

Review

Oncolytic viruses for malignant glioma: on the verge of success?

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Abstract: Glioblastoma is one of the most difficult tumor types to treat with conventional therapy options like tumor debulking, chemo and radiotherapy. Immunotherapeutic agents like oncolytic viruses, immune checkpoint inhibitors and chimeric antigen receptor T cells have revolutionized cancer therapy, but their success in glioblastoma remains limited and further optimization of immunotherapies is needed. Several oncolytic viruses have demonstrated ability to infect tumors and trigger anti-tumor immune responses in malignant glioma patients. Leading the pack, oncolytic herpesvirus, first in its class, awaits an approval for treating malignant glioma from MHLW, the federal authority of Japan. Nevertheless, some major hurdles like the blood brain barrier, immunosuppressive tumor microenvironment, and tumor heterogeneity can engender suboptimal efficacy in malignant glioma. In this review, we discuss the current status of malignant glioma therapies with a focus on oncolytic viruses in clinical trials. Furthermore, we discuss the obstacles faced by oncolytic viruses in malignant glioma patients and strategies that are being used to overcome these limitations to 1) optimize delivery of oncolytic viruses beyond the blood brain barrier; 2) trigger inflammatory immune responses in and around tumors; and 3) use of multimodal therapies in combination to tackle tumor heterogeneity, with an end goal of optimizing the therapeutic outcome of oncolytic virotherapy.

Keywords: Glioblastoma; Oncolytic Virus; Blood Brain Barrier; Tumor Microenvironment; Tumor Heterogeneity

1. Introduction

Glioblastoma (GBM) is a grade IV malignant glioma tumor that accounts for the majority (57%) of malignant glioma patients and remains the most common cause of death due to primary malignant brain tumors in humans [1, 2]. Radiotherapy, chemotherapy and surgical debulking remain the current standards of care for malignant glioma, but even tumor resection is difficult due to its location and potential neurological impairment [3]. GBM has one of the worst prognosis with a median survival of around 15 months [3, 4]. The highly aggressive nature, molecular heterogeneity, ability of resistant cancer stem cells to regrow post-therapy, invasion of critical regions of the brain, and inadequacy of achieving high therapeutic levels of chemotherapeutics in the brain due to the blood brain barrier (BBB), are some of the key factors that constitute the vast amount of unmet need in GBM patients (as reviewed in [5]).

The recent success of immunotherapy in clinic, especially with immune checkpoint inhibitors (ICIs) that impede the engagement of programmed cell death 1 (PD-1) or cytotoxic T lymphocyte-associated protein 4 (CTLA-4) with their ligands to boost anti-cancer immunity, has paved a way for these agents to become a part of standard treatments in many cancer types. ICIs have been shown to be effective in patients with an increasingly wide variety of tumors [6]; however the magnitude and duration of response to ICIs in solid tumors remains greatly variable. Although some of the hallmarks of cancer such as the degree of mutational burden [7], defective DNA-repair mechanisms [8], and checkpoint-ligand expression [9], have been helpful in predicting the potential efficacy of ICIs,

but the accuracy in predicting individual ICI responders using these hallmarks remains limited, owing to the complexity of interactions between cancer cells and the immune system [10]. Chimeric antigen receptor (CAR) T cell therapy is another option that appears promising for treating malignant glioma, however high molecular heterogeneity can lead to moderation of response to the treatment, similar to cancer vaccines, since both of these therapies are dependent on expression of specific antigen molecules by tumor cells.

Oncolytic virotherapy uses replication competent viruses that can selectively replicate and kill cancer cells [11-14]. Oncolytic viruses (OVs) lead to cancer cell death through different mechanisms including apoptosis, pyroptosis and necroptosis. Direct oncolysis releases a wide range of tumor-associated antigens (TAAs)/neoantigens or danger-associated molecular patterns and viral pathogen-associated molecular patterns, that trigger inflammatory immune responses in the tumor microenvironment (TME) [15]. A highly immunosuppressive TME is a characteristic of malignant glioma and other tumor types when they metastasize into the central nervous system (CNS) compartment. The local immunosuppression in and around malignant glioma tumors is due to deletion and development of tolerance against tumor specific T cells, in conjunction with systemic immunosuppression due to sequestration of T cells in the bone marrow [16, 17]. OVs can increase immune cell infiltration and trigger inflammation within the TME which could be crucial in breaking the immune tolerance and can improve tumor responsiveness to ICIs [18]. A wide range of OVs are being tested both at the preclinical and clinical level in malignant glioma. An increasing number of OVs are in various phases of clinical trials, amongst which some promising OV candidates are adenovirus (DNX-2401), poliovirus (PVS-RIPO), and retroviral vector (Toca 511) that induced a durable response in 20% of malignant glioma patients and has been put on a fast track to be reviewed by the US Food and Drug Administration (FDA) [19-21]. Furthermore, an application for oncolytic herpesvirus, G47 Δ has been submitted to Japan's Ministry of Health, Labour and Welfare (MHLW) for treatment of patients with malignant glioma [22]. This is the first instance that an application for an OV to treat malignant glioma has been filed to a regulatory authority in any country.

2. Current treatment options for malignant glioma

2.1 Standard therapy: surgery and chemoradiation

Surgical abscission remains at the core of treatment for malignant glioma along with adjuvant chemoradiation [3]. Temozolomide (TMZ) is an FDA approved chemotherapy agent for malignant glioma and is administered concomitantly with radiation as well as an adjuvant therapy [3]. However, TMZ has been linked with increasing mutation rates resulting in defective DNA repair mechanisms that can lead to the development of resistant malignant glioma cell subpopulations making the drug ineffective in previously responsive patients [23]. Poor overall survival with the existing standard treatments and emergence of resistant phenotypes has created an urgent need for newer therapeutics in malignant glioma patients. Several new therapies such as oncolytic virotherapy, immunotherapy, CAR T cell therapy and cancer vaccines are currently under investigation in preclinical and clinical studies and their approval for clinical application in malignant glioma patients is awaited.

2.2 Immunotherapy

A targeted treatment drug, Bevacizumab has also been approved by the FDA for treating recurrent malignant glioma patients [24]. Bevacizumab is a monoclonal antibody directed against vascular endothelial growth factor that acts by limiting angiogenesis in malignant glioma tumors restricting tumor growth, but eventually can lead to development of a resistant phenotype due to transition in mesenchymal gene expression [25]. The discovery of ICIs has been revolutionary, resulting in the approval of several ICIs blocking PD-1, CTLA-4 and programmed cell death receptor 1 ligand (PD-L1) to treat various

cancer types. However, the application of ICIs in solid tumors is challenging and their efficacy against GBM or brain metastases is limited [6]. Based on the response to ICIs, tumors are broadly classified as non-responding “cold” or responsive “hot” tumors [26]. Solid tumors are in a constantly transitional state with an increasing degree of heterogeneity and can develop adaptive resistance to therapies. The magnitude of innate and adaptive resistance to ICIs in tumors is the determining factor for efficacy of these therapies [27]. Among solid tumor types, malignant gliomas have been reported to have a high degree of both intrinsic and adaptive resistance to immunotherapies unlike melanoma that show a low level of both intrinsic and adaptive resistance [28–30]. Although the FDA has approved PD-1 blocker, pembrolizumab for pan-cancer application, including glioma and other solid tumors, concerns have been raised for its application in malignant glioma patients due to the distinct differences in the immunological attributes like local and systemic immunosuppression between glioma and other cancer patients [31].

