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Impact of Immunotherapy in the Treatment of Glioblastoma

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Abstract

Glioblastoma multiforme (GBM) is the most common and aggressive primary malignant brain tumor. Despite the efforts developed in the respective treatment, consisting of maximal surgical resection followed by adjuvant radiotherapy and chemotherapy, the prognosis remains very poor. This may be partly related to the resistance of GBM cells and their infiltrative and invasive nature into the surrounding brain tissue. Therefore, newer and challenging alternative approaches for the treatment have emerged, including immunotherapy. This anticancer therapy, based on the stimulation of the host's immune system, has been currently investigated and several advances in the clinical trial stage have already been reached. Immunotherapeutic strategies comprise a set of modalities, including vaccines (cell-free and cell-based), chimeric antigen receptor (CAR) T-cell therapy, immune checkpoint inhibitors, monoclonal antibodies (mAbs), and oncolytic viruses (OVs). In this chapter, we will review the principal concepts and the recent progress in immunotherapy for GBM.

Keywords: glioblastoma multiforme, immunotherapy, vaccine, antigen, dendritic cell, clinical trial

1. Introduction

Glioblastoma multiforme (GBM), histologically a World Health Organization (WHO) grade IV glioma, is the most common and aggressive malignant brain tumor, accounting for approximately 45–50% of all primary malignant brain tumors. Despite the efforts developed by its current standard therapy (maximal surgical tumor resection followed by concomitant

radiotherapy plus temozolomide (TMZ) chemotherapy), GBM remains an incurable disease with a poor prognosis that attains a median survival of 14.6 months and a mean survival rate of 0.05–4.7% at 5 years, which is partially due to its heterogeneous and invasive nature as well as to the tumor resistance [1–3]. In addition, it is well-known that the protective nature of the blood–brain barrier (BBB) limits the entry of therapeutic agents into the brain and consequently hampers the success of therapies [4].

Recently, new and promising immunotherapeutic approaches have emerged and evidenced a great impact in GBM treatment, harnessing the ability of the host's immune system to induce or enhance antitumor responses [2, 5]. These immunotherapeutic strategies are related with both active immunotherapy, such as vaccines (cell-free and cell-based), and passive immunotherapy, namely monoclonal antibodies (mAbs), immune checkpoint inhibitors, chimeric antigen receptor (CAR) T-cell therapy and oncolytic viruses (OVs). Despite the presence of the BBB, such strategies can be successful by considering some key points. If immunotherapy is intended to be given intravenously, the BBB is effectively a problem, but likely to be exceeded. On the one hand, it is noteworthy that patients with GBM tend to have a fenestrated endothelium with BBB disruption, which will possibly facilitate the passage of immunotherapeutic cells. On the other hand, there are already mechanisms to induce a reversible BBB opening with a transiently increase in the respective permeability. Nonetheless, there is currently an easier and more effective approach, which consists of direct intracranial injection of immunotherapeutic agents, thus overcoming the problems associated with the BBB [4, 6].

Regardless the low number of clinical trials (CT) that are completed to date, the early results reached for all of these strategies are generally related with positive patient outcomes, which has increased the interest in proceeding with the investigations. This chapter provides a brief description and the currently ongoing CT of all of these therapies, with particular emphasis on vaccines. In fact, vaccination represents a valuable therapeutic option in cancer since it can induce widespread and sustained antitumor effects, with less toxicity than standard chemotherapy [7, 8].

2. The spectrum of vaccine strategies in glioblastoma

Contrary to chemotherapy and passive immunotherapy, vaccination does not have a direct antitumor effect, but rather boosts the immune system to destroy tumor cells [9]. More precisely, vaccines aim at inducing tumor-specific immune responses, mainly based on CD8⁺ cytotoxic T lymphocytes (CTL), which are specific to tumor antigens [10]. According to the strategy used to present the antigens to the immune system, cancer vaccines can be divided mainly in two groups, cell-free or cell-based vaccines [11].

2.1. Cell-free vaccines

Treatment with cell-free vaccines consists on direct inoculation of single or multi antigens, later presented to host antigen-presenting cell (APC) that, upon such stimulation, migrate to lymph nodes where they boost immune response. In this group are included a peptide,

multi-peptide, and heat-shock protein (HSP) vaccines, whose applicability has already been developed for GBM [11–13].

