

Review

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# Immunotherapy and checkpoint inhibitors for gliomas

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## Abstract

Glioma treatments are faced with challenges, including the inability to fully eliminate cancer stem cells, the immunosuppressive tumor microenvironment, and the blood brain barrier. Although progress has been made with surgical, radiation, and chemotherapies, prognosis for patients remains poor. Rapidly emerging immunotherapies may be able to address the challenges that conventional techniques cannot. Immunotherapies manipulate the patient's immune system to selectively combat malignancies. Therapies often work to enhance T-cell and natural killer (NK) cell function, which can both eliminate tumor cells and enhance remission. Vaccines encourage *in vivo* development of anti-tumor T-cells and NK cells, while adoptive transfer techniques focus on engineering immune cells *ex vivo* before reintroducing them to patients. Vaccine and adoptive transfer therapies have been shown to induce enhanced immune responses in patients but have not always correlated with improved outcomes, likely because of the tumor immunosuppressive microenvironment. Checkpoint inhibitors can impair these tumor immunosuppressive capabilities. Although no one treatment has been able to consistently eliminate gliomas and maintain remission, combinations of vaccines or adoptive transfer techniques in conjunction with immune checkpoint inhibitors offers promise.

**Keywords:** Glioma, immunotherapy, checkpoint inhibitors, vaccines, T-cells, dendritic cells



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## INTRODUCTION

Gliomas arise from different glial cell populations and can manifest as astrocytic tumors (astrocytoma, anaplastic astrocytoma, and glioblastoma), oligodendrogliomas, ependymomas, and mixed gliomas. Glioblastoma multiforme (GBM) is the most malignant, invasive, and common glioma, accounting for over 50% of all diagnosed brain tumors. A patient with GBM possesses poor prognosis, as typical survival is 14-15 months following diagnosis. Standard treatment includes surgical resection, radiation therapy, and chemotherapy [temozolomide (TMZ)], but these often result in limited success<sup>[1]</sup>. This is partially due to treatment limitations, including infiltration of GBM cells to surrounding brain tissue, intratumoral heterogeneity, and therapy passage across the blood brain barrier (BBB)<sup>[2]</sup>.

The central nervous system (CNS) has long been considered an immune-privileged site, evidenced by the presence of the BBB, lack of lymphatic vessels, and absence of major histocompatibility complex (MHC) - positive antigen presenting cells (APCs)<sup>[3]</sup>. However, recent work suggests that the CNS has a closer relationship with the peripheral immune system. Soluble antigens of the cerebrospinal fluid drain into the cervical lymph nodes, providing T cells activation before transport to the site of inflammation<sup>[4]</sup>. Leukocytes are known to gain partial access to the CNS through the choroid plexus, across the superficial leptomeningeal vessels, and into the perivascular space<sup>[5,6]</sup>. Brain tumor progression further compromises the integrity of the BBB, allowing these T cells access to the brain<sup>[7]</sup>. Given that immune cells can permeate the BBB while maintaining cancer cell-specific targeting, immunotherapies have begun to establish their promise in GBM treatment.

In this review, we will examine recent advances made in immunotherapies for GBM, focusing on harnessing apoptotic functions in natural killer (NK) cells, blocking checkpoint inhibitors to unmask malignancies, and inducing systemic response by vaccine administration to target tumors. We will also highlight clinical trials using these immunotherapies. Finally, we will address therapy challenges and discuss the need for further refinement in applications specific to brain tumors.

## T-CELLS

The ratio of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells is a prognostic marker in many malignancies and has been found to inversely correlate with progression-free survival and overall survival (OS) in GBM<sup>[8]</sup>, demonstrating an avenue for immunotherapy intervention.

T-cells maturing in the thymus may develop into CD4<sup>+</sup> or CD8<sup>+</sup> T-cells, also known as helper T-cells or cytotoxic T-cells, respectively. They are activated upon APC interaction. APCs recognize pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors, such as toll-like receptors. Once PAMPs are recognized before or during endocytosis, antigens, usually peptides, are loaded onto MHC-I or MHC-II proteins. Antigens that are loaded onto MHC-II proteins activate CD4<sup>+</sup> T-cells, which further activate CD8<sup>+</sup> T-cell responses. This contrasts with antigens that are loaded onto MHC-I proteins, which activate only CD8<sup>+</sup> T-cells<sup>[9]</sup>.

Induction of helper T-cell differentiation from CD4<sup>+</sup> T-cells is orchestrated by cytokine stimulation<sup>[10]</sup>. Helper T-cells promote anti-tumor response by secreting pro-inflammatory cytokines interferon (IFN)- $\gamma$  and tumor necrosis factor (TNF). These cytokines then activate death receptors on the tumor cell surface, consequently triggering dendritic cell (DC) cytotoxic functions. It is these functions that eradicate tumor cells. T regulatory cells (Tregs) often modulate the immune response through the secretion of anti-inflammatory cytokines tumor growth factor- $\beta$  and interleukin (IL)-10. While Treg cytokine secretion is aimed to prevent a dramatic immune response, Tregs may inadvertently contribute to tumor development by suppressing pro-inflammatory anti-tumor responses<sup>[11]</sup>.

CD4<sup>+</sup> and CD8<sup>+</sup> T-cells can further differentiate into effector or memory T-cells, which are essential in delivering a robust secondary immune response after re-exposure to antigen<sup>[10]</sup>. CD8<sup>+</sup> effector T-cells attack tumor cells by releasing perforin and granzyme cytotoxic molecules and producing pro-inflammatory cytokines TNF and IFN- $\gamma$ . During antigen clearance, most CD8<sup>+</sup> effector T-cells undergo apoptosis, with a small margin surviving. When high levels of antigens persist in an environment, these remaining CD8<sup>+</sup> T-cells progress into a state of T-cell exhaustion. This situation is commonly seen in cancers with solid tumors like GBM. In the T-cell exhaustive state, inhibitory receptors are overexpressed, cytokine signaling pathways are dysregulated, and altered metabolic fitness occurs<sup>[12]</sup>, leading to altered T-cell functioning and unrestrained tumor growth. Novel strategies and new approaches have been developed in attempts to circumvent these hurdles to immunotherapy for gliomas.