Understanding the underlying mechanisms behind the development of innate and adaptive resistance to ICIs can help in designing better treatment strategies where ICIs can be used in combination with other therapeutic agents in tumors that are non-responsive to ICI monotherapies. Efficacy of ICIs is dependent on the degree of expression of the target checkpoint receptors on tumor and peritumoral cells and the heterogeneous nature of malignant glioma tumors can be a major hurdle to success. Systemic immunosuppression [17], poor immune cell infiltration of tumors and suboptimal delivery of systemically administered ICIs due to the blood brain barrier (BBB) [32], are some of the other factors that limit the efficacy of ICIs in malignant glioma. Although the list of FDA approved agents in this category is continually expanding, application of these agents in GBM patients will require caution and significant optimization.

2.3 Chimeric antigen receptor T cell therapy

CAR T cell therapy uses autologous T cells engineered to target specific tumor antigens expressed on the surface of tumor cells for tumor eradication. CAR T cell therapies have produced sustained therapeutic effects in refractory hematological cancers, but their success in the treatment of solid tumors has also been limited [22–24]. Efficacy of CAR T cells is restricted in malignant glioma mainly due to the high degree of tumor heterogeneity, the BBB and significantly immunosuppressive TME. Several CAR T cell therapies targeting a range of tumor antigens such as EGFR (NCT01454596, NCT03638167, NCT02844062, NCT02331693, NCT03726515), GD2 (NCT04196413, NCT04099797), HER2 (NCT03500991, NCT03389230, NCT01109095), IL13R α 2 (NCT02208362, NCT04661384, NCT04003649), B7-H3 (NCT04185038, NCT04077866, NCT04385173), and CD-147 (NCT04045847) are currently under clinical investigation in malignant glioma. The loss of target antigen expression by glioma cells renders the CAR T cell therapies ineffective as was evident by the decrease in or loss of IL13R α 2 [33] and epidermal growth factor receptor variant III (EGFRvIII) [34] antigens by tumor cells in patients with recurrent malignant glioma. CAR T cell therapies that can target multiple tumor antigens to avoid dependence on a single target antigen for efficacy or the simultaneous use of multiple CAR T cell cocktails, targeting different tumor antigens can help overcome tumor resistance due to variable antigen expression [35]. Furthermore, hypoxic environment in malignant glioma tumors has been linked to an increase in expression of hypoxia response elements which induce higher levels of PD-L1 expression in the TME, thus leading to suppression of T cell responses [36, 37]. High levels of immune suppressive cytokines like transforming growth factor (TGF)- β , interleukin (IL)-4, IL-10, Arg1, IDO and PD-L1 produced by tumor associated myeloid derived suppressor cells (MDSC), regulatory T (Treg) cells, and tumor associated macrophages/microglia (TAMs) further contribute to the subdued immune response in malignant glioma [38].

Immunomodulation in malignant glioma tumors blocks activation of immune response pathways important for successful CAR T cell therapy and leads to T cell exhaustion. Therefore, CAR T cell therapy needs further optimization in malignant glioma with

improved 1) accessibility to brain; 2) tumor cell targeting; 3) survivability in immunosuppressive TME; and 4) ability to proliferate and exert therapeutic effects with minimal immune-based toxicities [39].

2.4 Vaccines

There are two major types of vaccines that are under investigation for malignant glioma therapy, peptide vaccines and dendritic cell based (DC) vaccines. Peptide vaccines use small tumor specific antigen sequences up to 30 bases to induce anti-tumor immune responses. Several vaccines targeting single or multiple tumor antigens are under investigation and have shown some encouraging results in malignant glioma [40-42]. EGFRvIII is overexpressed in malignant gliomas. An EGFRvIII peptide vaccine, Rindopepimut, was tested in a phase II trial in newly diagnosed EGFRvIII-expressing GBM patients. The vaccine induced anti-EGFRvIII antibodies and resulted in 66% progression free survival (PFS) at 5.5 months, but 67% of tumor samples collected after more than 3 months of treatment showed loss of EGFRvIII expression [43]. Similar results were reported in a phase III study of Rindopepimut in GBM patients where the vaccine improved PFS, but 82% of tumor samples (n=11) from patients with recurrent disease showed loss of EGFRvIII expression [44]. Another peptide vaccine, IMA950 which is a multi-peptide vaccine, was well tolerated and the primary immunogenicity endpoint against tumor associated antigens exceeded in at least 30% of patients in combination with granulocyte monocyte-colony stimulating factor (GM-CSF) in newly diagnosed GBM patients [45]. However, IMA950 in combination with poly-ICLC, a synthetic toll-like receptor 3 ligand showed no improvement in PFS and overall survival in high grade glioma patients [46]. Among two other vaccines, isocitrate dehydrogenase 1 (IDH1) met the safety end point in a phase I study [47] and autologous heat-shock protein vaccine in combination with standard therapy improved overall survival in a phase II study in GBM patients [48]. Initial clinical studies with both these vaccines look encouraging and warrant further investigation.

DC vaccines are based on exposing autologous DCs to tumor antigens, *ex vivo* and administrating the activated DCs into patients. Several clinical studies are currently testing DC vaccine therapies in glioma patients (NCT02649582, NCT02709616, NCT01567202, NCT02772094, NCT02366728, NCT02465268, NCT01204684, NCT02754362, NCT03395587, NCT03400917). DCVax-L uses autologous phagocytic DCs exposed to immunologically enhanced glioma cells by interferon (IFN)- γ and heat-shock treatment, derived from patients, instead of using single or limited tumor antigens which helps DCVax-L to expand its targeting potential. DCVax-L showed an improved median survival in grade 4 glioma patients in a phase I/II study [49]. Additional clinical trials are currently underway to evaluate DCVax-L in glioma patients (NCT03014804, NCT00045968).

Considering the molecular heterogeneity in malignant glioma tumors, both peptide and DC based vaccines targeting a single tumor antigen are likely to mediate transient effects, but ultimately will lead to recurrent disease due to antigen escape and regrowth of tumor cells lacking the target antigen expression. DCVax-L attempts to compensate for this heterogeneity by exposing DCs to tumor cell lysates instead of specific tumor antigens but poses a risk of inducing an autoimmune reaction. Local and systemic immune suppression in glioma patients continues to be an obstacle in executing immune cell mediated effects of therapeutics like vaccines. Although clinical studies have provided us with evidence that both peptide and DC based vaccines have the potential to induce anti-tumor immune responses, vaccines will need to overcome the major hurdle of heterogeneity in glioma tumors to exert sustained efficacy and lower the recurrence rate. The immune stimulatory potential of vaccines can however be exploited in combination with other therapies to achieve a synergistic effect.