2.1.1. *Peptide and multi-peptide vaccines*

Cancer peptide vaccines take advantage of peptides to trigger a pharmacological activity through the mobilization of the immune system against tumor antigens [13]. Investigating the expression profile of antigens in human GBM thus becomes the most important step in the process of developing vaccine-directed immunotherapy [14]. In fact, multiple glioma-related antigens have been identified and even tested in vaccine trials over the last years, but only a few reached promising results given the known variable expression patterns of proteins/antigen among GBM patients [14, 15]. These vaccines may incorporate a single or multiple, long or short peptides acting as tumor antigens, which are often coupled to carrier proteins in order to potentiate their immunogenicity [15, 16]. Although being a recent treatment modality in oncology, GBM has already a varied range of successfully proven vaccines, many of them peptide/multi-peptide vaccines [11].

Considering the frequent amplification of epidermal growth factor receptor (EGFR) and its active mutant EGFRvIII in GBM, many researchers have focused their works on developing EGFRvIII vaccines with remarkable clinical results. For instance, rindopepimut (also called Rintega® and CDX-110) was subjected to a number of phase I/II CT since its introduction (VICTORI, pediatric pontine glioma pilot study (NCT01130077), ACTIVATE (NCT00643097), ACT II, ACT III (NCT00458601), and ReACT (NCT01498328)), where its clinical efficacy was clearly shown in patients with GBM. Such vaccination resulted in prolonged progression-free and overall survival (OS) with no safety concerns. However, a phase III CT (ACT IV (NCT01480479)) with rindopepimut was discontinued since the study failed to meet its primary OS endpoint [7, 17, 18]. Another example is a live attenuated *Listeria*-based vaccine (ADU-623) expressing both EGFRvIII and NY-ESO-1 antigens, which is currently being tested in patients with recurrent GBM through a phase I CT (NCT01967758) [11, 18].

Despite the increased interest in EGFRvIII, other antigens have been considered for the investigation of immunotherapy in GBM, with emphasis on SurVaxM, PEPIDH1M, and DSP-7888 vaccines. SVN53-67/M57 (SurVaxM), a peptide vaccine derived from survivin, not only has revealed promising results in preclinical studies with GL261-bearing mice but has also been investigated in CT (phase II so far (NCT02455557)) [11, 18, 19]. Since isocitrate dehydrogenase 1 (IDH1) is an enzyme commonly mutated in GBM, it has been developed IDH1 peptide vaccines for patients with glioma positive for IDH1 R132H mutation (presented in 5–12% of GBMs). Currently, two phase I CT are being conducted for that purpose, called RESIST trial (NCT02193347) with PEPIDH1M vaccine and NOA-16 (NCT02454634), both of which target patients with IDH1R132H-mutated gliomas [15, 20]. Another developing peptide vaccine uses the tumor-associated antigens (TAA) Wilm's tumor protein-1 (WT-1), recognized as an oncogene expressed in GBM responsible for tumor growth. WT1 peptide vaccination has been investigated through several phases I/II CT, alone or in combination with other therapeutics, including TMZ. Overall, the results of these WT1-based vaccines have been positive, confirming their safety profile along with good clinical

responses. By way of example, DSP-7888 vaccine revealed to induce a specific CTL and helper T-lymphocyte-mediated immune responses against WT1 expressing GBM, in a phase I CT (NCT02498665) [11, 18].

When not one but several antigens are incorporated in the same vaccine, multi-peptide vaccines are obtained as the case of IMA950, SL-701, and ERC-1671 vaccines. In fact, combining multiple peptides in a single mixture may offer therapeutic advantages bearing in mind the heterogeneous gene expression profiles in different GBM [15, 21]. IMA950 is a vaccine encompassing 11 peptides naturally presented in GBM tissue (brevican; chondroitin sulfate proteoglycan 4; fatty acid binding protein 7, brain; insulin-like growth factor 2 mRNA binding protein 3; neuroligin 4, X-linked; neuronal cell adhesion molecule; protein tyrosine phosphatase, receptor-type, Z polypeptide 1; tenascin C; Met proto-oncogene; baculoviral inhibitor of apoptosis protein repeat-containing 5; and hepatitis B virus core antigen). This multi-peptide vaccine has been subjected to numerous phase I/II CT (NCT02924038, NCT01920191, NCT01403285, and NCT01222221), alone or combined with other therapies, whose results are still somewhat inconclusive as to its clinical efficacy [11, 18]. Another case, the SL-701 vaccine composed of 3 peptides (a highly immunogenic mutant to target survivin, interleukin-13 receptor α -2 (IL-13R α 2), and ephrin A2) was investigated in adults with recurrent GBM through a phase I/II CT (NCT02078648), although the results have not yet been disclosed [11, 14, 18]. A slightly more complex vaccine concerns ERC-1671 (Gliovac) since it uses a combination of allogeneic tumor cells (derived from three different GBM donors), autologous GBM tumor cells (resultant from resected tumor of the patient) and GBM tumor lysates. Given the notable results obtained from the first clinical studies, this multi-peptide vaccine moved toward a phase II CT (NCT01903330), being examined in recurrent, bevacizumab naive GBM patients [11]. As it is well-known, O⁶-methylguanin-DNA-methyltransferase (MGMT) unmethylated GBM is correlated with TMZ resistance and worse prognosis of the tumor [22]. Such evidence led to the development of a personalized neoantigen cancer vaccine (NeoVax), which is currently being examined in a phase I CT (NCT03422094) along with radiotherapy in newly diagnosed GBM with exclusively unmethylated MGMT promoters [18].