## ADOPTIVE T-CELL TRANSFER

Adoptive T-cell transfer (ACT) involves the collection and *ex vivo* expansion of autologous anti-tumor T lymphocytes. These cells are then reinfused into the patient, delivering a potent and focused response. This approach provides the immune system and tumor microenvironment with an already abundant, activated T-cell population that can proliferate *in vivo* to maintain antitumor functions. ACT therapy can provide benefits to immunocompromised patients as it eliminates the need for self-induced antigen presentation. This feature, along with the ability for T-cells to bypass the BBB, suggests that ACT may be particularly effective in brain tumor treatment<sup>[13]</sup>.

There are several avenues to the ACT approach, but the furthest advanced is the chimeric antigen receptor T-cell (CAR T) therapy. After isolating T-cells from the patient, these cells are genetically engineered to express receptors that mediate tumor cell destruction after reinfusion to patients. CAR T targeting of B cell marker CD19 has shown great efficacy in lymphoblastic leukemia and B cell lymphomas, but applications in the treatment of solid tumors have only begun to be explored<sup>[14,15]</sup>. Progress has been made in CAR T treatments for brain tumor targeting, particularly utilizing epidermal growth factor receptor (EGFR)vIII [Table 1]. Given that EGFRvIII is known to be prevalent in gliomas, CAR was directed against EGFRvIII and used in a phase I clinical trial for recurrent GBM<sup>[16]</sup>. All patients given CAR T EGFRvIII intravenous infusions exhibited decreased expression of EGFRvIII in tumors, indicative of CAR on-target effects. Flow cytometric analysis of CD3<sup>+</sup> T-cells detected engraftment of CAR T-EGFRvIII cells in the peripheral blood. These findings demonstrated CAR T transient growth advantage as compared to endogenous lymphocytes, and that rescue of normal T-cell activity can be achieved. This is especially applicable to immunocompromised patients previously administered doses of TMZ and radiation. None of the infused subjects presented symptoms of tumor toxicity nor cytokine release syndrome, and there was no cross-reactivity of wild type EGFR<sup>[17]</sup>.

Other clinical trials have shown that through administration of glioma associated antigen IL13R $\alpha$ 2 in CAR T-cells, patients with recurrent GBM showed transient, anti-tumor responses. In metastatic GBM, significant tumor regression could be observed after 4-1BB co-stimulation and a mutated IgG4-Fc linker was incorporated into administered CAR T-cells which enhance antitumor potency and reduced off target interactions. While both intracavitary and intraventricular administration of CAR T populations was performed, intraventricular administration was found to achieve regression of all CNS tumors<sup>[18]</sup>. These preliminary findings illustrate the potential for CAR T mass manufacture, designed to address function and toxicity deficiencies.

Challenges in transitioning ACT to the clinic include the risk of inducing graft vs. host disease, which may arise through allogeneic T-cell transfusion. Identification of toxicity prior to human administration is another challenge, largely due to the lack of representative immune systems in pre-clinical models. Introduction of a robust T-cell population carries its drawbacks. Off-targeting can present as cross-reactivity of the T-cell receptor with an antigen that possesses similar structure to the target antigen. This can result in

**Table 1. Clinical trials using adoptive T-cell transfers to treat gliomas**

Identifier	Trial name	Treatment	Phase	Diagnosis (newly diagnosed or reoccurring)
NCT02844062	Pilot study of autologous anti-EGFRvIII CAR T-cells in recurrent GBM	Autologous anti-EGFRvIII CAR T	I	Reoccurring
NCT03283631	Intracerebral EGFRvIII CAR-T-cells for recurrent GBM (intercept)	Autologous anti-EGFRvIII CAR T	I	Reoccurring
NCT03389230	Memory-enriched T-cells in treating patients with recurrent or refractory grade III-IV glioma	Leukapheresis, autologous HER2(EQ)BBζ/CD19t+	I	Reoccurring
NCT03423992	Personalized CAR T immunotherapy for patients with recurrent malignant gliomas	Autologous anti-EGFRvIII, IL13Rα2, HER2, CD133, EphA2, GD2 CAR T	I	Reoccurring
NCT02208362	Genetically modified T-cells in treating patients with recurrent or refractory malignant glioma	IL13Rα2-specific, hinge-optimized, 41BB-costimulatory CAR/truncated CD19-expressing autologous T lymphocytes	I	Reoccurring
NCT02209376	Autologous T-cells redirected to EGFRvIII - with a chimeric antigen receptor in patients with EGFRvIII + GBM	CAR T-EGFRvIII T-cells	I	Patients with residual

EGFR: epidermal growth factor receptor; CAR T: chimeric antigen receptor T-cell; GBM: glioblastoma multiforme; HER2: human epidermal growth factor receptor 2

cytotoxic effects to otherwise healthy tissue<sup>[19]</sup>. Conversely, toxicities can also arise through on-target effects. This generally manifests as successful binding to the antigen expressed in environments not specific to the tumor<sup>[20]</sup>. Cytokine release syndrome is another condition that results from the release of mass quantities of cytokines, an indirect result of the release of mass quantities of T-cells<sup>[21]</sup>. However, downregulation in the prevalence of these cytokines can be achieved by receptor blockage. Specifically, in treatment focused on acute lymphoblastic leukemia, a patient administered tocilizumab (an IL-6 receptor inhibitor) was able to reverse cytokine storm syndrome symptoms yet still maintained the T-cell population and continued to derive benefits from ACT therapy<sup>[22]</sup>. Thus, while these side effects are serious, continued refinement protocols can be made so symptoms are less severe and more manageable.

Current efforts in ACT aim to target solid tumors and optimize gene transfer. Methods of achieving gene transduction to T lymphocytes include retroviral and lentiviral gene delivery. Positive efficacy in gene transduction may be observed, but integration of genes may prefer certain areas of the genome over others. Poor integration of genes could result in mutagenesis and overexpression or disruption of nearby genes in T-cells<sup>[23,24]</sup>. Additionally, patients' immune systems may react to the vectors themselves, and these genotoxic events may interfere with T-cell delivery. To circumvent these issues, engineering of CAR T-cells by piggyBac and Sleeping Beauty transposons has gained momentum<sup>[25,26]</sup>. These methods offer reduced manufacture cost, increased simplicity, and less good manufacturing practice requirements. Gene expression remains unperturbed and foreign proteins that could result in adverse effects are absent. Treatments utilizing Sleeping Beauty have just reached phase I clinical trials<sup>[27]</sup>. Optimization and precision of these transposon systems may prove crucial to improved ACT safety.