3. Oncolytic virotherapy for malignant glioma

OVs engineered to express immune-stimulatory proteins not only disrupt the immunosuppressive TME but can also recruit, activate, and promote pro-inflammatory immune cells at the tumor site. Engineering OVs to deliver a payload of therapeutic proteins at tumor sites has become a well-recognized strategy to optimize therapeutic efficacy, while minimizing the systemic toxicity afforded by these therapeutic proteins. Oncolytic virotherapy faces a unique set of challenges associated with malignant glioma, due to several roadblocks, including the BBB between vascular and CNS compartments, tumor protective immune environment and high variability in molecular attributes of tumor cells that are discussed in detail, later in this review. Most OVs, if not all that are in clinical trials in malignant glioma patients are being delivered locally to achieve an effective virus load in the tumors. At present, herpesvirus, adenovirus, vaccinia virus, reovirus, parvovirus, poliovirus, measles virus, replicating retrovirus vector and Newcastle disease virus (NDV) are being tested in malignant glioma patients for safety and efficacy at different clinical phases (Table 1).

3.1 Oncolytic herpesvirus

Conditionally replicating herpes simplex virus (HSV)-1 derivative, G207, contains deletions in the $\gamma 134.5$ and *ICP6/UL39* genes that prevent virus killing of normal brain cells. The safety of this virus was demonstrated following stereotactic inoculation of enhancing/actively growing sites of recurrent malignant gliomas and subsequent inoculation into the tumor bed cavity following tumor resection in phase I/II (NCT00028158) and phase Ib [50] studies. No toxicity or adverse events related to the virus were reported [50, 51]. Results of phase II studies are not available yet. The University of Alabama at Birmingham tested the safety of G207 in multiple phase I studies, both as a monotherapy and in combination with radiation in recurrent gliomas in adults. Even with a high dose of 3×10^9 plaque forming units (pfu), no virus related toxicities were reported [51, 52]. Another phase I study is testing the safety of G207 by itself or in combination with radiation in pediatric brain tumors, including malignant glioma (NCT02457845). Also, a phase II trial with G207 is testing the efficacy of virus alone or virus combined with a single low dose of radiation in pediatric patients with recurrent or progressive high-grade glioma (NCT04482933).

G47 Δ is a triple-mutated, third-generation oncolytic HSV-1, generated by introducing an additional genetic mutation in the viral genome of second-generation HSV-1, G207 [53]. This virus is being investigated in several tumor types, including malignant glioma. G47 Δ received designation as a breakthrough drug for treatment of malignant glioma by the MHLW, Japan, allowing its priority review for expedited approval by the Pharmaceuticals and Medical Devices Agency of Japan (PMDA) earlier in 2016 [54]. A phase I-IIa study in Japan tested G47 Δ safety in patients with progressive GBM which showed that patients tolerated the virotherapy well with no toxicity (UMIN000002661, Japan) [55]. A phase II study tested the efficacy of G47 Δ in malignant glioma patients, including GBM (UMIN000015995, Japan), using a dose of 1×10^9 pfu, injected stereotactically into the tumor at different coordinates twice within two weeks and every four weeks thereafter, with a maximum of six doses. The treatment is well tolerated and the interim analysis of results of this study showed a significantly higher 1-year survival rate of 92.3% in 13 patients as compared to the 15% in the control group, based on meta-analysis of historical data. The high efficacy of G47 Δ in this phase II study led to early termination of the trial as the evidence from the study was enough to submit a new drug application [56]. Based on the results of a phase II study, a new drug application for G47 Δ has recently been submitted for treating patients with malignant glioma to Japan's MHLW [22]. This is the only OV in any country that has reached this stage for treating malignant glioma and could well be the first OV to be approved for CNS tumor therapy.

Table 1. Oncolytic viruses in clinical trials for treatment of malignant glioma

Virus		Modification	Phase	Status	Reference	Results
HSV-1	G207	Deletions at both $\gamma 134.5$ and <i>ICP6</i> genes	I & II	Completed	NCT00028158 [51]	No toxicity or serious adverse events.
			Ib	Completed	[50]	No neurological adverse events after multiple virus dosages.
			I	Active, not recruiting	NCT02457845	
			II	Not yet recruiting	NCT04482933	
	G47Δ	G207 with triple mutations	I-IIa	Completed	UMIN000002661 (Japan)	No toxicity or serious adverse events.
			II	Ongoing	UMIN000015995 (Japan)	No toxicity with 1-year survival rate of 92.3% in 13 patients.
	rQNestin34.5v.2	Glioma selective transcriptional regulator for expression of <i>ICP34.5</i>	I	Recruiting	NCT03152318	
	M032	Deletions at both $\gamma 34.5$; expression of human <i>IL-12</i>	I	Recruiting	NCT02062827	

Table 1. Continued

Virus	Modification	Phase	Status	Reference	Results
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HSV-1	C134	Deletions at both γ 34.5; Expression of HCMV <i>IRS1</i> gene	I	Active, not recruiting	NCT03657576	
	HSV-1716	Deletion of both copies of <i>RL1</i> gene encoding ICP34.5 protein	I	Completed	[57]	No adverse effects with 4 out of 9 patients surviving 14-24 months after virotherapy.
			I	Completed	[58]	No toxicity with 3 out of 12 patients surviving over a year.
			I	Terminated	NCT02031965	NA
Adenovirus	DNX-2401	Deletion of <i>E1A</i>	I	Completed	NCT00805376 [20]	No dose limiting virus toxicities reported with enhanced long-term survival and T cell response to tumors.
			I	Active, not recruiting	NCT03178032	
			I	Completed	NCT01582516	NA
			I	Completed	NCT01956734	NA
			I	Completed	NCT02197169	NA

Table 1. Continued

Virus		Modification	Phase	Status	Reference	Results
Adenovirus	DNX-2401	Deletion of <i>E1A</i>	I	Recruiting	NCT03896568	
			II	Active, not recruiting	NCT02798406	
	DNX-2440	Deletion of <i>E1A</i> ; Expression of OX40L	I	Recruiting	NCT03714334	
	NSC-CRAAd-Survivin-pk7	<i>E1A</i> expression under the control of human <i>Survivin</i> promoter	I	Active, not recruiting	NCT03072134	
	Ad-RTS-hIL-12	Adenovirus vector encoding human <i>IL-12</i>	I/II	Recruiting	NCT03330197	
	ONYX-015	E1B-attenuated adenovirus	I	Completed	[59]	No serious adverse effects with 10 ¹⁰ pfu of virus; among 24 patients 1 patient each showed no progression and regression.
Vaccinia	TG6002	Deletions of <i>TK</i> and <i>14L</i> ; expression of transgene <i>FCU1</i>	I/II	Recruiting	NCT03294486	
Reovirus	Reolysin	None	I	Completed	NCT00528684	No dose limiting toxicity even with a highest does of 1X10 ¹⁰ TCID ₅₀ .

