Trends in Pharmacological Sciences

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Review

Immunotherapy for Malignant Glioma: Current Status and Future Directions

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Glioma is the most common intracranial primary malignancy, with limited treatment options and a poor overall survival (OS). Immunotherapy has been used successfully in various cancers, leading to the development of similar therapies that activate the patient's immune system to eliminate glioma. In this review, we introduce the diverse immunotherapeutic approaches available for treating glioma, highlighting the successes and challenges resulting from current clinical trials. Additionally, we emphasize the effect of multiple clinical factors on immunotherapy to help optimize individualized treatment regimens. Finally, we also highlight several novel concepts and technologies that could be used to design new and/or improve existing immunotherapies. Such approaches will delineate a new blueprint for glioma treatment.

Highlights

Malignant glioma is characterized by aggressive tumor growth, high heterogeneity, intricate oncogenic pathways, and intrinsic resistance to cell death, resulting in limited treatment options and low patient OS rates.

By manipulating the immune system, immunotherapeutic approaches have shown promise in achieving long-lasting tumor remission with minimal adverse effects in diverse types of cancer, including melanoma and leukemia.

Several trials utilizing checkpoint inhibitors, vaccines, chimeric antigen receptor (CAR) T cells, and oncolytic viruses for treating glioma reveal obstacles to achieving sustained responses, possibly due to a highly immunosuppressive tumor milieu, few and/or exhausted tumor-infiltrating lymphocytes, and a deficiency of specific and immunogenic tumor antigens.

Various clinical factors, including corticosteroids, isocitrate dehydrogenase (IDH) mutations, age, gender, obesity, and gut microbiota, may have an important impact on the efficacy of immunotherapy in patients with glioma, enabling the optimal design of individualized treatment.

The availability of data relating to the targeting of glioma stem cells, remodeling of the glioma microenvironment, CAR–natural killer cell interactions, shock and kill strategies, optogenetic immunomodulation, organoid use, and liquid biopsies of cerebrospinal fluid provide novel research directions for the development of glioma immunotherapy with optimal efficiency.

Malignant Glioma and Current Treatments

Gliomas represent the majority of malignant brain tumors. In adults, the disease mainly encompasses diffuse tumors ranging from Grade II to IV [1]. Unfortunately, there is a low OS rate for most patients with gliomas, with only 5% of patients with the most common subtype, glioblastoma (GBM), surviving beyond 5 years despite aggressive treatment. To address the limitations of the current treatment strategies (Box 1), **cancer immunotherapy** (see Glossary), which has resulted in exciting and significant treatment outcomes for multiple cancer types, has triggered unprecedented research interest as a treatment for gliomas. By manipulating the immune system, immunotherapy can achieve long-lasting tumor remission with minimal adverse effects [2]. Emerging studies have revealed that antitumor responses to immunotherapy could occur in the brain, paving the way for developing such strategies to treat malignant gliomas.

Herein, we review the use of immunotherapy as a treatment for malignant glioma (Figure 1), discuss the challenges and controversies underlying **cold tumor** status, and investigate novel approaches that may overcome refractory disease.

Current Cancer Immunotherapy Strategies against Malignant Gliomas

Immune Checkpoint Inhibitors

Two inhibitory immune pathways, programmed cell death protein and its ligand (PD-1 and PD-L1) and **cytotoxic T lymphocyte-associated antigen 4 (CTLA-4)**, have been identified as the main effectors impeding immune responses [9]. Monoclonal antibodies (mAbs) against PD-1 (nivolumab and pembrolizumab), PD-L1 (atezolizumab and durvalumab), and CTLA-4 (ipilimumab) act by reinvigorating the population of cytotoxic T lymphocytes (CTLs) to augment the antitumor response. However, a Phase III trial comparing nivolumab to bevacizumab in 369 patients with recurrent GBM (rGBM) failed to demonstrate the benefit of nivolumab, which conferred only a similar median OS (mOS, 9.8 vs 10.0 months) but a shorter progression-free survival time (PFS, 1.5 vs 3.5 months) compared with bevacizumab [10]. Nivolumab in combination with ipilimumab or as monotherapy for rGBM has been investigated further. Although nivolumab alone was better tolerated than combination schedules with comparable efficacy outcomes, only three

Box 1. Current Treatment Landscape of Malignant Gliomas

The current standard of care for malignant gliomas is surgical resection, followed by radiotherapy (RT) and chemotherapy. The goal of surgery is to maximally feasibly remove the tumor, which can be achieved with the help of operation devices and microsurgical skills, such as intraoperative functional monitoring and the subpial technique [3]. However, it is still difficult sometimes to completely resect the entire tumor due to its location in vital or inoperable areas of the brain and its growth into adjacent normal brain tissues.

Fractionated RT, administered at 50–60 Gy in 1.8–2.0 Gy fractions, is used principally to locally control the relapse of malignant gliomas. However, little clinical progress was made over the past few decades. Despite advances in modern RT technologies, including high-precision conformal RT and proton therapy, no improvement in OS has been gained, although these techniques are more likely to reduce treatment-related toxicity [4].

Cytotoxic chemotherapy containing systemic temozolomide (TMZ) or nitrosourea treatment and local implanted carmustine wafers have been added to RT for most patients with malignant glioma. However, the blood–brain barrier (BBB), which makes most agents unable to access the tumor site, and the existence of a population of stem-like cells, can contribute to tumor chemoresistance [5].

Significant advances in understanding the molecular basis of glioma provide rational options for treating the malignancy with targeted therapies against molecular drivers. Given that malignant gliomas harbor complex heterogeneity at both the molecular and cellular levels and are driven by intricate signaling cascades, the power of this modality appears to be seriously underused. Even though approved by the FDA, bevacizumab, an antibody targeting vascular endothelial growth factor (VEGF), cannot improve the OS of patients with glioma receiving either monotherapy or combination regimens [6,7].

Tumor-treating fields (TTF), suppressing the growth of cancer cells by applying alternating electric fields, is also an approved modality for the treatment of GBM. Despite clinical efficacy with an improved median OS of 4.9 months resulting from the combination of the modality and the current standard regimen, the mode of interaction in the brain, as well as its effects on iconographic or pathological changes upon tumor progression or regression, remain largely unknown [8]. Additionally, the incremental cost-effectiveness ratio of TTF as a first-line treatment is too high for individual patients and healthcare providers.