## NK CELLS

NK cells demonstrate potent anti-tumor immunity. Unlike T-cells, transfusions of NK cells are not complicated by graft-host-disease. NK cells detect and eliminate cell abnormalities and are found in lymphoid and non-lymphoid organs<sup>[28]</sup>. Activated NK cells secrete perforin and granzymes to induce apoptosis in target cells. Ligands like killer immunoglobulin-like receptors are expressed on healthy cells and can inhibit the destructive activity of NK cells. Antibodies, cytokines, natural cytotoxicity receptors and transmembrane protein NK2GD on infected or transformed cells provide activating signals to NK cells<sup>[29]</sup>. Conversely, downregulation of MHC-I in tumor cells depletes inhibitory signals to NK cells. It is these shifts between activating and inhibitory signals that allow NK cells to selectively target abnormal cells. This is the premise of anti-tumor NK cell immunotherapies<sup>[30]</sup>.

Studies aimed at heightening NK cell activity for immunotherapies have done so through genetic engineering or by stimulation of NK cells directly *in vivo* or *ex vivo* [Table 2]. The tumor microenvironment is known to be immunosuppressive which correlates with reduced NK cell activity<sup>[31]</sup>. Because of this, few NK cells achieve activation to carry out NK cell-mediated lysis. By stimulating NK cells, however, the immunosuppressive tumor microenvironment may be overcome. Efforts to stimulate NK cell proliferation and activity have focused on exposure of NK cells to cytokines including IL-2, 12, 15, 18 and 21. Once stimulated, NK cells become lymphokine-activated killer cells. These cells have demonstrated increased levels of cytotoxicity towards malignant tumors and proliferate at a greater rate<sup>[29]</sup>.

Clinically, NK cell stimulation with IL-2 was approved for treatment of metastatic renal cancer by the FDA in 1992. High-doses of IL-2 has demonstrated efficacy in treating various cancers, but increasing doses also increases the risk of severe adverse effects. A phase III trial comparing IL-2 doses for liver and bone metastases and primary tumors showed that the response rate for high-dose IL-2 was significantly higher than all other groups. It was concluded high-dose IL-2 were necessary for significant clinical benefits, despite the possible negative effects<sup>[32]</sup>.

IL-21 stimulates NK cells and CD8<sup>+</sup> T-cells while also increasing the production of IFN- $\gamma$ . Together with IL-2 and IL-15, IL-21 enhances cytotoxic effects of NK and CD8<sup>+</sup> T-cells<sup>[33]</sup>. A phase II clinical study treating melanoma patients with IL-21 found that antitumor efficacy of IL-21 is comparable to that of high-dose IL-2. The treatments were well tolerated among patients and resulted in few adverse actions<sup>[34]</sup>.

IL-15 has been tested in phase I trials to monitor the reactions among patients after administration. The cytokine was given as bolus intravenous infusions to patients with metastatic malignant melanoma and renal cell cancer. The treatments caused a large swing in the distribution of lymphocytes within the blood, suggesting its importance to the activation of NK cells and their cytotoxicity. Many adverse reactions were recorded, however, which is thought to be the result of the method of administration<sup>[35]</sup>.

IL-12 is another cytokine under investigation for use in immunotherapy. Studies in preclinical models using IL-12 have shown strong antitumor effects<sup>[36]</sup>. In one such study, the rejection of gliomas in mice was found to be significantly enhanced in those expressing IL-12 in the CNS, as compared to those without<sup>[37]</sup>. This gives evidence that the expression of IL-12 cytokine can be a major factor in anti-tumor response through stimulation of the immune system.

NK cell function is heavily dependent on cytokine support. But, even with administration of additional cytokines, the tumor microenvironment may limit NK cell activation. To overcome dependence on exogenous cytokines, genetically engineered NK cells have been explored for their ability to surpass the tumor microenvironment. One study examined whether transduced expression of a nonsecretory, membrane-bound form IL-15 (mbIL15) could sustain NK cells. The mbIL15 NK cells had enhanced survival and viability compared to mock-transduced NK cells and NK cells that expressed non-membrane bound IL-15. Because mbIL15 NK cells are less dependent on endogenous signaling molecules, their activity and cytotoxicity against solid tumors is resilient to immunosuppressant effects of the tumors<sup>[38]</sup>. Genetic engineering of NK cells to self-activate may prove more effective than stimulation from endogenous cytokines. Engineered NK cells avoid off targeting effects of cytokine administration to patients and may allow for NK cell antitumor functions to be enhanced. Patients with esophageal squamous cell carcinoma, squamous cell lung cancer, and gastric carcinoma have shown positive responses to NK treatments. Higher survival rates were correlated to CD57 positive cells at the site of the tumor. CD57 expression is associated with NK cells, as well as T-cells, and may serve as an additional target for enhancing NK cell effectiveness. Clinical trials to assess the efficacy of NK therapy for gliomas have now been initiated [Table 2] with peer-reviewed reports yet to be released.

**Table 2. Progress using natural killer cells against cancer clinical trials**

Identifier	Trial name	Treatment	Phase	Diagnosis
NCT03383978	Intracranial injection of NK- 92/5.28.z (HER2. taNK) cells in patients with recurrent HER2-positive GBM (Quilt 3.C001) (CAR2BRAIN)	NK-92/5.28.z (HER2. taNK) injection	I	GBM
NCT00909558	Safety and effectiveness study of autologous NK and NK T-cells on cancer	Autologous NK/NK T-cell immunotherapy	I	Glioma, squamous cell lung cancer, pancreatic cancer, colon cancer
NCT00823524	Donor NK cells after donor stem cell transplant in treating patients with advanced cancer	Donor NK cell infusion	I/II	Brain and central nervous system tumors
NCT03081780	Open label NK cell infusion (FATE-NK100) with Subq IL-2 in adults with AML	FATE-NK100	I	Refractory acute myelogenous leukemia, relapsed AML

NK: natural killer; GBM: glioblastoma multiforme; HER2: human epidermal growth factor receptor 2; AML: acute myelogenous leukemia

## CHECKPOINT INHIBITORS

Checkpoint inhibitors are a rapidly advancing field and involve the exploitation of tumor checkpoint regulators. Immune checkpoints regulate the life cycle of the cellular immune response by either activation of signals or by inhibition of activating processes. Tumor checkpoint regulators are mechanisms by which tumors evade immune system recognition through expression of neoantigens. These antigens emulate those of healthy tissue<sup>[39]</sup>. Checkpoint inhibition blocks tumor cell evasion and allows for T-cells to overcome the immunosuppressive tumor microenvironment. However, clinical trial outcomes and patient responses differ between cancer types. Thus, investigation of external influences on checkpoint mechanisms ought to be further explored.

