Review

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Immunotherapy

Current state and future prospects of immunotherapy for glioma

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There is a large unmet need for effective therapeutic approaches for glioma, the most malignant brain tumor. Clinical and preclinical studies have enormously expanded our knowledge about the molecular aspects of this deadly disease and its interaction with the host immune system. In this review we highlight the wide array of immunotherapeutic interventions that are currently being tested in glioma patients. Given the molecular heterogeneity, tumor immunoediting and the profound immunosuppression that characterize glioma, it has become clear that combinatorial approaches targeting multiple pathways tailored to the genetic signature of the tumor will be required in order to achieve optimal therapeutic efficacy.

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Glioma subtypes & molecular classification

Gliomas are the most common primary brain tumors with an estimated incidence of approximately 7 per 100,000 people in USA, representing 27% of all CNS tumors and 80% of malignant tumors [1]. Until 2016, the WHO classified gliomas based on histology and categorized them into three principal groups: astrocytoma, oligodendroglioma and oligoastrocytoma [2,3]. Gliomas are further separated based on their degree of anaplasia into WHO grades I-IV, of which WHO grade I is assigned to lesions with slow growth and better prognosis; and WHO grade IV is assigned to the most malignant tumors represented by high-grade gliomas (HGG) or glioblastomas [2–4].

With the advancement of genomics, transcriptomics and epigenomics, together with the incorporation of high-throughput technology and histological methods in the analysis of the glioma specimens, several molecular markers have been identified, for example, *TERT*, *PDGFR*, *PTEN*, *IDH*, *PI3K*, *ATRX*, *EGFR* and H3 histone family member 3A (Figure 1) [5–7]. These markers are associated with specific tumor phenotypes and indicate the need to define new glioma subtypes [6–10]. With the introduction of molecular data to tumor classification, the WHO 2016 classification underwent a notable improvement from the classical histological classification [3,11]. One of the more significant criteria is the mutation status of *IDH1*. Mutation of *IDH1* at arginine 132 (R132H) is present in around 80% of low-grade gliomas (LGG; WHO grade II) and anaplastic astrocytomas (WHO grade III), as well as in a subset of HGG (WHO grade IV) [12,13]. IDH1-R132H results in the production of the oncometabolite R-2-hydroxyglutarate, which can inhibit a variety of α-ketoglutarate-dependent dioxygenases, such as prolyl-4 hydroxylase, prolyl hydroxylase and the ten-eleven translocation family of DNA hydroxylases, which also function as histone demethylases [14,15]. 2-hydroxyglutarate also induces histone 3 hypermethylation, and is sufficient for formation of a glioma CpG island methylator phenotype thus causing a global hypermethylation phenotype in glioma cells [16,17]. Generally, patients with *IDH1* mutation have better prognosis and better response to treatment [7,9,10,18]. Therefore, gliomas can be separated into two large groups: mutant *IDH1* and wild-type *IDH1* (wt-IDH1) (Figure 1). In turn, mutant *IDH1* LGG can be further dissected into two subgroups according to 1p/19q or *ATRX* status, which are mutually exclusive (Figure 1) [3,9,10,12]. Mutant *IDH1* with 1p/19q co-

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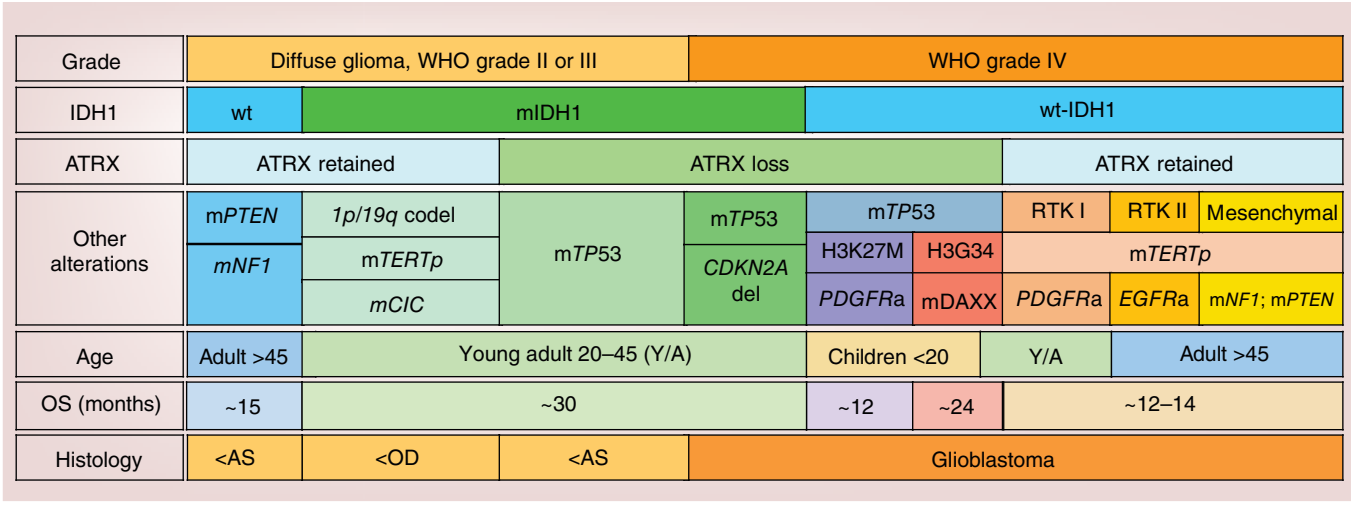

Figure 1. Overview of the major subtypes of glioma.
AS: Astrocytoma; OD: Oligodendroglioma; OS: Overall survival.

deletion is associated with oligodendroglioma phenotype in diffuse LGG [10,19]. In this subgroup, *TERTp* and *CIC* mutations are also present (Figure 1) [9,10,12]. Mutant *IDH1* with *ATRX* loss and *TP53* mutation is associated with astrocytoma and oligoastrocytoma phenotypes (Figure 1) [9,10,12,19]. This particular subtype of glioma can progress in malignancy to reach WHO IV grade [20]. For this reason, these molecular markers can also be found in the most aggressive forms of glioma [3]. On the other hand, gliomas harboring wt-*IDH1* represent most of the WHO grade IV gliomas. Gliomas expressing wt-*IDH1*, with loss of *TP53* and *ATRX*, and mutations in H3 histone family member 3A, including H3K27M and H3G34, are typically found in pediatric and young adult patients [19,21]. Gliomas with wt-*IDH1* that have retained *ATRX* typically co-express *TERTp* mutations and alterations in regulators of the RTK-RAS-PI3K signaling cascade and are typically encountered in adult patients (Figure 1) [3,4,6,11]. RTK I is a molecular subgroup of glioblastomas that generally arises in young adults, characterized by *TERTp* mutation and *PDGFRA* amplification [4,11]. Glioblastoma can also be divided in primary and secondary. Primary glioblastomas are generated *de novo* and represent almost 90% of glioblastoma patients [3,22]. Secondary glioblastomas develop from diffuse lower grade glioma [22]. They also harbor different molecular alterations. For example, *EGFR* overexpression is prevalent in primary glioblastoma, but is rare in secondary [23]. In contrast, *TP53* mutation is rare in primary glioblastoma; however, is a characteristic of secondary glioblastoma [23]. In addition, *IDH1* mutation and *ATRX* inactivation are typically found in secondary glioblastoma together with *TP53* mutation [3,22]. Therefore, primary and secondary glioblastoma correspond to a distinctive brain-tumor entities differing in origin and molecular characteristics.

In summary, gliomas represent a heterogeneous group of brain tumors that can be classified according to histology, malignancy, age range and genetic/epigenetic alterations. The molecular features of these tumors are crucial for accurate diagnosis, and also for designing therapeutic strategies tailored to tumor subtypes. We hypothesized specific molecular alterations can impact glioma responses to therapies.

