expression of PD-L1 could be observed in GBM cells, which was the negative indicator for GBM outcome (33). The overexpression of B7-H3 and CD47 was validated in our results. However, PD-L1 was rarely detected.

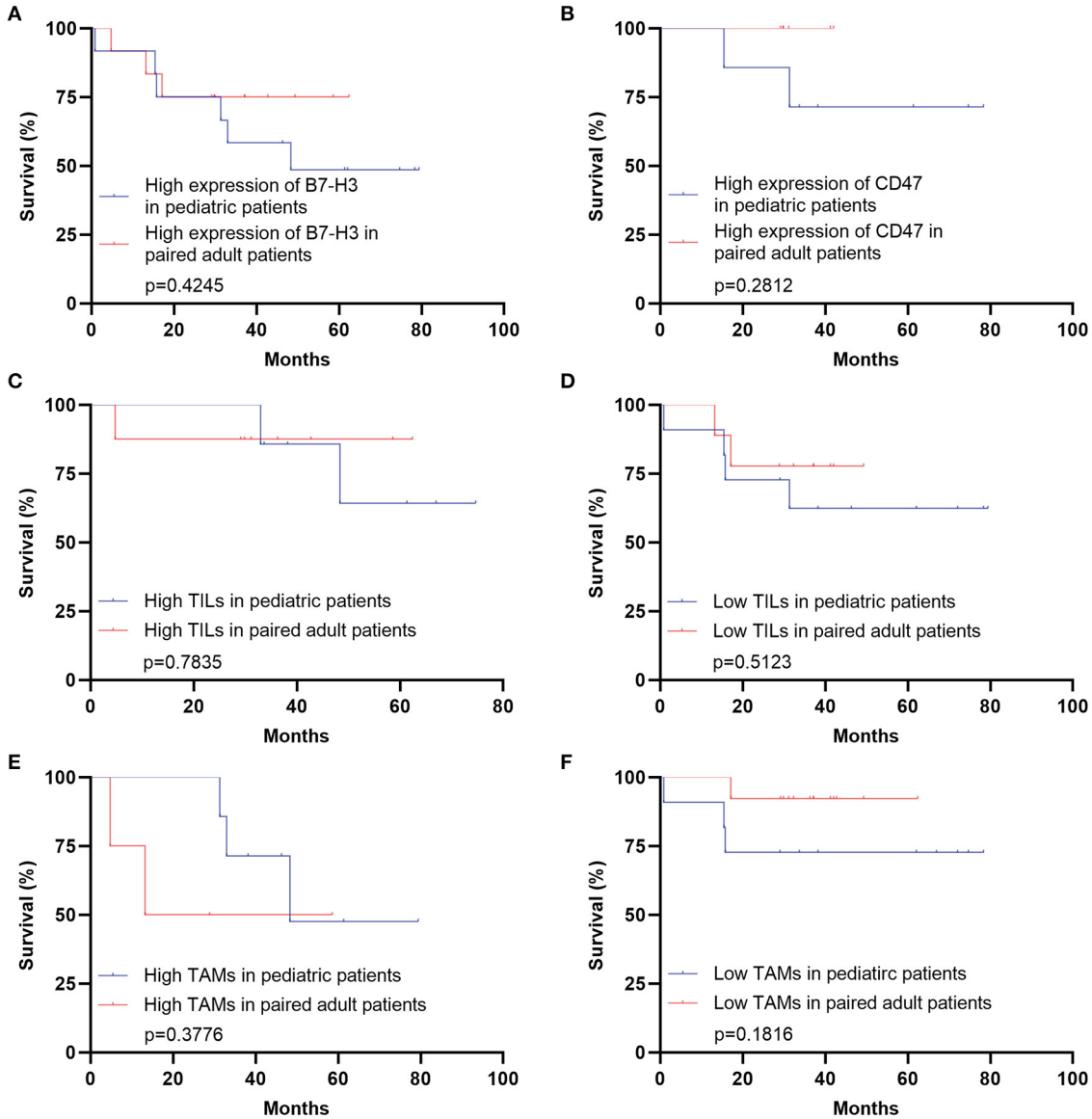

FIGURE 6 | Survival analyses comparing pediatric and paired adult patients with DGs under a similar expression level of immune checkpoint molecules or TILs/TAMs. **(A)** Survival analysis comparing the pediatric group with the paired adult group under the high expression of B7-H3 of DGs ($p = 0.4245$). **(B)** Survival analysis comparing the pediatric group with the paired adult group under the high expression of CD47 of DGs ($p = 0.2812$). **(C)** Survival analysis comparing the pediatric group with the paired adult group under the high TILs of DGs ($p = 0.7835$). **(D)** Survival analysis comparing the pediatric group with the paired adult group under the low TILs of DGs ($p = 0.5123$). **(E)** Survival analysis comparing the pediatric group with the paired adult group under the high TAMs of DGs ($p = 0.3776$). **(F)** Survival analysis comparing the pediatric group with the paired adult group under the low TAMs of DGs ($p = 0.1816$). TILs refer to the tumor-infiltrating lymphocytes; TAMs refer to the tumor-associated macrophages.

The difference in expression level between pediatric and adult DGs showed no statistical significance, indicating the similar expression patterns of immune checkpoints. The correlation analysis showed a potential association between the expression of PD-L1 and TAMs in pDGs, despite the low expression of PD-L1. It was described PD-L1 was expressed on TAMs in esophageal cancer and gastric cancer (34, 35). The positive relationship was also elucidated in pediatric cancers including Burkitt lymphoma, glioblastoma, and neuroblastoma (36). Therefore, in addition to B7-H3 and CD47, PD-L1 still remains to be the therapeutic target in pDGs. Interestingly, the activation of PD-L1^{+} NK cells with anti-PD-L1 inhibitor was the reason why some patients lacking PD-L1 expression on tumor cells still respond to anti-PD-L1 therapy (37).

Most importantly, the checkpoint-associated locations of DGs were found. Our results suggested the right posterior external capsule and the lateral side of the anterior horn of the left ventricle were predominant locations for the adult patients with high expression of B7-H3 and low expression of PD-L1 compared to pediatric ones, respectively. The external capsule is

anatomically located between the putamen and claustrum, and is composed of white matter fibers. The results that DGs with high expression of B7-H3 in adults located in this region might exactly reveal the difference in age and the association between B7-H3 and DGs. As the white matter is indicated to contribute to the malignant behaviors such as the spread of gliomas (38), mature white matter in adults rather than pediatric patients is assumed to be more conducive to this cancerous nature. Moreover, the anterior horn (also known as the frontal horn) of the ventricle is found to frequently affected by adult DGs (**Figure 1B**). However, the ADIFFI indicated, compared with pediatric patients, adult DGs with low expression of PD-L1 in this region were statistically significant. The results were supposed to predict the efficacy of immunotherapies targeting PD-L1 for adult DGs in this risky area. Therefore, the findings might be valuable for the design of immunotherapy strategies and clinical trials, as exemplified by the fact that local immunotherapies such as the local CAR-T delivery and local radiotherapy acting as immunosensitizer might benefit from these statistically significant sites for therapeutic priority. However, the detailed biological mechanisms warrant further investigation, especially the laterality.

The survival analysis comparing the pediatric and paired adult patients under a similar expression level of checkpoints or TILs/TAMs showed no significance. But high expression of B7-H3 lead to a decreased survival in pDGs though no statistical significance was found (**Figure 4C**). A previous study investigated 47 pediatric glioma patients and found a significant relation between high expression of B7-H3 and poor prognosis (39). More importantly, Haydar and his colleagues demonstrated that B7-H3 was consistently expressed in pediatric brain tumors and the subsequent use of B7-H3-CAR-T cells resulted in remarkable tumor regression in patient-derived orthotopic xenografts (40). These findings collectively showed the B7-H3 could be a promising candidate of immunotherapy in pediatric gliomas.

There were several limitations in the current study. Firstly, the sample size of pDGs was limited. This is probably due to the objectively low incidence of pediatric diffuse gliomas compared with pilocytic astrocytoma, followed by the fact that substantial pediatric patients receive diagnosis and therapy in the local tertiary children's hospital. Further cooperation among medical centers to expand the sample size is necessary. According to the previous work by Ellingson et al. the method of correction for cluster-size using random permutations was conducted to remove the scattered clusters after ADIFFI (17, 18). However, the correction was not performed in the present work, as the ADIFFI results indicated concentrated areas with statistical significance, which were not scattered (**Figure 3**). Furthermore, the sample size of this study was relatively small. Thus, the statistic correction may be too conservative to find possible clusters of specific immune markers' expression. Additionally, other quantitative methods such as flow cytometry, and novel technologies such as sing-cell sequencing and mass cytometry would help researchers better understand the immune difference between pediatric and adult DGs. Furthermore, the mechanisms of immune checkpoint-associated locations of DGs warrant further investigation as mentioned.

In conclusion, our study indicated that in the context of spatial location difference between pediatric and adult DGs, though the expression level and the prognostic role of immune checkpoint molecules and TILs/TAMs were not significantly different, the immune checkpoint-associated locations of DGs were found, which might be valuable for the design of immunotherapy strategies and clinical trials.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethical committee on clinical human research of the Second Affiliated Hospital of Zhejiang University, School of Medicine. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

JZ and CS contributed to the study design. LZ, BZ, and JW collected and analyzed the imaging data. BZ and ZD performed the IHC staining. YI performed survival and statistical analyses. LZ, BZ, and ZD wrote the manuscript. YI, BJ, CS, and JZ reviewed all the data, results, and manuscript. All authors read and approved the final version of the manuscript.

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REFERENCES

1. Ostrom QT, Cioffi G, Gittleman H, Patil N, Waite K, Kruchko C, et al. CBTRUS statistical report: primary brain and other central nervous system tumors diagnosed in the United States in 2012–2016. *Neuro Oncol.* (2019) 21(Suppl 5):v1–v100. doi: 10.1093/neuonc/noz150
2. Cacciotti C, Fleming A, Ramaswamy V. Advances in the molecular classification of pediatric brain tumors: a guide

to the galaxy. *J Pathol.* (2020) 251–249–61. doi: 10.1002/path.5457
3. Cherlow JM, Shaw DWW, Margraf LR, Bowers DC, Huang J, Fouladi M, et al. Conformal Radiation Therapy for Pediatric Patients with Low-Grade Glioma: Results from the Children's Oncology Group Phase 2 Study ACNS0221. *Int J Radiat Oncol Biol Phys.* (2019) 103:861–8. doi: 10.1016/j.ijrobp.2018.11.004
4. Jones C, Karajannis MA, Jones DTW, Kieran MW, Monje M, Baker SJ, et al. Pediatric high-grade glioma: biologically and clinically in need of new thinking. *Neuro Oncol.* (2017) 19:153–61. doi: 10.1093/neuonc/now101
5. Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-Branger D, Cavenee WK, et al. The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. *Acta Neuropathol.* (2016) 131:803–20. doi: 10.1007/s00401-016-1545-1
6. Jones DTW, Kieran MW, Bouffet E, Alexandrescu S, Bandopadhayay P, Bornhorst M, et al. Pediatric low-grade gliomas: next biologically driven steps. *Neuro Oncol.* (2018) 20:160–73. doi: 10.1093/neuonc/nox141
7. Yang RR, Aibaidula A, Wang WW, Chan AK, Shi ZF, Zhang ZY, et al. Pediatric low-grade gliomas can be molecularly stratified for risk. *Acta Neuropathol.* (2018) 136:641–55. doi: 10.1007/s00401-018-1874-3
8. Sadighi ZS, Curtis E, Zabrowksi J, Billups C, Gajjar A, Khan R, et al. Neurologic impairments from pediatric low-grade glioma by tumor location and timing of diagnosis. *Pediatr Blood Cancer.* (2018) 65:e27063. doi: 10.1002/pbc.27063
9. Roder C, Breitkopf M, Bisdas S, da Silva Freitas R, Dimostheni A, Ebinger M, et al. Beneficial impact of high-field intraoperative magnetic resonance imaging on the efficacy of pediatric low-grade glioma surgery. *Neurosurg Focus.* (2016) 40:E13. doi: 10.3171/2015.11.FOCUS15530
10. Ceschin R, Kocak M, Vajapeyam S, Pollack IF, Onar-Thomas A, Dunkel IJ, et al. Quantifying radiation therapy response using apparent diffusion coefficient (ADC) parametric mapping of pediatric diffuse intrinsic pontine glioma: a report from the pediatric brain tumor consortium. *J Neurooncol.* (2019) 143:79–86. doi: 10.1007/s11060-019-03133-y
11. Karsy M, Akbari SH, Limbrick D, Leuthardt EC, Evans J, Smyth MD, et al. Evaluation of pediatric glioma outcomes using intraoperative MRI: a multicenter cohort study. *J Neurooncol.* (2019) 143:271–80. doi: 10.1007/s11060-019-03154-7
12. Gunn ME, Lahdesmaki T, Malila N, Arola M, Gronroos M, Matomaki J, et al. Late morbidity in long-term survivors of childhood brain tumors: a nationwide registry-based study in Finland. *Neuro Oncol.* (2015) 17:747–56. doi: 10.1093/neuonc/nou321
13. Reardon DA, Brandes AA, Omuro A, Mulholland P, Lim M, Wick A, et al. Effect of Nivolumab vs Bevacizumab in Patients With Recurrent Glioblastoma: The CheckMate 143 Phase 3 Randomized Clinical Trial. *JAMA Oncol.* (2020) 6:1003–10. doi: 10.1001/jamaoncol.2020.1024
14. Wang SS, Bandopadhayay P, Jenkins MR. Towards Immunotherapy for Pediatric Brain Tumors. *Trends Immunol.* (2019) 40:748–61. doi: 10.1016/j.it.2019.05.009
15. Indelicato DJ, Rotondo RL, Uezono H, Sandler ES, Aldana PR, Ranalli NJ, et al. Outcomes Following Proton Therapy for Pediatric Low-Grade Glioma. *Int J Radiat Oncol Biol Phys.* (2019) 104:149–56. doi: 10.1016/j.ijrobp.2019.01.078
16. Fedorov A, Beichel R, Kalpathy-Cramer J, Finet J, Fillion-Robin JC, Pujol S, et al. 3D Slicer as an image computing platform for the Quantitative Imaging Network. *Magn Reson Imaging.* (2012) 30:1323–41. doi: 10.1016/j.mri.2012.05.001
17. Ellingson BM, Cloughesy TF, Pope WB, Zaw TM, Phillips H, Lalezari S, et al. Anatomic localization of O6-methylguanine DNA methyltransferase (MGMT) promoter methylated and unmethylated tumors: a radiographic study in 358 de novo human glioblastomas. *Neuroimage.* (2012) 59:908–16. doi: 10.1016/j.neuroimage.2011.09.076
18. Ellingson BM, Lai A, Harris RJ, Selfridge JM, Yong WH, Das K, et al. Probabilistic radiographic atlas of glioblastoma phenotypes. *AJNR Am J Neuroradiol.* (2013) 34:533–40. doi: 10.3174/ajnr.A3253
19. Inamura K, Takazawa Y, Inoue Y, Yokouchi Y, Kobayashi M, Saiura A, et al. Tumor B7-H3 (CD276) Expression and Survival in Pancreatic Cancer. *J Clin Med.* (2018) 7:172. doi: 10.3390/jcm7070172
20. Pi Castro D, Jose-Lopez R, Fernandez Flores F, Rabanal Prados RM, Mandara MT, Arus C, et al. Expression of FOXP3 in Canine Gliomas: Immunohistochemical Study of Tumor-Infiltrating Regulatory Lymphocytes. *J Neuropathol Exp Neurol.* (2020) 79:184–93. doi: 10.1093/jnen/nlz120
21. Lee C, Lee J, Choi SA, Kim SK, Wang KC, Park SH, et al. M1 macrophage recruitment correlates with worse outcome in SHH Medulloblastomas. *BMC Cancer.* (2018) 18:535. doi: 10.1186/s12885-018-4457-8
22. Tabash MA. Characteristics, survival and incidence rates and trends of pilocytic astrocytoma in children in the United States; SEER-based analysis. *J Neurol Sci.* (2019) 400:148–52. doi: 10.1016/j.jns.2019.03.028
23. Karremann M, Rausche U, Roth D, Kuhn A, Pietsch T, Gielen GH, et al. Cerebellar location may predict an unfavourable prognosis in paediatric high-grade glioma. *Br J Cancer.* (2013) 109:844–51. doi: 10.1038/bjc.2013.404
24. Lee JH, Lee JE, Kahng JY, Kim SH, Park JS, Yoon SJ, et al. Human glioblastoma arises from subventricular zone cells with low-level driver mutations. *Nature.* (2018) 560:243–7. doi: 10.1038/s41586-018-0389-3
25. Chapoval AI, Ni J, Lau JS, Wilcox RA, Flies DB, Liu D, et al. B7-H3: a costimulatory molecule for T cell activation and IFN-gamma production. *Nat Immunol.* (2001) 2:269–74. doi: 10.1038/85339
26. Lee YH, Martin-Orozco N, Zheng P, Li J, Zhang P, Tan H, et al. Inhibition of the B7-H3 immune checkpoint limits tumor growth by enhancing cytotoxic lymphocyte function. *Cell Res.* (2017) 27:1034–45. doi: 10.1038/cr.2017.90
27. Nehama D, Di Ianni N, Musio S, Du H, Patane M, Pollo B, et al. B7-H3-redirected chimeric antigen receptor T cells target glioblastoma and neurospheres. *EBioMedicine.* (2019) 47:33–43. doi: 10.1016/j.ebiom.2019.08.030
28. Tang X, Liu F, Liu Z, Cao Y, Zhang Z, Wang Y, et al. Bioactivity and safety of B7-H3-targeted chimeric antigen receptor T cells against anaplastic meningioma. *Clin Transl Immunology.* (2020) 9:e1137. doi: 10.1002/cti2.1137
29. Willingham SB, Volkmer JP, Gentles AJ, Sahoo D, Dalerba P, Mitra SS, et al. The CD47-signal regulatory protein alpha (SIRPa) interaction is a therapeutic target for human solid tumors. *Proc Natl Acad Sci USA.* (2012) 109:6662–7. doi: 10.1073/pnas.1121623109
30. Li F, Lv B, Liu Y, Hua T, Han J, Sun C, et al. Blocking the CD47-SIRPalpha axis by delivery of anti-CD47 antibody induces antitumor effects in glioma and glioma stem cells. *Oncoimmunology.* (2018) 7:e1391973. doi: 10.1080/2162402X.2017.1391973
31. Gholamin S, Mitra SS, Feroze AH, Liu J, Kahn SA, Zhang M, et al. Disrupting the CD47-SIRPα anti-phagocytic axis by a humanized anti-CD47 antibody is an efficacious treatment for malignant pediatric brain tumors. *Sci Transl Med.* (2017) 9:eaaf2968. doi: 10.1126/scitranslmed.aaf2968
32. Ribas A, Wolchok JD. Cancer immunotherapy using checkpoint blockade. *Science.* (2018) 359:1350–5. doi: 10.1126/science.aar4060
33. Nduom EK, Wei J, Yaghi NK, Huang N, Kong LY, Gabrusiewicz K, et al. PD-L1 expression and prognostic impact in glioblastoma. *Neuro Oncol.* (2016) 18:195–205. doi: 10.1093/neuonc/nov172
34. Yagi T, Baba Y, Okadome K, Kiyozumi Y, Hiyoshi Y, Ishimoto T, et al. Tumour-associated macrophages are associated with poor prognosis and programmed death ligand 1 expression in oesophageal cancer. *Eur J Cancer.* (2019) 111:38–49. doi: 10.1016/j.ejca.2019.01.018
35. Lin C, He H, Liu H, Li R, Chen Y, Qi Y, et al. Tumour-associated macrophages-derived CXCL8 determines immune evasion through autonomous PD-L1 expression in gastric cancer. *Gut.* (2019) 68:1764–73. doi: 10.1136/gutjnl-2018-316324
36. Majzner RG, Simon JS, Grosso JF, Martinez D, Pawel BR, Santi M, et al. Assessment of programmed death-ligand 1 expression and tumor-associated immune cells in pediatric cancer tissues. *Cancer.* (2017) 123:3807–15. doi: 10.1002/cncr.30724
37. Dong W, Wu X, Ma S, Wang Y, Nalin AP, Zhu Z, et al. The Mechanism of Anti-PD-L1 Antibody Efficacy against PD-L1-Negative Tumors Identifies NK Cells Expressing PD-L1 as a Cytolytic Effector. *Cancer Discov.* (2019) 9:1422–37. doi: 10.1158/2159-8290.CD-18-1259
38. Haydar D, Houke H, Chiang J, Yi Z, Odé Z, Caldwell K, et al. Cell surface antigen profiling of pediatric brain tumors: B7-H3 is consistently expressed and can be targeted via local or systemic CAR T-cell delivery. *Neuro Oncol.* (2020) 15:noaa278. doi: 10.1093/neuonc/noaa278
39. Maachani UB, Tosi U, Pisapia DJ, Mukherjee S, Marnell CS, Voronina J, et al. B7-H3 as a Prognostic Biomarker and Therapeutic Target in Pediatric central nervous system Tumors. *Transl Oncol.* (2020) 13:365–71. doi: 10.1016/j.tranon.2019.11.006