Inhibitors generated for therapeutic use are found as chemically synthesized monoclonal antibodies or recombinant forms of ligands or receptors. Such checkpoint targets include the programmed death receptor 1 (PD-1) and its ligand (PD-L1) or cytotoxic T-lymphocyte associated protein 4 (CTLA-4) receptor and its ligands CD80 and CD86. These pathways are responsible for restriction of T-cells in peripheral tissues during inflammatory response or for down-regulation of co-stimulatory T-cells, respectively<sup>[40-42]</sup>. Although the PD-1 and CTLA-4 pathways are not the only mechanisms which provide cancer cells protection from T-cell surveillance, PD-1 and CTLA-4 have exhibited profound outcomes in regard to tumor regression, appear to possess an immunodominant role as compared to other immune checkpoints, and their mechanisms are the most understood. It has been shown that PD-L1 is highly expressed on tumor cells and that coordination between PD-1/PD-L1 can inhibit CD8<sup>+</sup> T-cell function<sup>[43]</sup>. Administration of PD-L1 inhibitors results in regression of a number of tumor types<sup>[44-49]</sup>. CTLA-4 blockade has shown efficacy in murine melanoma, prostate cancer, and pancreatic carcinoma studies<sup>[50,51]</sup>. The latter demonstrated particular success when combined with PD-1 inhibition, as survival was prolonged even after tumor rechallenge<sup>[52]</sup>. This finding is applicable to cancer cells that remain concealed within the body following tumor resection.

Despite checkpoint inhibitor success in various cancer types, use of this therapy against brain tumors has yet to be extensively pursued. Preclinical assessments in orthotopic, immunocompetent murine models have identified the most effective checkpoint pathway against GBM. When administered alone, PD-1 inhibition has a 50% long term survival rate in mice. Combined treatment with PD-1 and CTLA-4 inhibition was found to achieve 75% long term survival<sup>[53]</sup>. These results paralleled those found in a melanoma clinical trial that utilized the same combination of inhibitors, indicating improved effectiveness<sup>[54]</sup>. Furthermore, checkpoint inhibitor OX-2 glycoprotein (CD200) has been found to be highly expressed in a number of human brain tissue samples, including astrocytomas, meningiomas, and GBM tumors<sup>[55]</sup>. This pathway has been investigated in canine models with high-grade gliomas. Although CD200 canine clinical trials are still ongoing, regression of tumors and absence of inhibitor toxicity has indicated therapeutic promise, and treated groups have already demonstrated an increase of 615 days of survival as compared to control subjects<sup>[56]</sup>. Another pursuit made to target meningioma and other rare CNS tumors is an ongoing, Phase II

**Table 3. Clinical trials using checkpoint inhibitors to treat central nervous system tumors**

Identifier	Trial name	Treatment	Phase	Diagnosis (newly diagnosed or reoccurring)
NCT02866747	A study evaluating the association of hypofractionated stereotactic radiation therapy and durvalumab for patients with recurrent GBM (STERIMGLI)	Durvalumab (PD-L1), radiotherapy	I/II	Reoccurring
NCT02311920	Immune-related DLTs	Pilimumab (CTLA-4), nivolumab (PD-1), TMZ	I	Both
NCT03173950	Immune checkpoint inhibitor nivolumab in people with select rare CNS cancers	Nivolumab (PD-1)	II	Newly diagnosed
NCT02617589	An investigational immuno-therapy study of nivolumab compared to TMZ, each given with radiation therapy, for newly-diagnosed patients with GBM (CheckMate 498)	Nivolumab (PD-1), radiotherapy, TMZ	III	Newly diagnosed
NCT02320058	An investigational immuno- therapy study to evaluate safety and effectiveness in patients with melanoma that has spread to the brain, treated with nivolumab in combination with ipilimumab, followed by nivolumab by itself (CheckMate 204)	Ipilimumab (CTLA-4), nivolumab (PD-1)	II	Both

GBM: glioblastoma multiforme; PD-1: programmed death receptor 1; PD-L1: programmed death receptor ligand 1; DLTs: dose limiting toxicities; CTLA-4: cytotoxic T-lymphocyte associated protein 4; TMZ: temozolomide; CNS: central nervous system

clinical trial utilizing PD-1 inhibitor nivolumab (NCT03173950). This progress emphasizes the importance of continued clinical efforts with checkpoint inhibitors.

Combinatorial methods using checkpoint inhibitors have also been under investigation [Table 3]. Mice implanted with GL261 gliomas treated with both stereotactic radiotherapy and PD-1 inhibitors have shown improved median survival compared to untreated mice. This is thought to be due to increased MHC-I expression and inhibited PD-1 expression, ultimately provoking an increased CD8<sup>+</sup> effector T and decreased Treg population. 15%-40% of mice became long-term survivors, and mice rechallenged with GL261 demonstrated systemic immunity<sup>[57]</sup>. Nivolumab coupled with radiotherapy presents a similar treatment combination, and the therapy is currently being explored in one of the first phase III clinical trials for GBM. The results have not yet been published (NCT02617589). Investigation of anti-PDL1 durvalumab combined with hypofractionated stereotactic radiotherapy to target recurrent GBM has paved the way for a phase II clinical trial, with an absence of serious adverse events and dose-related toxicity related to treatment in patients (NCT02866747). Combined administration of nivolumab and ipilimumab to patients with untreated melanoma metastatic to the brain demonstrated success in a phase II clinical study, with OS rates reaching 92.3% and 82.8% at 6 and 9 months respectively (NCT02320058). Four-1BB is another antibody that prompts CD8<sup>+</sup> and memory T cell proliferation upon activation. A study in mice combining radiation, CTLA-4 blockade, and 4-1BB activation achieved a minimum of 50% long-term tumor free survival, and the treatment increased populations of CD4<sup>+</sup> and CD8<sup>+</sup> tumor infiltrating lymphocytes. Tumor cells were also rejected after re-challenge<sup>[58]</sup>. Finally, a study utilized the catabolic tryptophan enzyme indoleamine 2,3 dioxygenase 1 (IDO), because it is upregulated in 90% of GBM cases, absent in healthy tissue and is also known to play a significant immunosuppressive role in the tumor microenvironment. Combined inhibitors for CTLA-4, PD-L1, and IDO (1-methyl-tryptophan) were administered to mice and resulted in 100% survival<sup>[59]</sup>. Because unperturbed CTLA-4, PD-L1, and IDO pathways greatly augment immunosuppression, it is thought that pathway inhibition should reduce Tregs and result in positive survival outcomes.