2.1.2. Heat-shock protein (HSP) vaccines

HSPs act as chaperones for intracellular proteins, so they have the ability to bind, fold and chaperone an antigenic representation of the cells from which they are originated. Based on this fact, HSPs isolated and purified from a patient's resected tumor can be subsequently reinfused, and then they will promote the presentation of antigenic peptides to APCs, which elicit antigen-specific CTL responses [11, 18]. Among the proteins of the HSP family, expressed in GBM, HSP70 and HSP90 stand out. Despite the dysregulation of these HSP families being reported to play a critical role in tumor proliferation, invasiveness, and metastasis, in addition to suppression of apoptosis, HSP70 and HSP90 have also shown ability to bind antigenic peptides, which can elicit tumor rejection responses [23]. HSP70 family inhibits cell stress-induced apoptotic pathways, facilitates protein folding, and guides protein transport across membranes, while HSP90 mainly assists in protein folding, protein stabilization, and peptide loading onto major histocompatibility complex (MHC) class I molecules. Particularly, HSP90

was found to bind to EGFRvIII, FAK, AKT, hTERT, p53, cdk4, MAPK, and PI3K in GBM, which are involved in key tumor initiation and proliferation signaling pathways. Although the studies have been conducted for other types of tumors, it is believed that the same may occur with GBM, with HSP presenting tumor-specific antigens to stimulate antitumor immune responses [11, 24, 25]. Autologous tumor-derived HSP-peptide complex 96 (HSPPC-96) have generated great interest over the last few years, reason why it has been the most used in HSP vaccine trials. In fact, HSPPC-96 has been extensively explored in several phases I/II CT (NCT02722512, NCT00905060, NCT01814813, NCT00293423, and among others), most of them for GBM patients, inducing strong tumor-specific immune responses with the improved median OS [11, 14, 18, 26].

2.2. Cell-based vaccines

Contrary to the vaccines previously presented, cell-based vaccines (as the name itself indicates) first resort to APCs *ex vivo*, most often dendritic cells (DCs) extracted from the patient, loading them with tumor antigens. After activation, they are injected into the host, presenting the antigens to naive T cells of the adaptive immune system [11, 27].

2.2.1. Dendritic cells (DCs) vaccines

As is already known, DC are considered the optimal APC of the immune system, due to their ability to stimulate T and B lymphocytes, in addition to promoting natural killer (NK) T cells activation [28, 29]. As such, a wide range of antigen sources has been explored for pulsing of DC, and include tumor peptides, autologous tumor lysates, tumor-derived mRNA (messenger RNA), glioblastoma stem cells (GSCs) and viral antigens. All of these strategies have already been properly tested in GBM, proving to be immunogenic with very promising outcomes [5, 11, 14, 15, 28].