Glioma prognosis & treatment

Glioma treatment modalities include surgical resection, radiation therapy and/or chemotherapy. Treatment strategies are influenced by the recently revised 2016 WHO brain tumor classification guidelines [3]. Maximal safe surgical resection is the primary treatment strategy for LGG. The most common LGG in adults is oligodendroglioma, a grade II tumor by the 2016 WHO classification. Molecular and genetic characteristics, such as *IDH* mutation and codeletion of the 1p/19q chromosomal arms are becoming increasingly important for stratifying patients based on response to treatment. In most cases, the standard treatment for oligodendroglioma beyond surgery is radiotherapy followed by procarbazine, lomustine and vincristine chemotherapy [24]. For those patients with anaplastic astrocytomas, the standard of care (SOC) involves maximal safe resection or biopsy followed by involved-field radiotherapy to 60 Gy given in 1.8-2 Gy fractions [25]. Median survival time is doubled in patients receiving adjuvant radiotherapy versus surgery alone in randomized trials [26]. However, whole brain radiation therapy can significantly

impact patient cognitive functions. First-line treatment also includes the use of chemotherapeutics with modest increases in 1- and 2-year survival times (58–63% and 31–37%, respectively) [27]. Glioma (WHO IV) carries the poorest prognosis, and surgical resection is a key element for initial management versus diagnostic biopsy. However, the benefit is mitigated if the patient is left with a neurologic deficit which significantly impairs daily function. Radiotherapy and adjuvant temozolomide (TMZ) have been the SOC for glioblastoma (GBM) patients based on the treatment protocol per Stupp *et al.* in a 2005 randomized controlled trial [28]. Patients receiving TMZ in addition to radiotherapy after surgery experienced a 2.5-month survival advantage compared with those receiving adjuvant radiotherapy alone [28]. Several important prognostic indicators exist, the most important of which include patient age and functional status, measured commonly on the Karnofsky Performance Scale and *MGMT* promoter methylation [29]. Current treatments for anaplastic oligodendroglioma and oligoastrocytoma, both WHO grade III tumors, represent a more rapidly evolving paradigm and insight into future glioma treatment effort. The molecular biology of these tumors and significance of well-recognized genetic aberrancies are leading to new treatment options and targeted therapies [25]. Evidence challenging the central dogma of the brain as an immune privileged organ and the success of immunotherapeutic approaches in other cancers has driven the investigation of several immune-based approaches in preclinical models and in numerous clinical trials [30–34].

Innate immune responses in glioma

Although the brain was originally considered an immune privileged organ, studies showed that due to disturbance of the blood–brain barrier integrity during inflammation or cancer and the presence of lymphatic outflow channels, the immune system is able to interact with cells within the CNS [30–32]. Immune cell infiltration has been demonstrated in patients with malignant glioma; however, endogenous immune responses fail to control the disease [35]. This is mainly due to glioma-mediated suppression of the infiltrating immune cells by mechanisms such as TGF-β secretion, release of LDH5 or expression of galectin-1 (gal-1) [36–38]. Immune surveillance by innate immune cells is the first line of defense against malignant cells. Natural killer (NK) cells are the main effector cells of the innate immune system mediating antitumor responses. Activation of NK cells is tightly regulated based on germline-encoded activating and inhibitory receptors [39,40]. Data from our laboratory showed that NK cells can mediate an antiglioma immune response which is suppressed by gal-1 expression by glioma cells [41]. Additional studies demonstrated that glioma-derived LDH5 and TGF-β abrogate the antiglioma activity of NK cells. LDH5 induces expression of activating receptor NKG2D ligands on myeloid cells, resulting in downregulation of NKG2D on NK cells and thereby decreased antiglioma reactivity [36]. Moreover, glioma cell-derived TGF-β has been shown to inhibit expression of activating NKG2D ligands MICA and ULBP2 on glioma cells, which facilitates downregulation of NKG2D receptor on immune cells, including NK cells and $CD8^+$ T cells [37,42].

It was recently demonstrated that a myeloid cell population ($Gr\text{-}1^+CD11b^+$) is required for NK cell-mediated glioma eradication in the absence of immunosuppressive gal-1 [43]. Immunodepletion of $Gr\text{-}1^+$ or $Ly6C^+$ expressing cells in an orthotopic glioma mouse model resulted in abrogation of tumor rejection, demonstrating the importance of this myeloid cell population for the innate antiglioma immune response [43]. Additionally, studies demonstrated a role for TLR-mediated immune activation in the context of glioma-targeting immunotherapeutic approaches. TLRs are germline-encoded receptors, some of which are expressed on the cell surface while others are expressed in the endosomal compartment [44]. Natural TLR ligands are common pathogen-associated molecular patterns, such as viral ssRNA (TLR7) or dsRNA (TLR3) or bacterial components like lipopolysaccharide (LPS; TLR4) or CpG-containing dsDNA (TLR9) [44]. TLR activation can also be induced by recognition of endogenous damage-associated molecular patterns released upon cell death [45]. Once activated, TLR signaling results in production of proinflammatory cytokines and upregulation of co-stimulatory molecules, thereby mediating activation of the adaptive immune response. We demonstrated *in vivo* that release of the endogenous TLR2 ligand HMGB1 (high mobility group box 1) from dying glioma cells mediates tumor regression following combined immunotherapy/cytotoxic therapy [46]. In this therapeutic approach, adenoviral vectors encoding for herpes simplex type 1-thymidine kinase (TK) and FMS-like Tyrosine kinase 3 ligand (Flt3L) are delivered into the brain where glioma cell death is induced upon systemic ganciclovir treatment [47]. This in turn facilitates the release of tumor antigens and the endogenous TLR2 ligand HMGB1, which then activates dendritic cells (DCs) that were recruited into the brain by Flt3L. Activation of TLR signaling in recruited DCs induces $CD8^+$ T cell-dependent glioma regression as well as antiglioma immunological memory [46]. In addition to TLR-induced immune activation by endogenous TLR ligands, administration of synthetic TLR ligands as single agents or as adjuvants in combination with peptide vaccines is under investigation. Studies in glioma mouse models showed that topical