40. Wang J, Xu SL, Duan JJ, Yi L, Guo YF, Shi Y, et al. Invasion of white matter tracts by glioma stem cells is regulated by a NOTCH1-SOX2 positive-feedback loop. *Nat Neurosci.* (2019) 22:91–105. doi: 10.1038/s41593-018-0285-z

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Investigation of Genetic Determinants of Glioma Immune Phenotype by Integrative Immunogenomic Scale Analysis

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The immunosuppressive mechanisms of the surrounding microenvironment and distinct immunogenomic features in glioblastoma (GBM) have not been elucidated to date. To fill this gap, useful data were extracted from The Cancer Genome Atlas (TCGA), the Chinese Glioma Genome Atlas (CGGA), GSE16011, GSE43378, GSE23806, and GSE12907. With the ssGSEA method and the ESTIMATE and CIBERSORT algorithms, four microenvironmental signatures were used to identify glioma microenvironment genes, and the samples were reasonably classified into three immune phenotypes. The molecular and clinical features of these phenotypes were characterized *via* key gene set expression, tumor mutation burden, fraction of immune cell infiltration, and functional enrichment. Exhausted CD8+ T cell (GET) signature construction with the predictive response to commonly used antitumor drugs and peritumoral edema assisted in further characterizing the immune phenotype features. A total of 2,466 glioma samples with gene expression profiles were enrolled. Tumor purity, ESTIMATE, and immune and stromal scores served as the 4 microenvironment signatures used to classify gliomas into immune-high, immune-middle and immune-low groups, which had distinct immune heterogeneity and clinicopathological characteristics. The immune-H phenotype had higher expression of four immune signatures; however, most checkpoint molecules exhibited poor survival. Enriched pathways among the subtypes were related to immunity. The GET score was similar among the three phenotypes, while immune-L was more sensitive to bortezomib, cisplatin, docetaxel, lapatinib, and rapamycin prescriptions and displayed mild peritumor edema. The three novel immune phenotypes with distinct immunogenetic features could have utility for understanding glioma microenvironment regulation and determining prognosis. These results contribute to classifying glioma subtypes, remodeling the

immunosuppressive microenvironment and informing novel cancer immunotherapy in the era of precision immuno-oncology.

Keywords: immunogenomic analysis, microenvironment, immune phenotype, glioma, biometrics

INTRODUCTION

Gliomas are the most common and malignant primary tumors in the central nervous system (CNS) and have a highly invasive nature (1, 2). The discovery of the lymphatic system in the CNS has aroused inspiration to provide a novel theoretical foundation and new prospects for immunotherapy in brain tumors, and previous literature has demonstrated the mutual interactions between gliomas and the immune system (3, 4). Multiple related biological processes influencing CNS immune surveillance, such as the PI3K/Akt pathway, FAK, the IGF pathway, the STAT3 pathway, chemokines, HIF-1α, IL-6, TGF-β, PD-1/PD-L1, and CTLA-4, could individually impact immunosurveillance (5–8). Since entering the era of precision oncology, the molecular profiles of gliomas have been well studied. Mutations in the isocitrate dehydrogenase 1 (IDH1) gene, 1p/19q codeletion, methylguanine methyltransferase (MGMT) promoter methylation, tumor protein 53 (TP53), and telomerase reverse transcriptase (TERT) promoters are becoming treatment targets or prognostic biomarkers (9–11). Monoclonal antibodies (mAbs) against PD-1/PD-L1 show satisfying overall survival (OS) in melanoma and non-small cell lung cancer (NSCLC), but there is limited survival benefit in glioma (12). The unique immune-privileged microenvironment due to the inherent expression of immunosuppressive cytokines, such as PD-1, TGF-β, and IL-10, and the lack of antigen-presenting cells (APCs) in the CNS present obstacles for the efficacy of immunotherapy in glioblastoma (GBM) (13). The development of more novel and effective therapies will require a deep understanding of the tumor immunosuppressive microenvironment.

Direct interactions between tumor and immune cells can result in suppression of natural killer (NK) cell activity mediated by HLA molecules (including HLA-E and HLA-G) (14), immune cell apoptosis *via* tumor necrosis factor receptor superfamily member 6 (TNFRSF6, known as FAS) and the FAS-ligand interaction (15), or triggering of inhibitory T cell checkpoints by PD-L1 (16). The hypofunctional state of T cells known as T-cell exhaustion was identified by the accumulation of coinhibitory checkpoints (17). Of note, the paucity of T cells in the glioma microenvironment is striking in contrast to the levels in other "hot tumors", and some studies have suggested that glioma-associated myeloid cells are immunosuppressive with an M2-like phenotype and require peripheral dendritic cells (DCs) to elicit an immune response (18). Indeed, the exact mechanism of immune suppression is still obscure. In this study, we employed 2,466 samples to properly classify glioma into immune phenotypes according to distinct immunogenomic features based on microenvironment-related genes. Then, we validated and identified microenvironmental processes, explored immune alterations, and characterized immunosuppressive mechanisms. The immune landscape may inspire glioma subtype classification, remodeling of the immunosuppressive microenvironment and development of new therapies.

METHODS

Data Acquisition and Filtration

Data from glioma patients from six mRNA databases were extracted from TCGA database (RNA-sequencing (RNA-seq) for GBM, n = 169, microarray, n = 539) (http://cancergenome.nih.gov/), the CGGA database (RNA-seq, n = 1018, microarray, n = 301) (http://www.cgga.org.cn), the GSE16011 database (microarray, n = 276), the GSE43378 database (microarray, n = 50), the GSE23806 database (microarray, n = 92) and the GSE12907 database (microarray, n = 21). Complete clinical information was obtained from TCGA (http://cancergenome.nih.gov/, n = 708) and GCCA (http://www.cgga.org.cn, n = 1319). Somatic mutations and single nucleotide polymorphisms (SNPs) of gliomas were obtained from the TCGA database (http://cancergenome.nih.gov/, n=901, gene number n = 13,389). RNA-seq data downloaded in FPKM values from TCGA were normalized and transformed into transcripts per kilobase million values. RNA expression of gliomas was assessed with the Affymetrix microarray platform in the Gene Expression Omnibus (GEO) database (GSE16011, GSE43378, GSE23806, and GSE12907). Data were filtered to exclude samples without mRNA expression or clear histology, and the genomic data were normalized and analyzed within lanes, between lanes, and per quantile using the "limma" and "DESeq2" R packages. In this study, TCGA and CGGA were mainly treated as the training sets, and GEO databases were regarded as the validation sets.

Immune Phenotype Classification

In the glioma microenvironment, immune and stromal cells are two key types of nontumor components and have been indicated to be significant for the diagnosis and prognosis of tumors. Yoshihara et al (19) designed the ESTIMATE algorithm to compute immune and stromal cell scores to predict the infiltration of these nontumor cells. The authors used ESTIMATE to evaluate immune scores, ESTIMATE scores, stromal scores and tumor purity scores in each tumor sample with the aim of determining the immune infiltration level.

Single-sample gene set enrichment analysis (ssGSEA), which assisted in quantifying the enrichment level of an immune cell/signature, pathway or biological process in a tumor sample, was used to assess the gene score of every gene set for every sample (20). The enrichment-related score represented the level at which the genes in the gene set were synchronously up- or downregulated in the sample. The infiltration of immune cells in the microenvironment was determined by 29 immune cell types: NK cells, effector memory CD4+ T cells, activated B cells, monocytes, memory B cells, activated CD4+ T cells, type 2 T helper cells, dendritic cells, neutrophils, macrophages, effector memory CD8+ T cells, myeloid-derived suppressor cells (MDSCs), immature B cells, mast cells, and regulatory T cells, and glioma samples were hierarchically clustered into "immune-high (immune-H)", "immune-middle

(immune-M)" and "immune-low (immune-L)" groups. Separation of gene expression patterns between immune phenotypes was evaluated by the principal component analysis (PCA) algorithm with the PCA1, PCA2, and PCA3 top three dimensions (21). Visualization was performed with the "GSVA", "GSEABase", "ComplexHeatmap", "estimate", and "ggplot" public packages.

Quantification of Molecular and Genomic Features

Tumor mutation burden (TMB) was defined as the total count of somatic mutations per megabase in each tumor sample. We used the MATH algorithm (22), which assessed the width of the allele frequency distribution, to evaluate the intratumor heterogeneity level of tumor samples. Further intratumor heterogeneity scores were quantified using the function "math. Score" in the "maftools" package with downloaded "maf" files based on the hg19 sequencing platform. Comparisons of the somatic mutations and SNP sites among immune phenotypes in distinct populations (low-grade glioma (LGG) and GBM samples) were displayed to investigate the discrepancies by the "maftools" and "GenVisR" packages.

Survival Analysis

Available clinicopathological factors (e.g., sex, age, treatment options, histological subtype, and classic mutations) were collected from the TCGA and CGGA datasets to estimate the association between these factors as well as the immune phenotypes and prognosis with univariable and multivariable Cox analysis (uniCox, multiCox) and proportional hazard models. We compared survival differences among immune-specific phenotypes of glioma in distinct groups using Kaplan-Meier curves and the log-rank test with normalized clinical data.

Estimation of the Proportions of Immune Cell Types

CIBERSORT is an algorithm designed to characterize the cell composition of complex tissues based on their gene expression profiles, and it is highly consistent with real-life estimations in many cancers. A leukocyte gene signature matrix employing 547 genes, which was defined as LM22, was used to quantify 22 immune cell types (23). These 22 types of immune cells mainly include myeloid subtypes, NK cells, plasma cells, naive and memory B cells and T cells. We used the CIBERSORT method to investigate the fraction of the 22 immune cell types in each derived phenotype and identify the characteristics of infiltrating cells in the glioma microenvironment.