Referring to tumor peptide-loaded DCs, we here present the two most important so far: autologous DC pulsed with an EGFRvIII peptide conjugated with keyhole limpet hemocyanin (PEPvIII-KLH), and the ICT-107 autologous vaccine [11]. The latter concerns patient DCs pulsed with six synthetic TAAs (AIM-2, MAGE1, TRP-2, gp100, HER2/neu, and IL-13R α 2, four of which are considered GSC-associated, and whose results obtained in phase I/II CT (NCT01280552) are quite encouraging for the ongoing phase III CT (NCT02546102) in GBM [5, 11, 14]. As a matter of fact, ICT-107 is also considered a tumor stem cell vaccine. It should be recalled that GBM possesses a small subpopulation of self-renewing, tumorigenic GSC, which drive invasive tumor growth and therapeutic resistance. Among the biomarkers studied, CD133 has been used extensively to identify and isolate tumor stem cells, given its overexpression on these malignant cells [7, 18, 30]. In this sense, ICT-107 vaccine was found to decrease CD133⁺ cells from recurrent GBM; moreover, the tumor stem cell vaccine ICT-121 consists of autologous DC pulsed with purified peptides from CD133, whose safety and clinical response will be assessed in a phase I CT now ongoing (NCT02049489) [18]. Still on the stemness, another way to take advantage of GSC is to develop tumor-derived mRNA-loaded DCs vaccines. In this case, autologous GSC cultures are established from resected

tumor, followed by isolation of RNA and amplification of mRNA, then transfected into DC [31, 32]. As such, a phase I CT (NCT00890032) studied CD133⁺ autologous brain tumor stem cell (BTSC) mRNA-loaded DC in patients with recurrent GBM, which revealed to be safe, feasible and well-tolerated [32].

An alternative approach involves using tumor cell lysates-loaded DCs, such as DCVax®-L vaccine in which cellular fragments (derived from the patient's own resected tumor) are pulsed into DCs. In fact, this technique offers the advantage of collecting a broad spectrum of patients' tumor antigens, known and unknown (given the heterogeneity of antigen expression among gliomas), thus triggering a polyclonal immune response [11, 14, 28, 33]. DCVax®-L, one of the most promising vaccines for GBM, consists of an autologous DC vaccine that is currently under evaluation in a phase III CT (NCT00045968). Previous studies (phase I/II CT) have proved its safety profile, also demonstrating that vaccine can increase progression-free and OS in newly diagnosed GBM (2 out of 39 patients survived more than 10 years) [2, 18, 34].

It has been reported that most GBM express exclusive human cytomegalovirus (CMV) proteins, as for example IE1, US28, pp65, gB, HCMV IL-10, and pp28. Such evidence made possible the use of immunodominant CMV antigens to produce viral antigen-loaded DCs, aimed at treating GBM patients [14, 15]. Exciting results were obtained in a study with patient-derived DCs pulsed with CMV pp65 RNA since the stimulation of CMV pp65-specific cytotoxic T cells resulted in recognition and destruction of autologous GBM tumor cells in an antigen-specific manner [35]. In the wake of this outcome, a phase II CT (NCT02366728) is currently ongoing testing the CMV pp65 RNA-pulsed autologous DC vaccine along with tetanus/diphtheria toxoid helper vaccine, for newly diagnosed GBM patients [18]. In addition, the DC vaccine PEP-CMV comprises two peptides, derived from human pp65 and gB, and has been investigated in some phase I CTs (NCT01854099, NCT02864368, and NCT03299309) without published results so far [7, 15].

2.2.2. Human umbilical vein endothelial cell (HUVEC) vaccines

Less commonly used in vaccines, but also with ongoing trials for GBM, are the glutaraldehyde-fixed human umbilical vein endothelial cells (HUVECs). [14, 36]. As it is well known, angiogenesis is a hallmark of GBM that facilitates tumor progression and invasiveness. Based on that, HUVEC vaccination consists of an endovascular targeting immunotherapy intended to trigger an antiangiogenic response, upon presentation of HUVEC antigen to the immune system [14, 37]. To the best of our knowledge, only two preliminary CT was conducted with HUVEC vaccines for recurrent GBM patients, whose clinical outcomes were promising without serious adverse events associated [14, 36, 38]. In addition, an interesting *in vivo* study assessed the effect of a combined vaccine, prepared from GBM and endothelial cells, on glioma-bearing mice. While tumor growth inhibition was seen only in the preventive use of the combined vaccine, a significantly decrease in vessel account was verified in the tumor upon the therapeutic experiment with that vaccine [37].

2.2.3. Autologous formalin-fixed tumor vaccines (AFTVs)

Last but not least, are whole tumor cell vaccines, which use autologous formalin-fixed tumor fragments obtained from surgical removal to trigger *in vivo* antigen-specific CTL responses [39, 40].