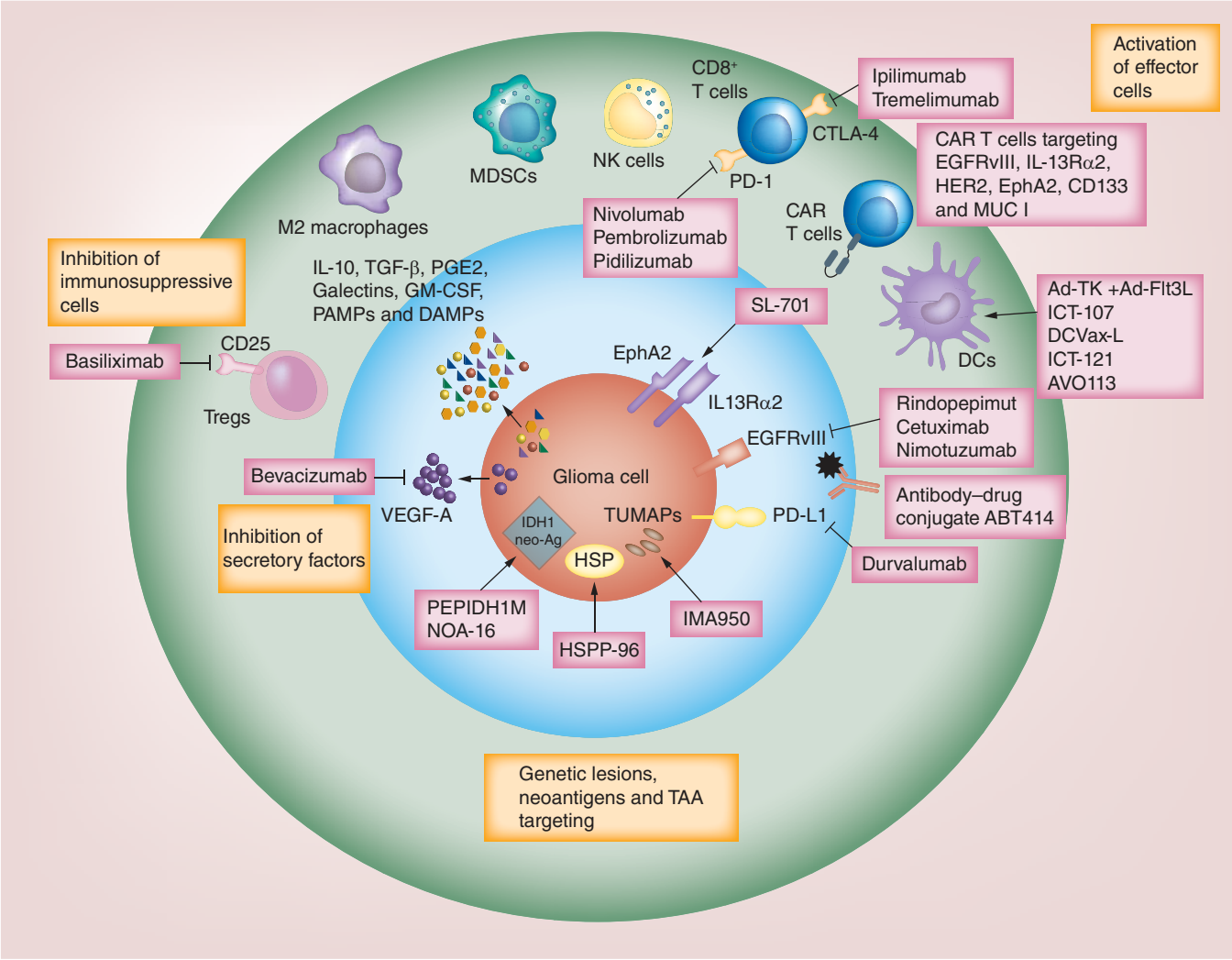

Figure 2. Immunotherapy strategies in clinical trials. Glioma evades the immune system through a variety of ways. Antiglioma immunotherapeutic strategies are targeting T-cell exhaustion, immunosuppressive cells and angiogenesis and testing peptide or dendritic cell vaccines-based approaches to enhance the antitumor adaptive immune response or the activity of effector cells using chimeric antigen receptor T cells.

application of imiquimod (TLR7/8 ligand) can convert plasmacytoid dendritic cells (pDCs) into antiglioma effector cells [48]. In a different glioma mouse model, topical imiquimod administration exerted therapeutic effect by inducing glioma-reactive $CD8^+$ T cells and reducing the number of $CD4^+Foxp3^+$ Treg cells [49]. Importantly, synthetic TLR agonists have entered clinical trials as adjuvant to amplify the therapeutic effect of peptide vaccines (NCT01920191, NCT02454634).

Together, these studies demonstrate that glioma can be infiltrated and recognized by cells of the innate as well as adaptive immune system; however, in most of the cases these immune interactions are effectively suppressed by glioma tumor cells. Therefore, counteracting glioma-mediated immune suppression is a prerequisite for the development of new and more effective immunotherapies for this devastating disease.

Immunosuppression in glioma

Glioma-mediated immunosuppression depends on the local production of cytokines and chemokines and the recruitment of regulatory, immunosupressive cells (Figure 2) [50]. TGF-β and IL-10 are central to maintaining the immunosuppressive glioma microenvironment. These cytokines are not only secreted by tumor-infiltrating immune cells, but also by glioma cells themselves [50]. IL-10 inhibits the activation and effector functions of DCs, macrophages and T cells, modulates the growth and differentiation of immune cells, and limits the expression of

MHC class II in monocytes [50,51]. IL-10 has also been shown to upregulate PD-L1 in circulating monocytes and tumor-associated macrophages in glioma [52]. Interestingly, IL-10 is not always a mediator of immunosuppression and also exerts proinflammatory and antitumor effects. It has been found that T cells' inhibition of glioma growth relies on high levels of IL-10 [53]. Furthermore, genetic ablation of IL-10 facilitates tumor growth and metastasis development in mouse models of colon cancer, an effect that was associated with the expansion of myeloid-derived suppressor cells (MDSCs) and Tregs [54]. In addition, IL-10 has been shown to stimulate macrophages to produce antiangiogenic cytokines and to promote antitumor NK-cell responses [50].

TGF-β is a pleiotropic cytokine involved in many biological functions, including the blockade of T-cell activation and proliferation, as well as the induction of Tregs [55,56]. It was first isolated from patients with glioma and is considered an important immunosuppressive factor [57–59]. TGF-β expression levels correlate with higher tumor grades and worse prognosis [60]. Nevertheless, this cytokine plays a complex role in glioma. In the early stages of tumor growth, TGF-β acts as a tumor suppressor [61]. At later stages, however, glioma cells stop responding to these TGF-β-mediated inhibitory signals and instead TGF-β enhances tumor progression through different mechanisms such as increasing angiogenesis and promoting the expansion of Tregs [62].

Glioma secrete other immunosuppressive factors such as CSF-1, VEGF, PGE2, NO, Arg I, IDO and Gal-1 [63] . VEGF does not only induce angiogenesis, but also inhibits the functional maturation of DCs [64]. PGE2 downregulates the production of Th1 cytokines (IL-2, IFN-γ and TNF-α) and upregulates Th2 cytokines (IL-10, IL-6, IL-4) [65,66]. Both TGF-β and PGE2 can act synergistically to induce a regulatory phenotype in DCs [67]. Studies performed in mice harboring subcutaneous glioma have shown that systemic immunosuppression associated with growing gliomas is partly mediated by the overproduction of NO in splenocytes [68]. Inhibition of inducible NO synthase, using either mercaptoethylguanidine or l-N(6)-(1-Iminoethyl)-l-lysine (l-NIL) could boost IFN-γ-based immunotherapy approach in rats with intracranial tumors [69,70]. Arg I has been proposed as a mechanism of monocyte-mediated inhibition of T-cell function in glioma patients and researchers have found an expanded population of circulating degranulated neutrophils, which is associated with elevated levels of serum Arg I and decreased T-cell CD3ζ expression in the peripheral blood of glioma patients [71]. IDO expression is higher in HGG than in LGG and correlates with worse overall survival rates [72]. IDO expression has been shown to increase the recruitment of immunosuppressive Tregs, promoting tumor escape [73]. Another immunosuppressive ligand expressed by glioma cells, Gal-1, enhances tumor cell migration, induces T-cell apoptosis and inhibition of T-cell proliferation, expansion and accumulation of Tregs and protumoral DCs, and its expression was found to correlate with the grade of astrocytic tumors and with a dismal prognosis [38,74,75].

Soluble factors that bind to pattern recognition receptors, such as TLRs on microglia and activate them, have also been involved in the maintenance of an immunosuppressive microenvironment in glioma [76,77]. Although glioma-derived chemokines (i.e., CSF-1 or CCL2) attract and activate microglia, locally produced TGF-β and PGE2 induces an anti-inflammatory phenotype in these cells, reducing their antigen-presenting activity and facilitating the infiltration of immunosuppressive cells such as Th2 cells and Tregs [78–81]. Glioma-derived CCL22 and CCL2 also recruit Tregs that express CCR4 into the tumor microenvironment [82,83]. Thus, blockade of these chemokines could improve antitumor immunity.

Tumor progression depends on the accumulation of genetic and epigenetic alterations in cancer cells resulting in a complex and heterogeneous cellular composition at the site of tumor growth [84,85]. This heterogeneous environment contains an immunosuppressive network of immune cells such as MDSCs, tumor-associated macrophages/microglia (TAMs) and Tregs [86]. Of particular interest are the MDSCs which promote tumor growth by variety of mechanisms including inhibition of T-cell proliferation and effector functions, suppression of NK and NKT activity, recruitment of Tregs, secretion of immune suppressive cytokines and upregulation of checkpoint receptor ligands such as PD-L1 [87,88]. MDSCs can also promote tumor angiogenesis via the secretion of VEGF as well as matrix metallopeptidase 9 [89,90].

In mice, MDSCs are characterized by the dual expression of CD11b and Gr-1 surface markers. They are further distinguished into polymorphonuclear (PMN-MDSCs) or monocytic (M-MDSCs). The two subtypes differ not only in the surface makers (i.e., $CD11b^+$, $Ly6G^+$, $Ly6C^{low}$ for the PMN-MDSCs, and $CD11b^+$, $Ly6G^-$, $Ly6C^{high}$ for the M-MDSCs) but also in the main mechanism involved in immunosuppression. The generation of Arg I and NO is the main suppressive mechanism of M-MDSC, whereas the PMN-MDSC suppresses $CD8^+$ T-cell responses mainly by producing reactive oxygen species [91–93]. There are multiple strategies for targeting MDSCs in glioma [94,95]. MDSCs depletion and/or blockade of their inhibitory mechanisms seem to be the most effective method. We have recently shown that depletion of immune-suppressive MDSCs in glioma-bearing mice markedly

enhances the efficacy of immunostimulatory/cytotoxic gene therapy [95]. Another possible strategy for targeting MDSCs is by promoting their differentiation into mature cells. Using an *in vitro* cell differentiation model, two groups showed that the macrophage migration inhibitory factor can be targeted to enhance DC differentiation from MDSCs [96,97]. TAMs are another dominant immune cell type in glioma [98,99]. Glioma can activate M2 polarized macrophages, which secrete IL-10 and TGF-β, and inhibit T-cell proliferation [100]. The exact mechanism for such effect is still not clear, although the CSF-1 receptor could be a possible mediator [101]. Gabrusiewicz *et al.* showed that in glioma patients, TAMs resembled the phenotype of nonpolarized M0 macrophages with partially immune-suppressive phenotype [98]. More recently, TAM recruitment has been connected to NF1 gene expression in glioma and the resistance to radiotherapy in some glioma patients may be associated with the presence of M2 macrophages [102].