Identification of a Gene Signature for Exhausted CD8+ T Cells

CD8+ T lymphocytes are regarded as a critical component of antitumor immunity, while immune invasion often occurs during the development of T cell exhaustion, characterized by the progressive accumulation of coinhibitory checkpoints, including PD-1, PD-L1, CTLA-4, TIM-3, and LAG-3 (17). We defined a gene expression signature of exhausted CD8+ T cells with integrative bioinformatics through publicly available NSCLC data considering the data quality and availability. We obtained an RNA-seq dataset of intratumoral CD8+ T cells showing high or no PD-1 (PDCD1) expression in a published study (24), and we generated an upregulated PD-1-positive gene list from another previous study (25). Pearson correlation analysis was conducted using the upregulated PD-1-positive gene list in the TCGA (microarray+ RNA-seq cohort) and CGGA (microarray+ RNA-seq cohort) datasets with an adjusted P-value < 0.05 and |correlation efficiency| > 0.25 as the eligibility criteria. In total, a 5-gene signature was identified in the glioma database, and an exhausted CD8+ T cell (GET) score was quantified in a tumor by conducting ssGSEA to obtain the ssGSEA score. In combination with clinical and molecular profiles, the prognostic and predictive values of the GET score were determined through different immune phenotypes.

Correlation and Functional Analysis

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed on genes differentially expressed between the immune-high and immune-low groups. Gene set enrichment analysis (GSEA) was carried out to identify the group of genes enriched either in the immune-high or immune-low group with cutoffs of a P-value < 0.1 and a false discovery rate (FDR) < 0.05 (26). Gene set variation analysis (GSVA) is a nonparametric and unsupervised method estimating the variations of samples in analyzed datasets in pathways and biological process (27). The gene sets of "c2.cp.kegg.v6.2.-symbols" used were captured from the MSigDB website for GSVA, with an adjusted P-value < 0.05 considered statistically significant. Correlation plots were constructed that primarily focused on the interactions between IDH1 and other key immune-related genes identified from the GSEA with a P filter = 0.001. A Sankey diagram was constructed to show the correlations between checkpoints and the GSEA-identified genes. Visualization of the unction analyses was realized *via* the "circlize" (28), "circus" (29), "clusterProfiler", and "ggalluvial" (30) packages.

Prediction of the Chemo/Targeted Therapy Response

Intended chemotherapeutic and targeted responses of glioma samples were evaluated by the largest publicly available pharmacogenomics database (Pharmaceutical Sensitivity Genomics in Cancer (GDSC) https://www.cancerrxgene.org/) (31). GDSC contains drug sensitivity information from nearly 75000 experiments and responses to 138 anticancer drugs across almost 700 cell lines. The database provides a unique source relevant to mainstream drug sensitivity and genomic datasets to inspire new discoveries on cancer therapeutic biomarkers. GDSC is also utilized due to its visualization capability. The evaluation procedure was conducted *via* the R software package "pRRophetic", half-maximal inhibitor concentration (IC50), and the evaluation accuracy was determined by ridge regression and 10-fold cross-validation using the GDSC dataset (32, 33). Different chemotherapeutic and targeted responses among the three phenotypes were analyzed by one-way analysis of variance (ANOVA) or the Kruskal-Wallis test (K-W test) based on the results of the normal distribution criteria test. The response to commonly used chemotherapy or targeted therapies was compared according to immune phenotype, although some drugs were not formally approved for utility in brain tumors.

Peri-Tumoral Edema Characteristics

To detect the variations in some radiomics features of classified immune phenotypes, MR images (MRIs) of patients from the TCGA dataset were obtained from the Cancer Imaging Archive. TCGA-GBM and TCGA-LGG cohorts in the Cancer Imaging Archive (http://www.cancerimagingarchive.net) were specifically selected and matched with existing results. Eligible tumor contrast enhancement images were determined after a discussion with three skilled neurosurgeons (Zhao B, Xing H, Wang Y) on the author list. Radiomics features of tumors included tumor size, enhancement, noncontrast-enhancing tumor (nCET), necrosis, edema, cysts, multifocality, contact with ventricles or neocortex and location based on a previous study (34). Features such as multifocality, enhancement, location and edema were revealed to have molecular signature correlations with glioma, such as IDH mutation or MGMT promoter methylation; edema and necrosis were regarded as poor survival imaging markers (34, 35). Edema associated with both molecular phenotypes and prognosis was the focus of investigation to facilitate identification of noninvasive acquired markers and features of the classified glioma phenotypes. A mild (or no) region of edema (-) was regarded as edema extending ≤ 1 cm from the margin of the tumor; otherwise, it was treated as moderate to severe (+) (36). The evaluations were all based on eligible T2-weighted images.

Statistical and Bioinformatics Analyses

Statistical analyses were conducted using R software (version 3.5.3), and other statistical methods are mentioned throughout the article. Bioinformatics analysis was conducted mainly following the methods of Thorsson et al (37). A two-sided $P < 0.05$ was considered to be significant unless otherwise specified. The public packages used are mentioned throughout this paper.

RESULTS

ssGSEA and Independent Immune Phenotype Classification

After excluding the normal tissues (5 normal samples in the TCGA RNA-seq database), tumor samples with distinct extension of inflammatory cell infiltration were classified into "immune-L", "immune-M" and "immune-H" phenotypes with ssGSEA incorporating 29 types of immune cell lineages, such as helper T cells, cytotoxic T cells, myeloid cells, monocytes, NK cells, dendritic cells, and T cells. The numbers of samples falling into the immune-L, immune-M, and immune-H phenotypes were 283, 234 and 21 in the TCGA microarray data; 129, 8 and 32 in the TCGA-GBM RNA-seq data; 105, 90 and 106 in the CGGA microarray cohort; 413, 425 and 180 in the CGGA RNA-seq cohort; 112, 162 and 2 in GSE16011; 28, 16 and 6 in GSE43378; 87, 2 and 3 in GSE23806; and 9, 10 and 2 in GSE12907, respectively (**Figure 1**).

Each Phenotype Has Distinct Immunogenetic Features

Four immune scores were employed. From the ESTIMATE algorithm, the immune-H phenotype was revealed to have a higher ESTIMATE score, immune score and stromal score and a lower tumor purity score than the immune-M and immune-L phenotypes. Statistical comparisons showed that there were significant differences between the immune-H and immune-L phenotypes (Wilcoxon P-value < 0.001) related to these immune signatures (**Figure 2**).

Checkpoint biomarkers are involved in tumor subtype classification, prognosis prediction and immunotherapy therapy

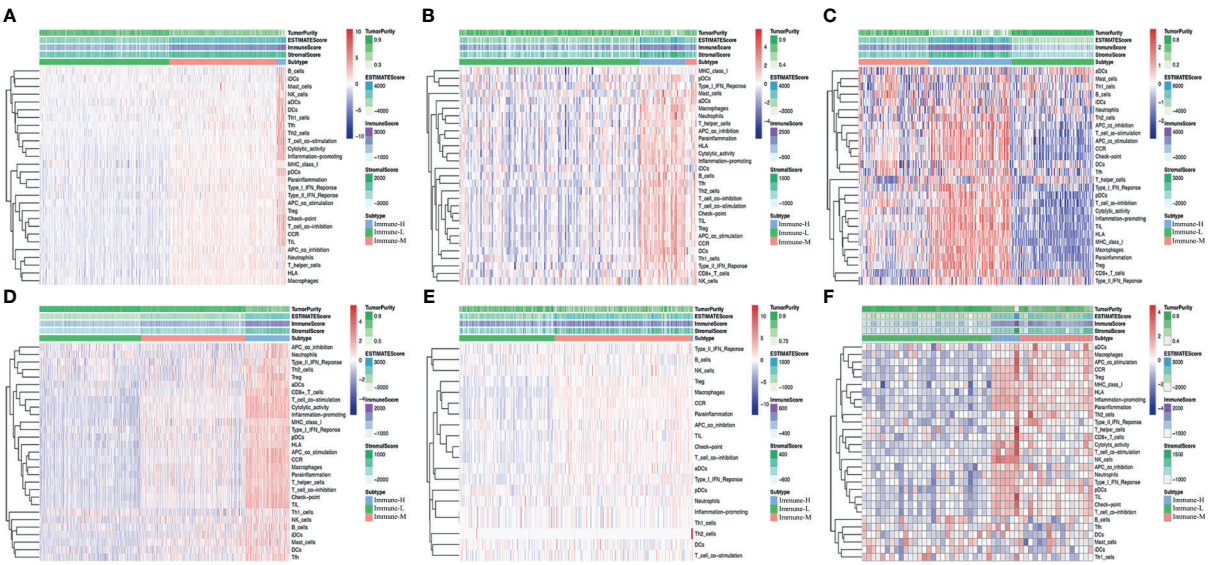

FIGURE 1 | Immune phenotype classification and four glioma immune microenvironment signatures identification. **(A–F)** Heatmaps showing three immune phenotypes, tumor purity, ESTIMATE, immune and stromal scores in the glioma microenvironment of samples from the TCGA microarray, TCGA GBM RNA-seq, CGGA microarray, CGGA RNA-seq, GSE16011, and GSE43378 cohorts.

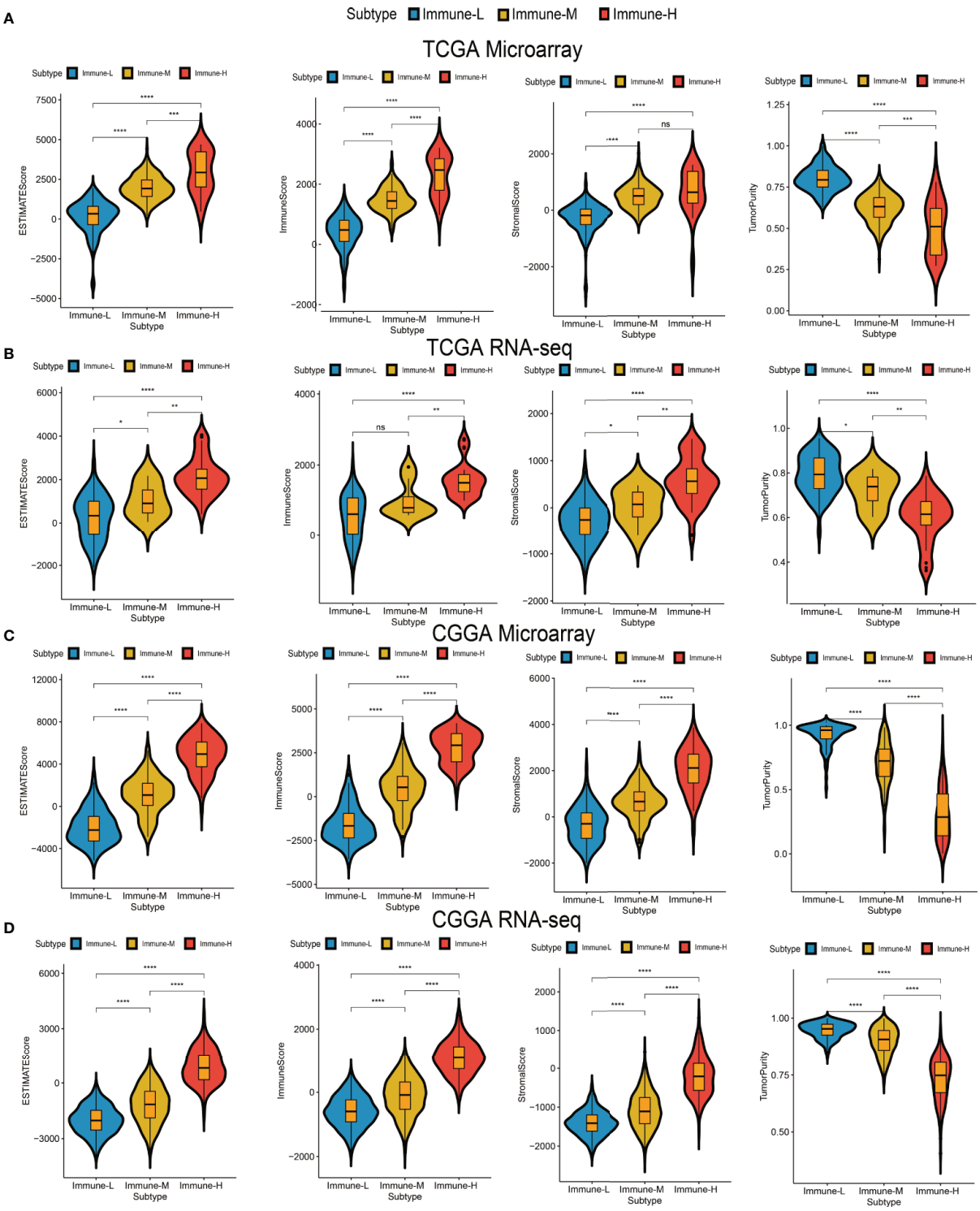

FIGURE 2 | Differences among immune phenotypes in terms of four glioma immune microenvironment signatures. **(A–D)** Violin plots comparing the ESTIMATE, immune and stromal scores and tumor purity among immune phenotypes in the TCGA microarray, TCGA GBM RNA-seq, CGGA microarray, and CGGA RNA-seq cohorts respectively. P values for Wilcoxon test were shown on the top of each violin plot. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. ns, not significant.

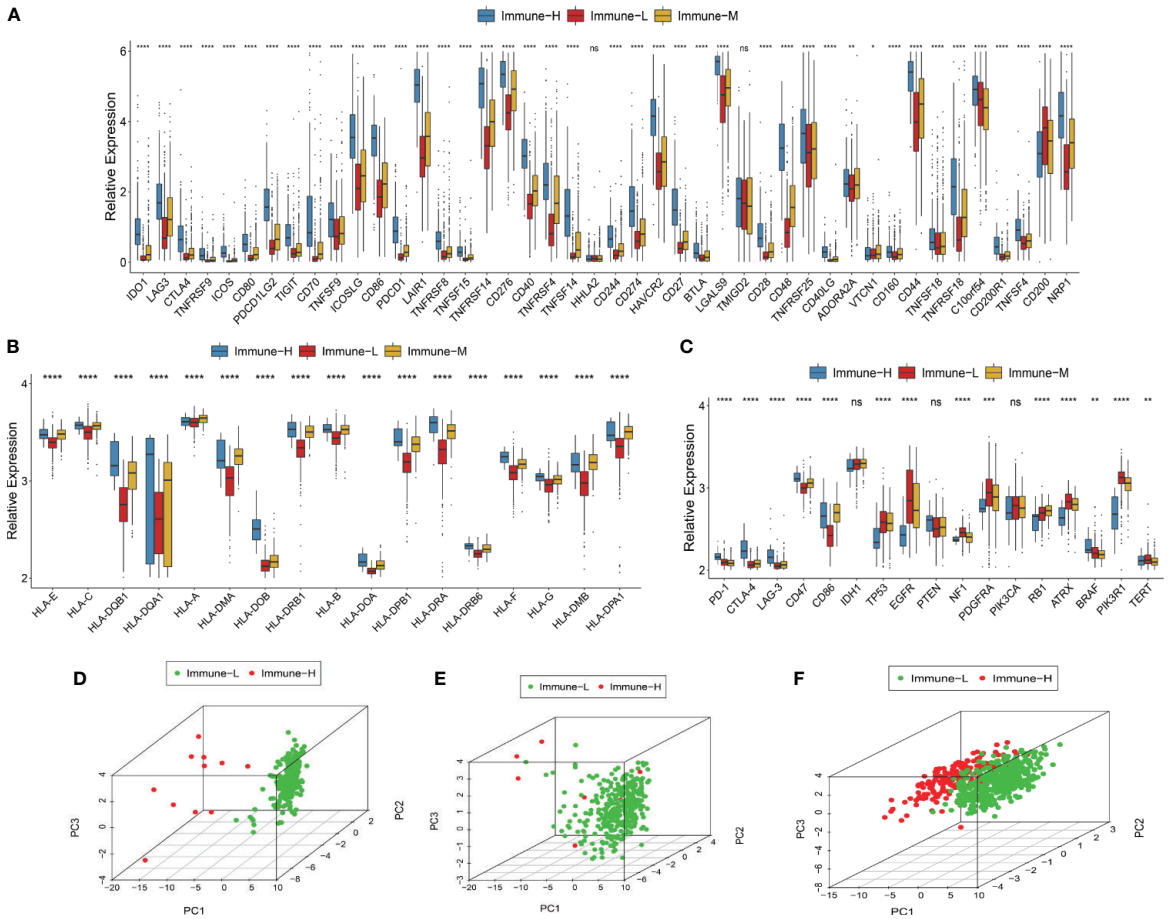

FIGURE 3 | Differences in checkpoints, HLA family and other key biomarkers between the immune phenotypes. **(A)** Expression of checkpoint family biomarkers of each phenotype in the CGGA RNA-seq cohort. **(B)** Expression of HLA family genes of each phenotype in the TCGA microarray cohort. **(C)** Expression of part T cells co-inhibitors checkpoints and key biomarkers relating to glioma biological behavior and pathways in the TCGA microarray cohort. The upper and lower ends of the boxes represented interquartile range of values. The lines in the boxes represented median value, and black dots showed outliers. The asterisks represented the statistical P value (*P < 0.05; **P < 0.01; ***P < 0.001; ***P < 0.0001, ns, not significant). **(D–F)** There was separation between the immune-H and immune-L phenotypes in the TCGA microarray **(D)**, TCGA GBM RNA-seq **(E)** and CGGA RNA-seq cohorts **(F)** according to PCA. PC1, PC2, PC3 represented three dimensions showing differential expression of markers related to immune cell lineage.