Actually, formalin fixation technique has been used since it allows admirable preservation of tissue morphology, and consequently, the antigenicity of tumor cells [14, 40]. A CT with an autologous formalin-fixed tumor vaccine (AFTV) being tested in 12 primary GBM patients demonstrated the safety and viability of the vaccine, along with promising clinical responses that were also achieved [40]. Another study, phase I/IIa CT, examined the impact of an AFTV concomitant with radiotherapy in 22 resected patients with newly diagnosed GBM. Such trial resulted in median OS of 19.8 months with an actuarial 2-year survival rate of 40%, in addition to not reporting serious adverse events [39]. Interestingly, an immunotherapy strategy combining cellular vaccines (prepared from autologous GL261 murine glioma cells and F-2 murine endothelial cells) was tested on glioma-bearing mice. Such preclinical study revealed that combined vaccine significantly decreased tumor growth and vessel account, thus representing an expecting strategy capable to target both GBM cells and their microenvironment [37].

2.3. Advantages and disadvantages of cell-free and cell-based vaccines

Throughout this chapter, several candidate vaccine approaches are presented for GBM treatment. Although most of them have demonstrated safety and clinical benefit in different

Strategy	Advantages	Disadvantages
Cell-free vaccines	<ul style="list-style-type: none"> • Easy to synthetize, purify, produce and standardize; [16, 41, 42] • Cost-effective approach; [41] • Safe with no biological contamination (in case of synthetic peptides); [42] • Stable in storage; [16] • Possibility of selecting one or more antigens (stimulation of an specific immune response); [16, 41] • Fully-defined composition; • Low risk of allergic and auto immune responses, as well as of oncogenicity effects [41, 42] 	<ul style="list-style-type: none"> • Prior and proper identification of immunogenic epitope(s); [41] • Existence of antigen-loss tumor variants; [16] • MHC restriction; [41] • Lack of helper activity and weak presentation of antigen by endogenous APC (contrary to the cross-presentation by DC); [16] • Instability of peptides <i>in vivo</i> being rapidly degraded by peptidases [16]
Cell-based vaccines	<ul style="list-style-type: none"> • Bypass the endogenous DC dysfunction in cancer patients; [43] • Cross-presentation of exogenous antigens by DC; [44] • Great ability of DC to prime T cells to attack the tumor; [44] • AFTV provide the entire spectrum of TAAs with no need to select the most proper antigen to target the tumor; [45] • Safe, multivalent and patient-specific [12] 	<ul style="list-style-type: none"> • High production costs; [42] • Quality concerns due to the manufacture highly variable; [12, 42] • Poor immunogenicity of the tumor cell themselves; [12] • Most of them are restricted to patients with a resectable tumor; [42, 46] • Some failure rate associated with culture of autologous tumor cells [46]

MHC: major histocompatibility complex; APC: antigen-presenting cell; DC: dendritic cell; TAA: tumor-associated antigens; AFTV: autologous formalin-fixed tumor vaccine.

Table 1. Overall assessment of cell-free and cell-based vaccines.

preclinical and clinical studies, it is essential to understand some intrinsic advantages and disadvantages of cell-free and cell-based vaccines in cancer therapy (**Table 1**).

3. Other immunotherapeutic strategies

Equally important, immunotherapeutic approaches, such as monoclonal antibodies, immune checkpoint inhibitors, adoptive T-cell therapy, CAR T-cell therapy and oncolytic viruses, have been investigated in the treatment of GBM [47]. Some related clinical data are presented in **Table 2**, with an indication of the CT ID.

3.1. Monoclonal antibodies (mAbs)

One of the most intensively explored passive immunotherapeutic approach resorts to mAbs. These are able to recognize cell surface receptors and ligands, which provide a successful strategy to target antigens highly expressed in tumor cells or receptors involved in tumorigenesis [6]. They can function through a set of ways, such as by blocking ligand-receptor binding and/or downstream signaling pathways, targeting the tumor microenvironment, immune cells or immunosuppressive tumor microenvironments, and modulating constant fragment (Fc) domain of antibodies [48].