Table 1. Continued

Virus		Modification	Phase	Status	Reference	Results
Reovirus	Reolysin	None	I	Completed	[60]	No high-grade adverse effects. One and 10 out of 12 patients had a stable and progressive disease, respectively.
			Ib	Completed	[61]	Reovirus is capable of infecting glioma tumors when injected i.v. and increase cytotoxic T cell infiltration in tumors.
		Reovirus + Sargramostim	I	Active, not recruiting	NCT02444546	
Parvovirus H-1	H-1PV		I/II	Completed	NCT01301430 [62]	Virus was safe and well tolerated. Induced cytotoxic T cell response.
			I/IIa	Completed	[63]	Enhanced immune response and improved median survival.
Poliovirus	PVSRIPO	Poliovirus IRES switched with HRV2 IRES	I	Recruiting	NCT03043391	
			I	Active, not recruiting	NCT01491893	Improved survival rate with no neurovirulence.
			I	Active, not recruiting	NCT02986178	

Table 1. Continued

Virus		Modification	Phase	Status	Reference	Results
Measles Virus	MV-CEA	Measles virus expressing carcinoembryonic antigen	I	Completed	NCT00390299	NA
Retroviral vector	Toca511	Replicating retroviral vector expressing cytosine deaminase	I	Completed	NCT01470794	Durable response rate in subgroup of malignant glioma patients.
			I	Completed	NCT01156584	NA
			II & III	Terminated	NCT02414165	Failed to improve survival and meet other efficacy endpoints.
Newcastle disease virus	NDV- HUI strain	Mutation at F1-F2 junction	I/II	Completed	[64]	No severe toxicity with complete remission in 1 patient.
	MTH-68/H		I	Completed	[65]	No adverse effects with improved survival of 4-9 years in 4 patients.

Abbreviations: IL-12, Interleukin-12; HMCV, Human Cytomegalovirus; HRV2, Human Rhinovirus type 2; IRES, Internal Ribosome Entry Site.

The rQNestin34.5v.2 virus is an oncolytic HSV-1, attenuated via a glioma-selective transcriptional regulator that controls expression of the neurovirulent *ICP34.5* gene, allowing selective replication of the virus in glioma cells [66]. This virus is now in a phase I clinical trial in malignant glioma patients to assess safety when delivered intracranially (NCT03152318). The M032 is a conditionally replicating HSV-1 engineered to express interleukin (IL)-12 to boost the immune responses against tumors. It is currently being tested in a phase I trial (NCT02062827). The C134 virus is a chimeric HSV-1 variant with a deleted $\gamma 134.5$ gene and expresses the human cytomegalovirus *IRS1* gene. The C134 virus is a replication competent virus that can infect and kill tumor cells and induce an anti-tumor immune response facilitated by the *IRS1* transgene which helps in evading PKR-mediated protein shutoff [67]. A phase I study is testing the safety of C134 in CNS tumors including malignant glioma where the virus will be delivered into tumors (NCT03657576). Another oncolytic HSV-1, HSV-1716 was tested in two phase I clinical trials where virus was delivered intracranially. Both trials showed no virus-related toxicity with a dose as high as 1×10^5 pfu and improved overall survival [57, 58]. However, a newer phase I study with HSV-1716 in younger patients with refractory or recurrent high-grade gliomas has been terminated due to unknown reasons (NCT02031965).

3.2 Oncolytic adenovirus

Oncolytic adenovirus DNX2401 was generated by deleting the *E1A* gene from the genome of adenovirus type 5. The *E1A* binds to the retinoblastoma (Rb) protein and deletion of the *E1A* gene is expected to allow selective replication of DNX2401 in cancer cells with disrupted *Rb* gene expression [68]. A phase I study tested the safety and maximum tolerated dose (MTD) when injected into recurrent malignant glioma tumors and surrounding brain tissue (NCT00805376). No dose limiting toxicities were observed in this study with the highest dose of 3×10^{10} virus particles (vps), and treatment increased cytotoxic T cell infiltration of tumors with an improved long-term survival [20]. Another phase I study tested tolerance of DNX2401, following injection of 3×10^{10} vps into brain parenchyma along with TMZ in malignant glioma patients but have not yet published the results (NCT01956734). Combination of DNX2401 with IFN- γ has also been studied in a phase I study in patients with recurrent malignant glioma, where virus was injected directly into tumors. Results of this study have not been published yet (NCT02197169). The MTD and toxicity of allogeneic bone marrow-derived human mesenchymal stem cells (BM-hMSCs) loaded with the DNX2401, when injected intraarterially will be investigated in an upcoming phase I trial. Homing and ability of BM-hMSCs to deliver DNX2401 will be tested in patients with recurrent high-grade glioma (NCT03896568). The DNX2401 is also being tested for efficacy in combination with pembrolizumab, an ICI in a phase 2 study in recurrent malignant glioma, where a single dose of virus ranging from 5×10^8 to 5×10^{10} will be delivered intratumorally followed by intravenous (i.v.) pembrolizumab every 3 weeks for up to 2 years or until disease progression (NCT02798406).

Oncolytic adenovirus DNX2440 is an engineered version of DNX2401 which expresses OX40 ligand (OX40L) for stimulation of T cell responses in tumors. This virus is being tested in a phase I study among patients with recurrent malignant glioma where the virus will be delivered stereotactically (NCT03714334). Adenovirus vector expressing human IL-12, Ad-RTS-hIL-12, is also being investigated in a phase I/II trial in malignant glioma patients (NCT03330197). Oncolytic adenovirus type 5, CRAd-Survivin-pk7, was generated by incorporating the *survivin* promoter to drive *E1A* gene expression and modification of the fiber protein to contain a poly-lysine (pk7) for enhancing the virus tropism in malignant glioma cells [69]. The MTD of CRAd-Survivin-pk7 loaded onto neural stem cells (NSCs) will be determined in a phase I study in newly diagnosed malignant glioma patients. Patients will receive the NSC-CRAd-Survivin-pk7 stereotactically along with chemoradiation (NCT03072134).

An *E1B* attenuated adenovirus, ONYX-015 has also been tested in a phase I study in patients with recurrent glioma and showed no serious virus associated adverse effects with a dose as high as 1×10^{10} pfu. Among 24 patients in the study one each showed non-

progression and regression of disease. Two patients who underwent a second resection 3 months after virus injection showed immune cell infiltration in the region [59].

3.3 *Oncolytic vaccinia virus*

Oncolytic vaccinia virus, TG6002 is an attenuated virus engineered to express the yeast *FCU1* gene, which encodes cytosine deaminase and uracil phosphoribosyl transferase, allowing local transformation of the pro-drug flucytosine (5-FC) into cytotoxic 5-fluorouracil (5-FU) and 5-fluoro-uridyl monophosphate at targeted sites by the virus, such as tumors. Combination of TG6002 with 5-FC showed tumor-selective viral replication, prolonged maintenance of therapeutic levels of 5-FU in tumors, and significant anti-tumor effects in multiple human xenograft tumor models [70]. TG6002 with 5-FC is currently being tested in patients with recurrent malignant glioma (NCT03294486). The phase I portion of the study will determine the MTD for TG6002, defining an appropriate dose of TG6002 for combination with 5-FC in the phase II study. The virus will be injected i.v. in these studies.