In addition to MDSCs and TAMs, glioma cells promote the accumulation of Tregs [103]. Tregs restrain antitumor immune response through numerous mechanisms [102]. They can mediate inhibition of functional T cells through the interaction of CTLA-4 (on Tregs) with CD80/86 (on T cells) [104], by the activation of the perforin/granzyme B pathway to cause target cell death [105], by suppressing the release of IL-2 and IFN-γ [106,107], or by expression of TGF-β [108] which can also inhibit NK cells activity [109]. Many reports have illustrated the correlation between blocking the activity of Tregs with more effective antiglioma effector response and improved survival [103,104]. This can be done by a variety of mechanisms such as blocking CTLA-4 [110], inhibiting the signaling pathways that lead to FoxP3 activation (i.e., STAT3) [111–113], or by targeting CD25 [114]. The use of chemotherapeutic alkylating agents (such as temozolomide and cyclophosphamide) has also been reported to inhibit Treg activity [113,115,116].

In summary, glioma cells secrete numerous chemokines, cytokines and growth factors that promote infiltration and expansion of MDSCs, microglia, macrophages and Tregs that directly enhance the invasion of glioma cells, dampen antitumor immune response and accelerate tumor progression. Such immunosuppressive networks are crucial targets for the development of effective immunotherapies.

T-cell exhaustion in glioma

Acquisition of effector functions by naive T cells in response to acute infections is accompanied by robust proliferation, transcriptional, metabolic and epigenetic reprogramming [117–119]. Memory T cells arise from a small subset of these activated T cells upon the resolution of infection, while the majority of the T cells die [117]. However, during chronic infections or inflammation, such as in cancer, an altered state of exhaustion is generated in T cells. Hallmarks of exhausted T cells include loss of effector functions, expression of various inhibitory receptors, metabolic and transcriptional derangements [117,120]. Typically as exhaustion develops, the ability of the T cells to release IL-2 and undergo proliferation is lost, followed by the failure to produce IFN-γ, TNFα and undergo degranulation which results in the release of cytolytic granules from the T cells and is essential for granzyme-dependent killing [117,120]. There is also an increase in the amount and diversity of inhibitory receptors expressed by the T cells. Some of the common inhibitory receptors associated with T-cell exhaustion are CTLA-4, PD-1, T-cell immunoreceptor with immunoglobulin and ITIM domains, LAG-3, 2B4, B and T lymphocyte attenuator and TIM3 [117,118,121,122]. It has been shown that patients with primary intracranial tumors have impaired cell-mediated immunity, with the majority of patients failing to respond to common recall skin test antigens and to neoantigens *in vivo* [123–125]. T-cell receptor (TCR)-mediated signaling in peripheral blood lymphocytes was also shown to be defective in T cells obtained from patients with primary brain tumors [126]. In addition, T cells from these patients showed a marked reduction in tyrosine phosphorylation in response to mitogens. Reduction in CD4 and CD8 T cells has been reported in the tumor and circulation of GBM patients [127]. Using immunohistochemistry and flow cytometry, PD-L1 expression was analyzed in 94 GBM patients. A total of 60.6% of GBM patients had tumors with at least 1% or more PD-L1-positive cells and 5.32% had 50% or greater PD-L1-positive cells. Higher PD-L1 expression was also observed to be correlated with a worse outcome [128]. Interestingly, since tumors with high mutational burdens may be more immunogenic, immune checkpoint inhibition using nivolumab was tested in two siblings with recurrent multifocal biallelic mismatch repair deficiency GBM with clinically significant responses [129].

Thus approaches targeting T-cell exhaustion could provide clinical benefit in glioma. CTLA-4 and PD-1 have been identified as the two major inhibitory receptors/checkpoints involved in T-cell exhaustion and monoclonal antibodies targeting CTLA-4 (ipilimumab and tremelimumab) and PD-1 (nivolumab and pembrolizumab) have been tested for safety and efficacy in clinical trials for melanoma [34,130,131], non-small-cell lung cancer [132,133] and

renal cell carcinoma [134]. Based on the impressive clinical benefit observed in the treatment of melanoma by the use of checkpoint inhibitors a number of preclinical and clinical studies are investigating their use in glioma.

Immunotherapeutic approaches in clinical trials

Various preclinical studies have demonstrated the success of immunotherapy-based approaches in animal models and many Phase I and II clinical trials have shown immunotherapy to be safe and in some cases improve progression-free survival (PFS) and overall survival (OS) [135–139]. Below, we provide an overview of the immunological approaches which yielded promising results in the preclinical setting and are currently being tested in the clinic (Figure 2).

Targeting immune checkpoints

Preclinical studies using murine models with orthotopic-transplanted gliomas have shown great benefit with checkpoint inhibitors used individually or with other immunotherapeutic strategies [95,110,140,141]. Administration of CTLA-4 blocking antibodies improved the survival of animals bearing intracranial SMA-560 tumors and in combination with IL-12 also caused tumor regression in GL261 tumor models [110,140]. Tumor eradication in both studies was accompanied by reduction of Tregs in the tumor microenvironment and an increase in effector CD8 T cells. Promising results from clinical trials for metastatic melanoma using ipilimumab in combination with gp100 vaccine or dacarbazine and the US FDA approval for ipilimumab's use in malignant melanoma have fueled research into its use for the treatment of other cancers [34]. Currently two clinical trials are assessing the use of anti-CTLA-4 antibodies (ipilimumab and tremelimumab) in the treatment of recurrent glioma (NCT02794883 and NCT02017717). The NCT02794883 trial evaluates the combination of tremelimumab with durvalumab, a PD-L1 blocking antibody and the NCT02017717 evaluates the combination of ipilimumab with nivolumab, a PD-1 blocking antibody. Preclinical studies investigating the PD-1 checkpoint blockade in glioma have shown antitumor efficacy [142]. Combination of PD-1 blocking antibodies with radiotherapy enhanced the survival of GL261 glioma-bearing mice [142,143]. PD-1 blocking antibodies, pembrolizumab and nivolumab were approved by the FDA for use in metastatic melanoma in 2014 and for non-small-cell lung cancer in 2015. Multiple clinical trials are investigating the efficacy of anti-PD-1 and anti-PD-L1 antibodies in malignant glioma. In addition to NCT02794883 and NCT02017717, two Phase I/II studies will test the use of pembrolizumab with or without bevacizumab (NCT02337491) or pembrolizumab in combination with laser ablation (NCT02311582) in patients with recurrent glioma. NCT01952769 will evaluate the use of pidilizumab (humanized anti-PD-1 monoclonal antibody) against diffuse intrinsic pontine glioma and recurrent glioma and NCT02336165 is testing MEDI4736 (anti-PD-L1 monoclonal antibody) in combination with radiotherapy and bevacizumab. As shown in Table 1, several other clinical trials are also testing the use of immune checkpoint inhibitors in malignant glioma.

Preclinical studies are investigating the potential of other checkpoints such as TIM-3, IDO, LAG-3 and adenosine A2a receptor as therapeutic targets in glioma [144]. Combination of anti-PD-1, anti-TIM-3 and focal radiation resulted in regression of murine gliomas and combination of IDO, CTLA-4 and PD-L1 blockade induced long-term survival in 100% of the glioma-bearing animals [145,146].