The mAbs have been applied as immunotherapeutic agents in GBM treatment. Two targets expressed on GBM cells are vascular endothelial growth factor (VEGF) and EGFR or its variant III mutation EGFRvIII. Bevacizumab, a humanized antibody against VEGF, was the first mAb studied in the treatment of GBM patients. This mAb has the ability to promote the blockade of VEGF pathway, intervening in neovascularization of the tumor and, consequently, in tumor growth, decreasing its size [49, 50]. It was approved by the food and drug administration (FDA) in 2009 for recurrent GBM. Two phase II multicenter and randomized CT were performed to evaluate the safety of bevacizumab with or without irinotecan (a cytotoxic prodrug which inhibits DNA replication and activates apoptotic cell death) in patients with recurrent GBM, where treatment-associated toxicity was documented in some of the patients [51]. For newly diagnosed GBM patients, this drug has been investigated together with standard therapy in comparative studies to assess the use of bevacizumab as first-line treatment. Two randomized double-blind placebo-control trials [52, 53] and one open-label single-arm phase II CT [54] showed that the addition of bevacizumab to standard therapy prolonged the progression-free survival (PFS), but did not improve OS. Additionally, serious adverse events related to bevacizumab were also reported in these trials. Thus, the efficacy of bevacizumab on quality of life of newly diagnosed GBM patients was not clearly specified and further well-designed CT should be performed. Other ongoing CT of bevacizumab agent in GBM treatment are present in **Table 2**. Inversely to bevacizumab, there are other mAbs agents that specifically target EGFR and/or EGFRvIII, which are the most common tumor-expressed targets explored in antibody therapy. EGFR is expressed in approximately 40% of GBM patients and 65% of them present EGFRvIII mutation [49]. These mAbs have the ability to blockade ligand binding or signaling through these receptors, interfering with tumor growth rates and inducing

Strategy	Drug/antigen	Other therapy	Clinical phase (status)	Clinical trial ID
mAbs	Bevacizumab	—	I/II (recruiting)	NCT01811498
		—	II (not recruiting)	NCT02157103
		TMZ	III (recruiting)	NCT02761070
		TMZ	II (not recruiting)	NCT01149850
	Nimotuzumab	RT plus TMZ	III (completed)	NCT00753246
		RT plus TMZ	II (completed)	NCT03388372
	Cetuximab	RT plus TMZ	I/II (recruiting)	NCT02861898
	ABT-414	RT plus TMZ	I (completed)	NCT01800695
		RT plus TMZ	III (recruiting)	NCT02573324
		TMZ or lomustine	II (recruiting)	NCT02343406
Immune checkpoint inhibitors	AMG595	—	I (completed)	NCT01475006
	Nivolumab (anti-PD-1)	RT and TMZ	III (recruiting)	NCT02617589
		RT plus TMZ	III (recruiting)	NCT02667587
	Pembrolizumab (anti-PD-L1)	—	Pilot (recruiting)	NCT02852655
		RT plus TMZ	I/II (not recruiting)	NCT02530502
		Bevacizumab	II (not recruiting)	NCT02337491
	Durvalumab (anti-PD-L1)	RT plus TMZ; HSPPC-96 vaccine	II (recruiting)	NCT03018288
		Bevacizumab or RT	II (not recruiting)	NCT02336165
		RT plus TMZ	II (recruiting)	NCT03047473
	Nivolumab (anti-PD-1) and ipilimumab (anti-CTLA-4)	TMZ	I (not recruiting)	NCT02311920
		Bevacizumab	III (not recruiting)	NCT02017717
Adoptive T-cell therapy	CMV-CTL	TMZ	I/II (recruiting)	NCT02661282
	CMV-ALT	RT plus TMZ	I (completed)	NCT00693095
CAR T-cell therapy	IL-13R α 2	—	I (recruiting)	NCT02208362
		—	Pilot (completed)	NCT00730613
	EphA2	—	I/II (completed)	NCT02575261
	HER2	CMV-CTL	I (not recruiting)	NCT01109095
		—	I/II (recruiting)	NCT02713984
		RT plus TMZ	I (recruiting)	NCT02442297
	EGFRvIII	Fludarabine and cyclophosphamide	Pilot (recruiting)	NCT02844062
		Fludarabine, cyclophosphamide and IL-2	I/II (recruiting)	NCT01454596
		—	I (not recruiting)	NCT02209376
		RT plus TMZ	I (recruiting)	NCT02664363