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out of 40 patients achieved a partial response (PR) [11]. Meanwhile, two retrospective studies provided evidence that anti-PD-1 **salvage therapy** was unable to confer a survival benefit in patients with recurrent high-grade glioma (HGG), with an mOS of only 4 months achieved in patients receiving pembrolizumab as part of a compassionate use program [12]; an mOS of 6.6 months was reported by another study using pembrolizumab or nivolumab with or without concurrent bevacizumab [13]. A **neoadjuvant** regimen of anti-PD-1 immunotherapy was also investigated for rGBM, whereby neoadjuvant pembrolizumab, with continued adjuvant therapy following surgery, resulted in significantly improved OS compared with postsurgical PD-1 blockade alone (mOS, 13.7 vs 7.5 months) [14]. However, no obvious benefit of neoadjuvant nivolumab was obtained in another cohort with resectable GBM, with an mOS of 7.3 months [15].

The reasons for the failure of different approaches are likely to be complicated, but appear to be related to a lack of biomarkers guiding **immune checkpoint** blockade (ICB) in individual patients. In noncentral nervous system (CNS) malignancies, PD-L1 expression within the tumor microenvironment (TME) has been evaluated as a predictive factor [9,16]. However, the incidence of PD-L1 expression is infrequent and the level appears to be variable in GBM [17]. As determined in the failed trial of CheckMate-143, only 27% of patients had PD-L1 expression levels ≥10%, and 32% of GBMs expressed PD-L1 in <1% of tumor cells [11]. A similarly important predictor is the **tumor mutational burden (TMB)**, which increases the amount of **neoantigens** and induces a robust antitumor response. Patients with GBM and germline, biallelic DNA repair defects showed a favorite response to nivolumab, supporting this hypothesis [18]. High TMB in another patient with GBM with mutations in the gene encoding DNA polymerase epsilon (POLE) also resulted in dramatic responses to pembrolizumab [19]. However, typical GBMs do not have a high rate of mutation [17], which is likely to reduce the efficacy of ICBs. More recently, a genomic study analyzing the molecular determinants of immunotherapeutic response to anti-PD-1 in GBM

revealed that enrichment of phosphatase and tensin homolog (PTEN) mutations was associated with immunosuppressive expression signatures in nonresponders, but with enrichment of mitogen-activated protein kinase (MAPK) pathway alterations in responders, suggesting a molecular, personalized approach for refining patient selection for initial immunotherapy. However, responsive tumors exhibited specific alterations and branched patterns of evolution, eventually leading to acquired resistance [20]. In addition, compared with tumors located in peripheral organs, such as melanoma and breast cancer, GBMs are considered to be poorly T cell-inflamed or T cell-exhausted tumors [21], which are less prone to respond to ICBs; high percentages of $CD8^+$ tumor-infiltrating lymphocytes (TILs) with terminally differentiated phenotypes are another possible reason for differences in treatment outcomes [22]. Furthermore, the access of antibodies to tumors in the CNS is less efficient due to the blood–brain barrier (BBB) [21]. Meanwhile, the clinical trial design should also be considered, given that different grouping criteria may influence the therapeutic response in unselected patients. For instance, recruited patients receiving high-dose corticosteroids, which significantly interfere with immunotherapy efficacy, contributed, in part, to negative outcomes of ICB treatment [10,14].

Peptide Vaccines

Antitumor vaccination strategies, broadly encompassing peptides and dendritic cells (DCs), aim to induce immune responses by augmenting the recruitment of antigen-specific effector T cells to tumor sites [23]. Peptide vaccines have mostly used **tumor-specific antigens (TSAs)**, such as epidermal growth factor receptor (EGFR)vIII and isocitrate dehydrogenase (IDH)-1 (R321H), or **tumor-associated antigens (TAAs)**, including interleukin (IL)-13Rα2 and gp100, as immunogenic targets [23–25]. Two studies investigating the effects of rindopepimut, which targets EGFRvIII, reported mOS of 23.6–26 months in patients with GBM [26,27], and the robust EGFRvIII-specific responses and promising OS rates were further confirmed in the ACT III trial [28]. However, a large Phase III study was terminated early due to the lack of effect of rindopepimut (mOS, 20.1 vs 20 months) for patients with newly diagnosed GBM (ND-GBM). Trial analysis indicated unstable EGFRvIII throughout the course of disease, given that ~60% of recurrent tumors lost EGFRvIII expression [24]. Spontaneous antigen loss creates the potential for the outgrowth of, and immune escape by, glioma cells lacking this single epitope, the risk of which may be overcome by multipeptide vaccines. However, an mOS of 15.3 months in a Phase I study of patients with ND-GBM did not support the superiority of the IMA950 vaccine, which comprises 11 peptides [29], although improved T cell responses were reported in Grade II–III gliomas [30], suggesting the preferential use of IMA950 in selected patients. Targeting the IDH1(R132H) neoantigen, which results from most IDH1 mutations and is expressed globally in GBM, may also offer a therapeutic advantage. Mutated-peptide vaccination efficiently triggered an immune response in IDH1(R132H)-mutated gliomas [25].

By contrast, the GAPVAC-101 trial used an approach comprising a vaccine (APVAC1) derived from a premanufactured library of unmutated antigens and a vaccine (APVAC2) preferentially targeting neoepitopes. Unmutated APVAC1 elicited sustained $CD8^+$ T cell responses that could recognize at least one protein, and APVAC2 induced $CD4^+$ T cell responses against predicted neoepitopes in eight out of ten cases. Patients with ND-GBM receiving vaccinations (N = 15) had an mOS of 29.0 months [31]. Another Phase I/Ib study also showed neoantigen-specific T cell responses after administration of a personal neoantigen-targeting vaccine containing up to 20 peptides in two patients with ND-GBM treated without dexamethasone [32]. Although both studies demonstrated boosted antitumor responses induced by personalized vaccines, most patients ultimately died as a result of their cancer, suggesting that the tumor-targeting T cells became exhausted and fell into a dysfunctional state [33].

Glossary

Cancer immunotherapy: a type of cancer treatment that manipulates the patient's immune system to fight the cancer. Immunotherapy methods currently under investigation include checkpoint inhibitors, peptide vaccines, DC vaccines, CAR-T cells, and oncolytic viruses.

Chimeric antigen receptor (CAR)-T cells: T cells are genetically engineered to produce a particular surface receptor comprising an extracellular domain recognizing a specific TSA and an intracellular signaling domain activating the cytotoxic function of T cells.

Circulating tumor DNA (ctDNA): tumor cell-derived fragmented DNA that occurs in plasma, urine, and cerebrospinal fluid.

Cold tumor: cancers that have not been recognized by the immune system and are insensitive to current immunotherapy.

Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4): also known as CD152; functions as an immune checkpoint that provides a negative signal to T cells when bound to CD80 or CD86 of APCs.

Glioma stem cells (GSCs): a small population of cells within a glioma characterized by their ability to self-renew, proliferate indefinitely, and their multidifferentiation; are held responsible for tumor formation, progression, and therapeutic resistance.