Immune checkpoint blockade appears to be an exciting avenue to develop based on the preclinical studies. An important consideration, however, is the use of GL261 as a tumor model in a variety of these studies. Tumors generated by implantation of GL261 cells mimic many of the features of GBM including neovascularization, pseudopalisading necrosis, perivascular organization and angiogenesis [147]. These characteristics have therefore prompted the use of this model to test a variety of antitumor strategies. With a high mutational burden, however, GL261 tumors may potentially generate neoantigens leading to the development of a large T-cell repertoire and a high response rate to immunotherapies. Human GBM is highly immunosuppressive though and it is therefore important to validate the efficacy of immunotherapeutic approaches including checkpoint inhibition with other rodent models (Table 2) and with tumors containing the mutations commonly found in human GBM (Figure 1). This would also allow for the development of personalized immunotherapy strategies.

Immunotherapy with peptide vaccines

Numerous glioma-associated antigens such as IL-13Rα2, HER2, EphA2, gp100 and AIM-2 are being targeted in glioma [148–150]. Additionally tumor-specific neoantigens such as EGFRvIII are being used to target tumor cells specifically [150,151]. EGFRvIII is expressed in about 20–30% of glioma patients [151]. Evaluation of a combination of EGFRvIII-specific peptide (PEP-3, rindopepimut) keyhole limpet hemocyanin conjugate vaccine plus GM-CSF with standard radiotherapy and chemotherapy in 18 patients expressing EGFRvIII showed a median survival of

Table 1. Current clinical trials evaluating checkpoint inhibition.

NCT#	Phase	Study title	Current status	Inclusion diagnosis	Intervention	Target
NCT02017717	Phase III	A Study of the Effectiveness and Safety of Nivolumab Compared to Bevacizumab and of Nivolumab With or Without Ipilimumab in Glioblastoma Patients (CheckMate 143)	Recruiting	Recurrent glioblastoma	Nivolumab alone vs bevacizumab or nivolumab alone or with ipilimumab	PD-1, CTLA-4
NCT02327078	Phase I/II with glioblastoma cohort in Phase II	A Study of the Safety, Tolerability, and Efficacy of Epacadostat Administered in Combination With Nivolumab in Select Advanced Cancers (ECHO-204)	Recruiting	Recurrent glioblastoma	Nivolumab + epacadostat	PD-1, IDO1
NCT02311582	Phase I/II	MK-3475 in Combination With MRI-guided Laser Ablation in Recurrent Malignant Gliomas	Recruiting	Recurrent malignant gliomas	MK-3475 in combination with mri-guided laser ablation	PD-1
NCT02313272	Phase I	Hypofractionated Stereotactic Irradiation (HFSRT) With Pembrolizumab and Bevacizumab for Recurrent High Grade Gliomas	Recruiting	Recurrent high grade gliomas	Pembrolizumab with radiation therapy and bevacizumab	PD-1 + VEGF
NCT02335918	Phase I/II with Phase II only for glioblastoma	A Dose Escalation and Cohort Expansion Study of Anti-CD27 (Varlilumab) and Anti-PD-1 (Nivolumab) in Advanced Refractory Solid Tumors	Recruiting	Glioblastoma	Combination of varlilumab and nivolumab	CD27 + PD-1
NCT02337686	Phase II	Pharmacodynamic Study of Pembrolizumab in Patients With Recurrent Glioblastoma	Recruiting	Recurrent glioblastoma	Pembrolizumab	PD-1
NCT02550249	Phase II	Neoadjuvant Nivolumab in Glioblastoma (Neo-nivo)	Recruiting	Primary and recurrent Glioblastoma	Nivolumab	PD-1
NCT02529072	Phase I	Nivolumab With DC Vaccines for Recurrent Brain Tumors (AVERT)	Recruiting	Recurrent grade iii and grade iv brain tumors	Nivolumab with DC vaccines	PD-1
NCT02526017	Phase Ia/Ib	Study of FPA008 in Combination With Nivolumab in Patients With Selected Advanced Cancers (FPA008-003)	Recruiting	Malignant glioma	FPA008 in combination with nivolumab	CSF-1R + PD-1
NCT02617589	Phase III	An Investigational Immuno-therapy Study of Nivolumab Compared to Temozolomide, Each Given With Radiation Therapy, for Newly-diagnosed Patients With Glioblastoma (GBM, a Malignant Brain Cancer) (CheckMate 498)	Recruiting	Newly diagnosed adults with unmethylated MGMT glioblastoma	Nivolumab + radiation vs temozolomide + radiation	PD-1
NCT02648633	Phase I	Stereotactic Radiosurgery With Nivolumab and Valproate in Patients With Recurrent Glioblastoma	Recruiting	Recurrent glioblastoma	Stereotactic radiosurgery with nivolumab and concurrent valproate	PD-1
NCT02658279	Proof-of-concept, pilot study	Pembrolizumab (MK-3475) in Patients With Recurrent Malignant Glioma With a Hypermutator Phenotype	Recruiting	Recurrent malignant glioma with a hypermutator phenotype	Pembrolizumab (MK-3475)	PD-1
NCT02658981	Phase I	Anti-LAG-3 or Urelumab Alone and in Combination With Nivolumab in Treating Patients With Recurrent Glioblastoma	Recruiting	Recurrent GBM	Anti-LAG-3 or urelumab alone and in combination with nivolumab	LAG-3 + CD137 + PD-1
NCT02667587	Phase III	An Investigational Immuno-therapy Study of Temozolomide Plus Radiation Therapy With Nivolumab or Placebo, for Newly Diagnosed Patients With Glioblastoma (GBM, a Malignant Brain Cancer) (CheckMate548)	Recruiting	MGMT-methylated glioblastoma	Temozolomide + radiation therapy with nivolumab or placebo	PD-1
NCT02798406	Phase II	Combination Adenovirus + Pembrolizumab to Trigger Immune Virus Effects (CAPTIVE)	Recruiting	Recurrent glioblastoma or gliosarcoma	DNX-2401 + Pembrolizumab	PD-1
NCT02829723	Phase I/II	Phase I/II Study of BLZ945 Single Agent or BLZ945 in Combination With PDR001 in Advanced Solid Tumors	Recruiting	Glioblastoma	BLZ945 single agent or BLZ945 in combination with PDR001	CSF1R + PD-1

DC: Dendritic cell; GBM: Glioblastoma.

Table 1. Current clinical trials evaluating checkpoint inhibition (cont.).

NCT#	Phase	Study title	Current status	Inclusion diagnosis	Intervention	Target
NCT02852655	Pilot	A Pilot Surgical Trial To Evaluate Early Immunologic Pharmacodynamic Parameters For The PD-1 Checkpoint Inhibitor, Pembrolizumab (MK-3475), In Patients With Surgically Accessible Recurrent/Progressive Glioblastoma	Recruiting	Recurrent/progressive glioblastoma	Pembrolizumab (MK-3475)	PD-1
NCT02336165	Phase II	Phase II Study of MEDI4736 in Patients With Glioblastoma	Not recruiting	Unmethylayed MGMT GBM and recurrent GBM	MEDI4736 alone or with radiotherapy or with bevacizumab	PD-L1
NCT02794883	Phase II	Tremelimumab and Durvalumab in Combination or Alone in Treating Patients With Recurrent Malignant Glioma	Recruiting	Recurrent malignant glioma	Tremelimumab and durvalumab (MEDI4736) alone and in combination	CTLA-4 + PD-L1
NCT02937844	Phase I	Pilot Study of Autologous Chimeric Switch Receptor Modified T Cells in Recurrent Glioblastoma Multiforme	Recruiting	Glioblastoma multiforme	Anti-PD-L1 CSR T cells + cyclophosphamide + fludarabine	PD-L1
NCT02866747	Phase I/II	A Study Evaluating the Association of Hypofractionated Stereotactic Radiation Therapy and Durvalumab for Patients With Recurrent Glioblastoma (STERIMGLI)	Recruiting	Recurrent glioblastoma	Durvalumab + radiation therapy	PD-L1
NCT02968940	Phase II	Avelumab With Hypofractionated Radiation Therapy in Adults With IDH Mutant Glioblastoma	Recruiting	Transformed IDH mutant glioblastoma	Avelumab + hypofractionated radiation therapy	PD-L1

DC: Dendritic cell; GBM: Glioblastoma.