response evaluation. We observed that most checkpoints were differentially expressed. Such biomarkers were more highly expressed in the immune-H phenotype than in the immune-M and immune-L groups. CD200 was highly expressed in the immune-L phenotype (K-W test P value < 0.001) (**Figure 3A**). HLA genes took important roles in innate immunity and tumor immune microenvironment regulation, these family genes had significantly different expression among phenotypes, with the immune-H group exhibiting significantly higher expression than the other two groups (**Figure 3B**). Furthermore, the immune-L showed higher expression of TP53, EGFR, NF1, PDGFRA, and RB1than immune-H phenotype, which suggested the converged axes of P53, tumor suppressive Rb and MAPK/PI3K were potentially activated in immune-L phenotype. IDH-mutant glioma with ATRX and TERT mutations was always associated with favorable survival (**Figure 3C**). Good separation between the immune-H and immune-L phenotypes was confirmed by PCA (**Figures 3D–F**). Based on the above results, the immune-H phenotype may be more sensitive to classic checkpoint immunotherapy than the others, while the immune-L phenotype was associated with longer survival and better prognosis.

The Immune-H Phenotype Is Associated With a Poor Prognosis

Clinical and molecular features of the immune-specific phenotypes of glioma are displayed in complex heatmaps (**Figures 4A–D**, **Supplementary Online Files 1–4**). Treatment options and histological characteristics seemed to have more prognostic influence, and patients who had received corresponding chemotherapy (including adjuvant temozolomide (TMZ) therapy) or radiotherapy or who had a lower tumor grade and malignancy were observed to have favorable survival. The results are summarized in standardized **Table 1**. In the TCGA (n = 701) (log-rank P-value = 0.031) and CGGA cohorts (n = 1281) (log-rank

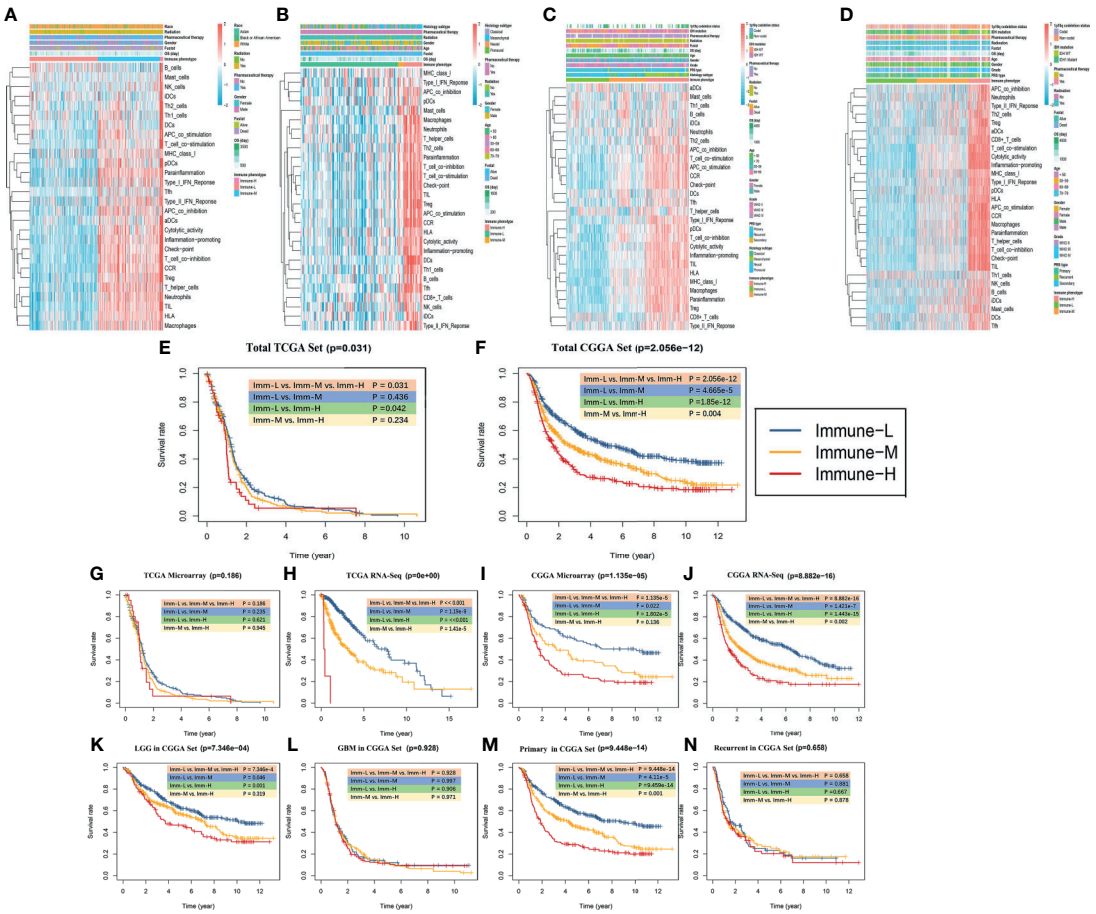

FIGURE 4 | Survival data showing that the immune-H phenotype is associated with a poor prognosis. **(A–D)** Complex heatmaps including ssGSEA results and clinical information from involved TCGA microarray, TCGA GBM RNA-seq, CGGA microarray, and CGGA RNA-seq cohorts. **(E, F)** Survival plots showed immune-H phenotype had poorer survival in all three immune phenotypes in total TCGA (P = 0.031) and CGGA (P = 2.056e-12) datasets. **(G–J)** Survival plots showing prognosis discrepancies among three immune phenotypes in TCGA microarray, TCGA RNA-Seq, CGGA microarray, CGGA RNA-Seq cohorts. **(K–N)** Survival plots for the LGG, GBM, primary glioma and recurrent glioma subpopulations in the total CGGA dataset. The log-rank test P value among three phenotypes and every two phenotypes are marked and shown.

P-value = 2.056e-12), the immune-H phenotype exhibited unfavorable survival compared with the immune-L phenotype (**Figures 4E, F**). Similar findings were consistent and confirmed in the TCGA RNA-seq (P-value < 0.001), CGGA microarray (P-value = 1.135e-5) and CGGA RNA-seq cohorts (P-value = 8.882e-16), but the results were not significant in the TCGA microarray cohort due to limitations derived from the sample number (P-value = 0.186) (**Figures 4G–J).** For subgroup analyses conducted in the CGGA cohort, in the LGG and primary glioma patients, there were significant survival differences between the immune-H, -M and -L phenotypes (log-rank P-value = 7.346e-4; P-value = 9.448e-14, respectively). The prognostic value was not obvious in the GBM (P-value = 0.928) or recurrent subpopulations (P-value = 0.658) (**Figures 4K–N**). These results were contrary to those of previous studies on other cancer types, including breast cancer (38), gastric cancer (39) and head and neck squamous cell cancer (40), which indicated the specificity of the association between tumor immunity and clinical outcomes in glioma, the microenvironment of which is regarded as rather immunosuppressive and refractory. Additionally, intrinsic limitations associated with sample size and variation of ethnicity among the used databases or cohorts should be acknowledged.

Infiltrating Immune Cell Fractions and Correlations

Through the CIBERSORT algorithm, M2, M1, and M0 macrophages, monocytes, DCs, and subsets of B and T cells (CD4+ and CD8+) were distinguished in the glioma microenvironment (**Figures 5A, B**). The results derived from ESTIMATE and CIBERSORT classified the glioma samples into three immune phenotypes, which had similar characteristics to those of the previously identified phenotypes. Correlations between each type of immune cell illustrated that the most negative correlations were found among M0 macrophages, monocytes, M2 macrophages, DCs (activated and resting) and helper T cells. These results suggested that myeloid cells highly

TABLE 1 | Results of univariable and multivariable analyses on overall survival of glioma patients from multiple cohorts.

	Univariable Cox		Multivariable Cox	
	HR (95% CI)	**P-value**	**HR (95% CI)**	**P-value**
***TCGA microarray* cohort**				
Gender (male vs. female)	1.09 (0.87-1.35)	0.457	1.22 (0.98-1.53)	0.080
Radiation (yes vs. no)	0.13 (0.09-0.18)	< 0.001*	0.15 (0.10-0.21)	< 0.001*
Chemotherapy (yes vs. no)	0.43 (0.33-0.54)	< 0.001*	0.56 (0.43-0.72)	< 0.001*
Ethnicity (not Hispanic or Latino vs. Hispanic or Latino)	0.90 (0.46-1.75)	0.750	0.87 (9,44-1.72)	0.685
Race				
White	NA	NA	NA	NA
Asian	0.97 (0.48-1.96)	0.935	1.05 (0.52-2.16)	0.885
Black or African American	0.82 (0.54-1.24)	0.350	0.96 (0.63-1.46)	0.845
Phenotype				
Immune-L	NA	NA	NA	NA
Immune-M	0.82 (0.43-1.56)	0.550	0.68 (0.35-1.30)	0.246
Immune-H	0.94 (0.49-1.79)	0.849	0.81 (0.42-1.55)	0.525
***TCGA GBM RNA-seq* cohort**				
Age (y)				
< 50	NA	NA	NA	NA
50-59	1.26 (0.69-2.31)	0.445	1.37 (0.70-2.68)	0.358
60-69	0.98 (0.56-1.72)	0.944	0.93 (0.48-1.80)	0.831
70-79	1.97 (1.03-3.79)	0.042*	2.20 (1.01-4.79)	0.048*
Gender (male vs. female)	0.89 (0.57-1.38)	0.599	1.19 (0.72-1.98)	0.497
Radiation (yes vs. no)	0.31 (0.15-0.65)	0.002*	0.31 (0.10-0.94)	0.039*
Chemotherapy (yes vs. no)	0.34 (0.18-0.66)	0.002*	0.76 (0.25-2.28)	0.620
Adjuvant TMZ chemotherapy (yes vs. no)	0.64 (0.41-0.99)	0.050*	0.91 (0.53-1.58)	0.746
Histology type				
Proneural	NA	NA	NA	NA
Neural	0.94 (0.49-1.84)	0.866	0.96 (0.45-2.03)	0.907
Classical	0.88 (0.44-1.52)	0.534	1.10 (0.54-2.24)	0.794
Mesenchymal	0.99 (0.56-1.75)	0.964	0.92 (0.45-1.87)	0.814
Phenotype				
Immune-L	NA	NA	NA	NA
Immune-M	0.77 (0.28-2.13)	0.619	0.88 (0.30-2.59)	0.817
Immune-H	1.68 (0.96-2.92)	0.067	2.00 (1.04-3.86)	0.038*
***CGGA microarray* cohort**				
Age (y)				
< 50	NA	NA	NA	NA
50-59	2.80 (1.96-4.01)	< 0.001*	1.70 (1.13-2.55)	0.011*
60-69	2.61 (1.67-4.08)	< 0.001*	1.60 (0.99-2.59)	0.055
70-79	16.69 (2.24-)	0.006*	6.42 (0.77-53.42)	0.085
Gender (male vs. female)	1.27 (0.94-1.72)	0.125	1.08 (0.78-1.49)	0.640
PRS type				
Primary	NA	NA	NA	NA
Recurrent	1.89 (1.11-3.22)	0.020*	2.19 (1.17-4.10)	0.014*
Secondary	4.44 (2.25-8.77)	< 0.001*	2.83 (1.31-6.14)	0.008*
Histology (GBM vs. LGG)	4.44 (3.24-6.09)	< 0.001*	4.69 (2.81-7.85)	< 0.001*
Grade				
WHO II	NA	NA	NA	NA
WHO III	3.08 (1.94-4.90)	< 0.001*	2.77 (1.62-4.71)	< 0.001*
WHO IV	6.83 (4.60-10.12)	< 0.001*	NA	NA
Radiation (yes vs. no)	0.49 (0.31-0.78)	0.003*	0.48 (0.28-0.81)	0.006*
Chemotherapy (yes vs. no)	1.57 (1.16-2.14)	0.004*	0.83 (0.57-1.20)	0.314
IDH1 status (IDH1 MT vs IDH1 WT)	0.42 (0.31-0.58)	< 0.001*	0.88 (0.59-1.31)	0.533
Histology type				
Proneural	NA	NA	NA	NA
Neural	0.80 (0.51-1.27)	0.343	0.95 (0.58-1.56)	0.845
Classical	2.67 (1.50-4.74)	< 0.001*	1.15 (0.59-2.25)	0.673
Mesenchymal	2.61 (1.81-3.77)	< 0.001*	1.75 (1.05-2.91)	0.031*
Phenotype				
Immune-L	NA	NA	NA	NA
Immune-M	1.77 (1.20-2.61)	0.004*	1.14 (0.71-1.82)	0.584
Immune-H	2.31 (1.59-3.36)	< 0.001*	0.83 (0.48-1.44)	0.512

(Continued)

TABLE 1 | Continued

	Univariable Cox		Multivariable Cox	
	HR (95% CI)	**P-value**	**HR (95% CI)**	**P-value**
***CGGA RNA-seq* cohort**				
Age (y)				
< 50	NA	NA	NA	NA
50-59	1.65 (1.33-2.05)	< 0.001*	1.11 (0.88-1.39)	0.376
60-69	2.40 (1.85-3.11)	< 0.001*	1.26 (0.96-1.67)	0.099
70-79	4.19 (2.53-6.95)	< 0.001*	2.35 (1.38-3.98)	0.002*
Gender (male vs. female)	1.01 (0.85-1.20)	0.922	1.12 (0.94-1.33)	0.217
PRS type				
Primary	NA	NA	NA	NA
Recurrent	2.23 (1.86-2.67)	< 0.001*	2.30 (1.90-2.79)	< 0.001*
Secondary	4.37 (2.92-6.54)	< 0.001*	3.11 (2.00-4.83)	< 0.001*
Histology (GBM vs. LGG)	4.38 (3.66-5.25)	< 0.001*	5.85 (4.25-8.06)	< 0.001*
Grade				
WHO II	NA	NA	NA	NA
WHO III	2.04 (2.24-3.87)	< 0.001*	2.68 (2.00-3.59)	< 0.001*
WHO IV	8.33 (6.39-10.85)	< 0.001*	NA	NA
Radiation (yes vs. no)	0.97 (0.77-1.23)	0.817	0.83 (0.64-1.06)	0.130
Chemotherapy (yes vs. no)	1.59 (1.30-1.94)	< 0.001*	0.72 (0.57-0.89)	0.003*
IDH1 status (IDH1 MT vs IDH1 WT)	0.32 (0.27-0.38)	< 0.001*	0.50 (0.40-0.62)	< 0.001*
Phenotype				
Immune-L	NA	NA	NA	NA
Immune-M	1.44 (1.18-1.74)	< 0.001*	1.04 (0.86-1.27)	0.685
Immune-H	1.94 (1.54-2.44)	< 0.001*	0.94 (0.73-1.20)	0.607

**represents the statistical test is significant (P < 0.05).*

HR, hazard ratio; TMZ, temozolomide; LGG, low grade glioma; GBM, glioblastoma; IDH1 MT, IDH1 mutant type; IDH1 WT, IDH1 wild type; NA, not available.

participated in the immunosuppressive glioma microenvironment (**Figures 5C, D**). Comparing the proportion of infiltrating immune cells, the immune-H phenotype was revealed to have higher proportions of all analyzed immune cells, except M2 macrophages, activated mast cells, monocytes, neutrophils and resting memory CD4+ T cells (**Figure 5E**).