IDH-mutant glioma: the presence of an IDH1 or IDH2 mutation is one of the most critical biomarkers for the molecular classification and prognostic prediction of adult diffuse gliomas. Patients with gliomas with a mutant *IDH1* gene have a better outcome.

Immune checkpoints: regulators of the immune system with a critical role in maintaining self-tolerance and preventing autoimmunity through balancing co-stimulatory and inhibitory signals.

Neoadjuvant: administration of drugs before a main therapy; mainly refers to surgical operations.

Neoantigens: antigens encoded by tumor-specific mutated genes but that are entirely absent from the normal human genome.

Optogenetics: biological technique combining genetic manipulation and optics that controls cells expressing light-sensitive ion channels in living tissue.

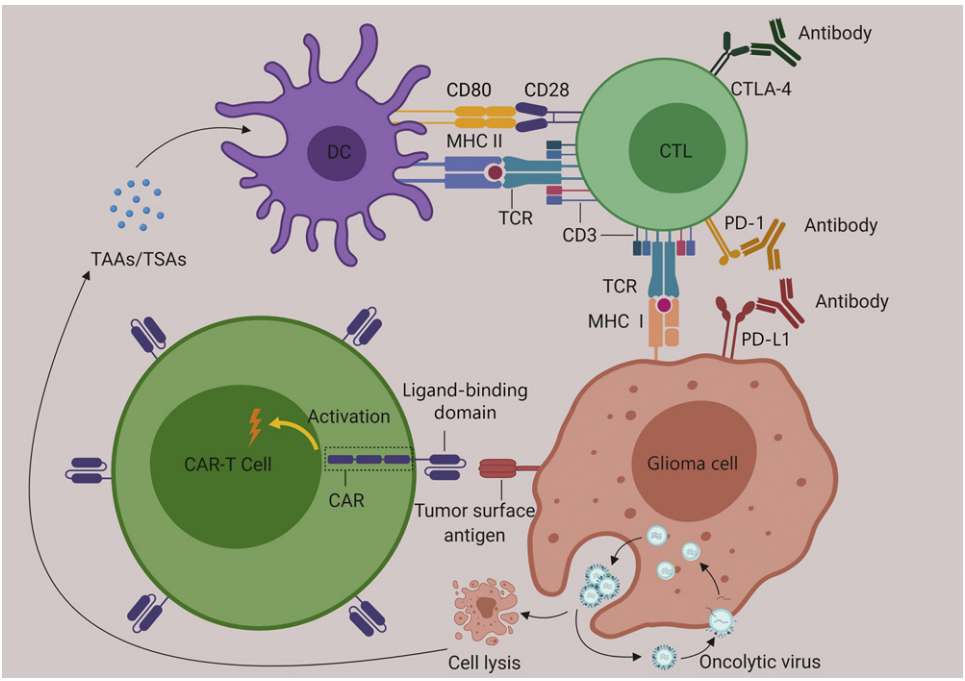

Figure 1. Schematics of Immunotherapy for Treating Malignant Glioma. Dendritic cells (DCs) loaded with tumor-associated antigens (TAAs)/tumor-specific antigens (TSAs) can directly induce the activation of cytotoxic T lymphocytes (CTLs). The immunosuppression status of CTLs is relieved by antibodies targeting inhibitory checkpoints, including cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), programmed cell death protein 1 (PD-1), and its ligand (PD-L1). Chimeric antigen receptor (CAR)-T cells produce an artificial T cell receptor that has high affinity to a tumor-specific surface antigen. Genetically engineered oncolytic viruses mediate tumor cell lysis and the subsequent immune response through selective targeting of cancer cells. Abbreviations: TCR, T cell receptor.

Organoid: cluster of cells that grow as a miniaturized and simplified version of an organ produced in a dish in 3D.
Salvage therapy: final resort therapy if the cancer has not responded to standard treatments.
T regulatory cells (Tregs): subpopulation of $CD4^+$ T cells expressing the transcription factor Foxp3; have a negative role in regulating other cells in the immune system.
Tumor-associated antigens (TAAs): native proteins shared by many patients with cancer and also expressed only limitedly in normal tissues.
Tumor-associated microglia and macrophages (TAMs): microglia are the resident macrophages of the CNS. TAMs account for most of the nonneoplastic cells in the tumor mass, and have a critical role in the creation of a TME that promotes tumor progression.
Tumor mutational burden (TMB): measures the number of mutations within the genome carried by tumor cells and is a biomarker being studied in the prediction of response to immunotherapy.
Tumor-specific antigens (TSAs): often the products of specific mutations; are exclusively expressed on cancer cells.

Thus, it is necessary to enhance the number and quality of TILs and combine multiple approaches to reawaken the exhausted T cells. In addition, the BBB, high heterogeneity, lack of professional antigen-presenting cells (APCs) within the brain, and low expression of MHCs remain the main challenges to successful peptide vaccine approaches.

DC Vaccines

Instead of injecting a peptide that is presented to an APC, autologous DCs can be primed *ex vivo* with specific antigens or cell lysates, followed by re-implantation into the patient to more effectively activate CTLs and boost antitumor responses [23]. A multiple-antigen-pulsed DC vaccine, ICT-107, comprising six epitopes (AIM-2, MAGE1, TRP-2, gp100, HER2, and IL-13Ra2) was tested in a pilot study in patients with ND-GBM. Robust responses to gp100 and HER2 were observed with a prolonged mOS (38.4 months) [34]. Although this promising result prompted further evaluation of ICT-107 in a Phase II trial enrolling 124 patients with ND-GBM, no improvement in OS was reported [23]. In a recent report, the autologous tumor-lysate-trained vaccine DCVaxL combined with standard therapy extended survival time of patients with ND-GBM over standard therapy alone (mOS, 23.1 vs 15–17 months) [35]; in addition, this personalized DC vaccination integrated with a neoantigen-based synthetic long-peptide vaccine resulted in an OS of 21 months in a patient with ND-GBM after initial diagnosis [36]. Fusing glioma cells with the patient's own DCs created a novel vaccine that also conferred favorable outcomes in a ND-GBM and a rGBM group (mOS, 25.3 and 12.6 months, respectively) [37]. Human cytomegalovirus (CMV) antigens have been identified in GBM but not in healthy brains, providing opportunities for DC

vaccines targeting these TSAs. In a pilot study, CMV pp65 RN- pulsed DCs preconditioned with a tetanus and diphtheria (Td) vaccine booster improved the survival of patients with GBM significantly [38]. In 2017, the same group published a second report of the favorable prognosis of 11 patients with ND-GBM receiving pp65-DCs, with a median PFS and OS of 25.3 and 41.1 months, respectively [39].