3.4 *Oncolytic reovirus*

A phase I study to determine the MTD, dose limiting toxicity (DLT) and anti-tumor effects of REOLYSIN, a therapeutic reovirus, in patients with malignant glioma when administered intralesionally has been completed (NCT00528684). Reovirus dose ranges from 1×10^8 to 1×10^{10} TCID₅₀ were tested in this phase I study and the highest dose tested will be used in the phase II study. The phase I study could not identify the DLT and MTD dose was not reached, however there was evidence of antitumor activity in some patients. This is the first report that demonstrated safety and tolerance of intratumoral infusion of reovirus in patients with recurrent malignant glioma [71]. Safety of oncolytic reovirus was also demonstrated in another phase I study, where no level 3 or 4 adverse effects due to treatment were observed with local administration of the virus [60]. A subsequent phase Ib study showed that the reovirus is capable of reaching and infecting glioma tumors when injected i.v. and enhanced leukocyte infiltration into tumors [61].

3.5 *Oncolytic parvovirus*

Two studies have tested oncolytic parvovirus in malignant glioma patients in a clinical set up. In a phase I/II study, the first dose of parvovirus H-1 was delivered intratumorally or intravenously and the second dose was administered after surgical removal of the tumor around the resection cavity after 10 days (NCT01301430). The treatment was safe and well tolerated by patients. Of note, the virus also demonstrated the ability to cross the BBB and infect tumors to trigger cytotoxic T cell responses [62]. In another phase I/IIa study, systemically administered oncolytic parvovirus was able to infect malignant glioma tumors and enhance recruitment of activated cytotoxic T lymphocytes and TAMs in malignant glioma patients [63]. These studies have demonstrated the ability of parvovirus to induce an immune response in immunosuppressive glioma tumors, even when administered systemically.

3.6 *Oncolytic poliovirus*

Oncolytic poliovirus, PVSRIPO has been generated by switching the original internal ribosome entry site (IRES) with the IRES from human rhinovirus 2 (HRV2). PVSRIPO has demonstrated excellent safety and efficacy in a wide range of tumor types, including malignant glioma [72-78]. The virus was tested in a phase I study where intratumoral infusion of PVSRIPO in patients with recurrent grade IV malignant glioma showed no neurovirulence and the survival rate among patients was higher at 24 and 36 months as compared to historical controls (NCT01491893). PVSRIPO is one of the only two oncolytic viruses along with oncolytic HSV-1, HSV-1716 which is presently being tested in a phase I study to determine safety and potential toxicity in young populations between 12-21 years

of age with malignant glioma. In this phase I study PVSRIPO will be delivered as a single intratumoral dose using intracerebral catheter (NCT03043391). Another phase II study is testing PVSRIPO in grade IV malignant glioma patients for safety and efficacy, where patients will be administered PVSRIPO intratumorally via convection-enhanced delivery (CED) in the enhancing portion of the tumor (NCT02986178).

3.7 Oncolytic measles virus

An engineered measles virus expressing carcinoembryonic antigen (MV-CEA) is being investigated in recurrent malignant glioma patients where the virus was delivered intratumorally or in the tumor bed. The study focused on safety, toxicity, MTD but also assessed efficacy in a preliminary manner (NCT00390299). The first group of patients in this study received direct MV-CEA escalating doses from 1×10^5 to 2×10^7 TCID₅₀, injected in the excised tumor cavity. The second group of patients received the MV-CEA after the dose escalation reached 1×10^7 TCID₅₀ in the first group. In the second group, the first dose of MV-CEA was injected directly into recurrent tumors, followed by resection of tumors 5 days post-first virus injection and the second dose of virus was administered into the tumor cavity. Preliminary results showed no DLT with use of intracranial MV-CEA doses as high as 2×10^7 TCID₅₀ (as reviewed in [79]).

3.8 Oncolytic retroviral vector Toca511

Vocimagene amiretrorepvec (Toca 511) is a gamma-retroviral replicating vector that encodes cytosine deaminase that converts prodrug 5-FC (Toca FC) to 5-FU in rapidly dividing cells, leading to targeted effects of the chemotherapeutic 5-FU. A phase I study with Toca511 administered with Toca FC (NCT01470794) showed a durable response rate in a subgroup which included both IDH-1 mutant and wild-type tumors. The Toca511 and Toca FC combination is also being investigated in recurrent malignant glioma patients, in another phase I study (NCT01156584) but the results are not yet available. A recent phase III study of Toca511+Toca FC combination (NCT02414165) has been terminated, since it failed to demonstrate improvement in survival or meet any other efficacy endpoints among patients with high grade glioma [80].

3.9 Oncolytic Newcastle disease virus

There are two NDV stains that are currently being investigated in clinical studies for glioma treatment. NDV-HUJ is an attenuated strain with mutation in the cleavage site between fusion proteins F1 and F2, while MTH-68/H is a pathogenic strain which differs in amino acid sequence at the F1-F2 junction from NDV-HUJ [81]. A phase I study showed that patients with recurrent malignant glioma tolerated i.v. injection of oncolytic NDV-HUJ and had minimal toxicity. One patient among 11 total who received the treatment achieved a complete response [64]. The pathogenic NDV stain, MTH-68/H resulted in increased survival time up to 5-9 years in 4 patients with high grade glioma, which was higher than the expected survival and enhanced the quality of life. These patients received only MTH-68/H as a non-surgical onco-therapy [65].

4. Challenges in treating malignant glioma with oncolytic virus

4.1 Getting beyond the blood brain barrier

Upon systemic delivery, OVs have to face several obstacles before reaching the tumors including neutralization by complement factors and/or antibodies, and anti-viral immune cell responses. Moreover, non-specific virus uptake in tissues such as liver, spleen, lung and tissue resident macrophages further reduce the viral load that can reach tumors (Figure 1A) [82]. Furthermore, an inefficient extravasation of virus from vascular to extravascular compartments due to physical barriers curtails the virus particles reaching tumors. The physical BBB in the CNS regulating passage of virus from vascular to

extravascular compartments is even more stringent (Figure 1B). The architecture of microvasculature in the CNS is unique where different cell types such as endothelial cells, pericytes, microglia, and astrocytes form a complexly interactive system. The continuous non-fenestrated blood vessels in the BBB tightly regulate transport of molecules, ions and cells across the blood vessel membrane to the brain which is critical for maintenance of homeostasis and optimum functioning of neurons. Additionally, the BBB plays a critical role in protecting the brain from inflammation, toxins, and injury (as reviewed in [32]). The BBB, however is a major obstacle in delivering systemic therapeutics to tumors located in the CNS compartment, including OV's [32].