26 months (Phase II multicenter study, ACTIVATe, NCT00643097) [152]. After adjustment for age and Karnofsky performance status, the OS of vaccinated patients was greater than that observed in a matched control group. Interestingly 82% of the tumors had lost EGFRvIII expression indicating treatment-induced immunoediting [152]. Immunoediting was also observed in a subsequent Phase II (ACT III, NCT00458601) trial using the combination of rindopepimut, GM-CSF and standard and dose-intensified TMZ with a decrease in EGFRvIII immunoreactivity in 67% of the patients and an OS of 21.8 months [138]. Phase III study of rindopepimut/GM-CSF with adjuvant TMZ in patients with newly diagnosed glioma (ACT IV, NCT01480479) has been discontinued as the median OS with rindopepimut was 20.4 months compared with 21.1 months in the control arm [153]. ReACT (NCT01498328) is a Phase II study of rindopepimut/GM-CSF plus bevacizumab in patients with relapsed EGFRvIII-positive glioma [154]. Interim analysis shows rindopepimut induces potent EGFRvIII-specific immune response and tumor regression, and appears to significantly prolong survival when administered with bevacizumab in patients with relapsed glioma [155].

To overcome the risk of immunoediting and disease recurrence following single peptide vaccinations, investigations testing peptide combinations are underway. Results from the NCT01130077 trial using the combination of EphA2, IL-13Rα2 and survivin with tetanus toxoid and Poly I:C in pediatric brain stem and HGG showed the development of peptide-specific immune responses and indications of immune cell infiltrates. Five out of twenty-six children showed pseudoprogression that was manageable, nineteen showed stable disease and two showed progressive disease [156]. The NCT02078648 study is testing the SL-701 vaccine (IL13Rα2, EphA2 and survivin) in combination with bevacizumab in patients with newly diagnosed glioma. A Phase I trial of IMA950 (consisting of peptides derived from the following proteins: brevican; chondroitin sulfate proteoglycan 4; fatty acid binding protein 7; insulin-like growth factor 2 mRNA binding protein 3; neuroligin 4, X-linked; neuronal cell adhesion molecule; protein tyrosine phosphatase, receptortype, Z polypeptide 1; tenascin C; Met proto-oncogene; baculoviral inhibitor of apoptosis repeat-containing 5; and HBV core antigen) in 45 patients with newly diagnosed glioma receiving maintenance TMZ showed 36 of 40 patients as single-peptide responders and 20 patients as multipeptide responders. However, the median OS was 15.3 months [157]. NCT01920191 tested the combination of intradermal IMA950 with intra muscular Poly-ICLC as an adjuvant in combination with TMZ in newly diagnosed HLA-A2 glioma patients. Preliminary results showed improvement in the median OS with two of the six patients showing induction of both peptide-specific CD4 and CD8 T cells [158].

Table 2. Rodent models for brain tumors.

Induction	Species/strain	Source	Pathology	Applications	Ref.
Cell line inoculation	C57BL/6	GL26 cells	GBM/ ependymoblastoma	DC vaccines engineered to express IL-12. Treg depletion. Metronomic chemotherapy. Combined conditionally cytotoxic and immunostimulatory gene therapy	[1–5]
		GL26.1 cells	GBM/ ependymoblastoma	Immunological checkpoint blockade. Antitumor DC vaccines + Treg blockade with anti-CD25 antibody. Peptide vaccinations + TGF-β neutralizing antibody	[6–10]
		CT-2A cells	Anaplastic astrocytoma	Genetically modified T cells targeting EGFRvIII and IL13Rα2	[11,12]
	VM-Dk	SMA-560 cells	Anaplastic astrocytoma	Overexpression of soluble CD70 ligand. Inhibition of TGF-β signaling. EGFRvIII CAR-modified T-cell therapy. Antitumor DC vaccines	[13–17]
	B6D2F1	4C8 cells	Oligodendroglioma, astrocytoma	Cationic liposome–DNA complexes. HSV vaccines encoding IL-12	[18,19]
Cell line inoculation	LEWIS	CNS-1 cells	GBM	Antitumor DC vaccines. TLR agonists. Metronomic chemotherapy. Combined conditionally cytotoxic and immunostimulatory gene therapy	[20–23]
	FISHER 344	F98 cells	GBM	Combined conditionally cytotoxic and immunostimulant gene therapy. Cellular vaccinations + GM-CSF. Upregulation of costimulatory molecules	[5,24,25]
		RG2 cells	Anaplastic astrocytoma	Gene therapy-mediated delivery of chemokines and cytokines. Metronomic chemotherapy	[26,27]
		9L cells	Gliosarcoma	Gene therapy-mediated delivery of proinflammatory cytokines. Tumor vaccination + TGF-β inhibition	[5,28,29]
Genetic engineering	GFAP-Cre	Lentiviral-mediated knock down of NF1 and p53	Mesenchymal GBM		[30]
	p53 KO	Lentiviral-mediated delivery of Ras and AKT	GBM		[31]
	C57BL/6FVB/nBalb/c	Sleeping beauty transposon plasmids encoding for NRAS, AKT, SV40-LgT, EGFRvIII, shp53	Grade III astrocytoma, GBM		[32,33]
	p53, Arf or Ink4a-Arf KO Gtv-a mice	RCAS-mediated delivery of PDGF	GBM/ oligodendroglioma	TAM targeting with a CSF-1R inhibitor	[34,35]
	Ntva-a mice	RCAS-mediated delivery of PDGF, Ras and AKT	GBM		[36]
	Ink4a-Arf KO Gtv-a mice	RCAS-mediated delivery of Ras and AKT	GBM/gliosarcoma		[37]
Genetic engineering	Sprague Dawley	Retroviral-mediated delivery of PDGF	GBM		[38]
		Lentiviral-mediated delivery of PDGF, H-RAS, AKT	GBM		[39]

DC: Dendritic cell; GBM: Glioblastoma; HSV: Herpes simplex virus; RCAS: Retroviral vectors derived from the SR-A strain of Rous sarcoma virus; TAM: Tumor-associated microglia.

NCT02149225 is a GAPVAC Phase I trial in newly diagnosed glioblastoma patients testing vaccines using both tumor-associated peptides and tumor-specific peptides, derived by expression profiling of tumors from individual patients. A Phase I clinical trial is testing the safety and efficacy of personalized neoantigen vaccines with radiotherapy for patients with MGMT unmethylated, newly diagnosed glioma (NCT02287428).

The genetic makeup of glioma seems to affect its response to immunotherapeutic strategies. Our lab has recently shown that tumors with *ATRX* loss have increased genetic instability [159]. Genome wide data analysis of human gliomas showed that *ATRX* mutation is associated with increased mutation rate at the single nucleotide variant level.

Such tumors may therefore be more immunogenic. *IDH1* and *IDH2* have been found to be mutated in >80% of WHO grade II/III astrocytomas, oligodendrogliomas and oligoastrocytomas [9,12]. The study by Schumacher *et al.* has shown that immunotherapeutic approaches can target this neoantigen [160].

Currently NCT02193347 (RESIST) is testing the use of IDH1 peptide vaccine (PEPIDH1M) in combination with GM-CSF and tetanus toxoid in recurrent grade II glioma positive for IDH1-R132H in adults. NOA-16 (NCT02454634) is another Phase I study testing an IDH1 peptide vaccine in *IDH1* mutant WHO grade III-IV tumors that also show *ATRX* loss without 1p/19q codeletion.

Certain HSP such as HSP70 and HSP90 have been shown to bind glioma antigens and induce innate and adaptive immune responses. Most trials using HSPs as vaccines have used the HSP–peptide complex 96. A Phase I trial (NCT00293423) using HSP vaccine in recurrent glioma showed tumor-specific responses [161]. A Phase II trial showed median PFS and OS as 19.1 and 42.6 weeks, respectively in patients given the vaccine postsurgical resection [135]. NCT01814813 is a Phase II study testing the combination of HSP–peptide complex 96 with bevacizumab postsurgical resection in patients with recurrent disease.

Immunotherapy with DC vaccines

Successful preclinical studies have prompted a number of clinical studies using DC vaccines. DCs can be loaded with peptides, tumor cell lysates, tumor-derived mRNA, viral antigens and cancer stem cells, all of which can be tailored to the individual makeup of a tumor. An autologous DC vaccine, ICT-107, consisting of six peptides (AIM-2, MAGE1, TRP-2, gp100, HER2 and IL-13Ra2) was tested in combination with radiochemotherapy in a Phase I trial in glioma patients [137]. An OS of 38.4 months was noted along with an increased production of IFNγ and TNFα in stimulated CD8 T cells, and a Phase III trial (NCT02546102) is currently recruiting patients to further investigate this treatment. In a Phase I/II trial of 22 patients with malignant glioma, administration of a type 1-polarized DCs pulsed with synthetic peptides (EphA2, IL-13Rα2, YKL-40 and gp100) and poly IC, 58% of the patients developed an immune response specific to at least one antigen, and IL-12 production by DCs was observed to positively correlate with PFS [162]. A Phase I study evaluated the safety and efficacy of an autologous tumor lysate-based DC vaccine. The median survival time for patients with recurrent glioma was determined to be 133 weeks with a significant increase in CD8 T cell activity [163]. An ongoing Phase III study (NCT00045968) is using an autologous DC vaccine (DCVax-L) prepared by loading the DCs with proteins from the patient's own tumor.