One challenge in checkpoint inhibition therapy is identifying which patients might derive the greatest benefit. Prognostic biomarkers must still be defined. The current means of predicting treatment outcome for the PD-1/PD-L1 pathway is by immunohistochemistry of cytologic tumor samples. This method is not completely reliable, as samples are susceptible to contamination and the interpretation of ambiguous findings<sup>[60,61]</sup>. CTLA-4 does not have clinically relevant biomarkers.

Further confounding the process, it is possible that the expression of checkpoint ligands or receptors on tumors may not always be reliable in determining treatment outcomes. In melanoma, for instance, PD-L1

presence on tumors is indicative of survival outcomes, because ligand expression is dependent on CD8<sup>+</sup> T-cells and IFN- $\gamma$  secretion. However, CD8<sup>+</sup> dependence may be specific to melanoma patients, as PD-L1 checkpoint inhibition therapy has demonstrated improved survival with non-small cell lung cancer even if classified as PD-L1 negative<sup>[62]</sup>. Similarly, expression of PD-L1 has not been definitively correlated with prognosis in GBM, suggesting the ligand is not a reliable biomarker<sup>[63]</sup>. Recent work has aimed to explain the immune-resistance of some tumors. Genes in  $\beta$ -catenin, peroxisome proliferator-activated receptor- $\gamma$ , and fibroblast growth factor receptor 3 pathways were found to be responsible for failed T cell priming and recruitment in the urothelial bladder tumor microenvironment in mice. This ultimately led to poor results in checkpoint inhibitor treatment<sup>[64]</sup>. Similar results were found in another study.  $\beta$ -catenin presence in murine BP-SIY tumors is responsible for preventing migration of effector T cells and a robust immune response succeeding ACT<sup>[65]</sup>. In application to transcriptome signatures, these data may allow for more reliable tumor-specific biomarkers options and could improve effectiveness in the total patient population. Because higher mutational load of the tumor has been associated with more effective immunotherapeutic outcomes using checkpoint inhibitors, assays exploring tumor mutational burden are also currently being pursued<sup>[66]</sup>.

These preclinical studies suggest that the mechanism of checkpoint inhibitors is more complex than once thought. Until recently, our gaps in understanding the mechanisms regarding checkpoint inhibition were mostly due to the absence of *in vivo* models representative of the human immune system. However, headway has been made in the development of new models. For instance, we know an exhausted CD8<sup>+</sup> T-cell population surrounds GBM tumors in humans and that this state is achieved through prolonged exposure to the tumor antigen<sup>[67]</sup>. To emulate these conditions in the laboratory, a murine model was generated by infection with chronic lymphocytic choriomeningitis virus followed by induction of murine glioma. This tumor positively responded to anti PD-1 treatment<sup>[68]</sup>. A model for human hematopoietic and immune systems was generated in nonobese diabetic Cg-PrkdcscidIL2rgtm1Wjl/Sz mice by transplantation of human CD34<sup>+</sup> hematopoietic progenitor and stem cells. Patient-derived tumor xenografts in this model responded positively to PD-1 checkpoint inhibitor pembrolizumab<sup>[69]</sup>. Studies utilizing these models should provide a more clear representation of the mechanisms and effects of checkpoint inhibition on human tumors. Through continued efforts, distinct biomarkers can be established for these therapies, and a push for additional clinical trials pursued.

## VACCINE THERAPIES

### Peptide Vaccines

Peptide vaccines have been widely studied for immunotherapy due to their cost-effectiveness, reproducibility, specificity, and low risk of generating an autoimmune response. Peptide vaccines stimulate the immune system by activating CD8<sup>+</sup> and CD4<sup>+</sup> T-cells via APCs. By developing peptides specific to tumors, peptide vaccines can be used to induce an anti-tumor immune response to combat GBM. A limitation of this therapy, however, is the capability of GBM cells to down-regulate MHC-I expression and increase prostaglandin E2 production, which in turn downregulates MHC-II expression on APCs. Furthermore, patient MHC heterogeneity and changes in MHC expression restrict the use of peptide vaccines. To overcome MHC-dependence, long synthetic peptides encoding multiple MHC class I and II epitopes have been developed which are more efficiently processed by DCs and associated with increased CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocyte activation<sup>[70,71]</sup>.

Antigens used in peptide vaccines can be tumor-specific antigens which are often the products of mutations or splice variants, or tumor-associated antigens which are overexpressed gene products that can be expressed in tumor cells. While tumor-specific antigens result in precise targeting of the tumor, they are not expressed by a majority of the patient population. Conversely, tumor-associated antigens are shared by a larger patient population and have been more preferentially used in vaccines as immunotherapies<sup>[72]</sup>. Adjuvants often supplement antigens to improve immunogenicity. Common adjuvants include PAMPs, damage-associated molecular patterns, or cytokines that can activate APCs and lymphocytes.