Although DC vaccines offer advantages over peptide vaccines in terms of antigen selection, trials of these approaches, especially personalized antitumor vaccinations, are time and cost intensive. Additionally, optimization of their efficacy remains particularly challenging because many vaccines are biologically active, and an apparent clinical benefit is not always obtained.

CAR-T Cells

Chimeric antigen receptor (CAR)-T cells specifically recognize tumor cells independent of MHC exposure; CAR-T cells accommodate infinite antigenic diversity and avoid some of the immune evasion mechanisms by which tumors downregulate MHC expression [2]. The identification of antigens used for vaccines, such as EGFRvIII and IL-13Rα2, also aids the development of CAR-T therapy. In an exploratory study, anti-EGFRvIII CAR-T cells penetrated the EGFRvIII-expressing tumor mass and triggered a modest response in nine out of ten patients with rGBM, although the remaining recipient lived for almost 3 years post treatment [40]. However, EGFRvIII antigen loss, upregulation of immunosuppressive factors, and recruitment of **T regulatory cells (Tregs)** were detected in the post-CAR-T-infused tumors [40], which implies the presence of multiple mechanisms of immune escape in GBM. Coincidentally, transient antitumor responses and decreased antigen expression were also found in studies treating rGBM with IL-13Rα2-specific CAR-T cells, despite different administration routes of this therapy [41,42]. As seen in a patient with recurrent multifocal GBM receiving CAR-T therapy via local and intraventricular infusions, outstanding improvement in the quality of life and dramatic radiographical responses in all intracranial and spinal tumors did occur, although the disease recurred 7.5 months later [42]. Following the benefits reported of HER2-CAR-T cells in preclinical models, HER2-specific CAR-modified virus-specific T cell therapies have been developed and have demonstrated tolerance in patients with progressive GBM (the mOS of all 17 participants was 11.1 months from the first T cell infusion and 24.5 months from diagnosis) [43].

While some results are encouraging, the main challenge to CAR-based strategies is that the single molecular target might not be sufficient to maintain this highly heterogeneous tumor in the cross hairs of CAR-T cells. Antigen escape is the main resistance mechanism of tumors, along with the emergence of compensatory immunosuppressive pressures within the TME, which requires combination therapies or targeting multiple antigens. Indeed, HER2 and IL13Rα2-directed tandem CAR-T cells, trivalent CAR-T cells targeting HER2, IL13Rα2 and EphA2, and CAR-T cells secreting bispecific T cell engagers against EGFR and EGFRvIII have been demonstrated to significantly mitigate tumor antigen escape and overcome antigenic heterogeneity in animal models of GBM [44–46].

Oncolytic Viruses

Oncolytic viruses can either hijack the replication of cancer cells or can be genetically altered to infect or kill neoplastic cells exclusively, which then engages the innate immune system to launch an adaptive antitumor immune response. This therapeutic strategy can break the stranglehold of a tumor on the microenvironment to shift the brain tumor from cold to hot and provoke a strong immune backlash [16]. In a Phase I trial, a genetically engineered herpes simplex virus (HSV) type-1 oncolytic virus (G207) achieved an mOS of 7.5 months in a cohort of patients with rGBM [47], and extended another rGBM patient's disease-free interval to 6 years [48]. More promisingly, the

modified adenovirus DNX2401 enabled 20% of 25 patients with recurrent HGG to survive >3 years and three patients to have a ≥95% reduction in the enhancing tumor that lasted for at least 3 years [49]. PSVRIPO is a modified poliovirus with a tropism for CD155, which is broadly upregulated in solid malignancies, including GBM [50]. This engineered poliovirus subverted the innate antiviral interferon (IFN) response to result in viral cytotoxicity and propagation in neoplastic cells and effectively reawakened the slumbering intratumor immune system [50,51]. Given the survival advantage conferred to patients without neurovirulent potential, confirmed by preliminary clinical data, PVSRIPO was granted breakthrough-therapy designation by the FDA in 2016. More recently, studies showed higher survival rates among 61 patients with rGBM receiving PVSRIPO at 24 and 36 months than among historical controls, with an mOS across all doses of PVSRIPO of 12.5 months [52].

Studies in glioma models also found that oncolytic viruses could exert synergistic functions with ICBs to elicit long-lasting therapeutic effects, supporting the potential of combination strategies in GBM treatment [53,54]. More recently, Zika virus (ZIKV) was found to preferentially infect and kill **glioma stem cells (GSCs)** relative to differentiated tumor progeny or normal neuronal cells, contributing to a longer survival time in GBM mice. These investigations are likely to trigger more efforts to develop oncolytic immunotherapy for genetically modified ZIKV with high efficiency and fewer adverse effects [55].

Novel Concepts for Developing Immunotherapy

Targeting Glioma Stem Cells

GSCs, regarded as 'root cells', contribute to tumor recurrence and therapeutic resistance [5,56]. There is building evidence demonstrating that GSCs directly modulate the immune system, thus acting as immunotherapy [53]; therefore, targeting GSCs would be more likely to eradicate the malignancy. Currently known markers for sorting and targeting the GSC population include cell surface molecules, such as CD133. A recent study used a recombinant AC133 (a stem cell-specific epitope of CD133) ×CD3 bispecific antibody (bsAb) that redirected polyclonal $CD3^+$ T cells to $AC133^+$ GSCs. This novel bsAb significantly prevented the outgrowth of orthotopic GSC-derived invasive brain tumors when locally administrated with human $CD8^+$ T cells, and also exhibited potent activity as a prophylactic treatment for this model [57]. The stem cell-determining transcription factor, Sox2, has also been reported as a novel target for active immunotherapy. Vaccinations with Sox2 peptides significantly induced specific antitumor effector responses and prolonged survival in mouse models with or without temozolomide (TMZ) [58]. These results support the development of vaccines targeting GSCs or GSC-associated antigens that further optimize safety for patients. Moreover, several advanced technologies, such as integrated proteomics-based approaches, have been utilized to identify T cell targets that are commonly expressed on GSCs and their differentiated counterparts but not in healthy donors. Stable expression of GSC-specific antigens associated with higher T cell infiltration and expression of positive immune modulators indicates both the reduced risk of autoimmune reactions and the suitability of such antigens for further clinical use [59].

Understanding the TME In Depth

One of the greatest hurdles to efficient immunotherapy is the immunosuppressive status of the glioma microenvironment due to tumor cell extrinsic components and intrinsic mechanisms. **Tumor-associated microglia and macrophages (TAMs)** comprise the bulk of infiltrating immune cells, manipulating immunosuppressive effects and promoting glioma progression by secreting growth and angiogenic factors as well as immune-suppressive cytokines, and enhancing the apoptosis of T cells [16,21]. Unlike the consistently mutating tumor cells, TAMs in the glioma microenvironment remain genetically stable, creating new opportunities for targeted therapeutics.