Strategy	Drug/antigen	Other therapy	Clinical phase (status)	Clinical trial ID
OVs	HSV-1 M032	—	I (recruiting)	NCT02062827
	DNX-2401	TMZ	I (completed)	NCT01956734
		IFN- γ	I (not recruiting)	NCT02197169
		—	I (recruiting)	NCT03178032
	AdV-tK	Pembrolizumab	II (recruiting)	NCT02798406
		RT and valacyclovir	II (completed)	NCT00589875
		RT and valacyclovir	I (completed)	NCT00751270
		Reolysin	I (completed)	NCT00528684

mAbs: monoclonal antibodies; TMZ: temozolomide; RT: radiotherapy; PD-1: programmed cell death protein 1; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; HSPPC-96: heat-shock protein peptide complex 96; CMV-CTL: cytomegalovirus cytotoxic T lymphocytes; CMV-ALT: cytomegalovirus autologous lymphocyte transfer; CAR: chimeric antigen receptor; IL-13R α 2: interleukin-13 receptor alpha 2; HER2: human epidermal growth factor receptor 2; EphA2: erythropoietin-producing hepatocellular carcinoma 2; EGFRvIII: epidermal growth factor receptor variant III mutation; IL-2: interleukin-2; Ovs: oncolytic viruses; HSV: herpes simplex virus (HSV); IFN- γ : interferon gamma; AdV-tK: adenovirus mutant thymidine kinase.

Table 2. Representative CT of immunotherapeutic strategies in GBM treatment, including mAbs, immune checkpoint inhibitors, adoptive T-cell therapy, CAR T-cell therapy and OVs.

apoptosis, as well as providing a better sensitization of tumors to chemotherapeutic agents [48]. Examples of these mAbs are nimotuzumab and cetuximab, which are tested in clinical stage with early promising outcomes in the majority of the cases. More details about their CT are displayed in **Table 2**.

A recent and promising strategy using mAbs is based on antibody drug-conjugate (ADC), where mAbs are linked to cytotoxic molecules that specifically target tumor cells, promoting delivery of drugs or toxins. ABT-414 and AMG595 are two examples of ADCs that specifically target EGFRvIII and are under investigation for the treatment of GBM (**Table 2**) [49, 50].

3.2. Immune checkpoint inhibitors

Immune checkpoints are molecules that can attenuate the strength and duration of the normal activity of CTLs and are responsible for preventing autoimmunity and mitigating collateral tissue damage [5, 55, 56]. Programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is the most studied immune checkpoints molecules that provide immune resistance mechanisms at different levels and by different mechanisms, leading to inhibition of T-cell proliferation and cytokine production, consequently resulting in a non-activation of T-cells [2, 56]. Both PD-1 and CTLA-4 are receptors expressed on the surface of T-cells [51]. PD-1 blocks T-cells at advanced stages of the immune response and interact with one of the PD-1 ligands expressed on the surface of tumor cells (such as PD-L1/B7.H1 or PD-L2/B7-DC), while CTLA-4 occurs early in T-cell immune response and binds to ligands expressed on the surface of APCs (such as B7.1/CD80 and B7.2/CD86) [2, 56].

A strategy based on the blockade of these inhibitory receptors and their ligands through the use of antibodies has been explored as a promising immunotherapeutic approach for diverse solid tumors, such as glioblastoma, inducing T-cell-mediated antitumor immunity [6]. PD-1 is expressed on activated T-cells, B-cells, DCs and macrophages and the expression of PD-L1 has been reported in glioma cell lines and tumor tissues, as well as in activated APCs in glioma patients, and its level of occurrence is associated with glioma grade [55]. Preclinical studies using antibodies to target PD-1/PD-L1 in animal glioma models showed encouraging results, especially in combination with radiotherapy [2, 57], which provided a strong support to proceed to clinical stage. Several CT are ongoing to evaluate the safety and efficacy of anti-PD-1/PD-L1 human antibodies, namely nivolumab (anti-PD-1 antibody) and pembrolizumab (anti-PD-L1 antibody), which have been tested alone or in combination with other agents in GBM patients. Some of these trials are in early phases, but two phase III trials are evaluating nivolumab and comparing it with standard therapy (NCT02617589 and NCT02667587). In a preclinical stage, the anti-CTLA-4 strategy has also demonstrated robust and effective response rates, with an increasing of long-term survival in 80% of treated mice [5]. Clinically, the safety and efficacy of nivolumab with or without ipilimumab, an anti-CTLA-4 human antibody, in GBM patients, are assessed in phase I and III trials (NCT02311920 and NCT02017717, respectively). This combinatorial PD-1 and CTLA-4 blockade demonstrated the most effective results. More information about immune checkpoint inhibitors trials is summarized in **Table 2**.

Despite their great impact on survival, this immunotherapeutic approach presents some limitations, reflected by the immune-associated side effects experienced by some patients, such as dermatological, endocrinological, gastrointestinal, and hepatic toxicities, which have been reported like associated with the abnormal infiltration of stimulated CD4⁺–CD8⁺ T-cells into normal tissues with the concurrent elevation of levels of pro-inflammatory cytokines [2].