A Phase I/II study (NCT00846456) was conducted using DCs loaded with mRNA amplified from the cancer stem cells isolated from the patient's tumor. No severe side effects were observed and encouragingly the PFS in treated patients was 2.9-fold longer than the matched controls [139]. An ongoing Phase I trial (NCT02049489) in recurrent glioma is testing the safety of ICT-121, a DC vaccine targeting CD133, the antigen expressed on glioma stem cells [164]. With the identification of cytomegalovirus (CMV) and its gene products in glioma, preclinical studies have utilized this feature to develop targeted immunotherapy. NCT00626483 is a study evaluating the combination of anti-CD25 antibody, basiliximab with autologous DCs loaded with CMV pp65-lysosomal-associated membrane protein mRNA. Another study (NCT00639639) showed that preconditioning with tetanus/diphtheria toxoid prior to vaccination with pp65 RNA-pulsed DCs improves DC migration and survival [136]. ELEVATE is a Phase II randomized study (NCT02366728) currently recruiting patients with newly diagnosed glioma, is investigating preconditioning with tetanus toxoid or basiliximab prior to a CMV-targeted DC vaccine.

Immunological checkpoint blockade could improve the efficacy of antitumor DC vaccines in glioma patients, as it has been shown in preclinical studies [95,145,146,165]. AVERT, a Phase I clinical trial (NCT02529072) is evaluating the combination of CMV-targeted DC vaccine with PD-1-blocking antibody, nivolumab in patients with recurrent HGG. Another Phase II study combining the SOC with the DC vaccine, AVO113 and bevacizumab showed an increase in the median OS compared with the vaccine alone or bevacizumab alone group [166].

Immunotherapy with antibodies

This form of immunotherapy relies on targeting antigens uniquely expressed on glioma cells or molecules that are overexpressed by tumor cells. Several Phase II trials have tested the efficacy of anti-VEGF therapies because gliomas are highly vascularized tumors that express high amounts of VEGF. Most commonly used anti-VEGF antibody is bevacizumab that has been tested either alone (NCT00345163) or in combination with irinotecan (NCT00345163), etoposide (NCT00612430) or with concurrent radiotherapy (NCT00595322) [155,167–170]. The RTOG0825 and the AVAglio studies are prospective Phase III studies that tested the efficacy of TMZ-based

radiochemotherapy with bevacizumab. No significant benefit in PFS or OS was seen in the RTOG 0825 study, while the AVAglio study showed an improvement of 4.4 months in the PFS with no change in the OS in the bevacizumab arm [171,172].

Monoclonal antibody therapy has been used to target EGFR using cetuximab. Combination of cetuximab with bevacizumab/irinotecan was not superior to bevacizumab/irinotecan alone [173]. A Phase I study (NCT01238237) showed that intra-arterial cerebral infusion of cetuximab and/or bevacizumab was safe for the treatment of recurrent gliomas in adults, and a Phase I/II trial is now evaluating the safety and efficacy of intra-arterial cetuximab and bevacizumab for the treatment of relapsed/refractory glioma in patients <22 years (NCT01884740). A Phase II study tested the combination of nimotuzumab (anti-EGFR monoclonal antibody) with concomitant radiation and vinorelbine in childhood diffuse pontine glioma [174].

A Phase III open label trial (NCT00753246) showed no significant benefit in OS by the addition of nimotuzumab to standard therapy for newly diagnosed glioma [175]. ABT414 is an antibody–drug conjugate that delivers the cytotoxic microtubule inhibitor, monomethyl auristatin F to cells with active EGFR or EGFRvIII [176]. A Phase I study (NCT02573324) tested the use of ABT414 alone or in combination with chemotherapy or chemotherapy and radiation and showed responses in 4 out of 12 patients [177].

Immunotherapy with adoptive T-cell transfer & chimeric antigen receptor T cells

Adoptive T cell transfer (ACT) involves the *ex vivo* production of autologous tumor reactive T cells that are directly transferred back to the patients. Initial studies using ACT for glioma involved the *ex vivo* expansion of T cells induced by culturing with tumor cells or the isolation of T cells from the draining lymph nodes (dLNs) following immunization with irradiated tumor cells and GM-CSF [178]. In a Phase I study in patients with recurrent glioma and CMV-positive serology, 4 out of 10 patients who received at least three T-cell infusions showed PFS at the time of data compilation and a median OS of 403 days, when infused with *ex vivo* expanded CMV-specific autologous T cells (Australia New Zealand Clinical Trial Registry; ACTRN12609000338268). While the therapy was deemed to be safe, no correlation was observed between the phenotype and functionality of T cells with PFS and further investigations are warranted [179].

Chimeric antigen receptor (CAR) T cells consist of the antigen-binding region of a monoclonal antibody fused with the signal transduction domain of CD3ζ or FcεR1γ, and thus combine the specificity and avidity of a monoclonal antibody to the signaling pathways for T-cell effector functions [180]. Preclinical studies have used CAR T cells to target EGFRvIII, IL-13Rα2, HER2 and EphA2. The approach has also been shown to be safe with minimal side effects in a first-in-human pilot safety and feasibility trial (NCT00730613) targeting IL-13Rα2 in recurrent glioma [181]. Two out of three patients also developed transient antiglioma responses. Based on these findings, the IL13Rα2-targeted CAR T cells were further modified to incorporate 4-1BB (CD137) costimulation and a mutated IgG4-Fc linker. Central memory T cells were lentivirally transduced to produce these IL13BBζ CAR T cells. Early findings from one patient who received intracavity and intraventricular infusions showed clinical regression that was sustained for 7.5 months post the initiation of the CAR T-cell therapy. Disease recurrence was, however, observed at new locations after the last cycle, possibly due to the decreased expression of IL13Rα2 may be responsible. Additionally accumulation and expansion of the CAR T cells in the CSF in later cycles and over the 7-day infusion cycle were limited. The results from this patient have prompted the expansion of the Phase I study to evaluate intraventricular administration in a larger cohort of patients [181].

Ongoing clinical trials are evaluating the safety and efficacy of CAR T cells against EGFRvIII, IL-13Rα2, HER2, EphA2, CD133 and MUC I in malignant glioma. A Phase I study (NCT02209376) evaluated the feasibility and safety of manufacturing and administering CAR T cells redirected to EGFRvIII (CART-EGFRvIII) to patients with EGFRvIII-expressing recurrent GBM. No evidence of off-target toxicity or cytokine release syndrome was observed. The data showed evidence of CART-EGFRvIII trafficking to the brain tumor, proliferation of the CAR T cells and antitumor activity; however, robust compensatory immunosuppressive mechanisms including upregulation of IDO1 and PD-L1 and recruitment of Tregs were observed to develop [182].

A Phase I trial is also investigating the use of CMV-specific cytotoxic T lymphocytes expressing a CAR targeting HER2 in patients with glioma (NCT01109095). Infusion of HER2-specific CAR-modified CMV-specific T cells was shown to be well tolerated with no dose-limiting side effects. The study also showed clinical benefit in 8 out of 17 patients (partial response in one and stable disease in seven patients) thus warranting further trials [183].

Numerous investigations are looking into enhancing the specificity and antitumor activity of CAR T cells including the generation of tandem CARs, balanced-signal CAR, dual-receptor circuit CARs and CARs containing

chimeric switch receptors [184–188]. The extracellular domain in chimeric switch receptor consists of PD-1 and the intracellular domain is stimulatory so that upon binding PD-L1 a stimulatory rather than inhibitory signal is generated [189]. Efforts are also being made to manage the toxicities associated with the administration of CAR T cells [190].