In a mouse glioma model, local ablation of TAMs *in vivo* decreased tumor size and improved survival curves (i.e., microglia/macrophages promote glioma progression). Furthermore, emerging evidence suggests that re-educating TAMs to adopt phenotypes that are likely to inhibit glioma progression is more effective than depletion strategies, because the polarization from a protumor M2-like TAM to antitumor M1-like TAM phenotype can remove the immunosuppressive constraints and elicit CTL immunity [60,61]. Pharmacological inhibition of colony-stimulating factor-1 receptor reduced protumor M2-like TAM polarization and dramatically increased the survival and regression of established tumors in another glioma model [62]. More recently, a dual-targeting biomimetic codelivery and treatment strategy was conducted to reprogram TAMs. After achieving biomimetic delivery to glioma using albumin nanoparticles modified with mannose, this system targeted M2-like TAMs by overexpressing the albumin-binding receptor SPARC and mannose receptors that occur on M2-like TAM, thus efficiently inhibiting glioma cell proliferation [60]. Additionally, ions in the TME have been shown to serve important roles in influencing TIL function [63,64]. Overabundance of potassium in TME, caused by the release of potassium ions from tumor cellular necrosis, reduced T cell nutrient uptake, suppressed effector programs, and maintained $CD8^+$ T cell stemness [63,64]. Augmenting potassium efflux in tumor-specific T cells by overexpressing the potassium channel $K_v1.3$ lowered intracellular potassium, whereas enforcing the expression of Acss1 metabolically reprogrammed antitumor T cells under conditions of increased extracellular potassium by driving enhanced oxygen utilization and autophagy, improved T cell effector function, enhanced tumor clearance, and prolonged host survival time in melanoma-bearing mouse models [63,64]. These findings could enable the development of reprogrammed TMA or T cell strategies for enhancing immunotherapeutic activity.

CAR-NKs

Natural killer (NK) cells are innate immune effectors with a short lifespan that kill targets without requiring human leukocyte antigen (HLA) matching, while expressing germline-encoded receptors that interact with ligands on target cells to induce cytotoxic functions. Thus, NK cells have become an allogenic candidate against malignancies and offer an attractive alternative to T cells for CAR engineering, because they do not cause graft-versus-host disease, cytokine release syndrome, or other long-term adverse events, but reduce the risk of relapse or resistance mediated by loss of CAR-targeted antigens [65]. In a preclinical study, the human NK cell line, NK-92 which expresses an ErbB2-specifc CAR, showed potent antitumor activity and contributed to a marked extension of symptom-free survival in GBM mice [66]. CARs carrying fragments for cell-binding EGFR and EGFRvIII were also engineered in NK-92 to control either EGFR wild-type or mutant clones. Dual targeting of CAR-NK cells resulted in extended survival without inducing rapid immune escape [66], suggesting their potential for adoptive immunotherapy. However, NK-92 cells require irradiation before infusion into patients due to chromosomal abnormalities and the risk of malignant transformation; in addition, primary NK cells are difficult to isolate, purify, and transduce [65]. Nevertheless, human induced pluripotent stem cells (iPSCs) provide an 'off-the-shelf' resource to produce NK cells, and iPSC-derived NK cells engineered with CARs have demonstrated enhanced antitumor activity *in vivo* [67]. Thus, iPSC-NK cells with novel CAR constructs could pave a practical way for the development of future antiglioma immunotherapy.

Shock and Kill

Multiple factors result in malignant gliomas falling into the category of cold tumors [68]. Current strategies mainly focus on alleviating the immunosuppression in the tumor milieu and the identification of TSAs and/or TAAs to improve immunotherapeutic efficacy. However, a novel regimen combining the Toll-like receptor 7 (TLR7) agonist GS-9620 and a neutralizing antibody PGT121 for treating HIV-1 infection may provide another way to transform glioma from cold to hot. GS-

9620 activates latently HIV-1-infected $CD4^+$ T cells, rendering them more susceptible to PGT121 binding and immune effector cells, facilitating antibody-mediated elimination of the infected $CD4^+$ T cells [69]. Activating glioma cells or upregulating specific epitopes in combination with boosted immune activation could elicit effective immune responses at tumor sites. In an experimental study, the adjuvant poly(I:C) stimulated expression of PD-L1/2 on GBM cells, but doubled the attraction of $CD8^+$ T cells and primed the TME for an immune response upon PD-L1 blockade [70]. Furthermore, specific DNA/RNA encoding TSAs or specific artificial non-functional proteins may be loaded on 5-ALA-based nanoparticles, which can selectively accumulate in glioma cells. Upon acquiring sufficient specific epitopes, tumor cells can be targeted and eliminated by corresponding vaccines or CAR-T cells. The combination of agents expressing different specific epitopes delivered sequentially may also alleviate resistance to immunotherapy. However, any potential adverse effects must be considered first.

Optogenetic Immunomodulation

Optogenetics is a novel technique widely applied in neuroscience to study the behavior of excitable cells with high spatiotemporal precision. Microbial opsin-based optogenetic approaches have been extended to the immune system to modulate lymphocyte trafficking, inflammasome activation, and DC maturation [71]. One proposal with great potential is to integrate optogenetics with antitumor immunotherapy. As reported, Ca^{2+} activation signals were boosted in adoptively transferred CTLs expressing CatCh, a new variant of channel rhodopsin with great light sensitivity, and, under highly selective optical control, overcame Treg-mediated immunosuppression in the TME, leading to significant tumor regression in a mouse melanoma model [72]. Moreover, an optogenetic tool using engineered chemokine receptor was designed to control T cell trafficking. The photoactivatable-chemokine C-X-C motif receptor 4 (PA-CXCR4), the CXCR4-associated $G\alpha_i$ which is swapped for the rhodopsin-coupled $G\alpha_t$, can transmit intracellular CXCR4 signals in response to light. Optical stimulation enhanced the recruitment of PA-CXCR4-expressing tumor-targeting CTLs at melanoma sites and improved the quality of T cell responses in mice [73]. For glioma optogenetic immunotherapy, CAR-T cells engineered by CatCh and PA-CXCR4 may not only accumulate feasibly in the TME, but also resist suppression of CTL killing by Tregs in the presence of a specific wavelength of light. To amplify the antitumor effect, this strategy also calls for a light delivery system with minimized invasiveness. Microscale light-emitting diodes (μLEDs) characterized by stretchable antennas and multichannel wireless operation implants, as well as cellular-scale inorganic wireless optofluidic neural probes, controlled by radio frequencies, have been implemented in the CNS of freely moving animals [74,75]. These exploratory efforts could help develop light devices implanted in the postoperative glioma cavity for wireless optogenetic manipulations.