Construction of the Exhausted CD8+ T Cell Signature

Exhausted CD8+ T cell levels were recognized to be uniquely regulated by distinct PD-1 upregulation. With transcriptional profiles of CD8+ T lymphocytes and upregulated PD-1-positive genes captured from previous studies (24, 25), correlation analyses were carried out in the involved datasets, in which five genes meeting the selection criteria were selected and termed GET signature. The GET signature included PDCD1, CD27, ICOS, RUNX2, and CXCR6, which are closely linked to T cell dysfunction and coregulation (**Figure 6F**). The GET score of each tumor sample was established with the ssGSEA method. To quantitatively illustrate the status of exhausted CD8+ T cells in each immune phenotype, we compared the distribution of the GET score in different phenotypes. We did not observe significant differences in the GET score between the immune-L, -M and -H phenotypes (**Figure 6A**, **Supplementary Online File 5**). Correlations between the defined GET score and immune score, ESTIMATE score, stromal score and tumor purity were assessed, and no tight correlation was found among these signatures (**Figures 6B–E**). The results from the TCGA microarray dataset seemed to vary slightly from the results in other datasets, and the lack of CD8+ T cells in the glioma microenvironment and the failure of immune surveillance against tumor cells were likely causes of these effects (41). Patients with a higher GET score in the total CGGA cohort had a more favorable prognosis than those with a lower GET score (HR: 1.38, 1.20-1.60; P-value = 1.25e-5), and the results were not significant in the total TCGA cohort (**Figures 6G, H**) (**Supplementary Online File 6**). Confirmatively, nearly all of the constructed GET signatures were mainly related to inflammatory components, lymphocyte functions and immune cell signaling (**Figure 6I**). To date, crosstalk between the GET signature and other molecular and clinicopathological factors is being warmly discussed in glioma, and more evidence is needed in the future.

Functional Enrichment Analysis of Phenotype-Associated Genes

In subsequent functional analyses of the biological processes of the identified microenvironment-related genes in the immune phenotypes, metagenes chosen as classifier gene sets for the immune-H over the immune-L phenotype in GSEA were significantly enriched in immune-related GO terms such as dendritic cell antigen processing and presentation, immunoglobulin processes, regulation of T cell chemotaxis, and T helper cell lineage (P-value and Benjamini P-value < 0.05); metagenes were significantly enriched in pathways related to immune-related graft-versus-host disease, the hematopoietic cell lineage, and the IL-17 signaling pathway (P-value and Benjamini P-value < 0.05) according to pathway GSEA (**Figures 7A–D**). Bubble plots can be found in **Appendix Figure A1**. The cluster maps display whole gene

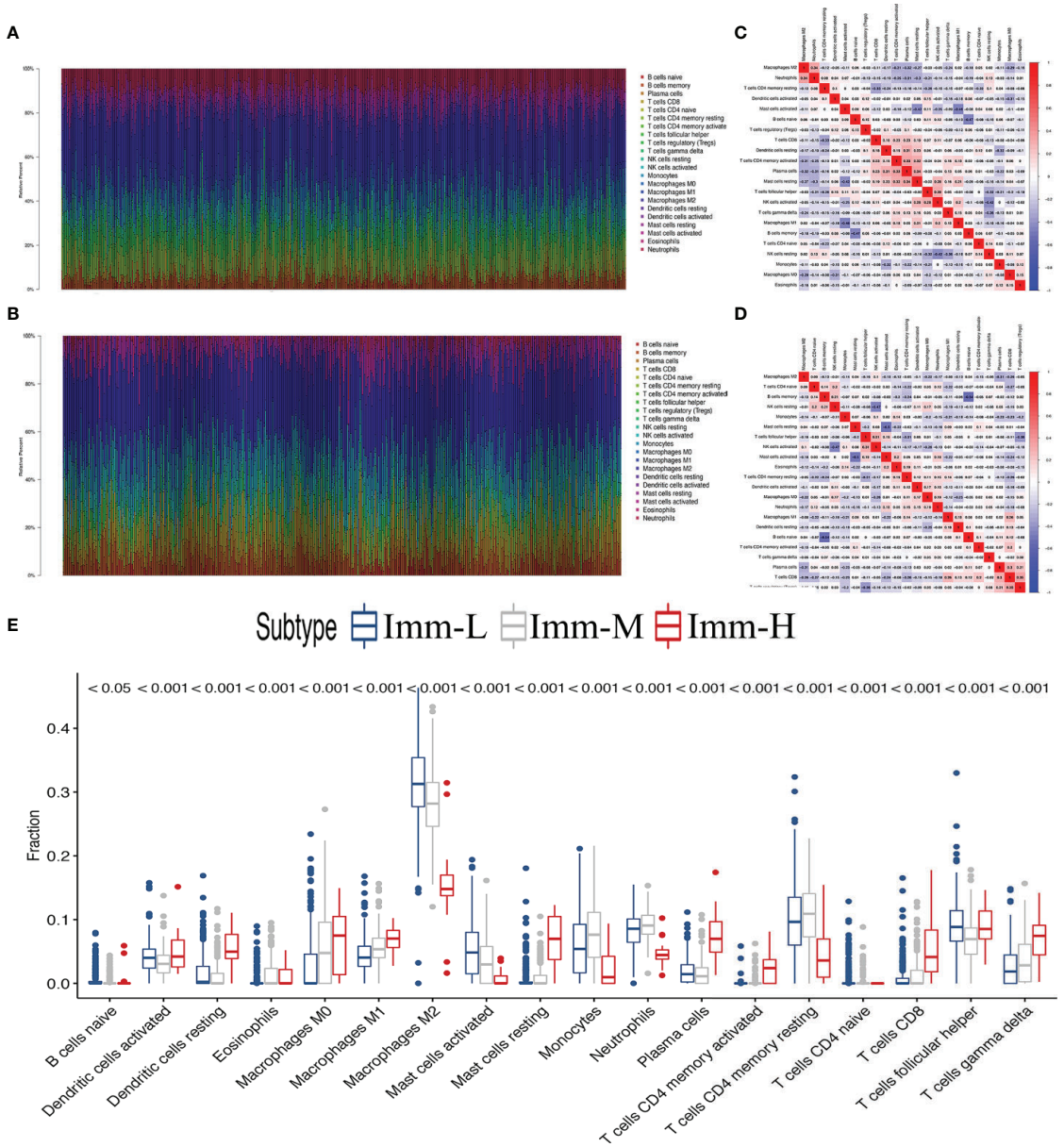

FIGURE 5 | The landscape of immune cell infiltration in the glioma microenvironment. **(A, B)** The proportions of 22 infiltrating immune cells in the glioma microenvironment in the TCGA microarray and CGGA RNA-seq cohorts respectively. **(C, D)** Correlation heatmaps of the TCGA microarray and CGGA RNA-seq cohorts respectively. **(E)** Immune cell infiltration level of glioma microenvironment among immune phenotypes in the TCGA microarray cohort based on the CIBERSORT algorithm.

clusters and enriched GO terms, and the GO chord plots show relevant GO terms for the classic PD1/PDCD1, CTLA-4, TIGIT, VISTA/VISR, and LAG-3 molecules (**Figures 7E–H**). GSVA showed enrichment discrepancies in several immune-related pathways, including antigen processing, primary immunodeficiency, the B/T cell receptor signaling pathway, NK cell cytotoxicity, and leukocyte transendothelial migration (**Figures 7I, J**). The Sankey diagram shows the links between checkpoint molecules and their correlated genes in glioma (**Figure 7K**).

Genomic Alterations, Tumor Mutation Burden, and Histological Characteristics

Compared with other immune phenotypes, immune-L was found to have a higher proportion of IDH-mutant patients (**Figures 8A, D**); the immune-H phenotype seemed to have a higher proportion of recurrent glioma but a lower rate of primary patients (**Figures 8B, E**); more GBM samples existed in immune-H, and more LGG was associated with the immune-L phenotype (**Figures 8C, F**). Detailed data are presented in **Table 2**.

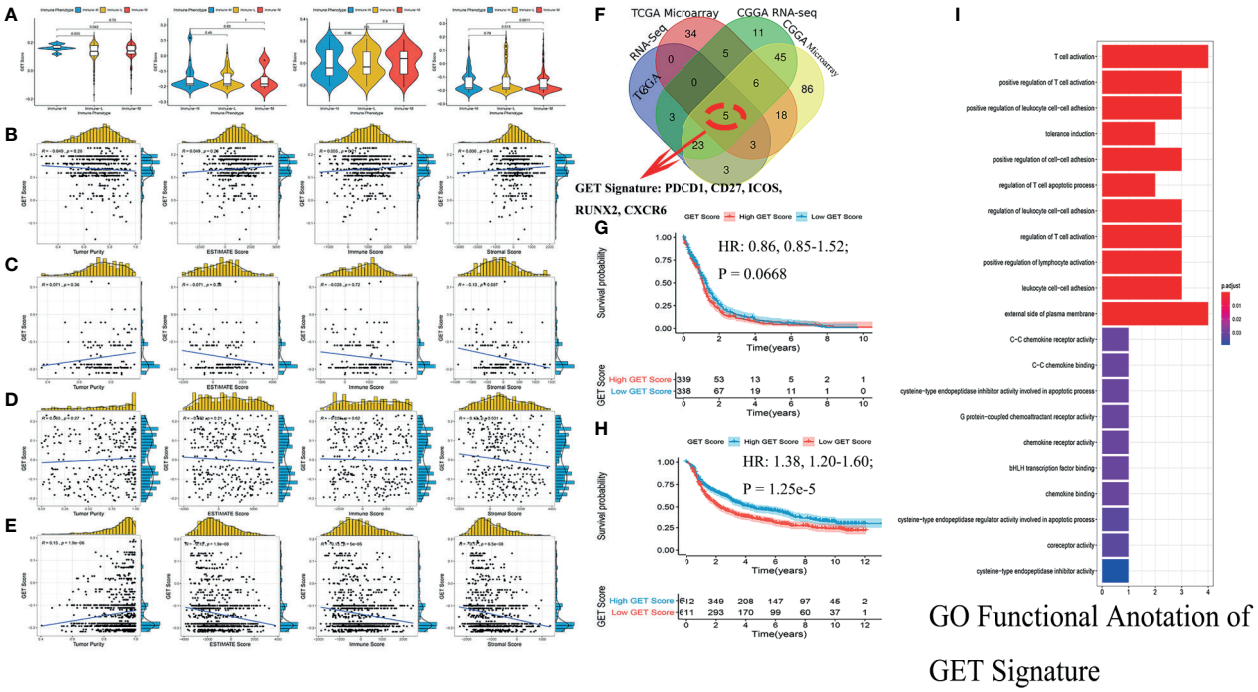

FIGURE 6 | Detection of a gene expression signature of exhausted CD8+ T cells in glioma. **(A)** Comparisons of GET Score among classified immune phenotypes in TCGA microarray, TCGA RNA-Seq, CGGA microarray, CGGA RNA-Seq four cohorts. **(B–E)** The correlation between GET Score and Tumor Purity, ESTIMATE Score, Immune Score and Stromal Score in above four cohorts respectively. **(F)** Venn diagram exhibited the five selected genes termed as GET Signature (PDCD1, CD27, ICOS, RUNX2, CXCR6). **(G, H)** Comparison of the prognosis of high GET Score and low GET Score group in total TCGA and CGGA datasets. The cut-off value was defined as the median GET Score of all involved samples. **(I)** Functional enrichment of GO terms relating to the GET Signature.

Surprisingly, no obviously significant discrepancies in TMB were found between the immune-H and immune-L phenotypes in the TCGA microarray cohort (P = 0.047, median $\log_2$(TMB), 0.385 vs. 0.464) and TCGA RNA-seq cohort (P = 0.100, median $\log_2$ (TMB), 0.357 vs. 0.447) (**Figures 8G, H**, **Supplementary Online File 7**).

We analyzed the distribution differences of somatic mutations and SNPs among the immune phenotypes using data from the TCGA project. **Figures 9A, B** displays recurrent SNP sites (N > 5) in chromosome models in LGG and GBM. Sites marked by orange and red are high-mutant SNP sites, while those marked by navy and green are low-mutant SNP sites. Major mutant genes and mutation types were different among immune phenotypes in combination with glioma grade (**Figures 9C, D**). In addition, GBM presented more extensive TMB than LGG, with details in the left bar plots and scatter plots in **Figure 9E**.

Phenotypes Predicting Response to Antitumor Drugs and Peritumor Edema

Chemotherapy and targeted therapy are both standard treatments for glioma. The response to commonly used antitumor drugs was evaluated among three immune phenotypes. The expected model using the GDSC dataset was trained by ridge regression, and the level of prediction accuracy was evaluated by 10-fold cross-validation. The treatment-related IC50 for each tumor sample in TCGA was properly estimated based on a predictive model of these drugs. There were significant differences in the response to several drugs, and the immune-L phenotype was more sensitive to bortezomib (K-W P < 2.2e-16), cisplatin (P = 5.3e-15), docetaxel (P < 2.2e-16), lapatinib (P < 2.2e-16), and rapamycin (P = 3.3e-8); however, the immune-H phenotype was more sensitive to paclitaxel (P = 3.1e-10) and sorafenib (P = 0.0053) (**Figure 10A**).

As a marker of inflammation, edema is a common pathophysiological entity surrounding gliomas. Herein, we compared the edema differences between the immune-L and immune-H phenotypes to assess the correlations. It was noted that immune-H phenotype gliomas displayed more severe edema than immune-L phenotype gliomas (**Figure 10B**). The present results suggested that peritumoral edema is also a probable marker to reflect the variations between immune phenotypes. The analysis process used in this study is shown as a flow chart in **Figure 11**.