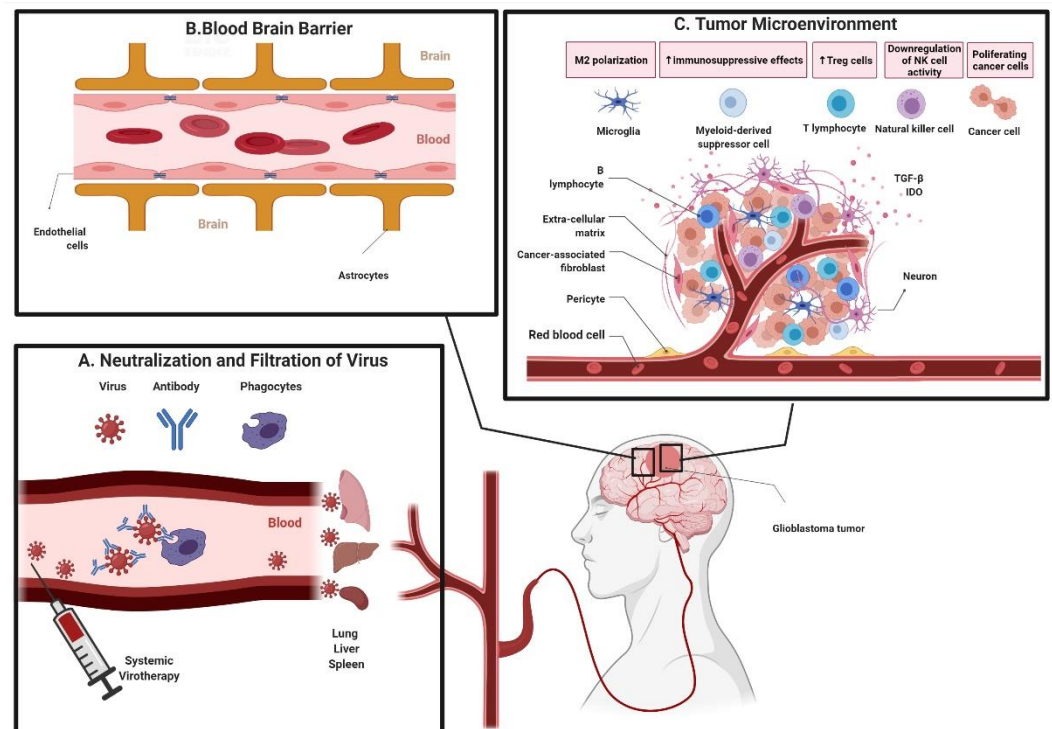


Figure 1. Overview of hurdles to oncolytic virotherapy in malignant glioma. A. Neutralization and non-specific filtration of virus. Systemically administered oncolytic viruses are prone to complement and antibody mediated neutralization and uptake by phagocytic macrophages. Virus particles further get non-specifically filtered in lungs, liver, spleen and other tissues as they pass through them reducing the overall number of virus particles that reach tumors. B. Blood Brain Barrier. Transportation across blood vessels into the brain tissue is tightly regulated by specialized perivascular architecture of cells, known as the “Blood Brain Barrier” which also hinders transport of oncolytic viruses to tumors within the central nervous system compartment, thereby reducing the virus load delivered into tumors. C. Immunosuppressive tumor microenvironment. Immunosuppressive cells like M2 phenotype microglia/macrophages, myeloid-derived suppressor cells, regulatory T cells are upregulated and maintained due to high levels of tumor necrosis factor (TGF)- β and indoleamine 2,3-dioxygenase (IDO) secreted from tumor cells which form a tumor-protective surrounding around a tumor (created with biorender.com).

Despite all the hurdles associated with the systemic delivery of OV's, some viruses have shown the ability to effectively cross the BBB to reach and infect tumors in animal models, such as Semliki Forest virus [83], vaccinia virus [84, 85], chimeric vesicular stomatitis virus (VSV) [86], parvovirus H-1 [87], Mengovirus [88], and Seneca Valley virus-001 [89] when administered systemically. Oncolytic parvovirus H-1 [62] has also been shown to reach malignant glioma tumors when delivered systemically in glioma patients. Oncolytic reovirus is another virus that has been shown to reach brain tumors when injected systemically in both animal models and patients. Reovirus is thought to be carried by immune cells across the BBB [61].

Most, if not all OV that are in clinical trials for treating malignant glioma are being administered locally, to circumvent the barriers associated with systemic delivery of viruses and maximize the virus load in tumors for optimum efficacy. The OV that are being injected systemically in glioma patients in the ongoing or completed trials, include vaccinia virus (NCT03294486), reovirus [61], parvovirus [62, 63] (NCT01301430), NDV [64] and adenovirus (NCT03896568). The adenovirus, however, is being loaded on carrier cells before systemic administration. Tumor tropism of neural and mesenchymal stem cells can be exploited by using them as carriers for OVs. Several preclinical studies have demonstrated that OV loaded on stem cell carriers can be effectively delivered to malignant glioma tumors when injected systemically (as reviewed in [90]). Use of carrier stem cells as 'trojan horses' can effectively deliver OV that are restricted by the BBB when injected systemically. Currently, two clinical trials are investigating this strategy to deliver the virus to tumors in malignant glioma patients. As described earlier, allogeneic BM-hMSCs and NSCs are being used to carry the oncolytic adenoviruses, DNX2401 (NCT03896568) and CRAd-Survivin-pk7 (NCT03072134), respectively in two different clinical trials in malignant glioma patients. Although the DNX2401 loaded BM-hMSCs will be delivered systemically and will need to overcome the BBB, the NSCs loaded with CRAd-Survivin-pk7 will be delivered directly into tumors with the goal of enhancing virus spread within the tumor. Furthermore, CED is a minimally invasive technique which establishes a pressure gradient using a catheter to locally deliver therapeutics in brain. CED helps to maximize the uptake of therapeutic agents by tumor cells, bypassing the BBB. [91]. A phase II study showed that intratumoral CED of oncolytic poliovirus, PVSRIPO, improved the overall survival in malignant glioma patients [19].

To summarize, some of the OV have a natural tropism for neuronal tissue or naturally use immune cells as carriers, enabling them to cross the BBB to infect and kill tumor cells in the CNS compartment, however most OV have difficulty in crossing the BBB, upon systemic delivery. The BBB poses as a major hurdle in ensuring efficacious levels of therapeutics are achieved in malignant glioma tumors, including OV. Carrier cells offer a promising alternative that can help improve the delivery of OV across the BBB, while local CED can potentially maximize the uptake of OV by tumor cells. Together these strategies can be used to achieve therapeutic levels of OV in CNS tumors for optimal efficacy.

4.2 Changing the tumor landscape: from cold to hot

There was an earlier notion that malignant glioma tumors are immunologically privileged due to the isolation from surrounding structures by the BBB [92], however there is growing evidence that immune cells can cross the BBB, especially in neuroinflammatory conditions. These evidences represent a window of opportunity that requires exploration as it may be exploited to potentiate immune- and virotherapies in malignant glioma [93, 94]. A thorough understanding of underlying mechanisms involved in enhanced immune cell infiltration in the CNS during neuroinflammatory conditions will be required to optimize anti-tumor immune responses in glioma patients. There is no dearth of immune cells in malignant glioma tumors, but rather an abundance of immune cells with TAMs constituting up to 30-50% of cellular mass in tumors [95]. It appears that a major part of local resistance to immunotherapeutics in malignant glioma tumors comes from their highly immunosuppressive TME (Figure 1C). Efficacy of immunotherapy is further limited in malignant glioma patients due to severe systemic immunosuppression [96].