Organoids

Organoids are 3D structures constructed from self-organizing stem cells. They almost fully recapitulate tumor heterogeneity and the TME *in vitro*, surpass the abilities of established cell lines, but are not as expensive or as time-consuming as patient-derived xenografts. Thus, organoid techniques successfully provide unique platforms to model glioma initiation and progression [76]. An air–liquid interface (ALI) method has been established to propagate patient-derived tumor organoids as tumor epithelia with endogenous and syngeneic TILs, in which the original tumor T cell receptor spectrum is completely preserved. Crucially, the ALI organoids recapitulate the PD-1-dependent immune checkpoint, and blocking PD-1 activated tumor antigen-specific TILs and elicited tumor cytotoxicity [77]. This ALI-based approach enables the feasibility of anti-PD-1 therapeutics for individuals to be tested, and is vital for the treatment of glioma with personalized PD-1 inhibition. Meanwhile, co-culture of matched tumor organoids and peripheral blood lymphocytes is a strategy to

obtain tumor-reactive T cells, which do not recognize autologous healthy organoids or tissues but strongly exert cytotoxic functions on tumor cells [78]. The generation of tumor-specific T cell products, derived from peripheral blood, provides a clinically feasible way for adoptive T cell transfer. This platform also enables the assessment of the sensitivity of tumor cells to T cell-mediated killing [78], which can be extended to analyze the efficacy at different time points during immunotherapy. For patients with glioma who initially respond to immunotherapeutic regimens but face eventual tumor relapse, the establishment of co-cultures based on paired tumor and blood specimens before and after relapse provides a valuable tool to mechanistically dissect the cause underlying recurrence, which could help develop drugs targeting pathways driving resistance to enhance tumor sensitivity to T cell attack.

Liquid Biopsies of Cerebrospinal Fluid

Repeat monitoring of tumor samples before and following treatment guides the optimization of the mode of immunotherapy, because heterogeneity exists in tumors from patients and antigenic epitopes may change throughout the course of the disease [79,80]. Liquid biopsies acquired by sequencing **circulating tumor DNA (ctDNA)** provide a way to genotype tumors with minimal invasion and low cost [79]. Detecting the molecular profiles of glioma using CSF ctDNA has been demonstrated to be more sensitive than sequencing ctDNA from blood [81]. More importantly, the genomic landscape of glioma in the CSF, including a broad spectrum of genetic alterations, closely resembles the genomes of tumor biopsies and enables the evolution of EGFR signaling pathways to be tracked [82]. These results highlight the potential of applying CSF biopsies for measuring TMB, which can stratify patients with glioma who might benefit from ICBs; for identifying targetable alterations, which facilitates the development of tumor-specific antigen-based immunotherapies; and for tracking the evolution of the glioma genome, which could help identify, and switch from immunotherapy approaches with acquired resistance.

Concluding Remarks and Future Perspectives

Explorations of drugs targeting genes or proteins pivotal for gliomagenesis and progression are at an impasse. Immunotherapy, which aims to awaken the immune system to generate antitumor immunity, could become the mainstay of glioma treatment, given that it results in dramatic and durable responses across various tumor types. Current trials of glioma immunotherapy predominantly focusing on immunosuppressive ICBs, vaccines, CAR-T cells, and oncolytic viruses have achieved some promising results (Table 1). However, sustained responses remain rare, the primary reasons for which are likely multifactorial and include: (i) a highly immunosuppressive TME; (ii) few and exhausted TILs and sequestration of systemic T cells in bone marrow [99]; (ii) deficiency of specific and immunogenic tumor antigens; and (iv) heterogeneity and plasticity at the single cell level [100]. Some secondary obstacles that will be equally difficult to be overcome include: (i) low permeability of drugs across the BBB; (ii) tumor and microenvironment evolution during immunotherapy; (iii) and lack of identified markers stratifying responders and nonresponders (see Outstanding Questions). Third, the standard of care and many clinical factors, including corticosteroid use and gut microbiota, need to be considered before the onset of immunotherapy, because they may affect the treatment efficacy to some extent (Box 2). In addition, the inaccuracy of preclinical models is related, in part, to the limited progress of immunotherapy, because they fail to consistently show responses to agents with therapeutic activity in patients and lack rigorous characteristics of human glioma [61]. For example, high TMB in GL-261 GBM models contributes to improved efficacy of ICB and untraceable U87 cells grow like a ball in immunocompromised mice [46,101]. The integration of immune-competent mouse models, genetically engineered mouse models, patient-derived xenografts,

Outstanding Questions

What are the core pathways mediating the immunosuppressive tumor milieu in glioma? Can we restrain or reawaken the TME?

How can we identify specific tumor antigens and avoid therapeutic resistance caused by antigen loss in highly heterogenic tumors treated with vaccines or CAR-T therapy?

Can we accurately monitor the genetic changes or clonal evolution in glioma during treatment to help adjust the immunotherapeutic regimen?

How can we develop biomarkers for immunotherapy to stratify patient responders or nonresponders?

What is the best way to combine radiochemotherapy and immunotherapy in a sequential therapy to treat gliomas?

Table 1. Clinical Studies with Immunotherapeutic Strategies for Treating Malignant Glioma[a]