DISCUSSION

Immunotherapy has been confirmed to be effective in other types of cancers except glioma, as glioma features a relatively immune-privileged microenvironment. With the aim of elucidating the immunosuppressive mechanism, in this research, we enrolled

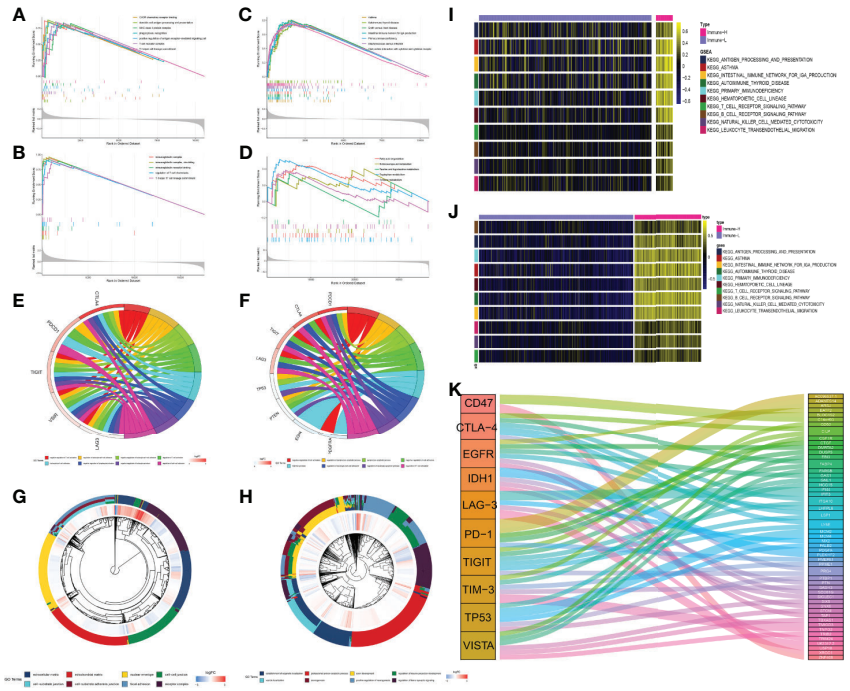

FIGURE 7 | Comprehensive functional analysis relating to the immune phenotypes. **(A, B)** GSEA of GO terms of metagenes co-expressed in the immune-H and immune-L phenotypes in the TCGA microarray and CGGA RNA-seq cohorts. **(C, D)** GSEA of pathways of metagenes co-expressed in the immune-H and immune-L phenotypes in the TCGA microarray and CGGA RNA-seq cohorts. **(E–H)** GO chord plots showing correlation and clusters of PDCD1, CTLA-4, TIGIT, LAG3, TP53, VSIR, PTEN, EGFR, PDGFRA checkpoints. **(I, J)** Variants in pathway categories demonstrated by GSVA relating to immune-H and immune-L phenotypes in TCGA microarray and CGGA RNA-seq cohorts. **(K)** The Sankey diagram showed multiple correlations between CD47, CTLA-4, EGFR, IDH1, LAG-3, PD-1, TIGIT, TIM-3, TP53, VISTA and their top-ranked correlated genes in glioma.

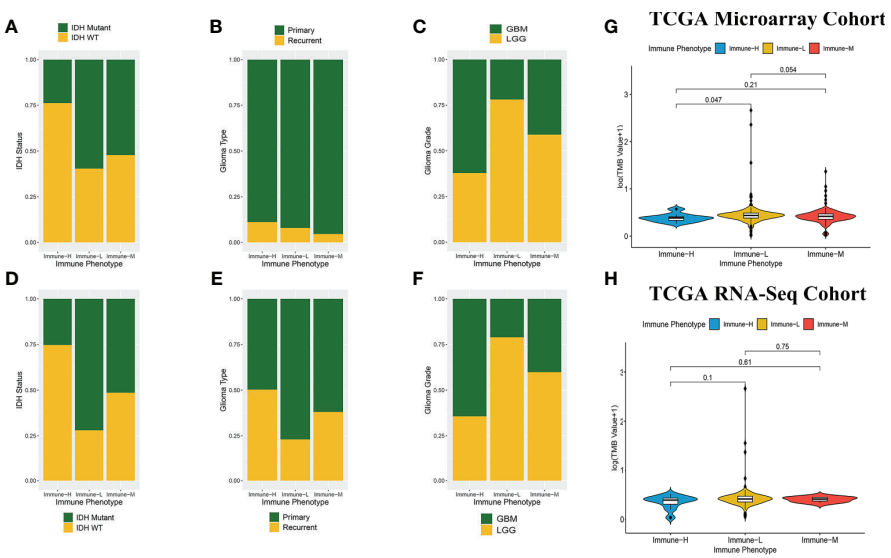

FIGURE 8 | Comparison on IDH status, glioma type, grade and tumor mutation burden among immune phenotypes. **(A)** Proportion of IDH-mutant and IDH-wild type glioma in three phenotypes in CGGA microarray cohort. **(B)** Proportion of primary and recurrent glioma in three phenotypes in CGGA microarray cohort. **(C)** Proportion of LGG and GBM in three phenotypes in CGGA microarray cohort. **(D)** Proportion of IDH-mutant and IDH-wild type glioma in three phenotypes in CGGA RNA-seq cohort. **(E)** Proportion of primary and recurrent glioma in three phenotypes in CGGA RNA-seq cohort. **(F)** Proportion of LGG and GBM in three phenotypes in CGGA RNA-seq cohort. **(G)** Violin plot showing comparison of TMB based on immune-phenotypes in TCGA microarray cohort. **(H)** Violin plot showing comparison of TMB based on immune-phenotypes in TCGA RNA-seq cohort. LGG, low grade glioma; GBM, glioblastoma.

TABLE 2 | Distribution of IDH status, type and grade of glioma among immune phenotypes in CGGA dataset.

		Immune-L Phenotype		Immune-M Phenotype		Immune-H Phenotype		Chi-square test [1]
CGGA RNA-seq cohort	IDH Status	IDH MT(%)	280 (72.0)	IDH MT(%)	203 (51.4)	IDH MT(%)	45 (25.3)	$\chi2 = 110.855$; $P < 0.001$
		IDH WT (%)	109 (28.0)	IDH WT (%)	192 (48.6)	IDH WT (%)	133 (74.7)	
	Glioma Type	Primary (%)	314 (77.0)	Primary (%)	249 (61.9)	Primary (%)	85 (49.7)	$\chi2 = 45.058$; $P < 0.001$
		Recurrent (%)	94 (23.0)	Recurrent (%)	153 (38.1)	Recurrent (%)	86 (50.3)	
	Glioma Grade	LGG (%)	322 (78.9)	LGG (%)	240 (59.9)	LGG (%)	61 (35.7)	$\chi2 = 101.384$; $P < 0.001$
		GBM (%)	86 (21.1)	GBM (%)	161 (40.1)	GBM (%)	110 (64.3)	
CGGA microarray cohort	IDH Status	IDH MT(%)	62 (59.6)	IDH MT(%)	47 (52.2)	IDH MT(%)	25 (23.8)	$\chi2 = 29.941$; $P < 0.001$
		IDH WT (%)	42 (40.4)	IDH WT (%)	43 (47.8)	IDH WT (%)	80 (76.2)	
	Glioma Type	Primary (%)	92 (91.1)	Primary (%)	83 (95.4)	Primary (%)	88 (88.9)	$\chi2 = 2.625$; $P = 0.269$
		Recurrent (%)	9 (8.9)	Recurrent (%)	4 (4.6)	Recurrent (%)	11 (11.1)	
	Glioma Grade	LGG (%)	82 (78.1)	LGG (%)	53 (58.9)	LGG (%)	39 (37.9)	$\chi2 = 34.592$; $P < 0.001$
		GBM (%)	23 (21.9)	GBM (%)	38 (42.2)	GBM (%)	64 (62.1)	

[1]Chi-square test was conducted to compare these differences between immune phenotypes.
IDH MT, IDH Mutant; IDH WT, IDH Wild Type; LGG, low grade glioma; GBM, glioblastoma.

2,466 glioma samples from 6 datasets and classified these samples into 3 immune phenotypes with distinct immunogenetic features. The immune-H phenotype has higher immune cell lineage infiltration and higher ESTIMATE, immune and stromal scores than the immune-L and immune-M phenotypes. Most HLA family genes and checkpoint molecules were significantly highly expressed in the immune-H phenotype; otherwise, some specific genes were highly expressed. Overall, patients with the immune-H phenotype will have a poor prognosis compared with those with the immune-L phenotypes, but this result was limited by the sample size. A five-gene GET signature including PDCD1, CD27, ICOS, RUNX2, and CXCR6 was established, and no significant differences in the GET score between immune phenotypes were observed. Patients with a high GET score seemed to have a better prognosis. A response difference was noticed among the phenotypes to several antitumor drugs. Immune-H was observed to have more severe peritumor edema than immune-L in representative T2 images.

Survival differences among the classified immune phenotypes of glioma were in contrast to those of some other cancer types reported previously, such as triple-negative breast cancer (38), gastric cancer (39), and head and neck squamous cancer (40). A potential reason is that the inflammatory microenvironment upregulated the tumor progressive nature and deteriorated glioma invasion and development (41, 42). The success of immunomodulatory therapy is widespread among diverse cancer types, which stimulates our interest in characterizing TME immune cell infiltration in glioma. The immune-H phenotype may be involved in immunosuppressive activities, including immunosuppressive checkpoints (**Table 3**), expression of tumor-supportive macrophage chemotactic and polarizing molecules and immune-suppressive pathway signaling (the IL-10 signaling pathway). The IL-10 pathway downregulates DC activation and IL-12 production and inhibits the cytotoxic T cell response during chemotherapy. Macrophage activation is also suppressed by IL-10 to inhibit the immune response (43). Importantly, there is large heterogeneity in the TME of different glioma genetic subtypes, and enriched tumor-associated macrophages (TAMs) participate in the promotion of glioma invasion, angiogenesis, tumor metastasis and immune suppression through intracellular and extracellular mediators (44). Glioma with IDH mutation status was shown to have low levels of infiltrating T cells and a higher ratio of TAMs derived from microglia (45). Although TAMs have distinct genetic profiles involving canonical M1 (antitumorigenic) and canonical M2 (protumorigenic) polarization, they show increased anti-PD-1 resistance-associated genes and predict poor survival (46, 47). Additionally, immunosuppressive chemokines/cytokines in the TME released by the tumor itself, such as through the TGF-β pathway, also block antitumor immunity activation (48). TIM-3 (T cell immunoglobulin mucin receptor 3) has an immunosuppressive effect in glioma, which may be due to the unique presence of TIM-3+ Tregs in tumor tissue (49). Furthermore, TIM-3 does not contain any immunoreceptor tyrosine-based inhibition motifs (ITIMs), which are necessary for avoiding major deficiencies in immunotherapy (50). VISTA (V-type immunoglobulin domain-containing suppressor of T cell activation) is a newly found checkpoint that restricts T cell activation by shaping the naive CD4+ T cell compartment (51). Therapeutics targeting VISTA curb the development of graft-versus-host disease and promote the death of naive CD4+ T cells; thus, VISTA can be regarded as a distinctive immunotherapy molecule (51, 52). Indeed, growing evidence suggests that dysfunctional CD8+ T cells incorporate heterogeneous subpopulations such as progenitor and terminally exhausted cells, and discrete functions in immunotherapy or the microenvironment need to be better elucidated (53). Clinical trials regarding Checkmate 143 (NCT02017717), Checkmate 498 (NCT02617589), and Checkmate 548 (NCT02667587) did not suggest a profound survival benefit from immunotherapy in glioma/GBM, with only some clinical advantages reported in some case reports; indeed, GBM typically has a relatively low mutational load and a paucity of T cell infiltration compared with other cancers (12, 54).

Similar to other studies, Chen and his colleagues (55) used ssGSEA to identify the immune microenvironment of glioma, and they did not classify glioma samples into immune phenotypes or detect the corresponding microenvironmental features of the phenotypes; however, they detected eight glioma microenvironment-associated genes, CCDC109B, EMP3, ANXA2,

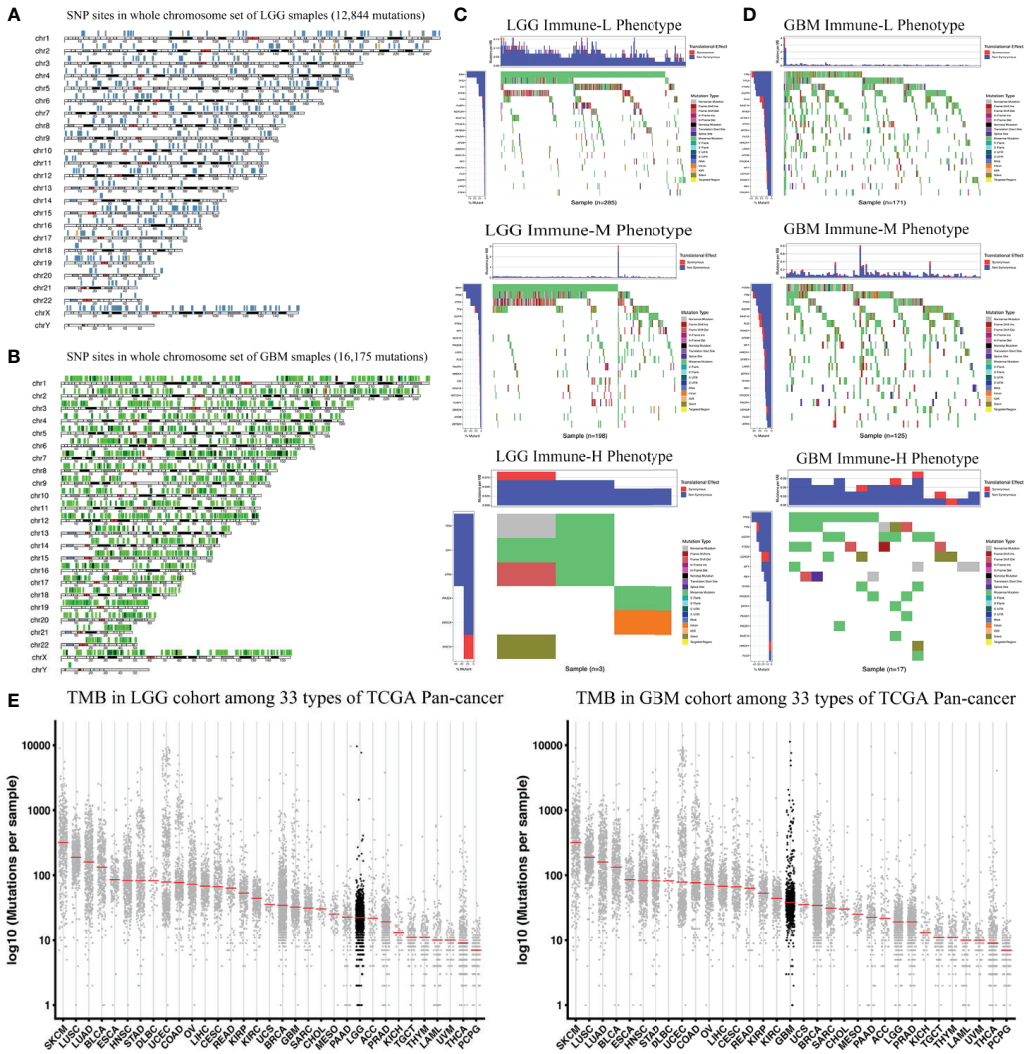

FIGURE 9 | Waterfall plots of genomic alternations associated with glioma immune phenotypes. **(A, B)** Recurrent SNP sites of LGG and GBM in chromosome models. Red and orange marked high-mutant SNP, navy and green marked low-mutant SNP. **(C)** The waterfall plots summarize the genomic alternations including somatic mutations and single nucleotide polymorphism in LGG of immune-L, immune-M and immune-H phenotypes respectively. **(D)** The waterfall plots summarize the genomic alternations in GBM of immune-L, immune-M and immune-H phenotypes respectively. **(E)** Scatter plots show tumor mutation burden of LGG and GBM among 33 types of Pan-cancer respectively. LGG, low grade glioma; GBM, glioblastoma.

CLIC1, TIMP1, VIM, LGALS1, and RBMS1, and constructed a prognostic model with them through integrative omics data points. They validated the immunosuppression of LGALS1 in *in vitro* experiments. Our findings based on large genomic data help characterize the glioma microenvironment and understand tumor immune complexity. The ESTIMATE, immune, stromal, and tumor purity scores can be used properly in both basic and translational medicine to help identify glioma subtypes. Work investigating the immunosuppressive mechanisms of glioma implies that microenvironments lacking T cells feature immunosuppressive biological processes carried out by a series of immune cells; more knowledge of immune cell infiltration will inform strategies to remodel the immunosuppressive microenvironment and will aid the identification of more therapeutic targets.