Oncolytic viral therapy

Viruses hijack host cells' replication, eventually leading to host cell death and infection of the surrounding healthy cells. Some studies have shown how viruses can be targeted specifically to tumor cells [191,192]. In addition to causing cell death, virus infection also leads to the activation of innate and adaptive immune responses and therefore become attractive immunotherapeutic agents [193]. Two variants of HSV-1 containing mutations in ICP34.5 and ribonucleotide reductase (RNR) have been shown to be safe in Phase I trials and are currently in Phase II testing [194,195]. Other variants of HSV-1, such as M032 and rQNestin34.5 are in preclinical testing. ONYX-015 is an E1B mutant adenovirus that was shown be safe in Phase I study [196]. Another variant AdDelta24-RGD is currently in preclinical and clinical development [197–200]. Reovirus selectively infects cells with activated Ras pathways and when tested in Phase I study with recurrent glioma demonstrated safety and antiglioma activity [201]. An attenuated poliovirus PVS-RIPO showed efficacy in preclinical testing and is currently being tested in Phase I study (NCT01491893) [202,203]. Interim analysis from this study using historical controls seems to confer a survival advantage to the patients infused with PVS-RIPO [204]. H-1 is a parvovirus variant, that was shown to be oncolytic in rat and human GBM cell lines and is in a Phase I/IIa study for recurrent glioma (NCT01301430) [205]. NCT00390299 is a Phase I trial testing the measles virus variant, MV-CEA, in patients with recurrent glioma. Since the receptor for measles virus is expressed on healthy brain tissue and T cells, further work is ongoing to enhance the selectivity and safety of this virus [206,207]. Avian Newcastle disease virus has been tested in a Phase I/II trial (NCT01174537), with no serious side effects and a complete response in one patient [208]. To further enhance the therapeutic efficacy of the oncolytic virus and to reverse tumor-induced immunosuppression, NCT02798406 (CAPTIVE/KEYNOTE-192) is a Phase II trial of a conditionally replicative adenovirus, DNX-2401, in combination with anti-PD-1 monoclonal antibody, pembrolizumab in patients of recurrent glioma or gliosarcoma.

Immunostimulatory gene therapy

The aim of immune stimulatorygene therapy is to modulate the tumor environment such that a robust and effective antitumor immune response can be generated. In a Phase I trial, combination of suicide gene therapy using HSV-TK and IL-2 resulted in minimal side effects and partial response in 2 out of 12 patients [209]. IL-4 has been tested in Phase I study of IL-4-HSV-TK gene-modified autologous tumor to elicit an immune response [210]. A Phase I study conducted in Japan in patients with malignant glioma showed minimal toxicity and a 50% reduction in tumor size in two out of five patients that were given liposomal-mediated delivery of IFNβ [211]. A second Phase I trial that directly delivered Ad-hIFNβ into the tumor cavity and the surrounding area demonstrated safety and tumor cell apoptosis [212]. Adeno-associated viral vectors have been also developed to locally deliver adeno-associated virus (AAV)-IFN-β and tested in combination with chemotherapy. Since DNA replication is required for the synthesis of the second strand of DNA in order to activate the transcription of single-stranded AAV vector, chemotherapy was administered after viral gene therapy, improving the median survival of murine GBM models when compared with single treatments [213]. AAV vectors have been also employed to deliver IL-12 in rodent GBM models [214]. Our team has developed a conditionally cytotoxic immune stimulatory gene therapy mediated through the delivery of adenoviruses encoding HSV1-TK and Flt3L. This therapeutic approach results in tumor regression, long-term survival and a robust memory T-cell response in numerous preclinical glioma models. Importantly, concomitant treatment with temozolomide enhances the efficacy of this gene therapeutic approach in murine models of brain cancer [215]. This strategy is currently under investigation in a Phase I clinical trial in GBM patients (NCT01811992) [47,216–218].

The combination of immune gene therapeutic strategies with the blockade of immunosuppressive mechanisms could improve their efficacy in GBM patients. We have recently found that antibody-mediated blockade of immunological checkpoints and depletion of MDSCs, which constitute 40% of tumor-infiltrating immune cells, enhances the antitumor immune response induced by TK/Flt3L gene therapy in GBM mouse models [95]. Armed oncolytic HSV (oHSV G47Δ), which encodes for IL-12 has been shown to exhibit robust antitumor effects in murine models of GBM when combined with anti-CTLA-4, anti-PD-1 antibodies [219].

Conclusion

The extensive molecular characterization of gliomas, coupled with the 2016 WHO histological classification, have been instrumental in improving our understanding of glioma progression and the response to therapeutics. Also, the genetic lesions encountered within the glioma cells, play a critical role in reprogramming the immune TME. This has opened the horizon for scientists to investigate novel glioma treatment strategies. Work in the field has led to the conclusion that there is a need for combinatorial treatments, in order to elicit higher efficacy and better outcomes in the clinic. In particular, immunotherapies offer very promising approaches for prolonging patient survival; in several ongoing clinical trials immunotherapies have shown evidence of significant anti-tumor outcomes, i.e., circulating specific anti-glioma T cells and higher infiltration of activated immune cells into the TME.

Future perspective

Glioma is a devastating disease and despite many years of research the prognosis remains dismal. Significant progress has been made in developing immunotherapeutic regimens and these may soon be included in the SOC. Several challenges, however, need to be overcome, the chief among which is the intratumor heterogeneity [220].

Given the enormous increase in availability of gene expression, epigenetic and molecular pathway analysis, a personalized therapeutic approach tailored to the tumor would be ideal. A second point to consider is the standardization of diagnostic, therapeutic response and efficacy criteria for clinical trials, making it easier to interpret results and compare outcomes across different clinical trials. The immunotherapy response assessment in neuro-oncology criteria is being established in this regard [221]. Repeat tissue sampling is extremely challenging for CNS tumors and the assessment of therapeutic efficacy is further complicated by the associated edema and pseudoprogression. Efforts are also being made to identify unique biomarkers to serve as inclusion criterion or that can be of prognostic value to predict the response to a particular therapy using tumor-derived DNA from the cerebrospinal fluid [222]. It is also apparent that given the tumor heterogeneity and immunoediting resulting from treatment, a single approach will not be sufficient and successful treatment will require the combination of multipronged therapies such as those combining multiple checkpoint inhibitors with radiation, the combination of checkpoint inhibitors with IDO inhibitors or the combination of checkpoint inhibitors with immune stimulatory gene therapy or with vaccination strategies [95,145,146]. Ongoing clinical trials are testing combinatorial approaches to achieve broad and durable clinical efficacy. Another important factor to consider for the integration of immunotherapy with the current SOC, is the effect of radiation and TMZ on cells of the immune system. Hyperfractionated radiation has been found to correlate with CD4 T-cell depletion [223]. TMZ also causes lymphopenia and it is therefore critical to evaluate the novel immunotherapeutic approaches in the context of SOC [224,225]. Of note, our lab has shown that TMZ administration does not affect the therapeutic efficacy of the TK/Flt3L immunotherapy approach currently in a Phase I study [215].

Executive summary

- Recent molecular characterization of several glioma subtypes, raises the possibility of tailoring treatments to specific genetic lesions encountered in these tumors. This will give rise to precision medicine-based therapies for glioma patients.
- The presence of the blood–brain barrier hampers the efficacy of chemotherapies for brain tumors. Immunotherapies, which rely on the migration of activated, tumor antigen-specific cytotoxic T cells, could yield efficacious therapeutic options, as activated immune cells can migrate across the blood–brain barrier.
- The use of oncolytic therapeutic approaches, which induce the release of tumor-derived ligands capable of stimulating the immune system of the host, provides an exciting therapeutic modality, triggering immunogenic tumor cell death.
- Improvements in genetically engineered chimeric antigen receptor T cells, in order to improve their survival, tumor penetration and *in vivo* expansion, may provide an attractive therapeutic modality.
- Combination therapies, including standard of care together with immunotherapies provide improved efficacy.
- Adding immune checkpoint blockade to immunotherapies, would provide another layer of enhancement of therapeutic efficacy.
- A coordinated, multi-institutional approach would be required to analyze the results from multicentric Phase I clinical trials, which would enable to move these exciting novel therapies in a timelier fashion into the clinical arena in order to benefit glioma patients.

Supplementary data

Supplementary information includes one table. To view the supplementary data that accompany this paper please visit the journal website at: **www.futuremedicine.com/doi/suppl/10.2217/imt-2017-0122**

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• **Significant advance in the classification of CNS tumors after taking into consideration the molecular patterns encountered.**

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