Local immunosuppression in malignant glioma tumors is mediated through both suppression of immune effector cells and stimulation of immunosuppressive immune cell types. T cell dysfunction in the TME of malignant glioma is mediated via multiple mechanisms that lead to T cell senescence [97], exhaustion [98], tolerance [99] and anergy [100]. Furthermore, immunoreactive cells like cytotoxic T cells, natural killer (NK) cells and M1 macrophages are downregulated, in addition to pacifying the functionality of antigen presenting cells (APCs) by reduction of co-stimulatory cell surface receptors.

Immunosuppressive cell phenotypes such as Treg cells, M2 macrophages/microglia and MDSCs are promoted in TME of malignant glioma via secretion of immunosuppressive cytokines [101, 102]. Secretion of TGF- β and indoleamine 2,3-dioxygenase (IDO) from malignant glioma tumors promote recruitment, survival and maintenance of Treg cells along with reduced activation and proliferation of cytotoxic T cells and inhibition of APCs [103, 104]. The NK cell activity in TME of malignant glioma is not only inhibited directly by tumor cells which express an inhibitory ligand, HLA-G that binds to NK receptors, reducing NK cytotoxicity but also via secretion of TGF- β which downregulates NK cells [105, 106]. The upregulated Treg cells in the TME can further inhibit NK cell functions like cytotoxic activity, cytokine production, proliferation and tumor rejection [107]. Both MDSCs and M2 macrophages/microglia, also known as TAMs exert immunosuppressive effects in the TME and have been correlated with poor survival in malignant glioma patients [108-111].

Systemic immunosuppression in malignant glioma patients is due to T cell lymphopenia caused by sequestration of T cells in bone marrow, spleen and lymphoid organs. Loss of surface expression of S1P1 on T cells which is critical for egress of T cells from the bone marrow or lymphoid tissues into systemic circulation is believed to be responsible for severe T cell dysfunction in malignant glioma patients. Tumors located in the CNS, including malignant glioma, disrupt the S1P1-S1P axis gradient to trap T cells in peripheral organs, while causing contracture of spleen and lymphoid organs [17].

As discussed earlier, oncolytic virotherapy can lead to tumor cell lysis, release of TAAs, disruption of the immunosuppressive TME and induction of innate immune responses in and around tumors. Induction and maintenance of immune responses against tumors is critical for extended therapeutic effects of OV, post their immune clearance (as reviewed in [112-116]). Several OVs that are currently in clinical studies in malignant glioma patients have been shown to induce immune cell responses in tumors. Oncolytic adenovirus, DNX-2401 treatment enhanced CD8⁺ and T-bet⁺ cell infiltration in tumors [20], while ONYX-015 treatment increased lymphocytic and plasmacytoid infiltration in peritumoral region [59]. Increased cytotoxic T cell infiltration of tumors treated with oncolytic reovirus as compared to the control samples was also seen in malignant glioma patients [61]. Oncolytic parvovirus, also increased infiltration of activated cytotoxic T cells and TAMs with inducible nitric oxide synthase expression in tumors in malignant glioma patients [62, 63].

As discussed before, malignant glioma tumors secrete immunosuppressive cytokines that help to maintain a tumor protective environment, hence more immunostimulatory factors in the TME can help to shift the cytokine balance to boost and maintain anti-tumor immune responses. Oncolytic virotherapy uses two main approaches to achieve the higher levels of pro-inflammatory factors in the TME; 1) engineering OVs to express immunostimulatory proteins; and 2) administration of immune stimulators and OVs in combination. Several OVs have been designed to express immunostimulatory cytokines such as IL-12 [117-119], IL15R α -IL15 fusion protein [120], IL-4 [121], or GM-CSF [122, 123], and have shown promising results in pre-clinical studies in malignant glioma tumor models, however this review will focus on OVs that are in clinical studies. At least three OVs, M032, Ad-RTS-hIL-12 and DNX-2440 expressing immunostimulatory proteins to potentiate anti-tumor immune responses are currently in clinical trials in malignant glioma patients. Both oncolytic herpesvirus, M032 (NCT02062827) and adenovirus, Ad-RTS-hIL-12 (NCT03330197) are engineered to express IL-12. IL-12 is a pleiotropic pro-inflammatory cytokine that plays a role in activating a diverse population of pro-inflammatory immune cells. Some of the known pro-inflammatory processes that IL-12 is involved in are 1) differentiation of T helper 1 (TH1) cells; 2) generation of cytotoxic T and lymphokine activated killer cells; and 3) augmentation of the cytotoxic activity of NK and cytotoxic T cells [124-127]. Another oncolytic adenovirus in clinical trial for treating malignant glioma, DNX-2440, is engineered to express OX40L (NCT03714334), the ligand for the T-cell activating receptor OX40 on the surface of T cells. The OX40L-OX40 interaction promotes

survival of activated T cells [128] and is critical for development of memory T cell response [129]. Lastly, oncolytic reovirus is being tested in combination with a recombinant GM-CSF, also known as Sargramostim in a malignant glioma clinical trial, where the latter is expected to boost the production of blood cells and possibly promote the tumor cell killing effects of reovirus (NCT02444546).

The OV's expressing inflammatory cytokines or immune stimulatory factors can effectively turn tumors from 'cold' to 'hot', while limiting the toxicities due to systemic administration of pro-inflammatory factors. Furthermore, cancer stem cells that are responsible for the recurrence of disease after chemo-radiation therapy can be effectively killed by OV's [130]. Oncolytic adenovirus Delta-24-RGD demonstrated the ability to infect, replicate and kill glioma stem cells (GSC) derived from patients [131]. TAMs can engulf and trap OV's, preventing the efficient spreading of virus in and around glioma tumors [132, 133]. It is important to consider the fact that viruses differ in their cellular tropism and can be restricted at cell entry or post-entry levels which can limit infection of specific cell types in the TME [134, 135]. Overall, the complex interaction between OV's and different cells in the TME needs to be better understood to optimize the virus spread in tumors to maximize therapeutic efficacy.

4.3 Innate immunity and oncolytic viruses

Infection of host cells by virus can trigger an innate anti-viral inflammatory response, releasing a range of stimulatory cytokines like type I IFN, tumor necrosis factor (TNF) and IL-1 which can be crucial in triggering immune responses against TAAs [136]. Although tumor cells with defective IFN response pathways allow unrestricted virus replication and can be more susceptible to OV's, infection of peri-tumoral cells by OV's can trigger an anti-viral state in the TME to limit virus spread and negatively impact the efficacy of oncolytic virotherapy [137]. Modulation of the type I IFN response by using FDA approved JAK/STAT pathway inhibitors, such as Ruxolitinib, has been shown to enhance the titers of oncolytic measles virus in patient derived GBM xenografts [138].