Patients with the immune-H phenotype were more prone to developing a poor prognosis compared with others; thus, we may properly predict the prognosis of glioma patients with immune phenotypes. Our findings also suggest that immunotherapy will be effective in immune-H patients, who are more sensitive to checkpoint-related immunotherapy (56). Recent evidence showed that samples with high TMB could exhibit a durable response to PD-1/PD-L1 immunotherapy (57), and current findings indirectly confirmed the value of TMB in predicting immunotherapeutic outcomes of established immune phenotypes. Translational research indicated that a high TMB status may yield a long-term response and durable survival benefit (58). The presented results provide a novel perspective on immune signatures in the genetic TMB, the microenvironment and roles in immune checkpoint

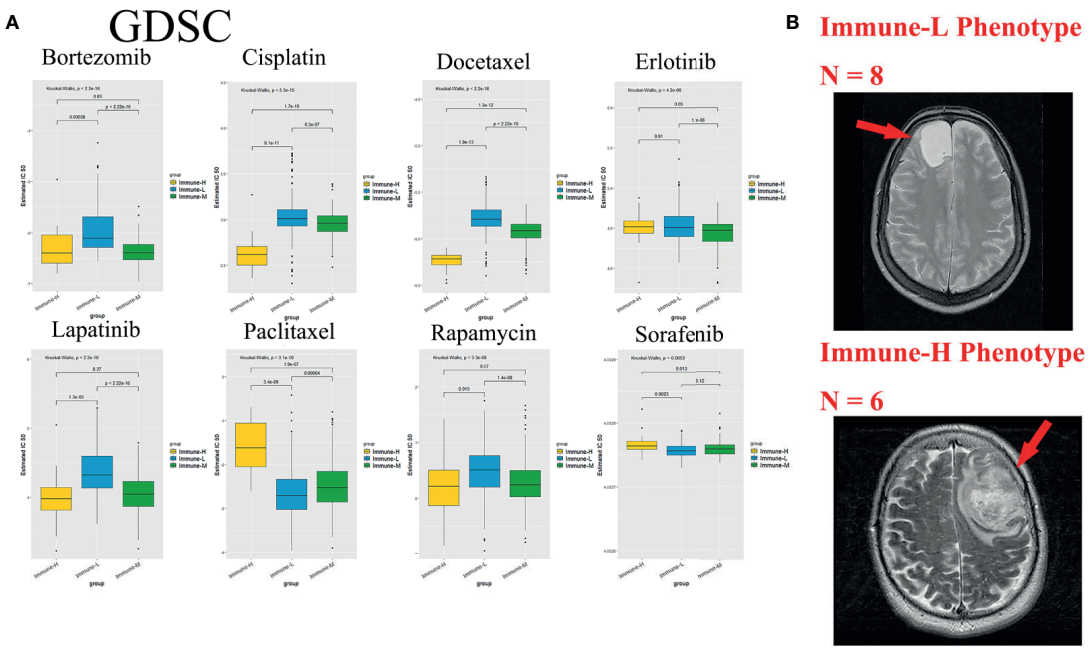

FIGURE 10 | Role of phenotype in predicting anti-tumor drugs response and peri-tumoral edema. **(A)** The immune-L phenotype was more sensitive to bortezomib (P < 2.2e-16), cisplatin (P = 5.3e-15), docetaxel (P < 2.2e-16), lapatinib (P < 2.2e-16), rapamycin (P = 3.3e-8); the immune-H phenotype was more sensitive to paclitaxel (P = 3.1e-10) and sorafenib (P = 0.0053). **(B)** Representative images of the differences in the extent of peri-tumoral edema in TCGA cohort patients. Immune-H phenotype significantly possessed more-severe edema than immune-L.

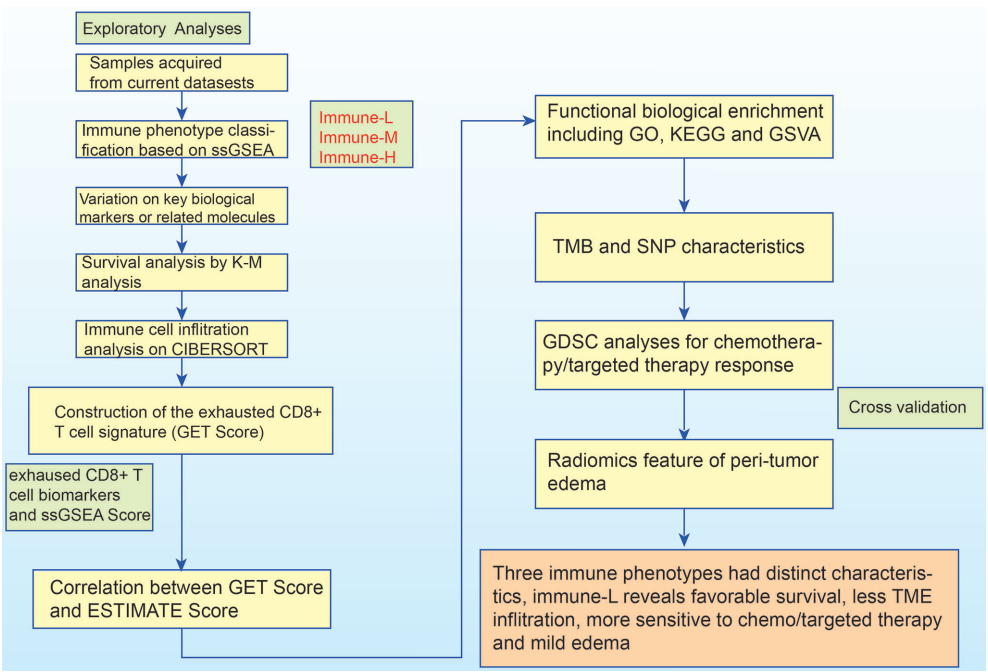

FIGURE 11 | The logic flow chart of current study.

TABLE 3 | Summary of the molecular and biological functions of T cell costimulatory molecules.

Molecular marker	Aliase(s)	Ligand(s)	Receptor expression pattern	Biological function	Molecular function
Coinhibitory					
PD-1	PDCD1, CD279, SLEB2, hPD-1	PD-L1, PD-L2	Activated T cells, NK cells, NKT cells, B cells, macrophages, subsets of DCs	Negative T cells costimulation (primarily in periphery), attenuate peripheral activity, preserve T-cell function in the context of chronic antigen	Inhibition of proximal TCR signaling, attenuate CD28 signaling
CTLA-4	CD152, ALPS5, CELIAC3, GRD4	B7-1 (CD80), B7-2 (CD86)	Activated T cells, Tregs	Negative T-cell costimulation (primarily at priming); prevent tonic signaling, attenuate high-affinity clones	Competitive inhibition of CD28 costimulation (binding to B7-1 and B7-2)
PD-L1	CD274, PDCD1L1, B7-H, B7H1	PD-1, B7-1 (CD80)	Monocytes, macrophages, mast cells, inducible in DCs, T cells, B cells, NK cells	Attenuate T cells activity in inflamed peripheral tissues	PD-1 ligation; cell-intrinsic mechanism unclear
LAG-3	CD223, Ly66	MHC-II, LSECtin	Activated CD4+ and CD8+ T cells, NK cells, Tregs	Negative regulator of T cells expansion; control T cells homeostasis; DCs activation	Competitive binding to MHC-II; proximal LSECtin mechanism unclear
TIM-3	HAVCR2, CD366, KIM-3, SPTCL, TIMD-3	Galectin-9, PtdSer, HMGB1, CEACAM-1	Th1 CD4+ and Tc1 CD8+, Tregs, DCs, NK cells, monocytes	Negative regulation of Type immunity; preserve peripheral tolerance	Negative regulation of proximal TCR components; differences between ligands unknown
TIGIT	VSIG9, VSTM3, WUCAM	PVR (CD155), PVRL2 (CD112)	CD4+ and CD8+ T cells, Tregs, TFH, NK cells	Inhibition of T cells activity; DC tolerization	Competitive inhibition of DNAM1 (CD226) costimulation (binding of PVR), binding of DNAM1 in cis; cell-intrinsic ITIM-negative signaling
VISTA	VSIR, B7-H5, B7H5, C10orf54, PD-1H	Counterreceptor unknown	T cells and activated Tregs, myeloid cells, mature APCs	Negative regulation of T cells activity; suppression of CD4+ T cells, shaping naive CD4+ T cells compartment	Increase threshold for TCR signaling, induce FOXP3 synthesis; proximal signaling unknown
Costimulatory					
ICOS	AILIM, CCLP, CRP-1	ICOSL	Activated T cells, B cells, ILC2	Positive costimulation; Type I and II immunity; Tregs maintenance; TFH differentiation	p50 PI3K recruitment (AKT signaling); enhance calcium signaling (PLCγ)
OX40	TNFRSF4, ACT35, CD134, TXGP1L	OX40L	Activated T cells, Tregs, NK cells, NKT cells, neutrophils	Sustain and enhance CD4+ T cell immunity; role in CD8+ T cells and Tregs	Regulation of BCL2/XL (survival); enhance PI3K/AKT signaling
GITR	TNFRSF18, AITR, CD357, ENERGEN, GITR	GITRL	Activated T cells, Tregs, B cells, NK cells, macrophages	Attenuate Tregs; costimulation of activated T cells, NK cell activation	Signal through TRAF5
CD137	TNFRSF9, 4-1BB, CDw137, ILA	4-1BBL (CD137L)	Activated T cells, Tregs, NK cells, monocytes, DCs, B cells	Positive T cells costimulation; DC activation	Signal through TRAF1, TRAF2
CD40	TNFRSF5, Bp50, CDW40, p50	CD40L	APCs, B cells, monocytes, non hematopoietic cells (e.g., fibroblasts, endothelial cells)	APC licensing	Signal through TRAF2, 3, 5, 6; TRAF-independent mechanisms unclear
CD27	TNFRSF7, S152, LPFS2, Tp55	CD70	CD4+ and CD8+ T cells, B cells, NK cells	Lymphocyte and NK cell costimulation; generation of T-cell memory	Signal through TRAF2, TRAF5

A summary of the ligands, immune-related expression pattern, biological function, and molecular mechanisms is reviewed for selected costimulatory and coinhibitory receptors. Molecular functions (i.e., downstream signaling) reflect predominant currently known mechanisms, but additional mechanisms are likely to contribute significantly.
NK, natural killer; NKT, natural killer T cell; TFH, T follicular helper; TRAF, tumor necrosis factor receptor–associated factors; DC, dendritic cell.

blockade treatment and inspired the exploration of fresh neoepitopes. Immune phenotype classification highlights the importance of individualized treatments and provides potential methods to be used in further clinical trials related to glioma immunotherapy. We believe that with the current Pan-Cancer Analysis of Whole Genomes (PCAWG) project involving classic glioma microenvironment biomarkers (i.e., IDH1), researchers will identify more specialized features of cancer immune genomes (59).

CONCLUSIONS

Glioma samples can potentially be classified into "immune-H", "immune-M" and "immune-L" phenotypes, which exhibit distinct immunogenetic features. The immune-H phenotype is associated with higher ESTIMATE, immune and stromal scores but poorer survival than the immune-L phenotype. HLA and checkpoint family genes are relatively highly expressed in patients with the

immune-H phenotype. The GET signature cannot effectively reveal the discrepancies among immune phenotypes, and aggressive peritumor edema was displayed in immune-H compared with immune-L phenotypes. Our immunogenetic pipeline characterizes the glioma microenvironment and properly identifies patients who are more sensitive to chemo/targeted therapy and are likely to have better survival. These results possibly facilitate new therapeutic development and advance precision oncology, limited by the observational nature, the experimental profile should be highlighted in the future.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

All authors designed and conducted this review. BZ, YKW, and YaW, wrote the paper. YKW helped the study design. CD and YuW revised the statistical methodology. YuW and WM had primary responsibility for the final content. All authors contributed to the article and approved the submitted version. Notably, YuW and WM equally share the corresponding authorship.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.557994/full#supplementary-material

Supplementary Figure 1 | GO bubble plots of metagenes in Immune-H over immune-L phenotypes. **(A–C)** Bubble plots for TCGA microarray, TCGA GBM RNA-seq, CGGA RNA-seq databases.

REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer Statistics, 2020. *CA Cancer J Clin* (2020) 70(1):7–30. doi: 10.3322/caac.21590
2. Jiang T, Mao Y, Ma W, Mao Q, You Y, Yang X, et al. CGCG Clinical Practice Guidelines for the Management of Adult Diffuse Gliomas. *Cancer Lett* (2016) 375(2):263–73. doi: 10.1016/j.canlet.2016.01.024
3. Louveau A, Smirnov I, Keyes TJ, Eccles JD, Rouhani SJ, Peske JD, et al. Structural and Functional Features of Central Nervous System Lymphatic Vessels. *Nature* (2015) 523(7560):337–41. doi: 10.1038/nature14432
4. Saha D, Martuza RL, Rabkin SD. Macrophage Polarization Contributes to Glioblastoma Eradication by Combination Immunovirotherapy and Immune Checkpoint Blockade. *Cancer Cell* (2017) 32(2):253–67.e255. doi: 10.1016/j.ccell.2017.07.006
5. Li G, Wang Z, Zhang C, Liu X, Cai J, Wang Z, et al. Molecular and Clinical Characterization of TIM-3 in Glioma Through 1,024 Samples. *Oncoimmunology* (2017) 6(8):e1328339. doi: 10.1080/2162402X.2017.1328339
6. Kravchenko J, Corsini E, Williams MA, Decker W, Manjili MH, Otsuki T, et al. Chemical Compounds From Anthropogenic Environment and Immune Evasion Mechanisms: Potential Interactions. *Carcinogenesis* (2015) 36(Suppl 1):S111–127. doi: 10.1093/carcin/bgv033
7. Wang Z, Zhang C, Liu X, Wang Z, Sun L, Li G, et al. Molecular and Clinical Characterization of PD-L1 Expression at Transcriptional Level Via 976 Samples of Brain Glioma. *Oncoimmunology* (2016) 5(11):e1196310. doi: 10.1080/2162402X.2016.1196310
8. Cai J, Chen Q, Cui Y, Dong J, Chen M, Wu P, et al. Immune Heterogeneity and Clinicopathologic Characterization of IGFBP2 in 2447 Glioma Samples. *Oncoimmunology* (2018) 7(5):e1426516. doi: 10.1080/2162402X.2018.1426516
9. Eckel-Passow JE, Lachance DH, Molinaro AM, Walsh KM, Decker PA, Sicotte H, et al. Glioma Groups Based on 1p/19q, IDH, and TERT Promoter Mutations in Tumors. *N Engl J Med* (2015) 372(26):2499–508. doi: 10.1056/NEJMoa1407279
10. Verhaak RG, Hoadley KA, Purdom E, Wang V, Qi Y, Wilkerson MD, et al. Integrated Genomic Analysis Identifies Clinically Relevant Subtypes of Glioblastoma Characterized by Abnormalities in PDGFRA, Idh1, EGFR, and NF1. *Cancer Cell* (2010) 17(1):98–110. doi: 10.1016/j.ccr.2009.12.020
11. Meng X, Duan C, Pang H, Chen Q, Han B, Zha C, et al. DNA Damage Repair Alterations Modulate M2 Polarization of Microglia to Remodel the Tumor Microenvironment Via the P53-Mediated MDK Expression in Glioma. *EBioMedicine* (2019) 41:185–99. doi: 10.1016/j.ebiom.2019.01.067
12. Lim M, Xia Y, Bettegowda C, Weller M. Current State of Immunotherapy for Glioblastoma. *Nat Rev Clin Oncol* (2018) 15(7):422–42. doi: 10.1038/s41571-018-0003-5
13. McLendon R, Friedman A, Bigner D, Friedman A, Bigner D, Van Meir EG, et al. Comprehensive Genomic Characterization Defines Human Glioblastoma Genes and Core Pathways. *Nature* (2008) 455(7216):1061–8. doi: 10.1038/nature07385
14. Wiendl H, Mitsdoerffer M, Hofmeister V, Wischhusen J, Bornemann A, Meyermann R, et al. A Functional Role of HLA-G Expression in Human Gliomas: An Alternative Strategy of Immune Escape. *J Immunol* (2002) 168 (9):4772–80. doi: 10.4049/jimmunol.168.9.4772
15. Didenko VV, Ngo HN, Minchew C, Baskin DS. Apoptosis of T Lymphocytes Invading Glioblastomas Multiforme: A Possible Tumor Defense Mechanism. *J Neurosurg* (2002) 96(3):580–4. doi: 10.3171/jns.2002.96.3.0580
16. Parsa AT, Waldron JS, Panner A, Crane CA, Parney IF, Barry JJ, et al. Loss of Tumor Suppressor PTEN Function Increases B7-H1 Expression and Immunoresistance in Glioma. *Nat Med* (2007) 13(1):84–8. doi: 10.1038/nm1517
17. Wherry EJ. T Cell Exhaustion. *Nat Immunol* (2011) 12(6):492–9. doi: 10.1038/ni.2035
18. Greter M, Heppner FL, Lemos MP, Odermatt BM, Goebels N, Laufer T, et al. Dendritic Cells Permit Immune Invasion of the CNS in an Animal Model of Multiple Sclerosis. *Nat Med* (2005) 11(3):328–34. doi: 10.1038/nm1197