Therapeutic approach	Immune target(s)	Type of glioma	Type of study	Number of subjects	Median OS	Median PFS	Other endpoints	Clinical trial identifier	Refs
Immune checkpoint inhibitors									
Nivolumab vs bevacizumab	PD-1	Recurrent GBM	Phase III	369	9.8 vs 10.0 months	1.5 vs 3.5 months	N/A	NCT02017717	[10]
Nivolumab ± Ipilimumab	PD-1, CTLA-4	Recurrent GBM	Phase I	40	N/A	N/A	3/40 PR, 8/40 SD ≥12 weeks	NCT02017717	[11]
Pembrolizumab	PD-1	Refractory HGG	Retrospective study	25	4 months	1.4 months	N/A	N/A	[12]
Pembrolizumab/ nivolumab ± bevacizumab	PD-1	Recurrent HGG	Retrospective study	31	6.6 months	3.2 months	N/A	N/A	[13]
Neoadjuvant pembrolizumab	PD-1	Recurrent HGG	Phase II	35 (neoadjuvant 16; adjuvant 19)	Neoadjuvant 13.7 months; adjuvant 7.5 months	Neoadjuvant 3.3 months; adjuvant 2.4 months	N/A	N/A	[14]
Neoadjuvant nivolumab	PD-1	GBM	Phase II	30 (27 recurrent GBM, 3 ND-GBM)	7.3 months	4.1 months	N/A	NCT02550249	[15]
Peptide vaccines									
IMA950	BCAN, CSPG4, FABP7, IGF2BP3, NLGN4X, NRCAM, PTPRZ1, TNC, MET, BIRC5, HBcAg	ND-GBM	Phase I	45	15.3 months	N/A	PFS-6 74%	NCT01222221	[29]
PEPvIII-KLH	EGFRvIII	ND-EGFRvIII-expressing GBM	Phase II	22	23.6 months	15.2 months	N/A	BB-IND-9,944	[27]
Rindopepimut	EGFRvIII	ND-EGFRvIII-expressing GBM	Phase II	18	26 months	N/A	PFS-6 67%	NCT00643097	[26]
				65	21.8 months	N/A	OS-36 26%	NCT00458601	[28]
			Phase III	Rindopepimut 371; Control 374	20.1 vs. 20 months	N/A	N/A	NCT01480479	[24]
Neoantigen vaccine + poly-ICLC	Personalized	ND-MGMT-unmethylated GBM	Phase I/Ib	10	16.8 months	7.6 months	N/A	NCT02287428	[32]
APVAC1 APVAC2 + poly-ICLC/GM-CSF	Personalized	ND-GBM	Phase I	15	29 months	15.2 months	N/A	NCT02149225	[31]

DC vaccines									
ICT-107	AIM-2, MAGE1, TRP-2, gp100, HER2 and IL-13Rα2	ND-GBM	Phase I	17	38.4 months	16.9 months	N/A	N/A	[34]
DCVax-L	Autologous tumor lysate	ND-GBM	Phase III	331	23.1 months	N/A	N/A	NCT00045968	[35]
Fusions of DC and glioma cells	Autologous glioma cells	GBM	Phase I/II	32 (10 recurrent GBM; 22 ND-GBM)	Recurrent GBM 18.3; ND-GBM 30.5 months	Recurrent GBM 10.3; ND-GBM 18 months	N/A	16-184-4412	[37]
DCVax-L + GBM. Pvax	Autologous tumor lysate + personalized neoantigens	ND-GBM	Case report	N/A	21 months	N/A	N/A	NCT02510950	[36]
Pp65-DCs + GM-CSF	Cytomegalovirus pp65	ND-GBM	Phase I	11	41.1 months	25.3 months	N/A	NCT00639639	[39]
CAR-T therapy									
CART-EGFRvIII	EGFRvIII	EGFRvIII-expressing recurrent GBM	Phase I	10	251 days (~8 months)	N/A	N/A	NCT02209376	[40]
IL-13Rα2-specific CAR	IL-13Rα2	Recurrent GBM	Pilot trial	3	11 months (8.6-10.3-13.9)	N/A	N/A	NCT00730613	[41]
IL13Rα2-targeted CAR	IL-13Rα2	Recurrent multifocal GBM	Case report	1	N/A	7.5 months	N/A	NCT02208362	[42]
HER2-CAR VSTs	HER2	Progressive HER2-positive GBM	Phase I	17	11.1 months (from first T cell infusion); 24.5 months (from diagnosis)	N/A	1 PD> 9 months, 7 SD = 8 weeks–29 months	NCT01109095	[43]
Oncolytic viruses									
G207 (genetically engineered HSV type I)	N/A	Recurrent GBM	Phase I	9	7.5 months	2.5 months	N/A	NCT00157703	[47]
G207 (genetically engineered HSV type I)	N/A	Recurrent GBM	Case report	N/A	7.5 years	6 years	N/A	N/A	[48]
DNX-2401 (modified adenovirus)	N/A	Recurrent malignant glioma	Phase I	37 (25 treatment-only; 12 treat–resect–treat)	Group A 9.5 months; Group B 13 months	N/A	N/A	NCT00805376	[49]
PVSRIPO (recombinant nonpathogenic polio–rhinovirus chimera)	CD155	Recurrent GBM	Phase I	61	12.5 months	N/A	OS-24/36 21%	NCT01491893	[52]

[a]Abbreviations: AA, anaplastic astrocytoma; AO, anaplastic oligodendroglioma; AOA, anaplastic oligoastrocytoma; PD, progressive disease; SD, stable disease.

Box 2. Factors Influencing Immunotherapy

Chemoradiotherapy

RT/TMZ treatment usually contributes to systemic immune suppression, including myelosuppression and a reduction in both T cell and B lymphocyte counts, but promotes tumor infiltration of CTLs and DCs and PD-L1 expression in glioma, potentially transforming the cold milieu to a hot status by inducing a higher mutational load and release of massive antigens [16,83,84].Combining chemoradiotherapy with immunotherapy should be optimized to achieve greater clinical efficacy.

Corticosteroids

Corticosteroids adversely impact the effectiveness of immunotherapies and compromise survival in glioma. This may be related to corticosteroid-induced immunosuppression via the upregulation of CTLA-4 expression in $CD4^+$ and $CD8^+$ T cells and blocking of naïve T cell proliferation and differentiation [85]. Thus, the prudent and restricted use of corticosteroids avoids abrogating the efforts of immunotherapy.

IDH Mutation

IDH-wild-type gliomas display more prominent tumor infiltration of lymphocytes and higher PD-L1 expression, while mutated IDH suppresses the accumulation of $CD8^+$ T cells in tumor sites and enhances glioma cell evasion [86,87]. Targeting mutant IDH together with immunotherapy is suggested to improve the efficacy of immunotherapy in **IDH-mutant gliomas**.

Age

Antitumor immunity may be compromised in older patients due to their low levels of naïve T cells, exhaustion of potentially tumor-specific memory T cells, and higher amounts of suppressive cells [88]. Poorer outcomes were predicted in older patients with GBM receiving DC vaccines, despite more efficient responses to anti-PD-1/PD-L1 occurring in older patients with other cancers [88,89].

Gender

Immune components of both innate and adaptive immunity are differently regulated in females and males [90], indicating different responses to immunotherapy. For example, high levels of estrogen upregulate PD-1 on T cells, suggesting the higher efficacy of ICBs in female patients [91]. However, the ways in which sex intersects with immune function needs more research.

Obesity

Obesity relates to certain malignancies and promotes tumor progression, possibly because of increased immune aging and PD-1-mediated T cell dysfunction, although the latter can render tumors more responsive to ICBs [92]. However, immunotherapeutic efficacy can be reduced by elevated leptin [93], suggesting that leptin inhibition could increase the window for successful immunotherapy in obese patients.