**Table 4. Clinical trials using peptide vaccines to treat glioblastoma multiforme**

Identifier	Trial name	Treatment	Status	Diagnosis (newly diagnosed or reoccurring)
NCT02149225	GAPVAC Phase I trial in newly diagnosed GBM patients	Patient-tailored APVAC vaccine plus poly-ICLC and GM-CSF	I	Newly diagnosed
NCT01250470	Phase I study of safety, tolerability and immunological effects of SVN53-67/M57-KOH in patients with survivin-positive malignant gliomas	Montanide ISA-51/survivin peptide vaccine with GM-CSF	I	Both
NCT01222221	A cancer research UK Phase I trial of IMA950 (a novel multi-peptide vaccine) plus GM-CSF in patients with newly diagnosed GBM	IMA-950 vaccine with GM-CSF	I	Newly diagnosed
NCT00626015	Zenapax®-activated peptide immunotherapy	PEP-3 KLH conjugate vaccine with daclizumab	I	Newly diagnosed
NCT00069940	A Phase I study of vaccination with telomerase peptide plus GM-CSF	540-548 telomerase peptide vaccine with GM-CSF	I	Both
NCT00643097	A complementary trial of an immunotherapy vaccine against tumor-specific EGFRvIII	PEP-3 KLH conjugate vaccine with GM-CSF	II	Newly diagnosed
NCT01498328	A Phase II study of rindopepimut/GM-CSF in patients with relapsed EGFRvIII-positive GBM	PEP-3 KLH conjugate vaccine with GM-CSF and bevacizumab	II	Reoccurring
NCT00458601	A Phase II study of CDX-110 with radiation and temozolomide in patients with newly diagnosed GBM	PEP-3 KLH conjugate vaccine with GM-CSF	II	Newly diagnosed
NCT01920191	Phase I/II study of intradermal IMA-950 peptide-based vaccine adjuvanted with intra muscular poly-ICLC in combination with temozolomide in newly diagnosed HLA-A2 GBM patients	IMA-950 vaccine with poly-ICLC	II	Newly diagnosed
NCT01480479	An international, randomized, double-blind, controlled study of rindopepimut/GM-CSF with adjuvant temozolomide in patients with newly diagnosed, surgically resected, EGFRvIII-positive GBM	PEP-3 KLH conjugated vaccine with GM-CSF	III	Newly diagnosed
NCT03422094	A pilot study to assess the safety, feasibility, and immunogenicity of a neoantigen-based personalized vaccine combined with immune checkpoint blockade therapy in patients with newly diagnosed, unmethylated GBM	Neovax with poly-ICLC, nivolumab, and ipilimumab	Active, recruiting	Newly diagnosed
NCT03223103	Phase I study of tumor treatment fields and a personalized mutation-derived tumor vaccine in patients with newly diagnosed GBM	MTA-based vaccine with poly-ICLC and TTF	Active, not recruiting	Newly diagnosed
NCT02455557	A Phase II study of the safety and efficacy of SVN53-67/M57-KLH (SurVaxM) in survivin-positive newly diagnosed GBM	SVN53-67-KLH peptide vaccine with GM-CSF	Active, not recruiting	Newly diagnosed

GBM: glioblastoma multiforme; GM-CSF: granulocyte-macrophage colony-stimulating factor; KLH: keyhole limpet hemocyanin; EGFR: epidermal growth factor receptor; TTF: tumor treating fields; poly-ICLC: polyriboinosinic-polyribocytidylic acid-polylysine carboxymethylcellulose

One of the most successful tumor-specific antigen peptide vaccines against GBM, rindopepimut, uses the EGFRvIII peptide which is expressed in 25%-64% of GBM patients<sup>[17]</sup>. Rindopepimut is commonly co-administered with the keyhole limpet hemocyanin (KLH) and granulocyte-macrophage colony-stimulating factor (GM-CSF) adjuvants. Adjuvants are commonly used to enhance cross-presentation of antigens to improve immunogenicity. A promising phase II trial (ACTIVATE) conducted with rindopepimut and TMZ treatment demonstrated a median OS of 26.0 months [Table 4]. This compared well to controls with a median OS of only 15.0 months (TMZ treatment). Antibody formation against the EGFRvIII peptide was observed in a small subset of patients which correlated with improved median OS, 47.7 months as compared to 22.8 months. However, EGFRvIII negative tumor recurrence was observed in 82% of patients<sup>[73]</sup>. A larger, phase III clinical trial (ACT IV) was later performed that did not show significant improvement in OS following rindopepimut treatment<sup>[74]</sup>. KLH, an adjuvant given with the peptide vaccine, was administered to the phase III control group but not the phase II control group. This suggests that the improved OS may be attributable to the immunogenicity generated by KLH alone and not the peptide. Additionally, at larger scales, peptide vaccinations may be limited by heterogeneity of the patient population. Co-administration of rindopepimut with bevacizumab, however, improved OS for patients which suggests combination with anti-angiogenic therapies may improve efficacy of immunotherapies<sup>[75]</sup>.

Clinical trials involving tumor-associated antigens have not shown significant benefit. Survivin, an inhibitor of apoptosis protein family, is highly expressed in all four subtypes of GBM. A phase I clinical trial found that SurVaxM, a survivin peptide vaccine, did not improve OS, though it was shown to induce cellular and humoral immune responses<sup>[76]</sup> and has moved on to a phase II trial. Similarly, a phase I/II clinical trial using IMA-950, a multi-peptide vaccine, did not significantly improve OS in combination with polyribinosinic-polyribocytidylic acid-polylysine carboxymethylcellulose and TMZ treatment<sup>[77]</sup>. Similar to SurVaxM, IMA-950 induced a measurable increase in CD8<sup>+</sup> and CD4<sup>+</sup> T-cell response. Lack of correlation between improved outcomes and a peripheral immune response is a common theme among peptide vaccines, and suggests a disrupted interaction between peripheral immune cells and GBM cells due to the immunosuppressive tumor microenvironment.

Recently, efforts have turned to developing personalized peptide vaccines based on analysis of patients' resected tumors. A phase I clinical trial assessed actively personalized vaccination (APVAC) for improving immunogenicity and survival in GBM patients. APVAC induced a CD4<sup>+</sup> T-cell driven immune response in 90% of patients, with a median OS of 29 months<sup>[78]</sup>. However, APVAC was less-tolerated than previous peptide vaccines, with adverse events including anaphylactic reactions and cerebral edema which must be addressed for personalized vaccines to advance.