19. Yoshihara K, Shahmoradgoli M, Martinez E, Vegesna R, Kim H, Torres-Garcia W, et al. Inferring Tumour Purity and Stromal and Immune Cell Admixture From Expression Data. *Nat Commun* (2013) 4:2612. doi: 10.1038/ncomms3612
20. Verhaak RG, Tamayo P, Yang JY, Hubbard D, Zhang H, Creighton CJ, et al. Prognostically Relevant Gene Signatures of High-Grade Serous Ovarian Carcinoma. *J Clin Invest* (2013) 123(1):517–25. doi: 10.1172/JCI65833
21. Yang J, Zhang D, Frangi AF, Yang JY. Two-Dimensional PCA: A New Approach to Appearance-Based Face Representation and Recognition. *IEEE Trans Pattern Anal Mach intell* (2004) 26(1):131–7. doi: 10.1109/TPAMI.2004.1261097
22. Mayakonda A, Lin DC, Assenov Y, Plass C, Koeffler HP. Maftools: Efficient and Comprehensive Analysis of Somatic Variants in Cancer. *Genome Res* (2018) 28(11):1747–56. doi: 10.1101/gr.239244.118
23. Newman AM, Liu CL, Green MR, Gentles AJ, Feng W, Xu Y, et al. Robust Enumeration of Cell Subsets From Tissue Expression Profiles. *Nat Methods* (2015) 12(5):453–7. doi: 10.1038/nmeth.3337
24. Thommen DS, Koelzer VH, Herzig P, Roller A, Trefny M, Dimeloe S, et al. A Transcriptionally and Functionally Distinct PD-1(+) Cd8(+) T Cell Pool With Predictive Potential in non-Small-Cell Lung Cancer Treated With PD-1 Blockade. *Nat Med* (2018) 24(7):994–1004. doi: 10.1038/s41591-018-0057-z
25. Cai MC, Zhao X, Cao M, Ma P, Chen M, Wu J, et al. T-Cell Exhaustion Interrelates With Immune Cytolytic Activity to Shape the Inflamed Tumor Microenvironment. *J pathol* (2020) 251(2):147–59. doi: 10.1002/path.5435
26. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene Set Enrichment Analysis: A Knowledge-Based Approach for Interpreting Genome-Wide Expression Profiles. *Proc Natl Acad Sci USA* (2005) 102(43):15545–50. doi: 10.1073/pnas.0506580102
27. Hänzelmann S, Castelo R, Guinney J. Gsva: Gene Set Variation Analysis for Microarray and RNA-Seq Data. *BMC Bioinf* (2013) 14(1):7. doi: 10.1186/1471-2105-14-7
28. Gu Z, Lei G, Roland E, Matthias S, Benedikt B. N Circlize\N Implements and Enhances Circular Visualization in R\N. *Bioinformatics* (2014) 19:19. doi: 10.1093/bioinformatics/btu393
29. Krzywinski M, Schein J, Birol I, Connors J, Gascoyne R, Horsman D, et al. Circos: An Information Aesthetic for Comparative Genomics. *Genome Res* (2009) 19(9):1639–45. doi: 10.1101/gr.092759.109
30. Riehmann P, Hanfler M, Froehlich B. Interactive Sankey Diagrams. Paper Presented at: IEEE Symposium on Information Visualization, 2005. *INFOVIS 2005* (2005) 2005.
31. Yang W, Soares J, Greninger P, Edelman EJ, Lightfoot H, Forbes S, et al. Genomics of Drug Sensitivity in Cancer (GDSC): A Resource for Therapeutic Biomarker Discovery in Cancer Cells. *Nucleic Acids Res* (2013) 41(Database issue):D955–961. doi: 10.1093/nar/gks1111
32. Geeleher P, Cox NJ, Huang RS. Clinical Drug Response can be Predicted Using Baseline Gene Expression Levels and In Vitro Drug Sensitivity in Cell Lines. *Genome Biol* (2014) 15(3):R47. doi: 10.1186/gb-2014-15-3-r47
33. Iorio F, Knijnenburg TA, Vis DJ, Bignell GR, Menden MP, Schubert M, et al. A Landscape of Pharmacogenomic Interactions in Cancer. *Cell* (2016) 166(3):740–54. doi: 10.1016/j.cell.2016.06.017
34. Pope WB, Sayre J, Perlina A, Villablanca JP, Mischel PS, Cloughesy TF. MR Imaging Correlates of Survival in Patients With High-Grade Gliomas. *AJNR Am J neuroradiol* (2005) 26(10):2466–74.
35. Bruzzone MG, Eoli M, Cuccarini V, Grisoli M, Valletta L, Finocchiaro G. Genetic Signature of Adult Gliomas and Correlation With MRI Features. *Expert Rev Mol diagn* (2009) 9(7):709–20. doi: 10.1586/erm.09.44
36. Carrillo JA, Lai A, Nghiemphu PL, Phillips HS, Kharbanda S, Moftakhar P, et al. Relationship Between Tumor Enhancement, Edema, IDH1 Mutational Status, MGMT Promoter Methylation, and Survival in Glioblastoma. *AJNR Am J neuroradiol* (2012) 33(7):1349–55. doi: 10.3174/ajnr.A2950
37. Thorsson V, Gibbs DL, Brown SD, Wolf D, Bortone DS, Yang T-HO, et al. The Immune Landscape of Cancer. *Immunity* (2018) 48(4):812–30.e814. doi: 10.1016/j.immuni.2018.03.023
38. Liu Z, Li M, Jiang Z, Wang X. A Comprehensive Immunologic Portrait of Triple-Negative Breast Cancer. *Trans Oncol* (2018) 11(2):311–29. doi: 10.1016/j.tranon.2018.01.011
39. Jiang Z, Liu Z, Li M, Chen C, Wang X. Immunogenomics Analysis Reveals That TP53 Mutations Inhibit Tumor Immunity in Gastric Cancer. *Trans Oncol* (2018) 11(5):1171–87. doi: 10.1016/j.tranon.2018.07.012
40. Lyu H, Li M, Jiang Z, Liu Z, Wang X. Correlate the TP53 Mutation and the HRAS Mutation With Immune Signatures in Head and Neck Squamous Cell Cancer. *Comput Struct Biotechnol J* (2019) 17:1020–30. doi: 10.1016/j.csbj.2019.07.009
41. Nakashima H, Alayo QA, Penaloza-MacMaster P, Freeman GJ, Kuchroo VK, Reardon DA, et al. Modeling Tumor Immunity of Mouse Glioblastoma by Exhausted CD8(+) T Cells. *Sci Rep* (2018) 8(1):208–8. doi: 10.1038/s41598-017-18540-2
42. Pombo Antunes AR, Scheyltjens I, Duerinck J, Neyns B, Movahedi K, Van Ginderachter JA. Understanding the Glioblastoma Immune Microenvironment as Basis for the Development of New Immunotherapeutic Strategies. *eLife* (2020) 9. doi: 10.7554/eLife.52176
43. Mumm JB, Emmerich J, Zhang X, Chan I, Wu L, Mauze S, et al. Il-10 Elicits IFNgamma-dependent Tumor Immune Surveillance. *Cancer Cell* (2011) 20(6):781–96. doi: 10.1016/j.ccr.2011.11.003
44. Mantovani A, Marchesi F, Malesci A, Laghi L, Allavena P. Tumour-Associated Macrophages as Treatment Targets in Oncology. *Nat Rev Clin Oncol* (2017) 14(7):399–416. doi: 10.1038/nrclinonc.2016.217
45. Klemm F, Maas RR, Bowman RL, Kornete M, Soukup K, Nassiri S, et al. Interrogation of the Microenvironmental Landscape in Brain Tumors Reveals Disease-Specific Alterations of Immune Cells. *Cell* (2020) 181(7):1643–60.e1617. doi: 10.1016/j.cell.2020.05.007
46. Hugo W, Zaretsky JM, Sun L, Song C, Moreno BH, Hu-Lieskovan S, et al. Genomic and Transcriptomic Features of Response to Anti-PD-1 Therapy in Metastatic Melanoma. *Cell* (2016) 165(1):35–44. doi: 10.1016/j.cell.2016.02.065
47. Zha C, Meng X, Li L, Mi S, Qian D, Li Z, et al. Neutrophil Extracellular Traps Mediate the Crosstalk Between Glioma Progression and the Tumor Microenvironment Via the HMGB1/RAGE/IL-8 Axis. *Cancer Biol Med* (2020) 17(1):154–68. doi: 10.20892/j.issn.2095-3941.2019.0353
48. Rodrigues JC, Gonzalez GC, Zhang L, Ibrahim G, Kelly JJ, Gustafson MP, et al. Normal Human Monocytes Exposed to Glioma Cells Acquire Myeloid-Derived Suppressor Cell-Like Properties. *Neuro-Oncology* (2010) 12(4):351–65. doi: 10.1093/neuonc/nop023
49. Cipolletta D, Feuere M, Li A, Kamei N, Lee J, Shoelson SE, et al. Ppar-γ is a Major Driver of the Accumulation and Phenotype of Adipose Tissue T_(reg) Cells. *Nature* (2012) 486(7404):549–53. doi: 10.1038/nature11132
50. Rangachari M, Zhu C, Sakuishi K, Xiao S, Karman J, Chen A, et al. Bat3 Promotes T Cell Responses and Autoimmunity by Repressing Tim-3-mediated Cell Death and Exhaustion. *Nat Med* (2012) 18(9):1394–400. doi: 10.1038/nm.2871
51. ElTanbouly MA, Zhao Y, Nowak E, Li J, Schaafsma E, Mercier IL, et al. VISTA is a Checkpoint Regulator for Naïve T Cell Quiescence and Peripheral Tolerance. *Science* (2020) 367(6475):eaay0524. doi: 10.1126/science.aay0524
52. Topalian SL, Taube JM, Pardoll DM. Neoadjuvant Checkpoint Blockade for Cancer Immunotherapy. *Science* (2020) 367(6477):eaax0182. doi: 10.1126/science.aax0182
53. Im SJ, Hashimoto M, Gerner MY, Lee J, Kissick HT, Burger MC, et al. Defining CD8+ T Cells That Provide the Proliferative Burst After PD-1 Therapy. *Nature* (2016) 537(7620):417–21. doi: 10.1038/nature19330
54. Li B, Severson E, Pignon JC, Zhao H, Li T, Novak J, et al. Comprehensive Analyses of Tumor Immunity: Implications for Cancer Immunotherapy. *Genome Biol* (2016) 17(1):174. doi: 10.1186/s13059-016-1028-7
55. Chen Q, Han B, Meng X, Duan C, Yang C, Wu Z, et al. Immunogenomic Analysis Reveals LGALS1 Contributes to the Immune Heterogeneity and Immunosuppression in Glioma. *Int J Cancer* (2019) 145(2):517–30. doi: 10.1002/ijc.32102
56. Sanmamed MF, Chen L. A Paradigm Shift in Cancer Immunotherapy: From Enhancement to Normalization. *Cell* (2018) 175(2):313–26. doi: 10.1016/j.cell.2018.09.035
57. Jin X, Kim LJY, Wu Q, Wallace LC, Prager BC, Sanvoranart T, et al. Targeting Glioma Stem Cells Through Combined BMI1 and EZH2 Inhibition. *Nat Med* (2017) 23(11):1352–61. doi: 10.1038/nm.4415
58. Yarchoan M, Hopkins A, Jaffee EM. Tumor Mutational Burden and Response Rate to PD-1 Inhibition. *N Engl J Med* (2017) 377(25):2500–1. doi: 10.1056/NEJMc1713444

59. Campbell PJ, Getz G, Korbel JO, Stuart JM, Jennings JL, Stein LD, et al. Pan-Cancer Analysis of Whole Genomes. *Nature* (2020) 578(7793):82–93. doi: 10.1038/s41586-020-1969-6

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GLOSSARY

CNS	central nervous system
PI3K	phosphatidylinositol-3-kinases
Akt	protein-serine-threonine kinase
FAK	focal adhesion kinase
IGF	insulin like growth factor
STAT3	signal transducer and activator of transcription
HIF-1α	hypoxia inducible factor-1α
IL-6	interleukin-6
TGF-β	transforming growth factor-β
PD-1	programmed death 1
PD-L1	programmed death-ligand 1
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
IDH1	isocitrate dehydrogenase 1
MGMT	methylguanine methyltransferase
TP53	tumour protein 53
TERT	telomerase reverse transcriptase
mAb	monoclonal antibody
OS	overall survival
NSCLC	non-small cell lung cancer
APC	antigen-presenting cell
GBM	glioblastoma
LGG	low grade glioma
NK cell	natural killer cell
HLA	human leukocyte antigen
TNFRSF6	tumour necrosis factor receptor superfamily member 6
DC	dendritic cell
TCGA	The Cancer Genome Atlas
CGGA	Chinese Glioma Genome Atlas
GEO	Gene Expression Omnibus
SNP	single nucleotide polymorphism
ssGSEA	Single-sample Gene Set Enrichment Analysis
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
GSEA	Gene Set Enrichment Analysis
GSVA	Gene set variation analysis
FDR	false discover rate
PCA	principal component analysis
TMZ	temozolomide
TMB	tumor mutation burden
GET	exhausted CD8+ T cells
TAMs	tumor-associated macrophages
TIM-3	T cell immunoglobulin and mucin domain-containing protein 3
LAG-3	lymphocyte activation gene-3
TIGIT	T cell immunoreceptor with Ig and ITIM domains
VISTA	V-type immunoglobulin domain-containing suppressor of T cell activation
TAM	Tumour-associated macrophages
CHI3L1	Chitinase-3-like protein 1
IL-13Ra2	interleukin-13 receptor α2 chain
VEGFR	Vascular Endothelial Growth Factor Receptor
VEGFA	vascular endothelial growth factor A
PCAWG	Pan-Cancer Analysis of Whole Genomes

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