Gut Microbiota

The gut microbiota has been shown to affect cancer responses to immunotherapy. For example, *Bacteroides fragilis* favored the antitumor immunity of CTLA-4 blockade, and an abundance of *Bifidobacterium* and *Akkermansia muciniphila* facilitated anti-PD-L1 efficacy [94–97]. Fecal microbiota transplantation from responders or supplementation with these beneficial microbial populations could significantly improve tumor control in nonresponders [94–98].

and models that more accurately recapitulate human glioma is an urgent need. Along with translating our proposed schemes into actions (Figure 2), efforts should also be made towards understanding the glioma–immune interaction parameters to assess the individual tumor immune status to explore resistance mechanisms at different stages of treatment and response [68], to find drugs that can penetrate the BBB, that can therapeutically induce intratumoral vessel normalization and high endothelial venule formation to improve immune cell trafficking and the sensitivity of immunomodulating therapies [102], to identify key features immediately before treatment to stratify patients and predict treatment efficacy, and to design combination strategies involving immunotherapies, molecular-targeted therapies, and chemoradiotherapy to achieve maximal efficiency and alleviate acquired immunotherapeutic resistance. Moreover, attention should also focus on any adverse effects, especially ICB-associated edema and T cell immunotherapy-associated neurotoxicity [103–105]. A deeper understanding of the biological basis of such complications will help manage drug discontinuation or dose adjustment and differentiate treatment-related symptoms from tumor progression. With this in

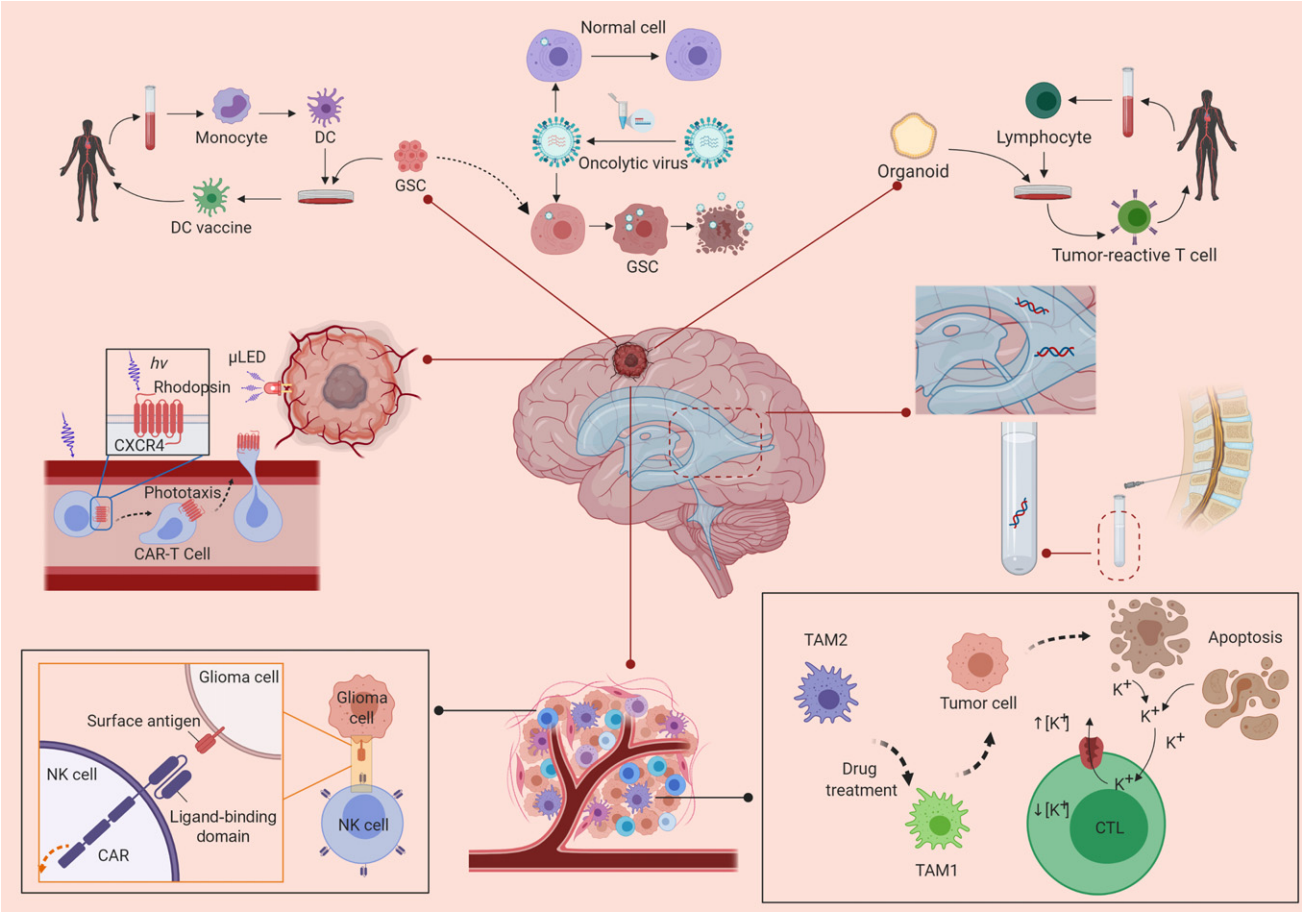

Figure 2. Development of an Immunotherapeutic Blueprint for Treating Malignant Glioma. Fresh tumor tissue-derived glioma stem cells (GSCs) can be lysed for loading dendritic cells (DCs) to generate DC vaccines, and genetically modified oncolytic viruses have the potential to specifically attack tumor seed cells. GSCs also can be induced to form patient-derived organoids (PDO), and the *in vitro* co-culture of glioma PDO and matched peripheral blood lymphocytes would generate tumor-specific T cell products. Natural killer (NK) cells armed with chimeric antigen receptors (CAR) specifically recognize surface antigens on glioma cells, but do not cause severe adverse events. The polarization from protumor M2-like tumor-associated microglia and macrophages (TAM) to an antitumor M1-like TAM phenotype induced by drug treatment and augmentation of potassium efflux in cytotoxic T lymphocytes (CTLs) helps to relieve immune suppression in the tumor microenvironment (TME) and enhances the antitumor activity of immunotherapy. T cells, such as CAR-T cells, engineered with chemokine receptors and photoactivatable proteins, can be recruited by optical stimulation at a specific wavelength and accumulate in the tumor site to control glioma progression effectively. Liquid biopsies by sequencing circulating tumor DNA in the cerebrospinal fluid obtained by lumbar punctures is an approach to detect and monitor genomic alterations, which helps to design individualized immune treatment programs. Abbreviations: μLED, microscale light-emitting diode.

mind, immunotherapy holds the promise of a new era in glioma treatment, resulting in long-lasting tumor remission with minimized toxicity.

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