Overall, peptide vaccines have been shown to induce an immune response without a corresponding improvement in OS. The lack of correlation may be attributed to the immunosuppressive tumor microenvironment. Combination therapies with checkpoint inhibitors may provide a more robust response with better survival outcomes. Personalized vaccine therapies offer a unique and potentially effective way to not only prevent initial tumor progression but also recurrent tumor development, and warrant further investigation. An active clinical trial is currently pursuing combining personalized peptide vaccines with checkpoint inhibitors, and will hopefully elucidate the benefit of these combined therapies (NCT03422094).

### **Induced pluripotent stem cells vaccines**

Stem cell vaccines comprised of embryonic stem cells (ESCs) have been studied for their ability to generate antitumor immunity. This is largely attributed to the common markers expressed by both tumors and ESCs<sup>[79]</sup>. Studies investigating administration of ESC vaccines prior to tumor induction demonstrated that pre-vaccination could effectively halt tumor growth. However, ethical concerns regarding ESCs limit progress. Focus has now shifted towards induced pluripotent stem cells (iPSCs), which are stem cells derived from somatic cells in combination with Oct3/4, Sox2, c-Myc, and Klf4 transcription factors. The exposure of somatic cells to these transcription factors promotes oncogenic transformation and tumor antigen expression<sup>[80]</sup>. This can lead to improved immunogenicity and more precise targeting of tumor cells. Additionally, iPSCs can be generated from a patient's own tissue and may provide a better representation of a patient's tumor immunogens, although this procedure is not cost-effective.

The use of non-autologous iPSC vaccines can provide a more commercially viable option for iPSC based vaccines. Vaccines generated from iPSCs genetically engineered to express GM-CSF have been shown to suppress lung tumor growth in mice prior to tumor induction as well as in pre-established tumors<sup>[81]</sup>. More recently, an iPSC vaccine comprised of iPSCs with DNA adjuvant CpG demonstrated tumor regression and significantly longer survival in murine models of breast cancer and melanoma. Additionally, treated mice developed antibody titers against iPSCs and tumor cells and were able to protect against tumor rechallenge<sup>[82]</sup>. These data suggest iPSC vaccines may be applicable to other solid tumors, such as GBM.

### **DC vaccines**

DCs act as the bridge between the innate and adaptive immune system, collecting antigens and presenting them to lymphocytes. DC presentation of antigens to lymphocytes leads to activation of various T-cell populations. These T-cells' respective types and specificity are dependent on the antigens presented by

the DCs and the context in which the DC presents the antigen. To elicit the appropriate lymphocyte and immune response, DCs must present tumor-specific or tumor-associated antigens, upregulate expression of MHC-I and II in conjunction with adhesion and co-stimulatory molecules, and induce secretion of stimulatory and anti-tumor cytokines including IL-12, IL-15, IL-18, and IFN- $\gamma$ <sup>[83]</sup>.

Certain lymphocytes possess inherent capabilities to fight cancer. However, cancers may lack a sufficient supply of tumor antigens to stimulate this immune response. DC vaccines address the lack of tumor antigen by supplying antigen and stimulation to DCs *ex vivo*. Patients can then be vaccinated with these tumor-specific DCs. By manipulating DCs, both in terms of the antigens they present and the context by which they do so, activation of lymphocytes can be manipulated in order to yield an anti-tumor response.

Clinical trials using DC vaccines have employed a variety of antigen loading strategies which include pulsing maturing DCs with autologous-tumor lysate<sup>[84]</sup>. In this technique, DCs must be enriched and matured from monocytes obtained from individual patient's peripheral blood mononuclear cells<sup>[84-87]</sup>. The monocytes are then expanded and differentiated into immature DCs through exposure to GM-CSF and IL-4<sup>[88]</sup>. Immature DCs are loaded with antigens and matured before being administered to patients as a vaccine.

Pulsing maturing DCs with autologous-tumor lysate has made the most progress in GBM therapeutic outcomes. Northwest Biotherapeutics' DCVax<sup>®</sup>-L has recently shown positive results in a phase III clinical trial [Table 5]. Patients selected were between 18 and 70 years of age, and had just been newly diagnosed with GBM. Following surgical resection (the source of autologous tumor-lysate) and chemoradiotherapy, patients were given a series of DCVax<sup>®</sup>-L injections in addition to monthly administration of TMZ. Median OS was 23.1 months, with 25.4% of patients surviving for more than three years post-surgery. This therapy was also well tolerated, with only 2.1% of patients demonstrating grade 3 or 4 adverse events which may have been related to surgery and chemoradiotherapy<sup>[89]</sup>.

Smaller trials using autologous-tumor lysate pulsed vaccines have demonstrated similar safety and efficacy. In the University of Navarra phase II trial, autologous DC vaccines were administered to 31 patients who had a median OS of 23.4 months<sup>[90]</sup>. The high grade glioma-2006 phase I and II trials administered autologous DC vaccines to 77 patients in total with a median OS of 19.4 months<sup>[91]</sup>, and the phase II DENDR1 trial administered autologous tumor lysate vaccines to 24 patients demonstrated a median OS of 20.1 months<sup>[92]</sup>. Compared to the 14 months OS following GBM diagnosis and standard of care, DC vaccines pulsed with autologous tumor lysate have demonstrated a substantial and consistent increase in OS, with little to no adverse events.

More recent endeavors have sought to tailor protein-specific DC vaccines through lysates composed of select tumor-associated antigens<sup>[87]</sup> or by transfecting DCs with nucleic acids for tumor-specific or tumor-associated antigen<sup>[93]</sup>, or with cytomegalovirus RNA<sup>[94]</sup>. A phase I study pulsed DC with lysate containing tumor-associated antigens including human epidermal growth factor receptor 2 (HER2), tyrosinase related protein-2, gp100, melanoma-associated antigen 1 (MAGE-1), IL13R $\alpha$ 2, and absent in melanoma 2 (AIM-2), proteins which are enriched in GBM cancer stem cells (GCSC). The multi-epitope-pulsed DC vaccine was used to vaccinate 16 patients with newly diagnosed GBM. Median OS was 38.4 months and improved OS correlated with expression of AIM-2 and MAGE-1 in the tumor. Notably, a decrease was seen in GCSC marker CD133, suggesting that the multi-epitope-pulsed DC vaccine had successfully reduced the population of cancer stem cells<sup>[87]</sup>, potentially accounting for the significant increase in median OS, nearly three times that seen with the standard of care.