Another study showed that oncolytic HSV in a murine malignant glioma model enhanced macrophage/microglia infiltration into tumors and polarized them to the pro-inflammatory M1 phenotype that triggered the apoptosis of virus infected cells, limiting virus spread [139]. Microglia and TAMs can restrict the virus spread and reduce efficacy of OV's in glioma. Depleting innate immune cells like microglia and peripheral macrophages has been shown to improve the OV titers in brain tumors [132]. Combination therapies where immunomodulatory drugs like rapamycin and cyclophosphamide can boost initial virus replication in tumor cells have been used synergistically to improve therapeutic outcomes in malignant glioma tumor models [84]. It can be advantageous to negate the innate anti-viral immune response in tumors and TME to promote viral replication, leading to direct tumor cell lysis, however strong anti-tumor immune responses are critical for the immune mediated tumor clearance. Setting a fine-tuned balance between the direct tumor cell lysis by OV's and immune mediated clearance of tumor cells is essential for the best treatment outcome. Nevertheless, application of IFN modulators with OV's in malignant glioma patients is extremely tricky due to heterogeneous IFN responses within a tumor, systemic immunosuppression, and lack of reliable translatable animal models.

4.4 Overcoming immune checkpoint mediated immune resistance

Immune checkpoint receptors are critical to prevent an over-reactive T cell immune response by fine tuning the activation and maintenance of T cell responses. Binding of immune checkpoint receptors expressed on the T cell surface, like PD-1 and CTLA-4 with their ligands sends inhibitory signals to T cells, negatively modulating T cell activity [140, 141]. Several cancer types, including malignant glioma have upregulated expression of immune checkpoint receptor ligands like PD-L1, which potentiates immunosuppression in the TME by modulating T cell activation. High PD-L1 expression has been linked with

poor survival [142, 143]. ICIs are an important milestone on the path of optimizing cancer therapy. ICIs can promote activation and maintenance of T cell responses by blocking/interfering with the immune checkpoint inhibitory axis that constrains T cell responses. Three recent clinical trials testing ICI therapies in malignant glioma patients showed limited efficacy [144]. In the wake of established immunosuppressive TME and low T cell activity, the ICIs are unlikely to provide desired therapeutic benefits in malignant glioma patients.

OVs can kickstart inflammatory T cell responses in malignant glioma tumors that can be harnessed with ICIs. Combination of OVs and ICIs is a natural next step and can exert synergistic effects to optimize the therapeutic outcome in malignant glioma patients. Oncolytic adenovirus, DNX-2401 in combination with pembrolizumab, an anti-PD1 antibody, is currently in a phase II clinical study (NCT02798406), where the latter is expected to boost the anti-tumor immune response initiated by DNX-2401. There are two other ongoing clinical trials testing efficacy of nivolumab (anti PD-1 antibody) (NCT02017717, NCT02617589) in GBM patients. ICIs are being delivered systemically in malignant glioma patients in all current clinical studies and face the obstacle of the BBB which may reduce their delivery into CNS compartment. Preclinical studies have shown that the delivery of ICIs across the BBB can be improved by loading them onto nanoparticles resulting in improved overall survival [145, 146]. In conclusion, considering the limited success of ICIs as monotherapy in highly immunosuppressive glioma tumors, there is a strong rationale for combining ICIs with OVs to augment immunotherapeutic effects.

4.5 Tumor heterogeneity and oncolytic viruses

Malignant glioma tumors, similar to other solid tumors, show both intra and inter-tumoral molecular heterogeneity [147]. The level of heterogeneity is negatively correlated with the response to therapeutics among malignant glioma patients [148, 149]. Pre-screening of patients for molecular patterns that are likely to be benefited by a given treatment in clinical trials is very challenging due to loco-regional heterogeneity among tumor sub-clones which makes the selection of tumors for sampling difficult. Similar to other therapeutics, tumor heterogeneity also affects the outcome of oncolytic virotherapy. Differential expression of virus entry receptors among tumors has been shown to affect the ability of oncolytic reovirus [150] and adenovirus [151] to infect patient derived malignant glioma cells. OVs that are retargeted against multiple receptors instead of being dependent upon the expression of a single entry receptor can expand their ability to infect more cells in a tumor despite the underlying heterogeneity [152]. Another study showed that patient derived primary malignant GSCs showed differential levels of resistance to oncolytic HSV due to heterogeneity. An HSV engineered to express tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) allowed targeting of a broader tumor cell population by combining the effect of direct HSV mediated oncolysis and TRAIL mediated apoptosis induction, irrespective of differential susceptibility of tumor cells to either modes of cell death [153]. Lastly, OVs that are engineered to exploit specific gene mutations like mutated *P53*, *Ras* and *Rb* genes in tumors [152] or use tumor specific promoters to enhance their tumor-selectivity may result in a sub-optimal therapeutic efficacy due to the diversity in the level of expression of these specific mutated genes in tumor cells [154-156].

In the present scenario, a practical way to tackle the tumor heterogeneity and maintain or improve the therapeutic outcome of OVs is to use multimodal combination therapies to achieve synergistic effects. Furthermore, continuous efforts will be needed to develop molecular markers that can be reliably used to identify potential responders to a particular treatment, including OVs. Multifaceted approaches will be crucial when engineering OVs that can exert multimodal effects leading to tumor cell killing to tackle the heterogeneity in glioma tumors.

4. Conclusions

New treatment options will be critical for improving the formidable situation of poor survivability with standard treatment options in malignant glioma patients [3, 4]. Approval of oncolytic herpesvirus, T-vec, for melanoma treatment has catapulted several experimental virotherapies into clinical studies for various tumor types, including malignant glioma [95, 157, 158]. Results from some of the clinical trials testing OV in malignant glioma patients found evidence that viruses are capable of infecting tumor cells and enhancing immune cell recruitment, despite the immunosuppressive TME [20, 61, 63]. Although Toca511 therapy failed to meet the end point for survival in phase III clinical trial in malignant glioma patients (NCT02414165), the recent report from Japan about submission of a new drug application seeking an approval to use the first OV, G47Δ for malignant glioma treatment is an important milestone in the advancement of oncolytic virotherapy for malignant glioma [22]. The phase II study with oncolytic herpesvirus, G47Δ that led to the application for a new drug for malignant glioma (UMIN000015995, Japan) used multiple stereotactic injections of the virus with a maximum of 6 dosages [56]. These results are undoubtedly exciting, but the economic feasibility of injecting multiple dosages of virus stereotactically remains a concern. Although some OVs have shown the ability to cross the BBB to infect malignant glioma tumors, systemic use of OVs is still limited. GBM patients present with a unique set of problems like the BBB, immunosuppressive TME and high level of molecular heterogeneity which warrants persistent efforts to optimize oncolytic virotherapy among these patients. In the meantime, use of OVs in synergistic combination therapies to maximize therapeutic outcome is a practical approach.

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