Transfection of DC to induce expression of tumor-specific or tumor-associated proteins has also demonstrated success in treating GBM. In a phase I clinical trial GCSC mRNA was isolated from brain

**Table 5. Clinical trials using dendritic vaccines to treat gliomas**

Identifier	Trial name	Treatment	Phase	Diagnosis (newly diagnosed or reoccurring)
NCT00107185	Vaccine therapy in treating young patients who are undergoing surgery for malignant glioma	Autologous DC vaccine	I	Both
NCT01171469	Vaccination with dendritic cells loaded with brain tumor stem cells for progressive malignant brain tumor	Autologous DC vaccine	I	Both
NCT00639639	Vaccine Therapy in treating patients with newly diagnosed GBM (ATTAC)	DC vaccine with mRNA from human cytomegalo-virus	I	Newly diagnosed
EudraCT 2006- 002881-20	HGG-2006 phase I/II trial	Autologous DC vaccine	I/II	Newly diagnosed
NCT00846456 (EudraCT 2007- 006171-37)	Safe study of DC based therapy targeting tumor stem cells in GBM	DC vaccine with mRNA from tumor stem cells	I/II	Both
NCT00323115	Phase II feasibility study of DC vaccination for newly diagnosed GBM	Autologous DC vaccine	II	Newly diagnosed
EudraCT 2008- 005035-15	DENDR1	Autologous DC vaccine	II	Newly diagnosed
EudraCT 2008- 005038-62	DENDR2	Autologous DC vaccine	II	Reoccurring
NCT03395587	Efficiency of vaccination with lysate-loaded DCs in patients with newly diagnosed GBM (GlioVax)	Autologous DC vaccine	II	Newly diagnosed
NCT01006044 (EudraCT 2009- 009879-35)	Efficacy & safety of autologous DC vaccination in GBM after complete surgical resection	Autologous DC vaccine	II	Newly diagnosed
NCT01280552	A study of ICT-107 immunotherapy in GBM	Multi-epitope pulsed DC vaccine	II	Newly diagnosed
NCT02546102	Phase 3 randomized, double-blind, controlled study of ICT-107 in GBM	Multi-epitope pulsed DC vaccine	III	Newly diagnosed (and in remission)
NCT00045968	Study of a drug [DCVax®-L] to treat newly diagnosed GBM brain cancer	Autologous DC vaccine	III	Newly diagnosed

DC: dendritic cell; GBM: glioblastoma multiforme; HGG: high grade glioma

tumor biopsies and transfected into DC cells used to vaccinate patients. To isolate GCSC, patient tumor cells were selected for their ability to form spheres *in vitro* as a proxy for identifying cancer stem cells. From the sphere-forming tumor cells, mRNA was isolated and transfected into patient derived DCs, which were used as vaccinations for seven patients. Patients showed no adverse effects and had a median OS of 23 months<sup>[95]</sup>, comparable to that seen in DC vaccines loaded with autologous tumor-lysate.

In addition to cellular proteins, recent studies have shown that a high percentage of GBM express cytomegalovirus proteins, a potential target for DC vaccines. In a phase I clinical trial, 11 patients were vaccinated with DCs transfected with cytomegalovirus pp65 lysosome-associated membrane glycoprotein mRNA, following resection and radiochemotherapy. These patients demonstrated a median OS of 41.1 months, with no adverse effects attributed to the cellular component of treatment<sup>[94]</sup>. If larger studies confirm that a significant number of GBM patient tumors exhibit cytomegalovirus, cytomegalovirus proteins offer a clear target for DC vaccines, and potentially peptide vaccines as well.

One challenge of evaluating DC vaccines has been the lack of consistency across trials to measure immune response. While median OS provides a good measure of general effectiveness, trials vary in measurements when evaluating immunological outcomes, such as cytokine levels or immune cell counts. Trials have shown mixed results as to whether or not DC vaccines increase cytotoxic T-cell and TH1 responses, which is further confounded by trials that did not measure these criteria. Trials that have measured NK cell populations have shown that an increase in NK cell activity and number following vaccinations has been correlated with improved outcomes<sup>[86,92]</sup>. In order to improve the development of DC vaccinations, consistent immunological evaluations (patient T-cell, NK cell, and cytokine production, *etc.*) offer a clear target so that the mechanisms underlying the success of various aspects of DC vaccinations can be elucidated and better applied going forward.

While DC vaccines have shown their ability to prolong OS, correlation of immune response with long-term outcomes remains unclear, and prognostic markers for determining patients that will best respond to DC vaccines needs to be further elucidated. DC vaccines targeting specific proteins, the multi-epitope trial and cytomegalovirus trial, show great clinical potential, but must be evaluated on a larger scale before conclusions can be drawn. Whole-tumor lysate vaccines are the most advanced in terms of clinical trial progress and demonstrate clear improvements, with 25% of patients achieving above a three-year survival rate in the largest phase 3 trial to date. This trial further demonstrated increased efficacy in patients with methylated O6-methylguanine-DNA methyltransferase, offering another potential prognostic marker for identifying patients who might best respond to autologous tumor-lysate DC vaccines<sup>[89]</sup>. For patients whose tumors can be biopsied, DC vaccines offer a significant improvement in survival outcome, which will be enhanced as prognostic markers become clearer.

## CONCLUSION

Glioma-targeted immunotherapy is still in its infancy. Although ACT, NK cells, checkpoint inhibitors and vaccines have proven their efficacy in other cancers, a deeper understanding of the features specific to solid gliomas is necessary for refined therapy adjustment. Furthermore, improved human preclinical models can more accurately illustrate the human CNS microenvironment and immune cell relationships with the BBB. Studies utilizing these models can deepen our understanding of immune function, ultimately revealing ways to enhance combined treatment modalities. Yet as these ambiguities are made clear, the future of these treatments against GBM remains bright. These methods of tumor eradication address limitations posed by conventional surgical, radiation, and chemotherapies.

## DECLARATIONS

### Authors' contributions

Conceptualize the project, write and edit the manuscript: Low WC, Crane AT, Pearce CM, Chrostek MR  
Write various sections of the manuscript: Fellows EG, Toman NG, Tran S

### Availability of data and materials

Not applicable.

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### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

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