3.3. Chimeric antigen receptor (CAR) T-cell therapy

Adoptive T-cell therapy has also been widely applied in treatment of highly aggressive and advanced tumors, including GBM [58]. This therapy is based on direct T-cells activation and can be described in a simple way through the isolation of tumor-specific autologous T-cells and *in vitro* expansion followed by their injection into the patient, in order to improve anti-tumor activity [5, 6]. As referred previously, CMV has been suggested as a potential GBM therapeutic target, due to the expression of its antigens in GBM cells, but not in surrounding healthy brain tissue. In a phase I CT for recurrent GBM patients, the feasibility and safety of a combinatorial therapy of autologous adoptively CMV-specific T-cell therapy and chemotherapy were demonstrated, highlighting the power of this antiviral therapy to improve GBM prognosis [59]. Other examples of CTs regarding CMV adoptive T-cell therapy are depicted in **Table 2**. However, it has been observed that this immunotherapeutic strategy is limited by the immunosuppressive tumor microenvironment, and the need of T-cells to recognize tumor antigens presented by the MHC class I [2, 60].

A novel type of adoptive T-cell therapy that has been proposed to overcome these shortcomings, is based on the transference of autologous T-cells genetically modified with CARs [5]. CARs are cell surface receptors selected to establish a high specificity with tumor cells and provide T-cell

activation through a mechanism where the antigen-binding region of a mAb is fused with the T-cell receptor (TCR) signaling domain CD3. They allow an antibody-like antigen recognition in a non-MHC-restricted pathway and a more effective T-cell penetration and persistence into the tumor microenvironment than mAbs [60, 61]. The implementation of CAR T-cell therapy in GBM treatment has demonstrated to be highly promising, where several GBM-specific antigens have been investigated as targets, such as (IL-13R α 2), human epidermal growth factor receptor 2 (HER2), erythropoietin-producing hepatocellular carcinoma 2 (EphA2) and EGFRvIII [50, 61]. Preclinical studies that involve these targets are associated with positive outcomes, and all of them are also ongoing in a clinical stage [5]. A pilot study of intracranial delivery of CAR T-cells targeting IL-13R α 2 into the resection cavity of three patients with recurrent GBM (NCT00730613) provided preliminary results about its safety and feasibility, showing that the strategy was well-tolerated without the emergence of serious side effects. Further, a transient antitumor activity was developed in two of the patients and the decrease of IL-13R α 2 expression within the tumor was confirmed in one patient [62]. Regarding the application of CAR T-cells therapy for EphA2 positive malignant glioma patients, a phase I/II CT (NCT02575261) was completed, where the effectiveness, safety, and clinical response were evaluated, but the results have not yet been published. A phase I trial of CMV-specific CAR T-cells therapy targeting HER2 (NCT01109095), considered a more active strategy that reacts with the virus and tumor cells is also in progress to investigate its safety and efficacy in the treatment of patients with GBM. Several clinical trials exploring CAR T-cells targeting EGFRvIII in GBM are underway to test the safety and effectiveness of this approach. More detailed information about these CAR T-cell clinical trials, as well as other examples, can be found in **Table 2**. Phase I/II clinical trials have provided early results of potential efficacy with an acceptable toxicity profile, but more efforts need to be carried out to assess the efficacy of the application of CAR T-cell therapy in GBM treatment. Despite the tolerable toxicity, cytokine release syndrome (CRS) is the most frequent adverse effect due to the extreme immune activation, especially evidenced in the case of intravenous administration. Furthermore, additional disadvantages are the high cost of the therapy and the possibility of expression of the target antigen on healthy tissues [61].

3.4. Oncolytic viruses (OVs)

Oncolytic virotherapy is another emerging immunotherapeutic strategy that has been investigated in the treatment of GBM, evidencing promising results. OVs are replication-competent viral vectors used to induce effective antiviral and antitumor immune responses [2, 51]. They are able to selectively replicate in tumor cells through cell surface marker identification, induce tumor cell death, spread within the tumor and indirectly recruit immune cells to promote immune responses against themselves and infected and uninfected tumor cells [58, 63]. They can naturally occur or are genetically manipulated to specifically infect and destroy tumor cells overexpressing tumor antigens [50, 51]. Once all the tumor cells are eradicated, the excess of virus can be removed using an anti-viral medication [49]. Several OVs are under investigation for GBM targeting. Some of them have been tested at different stages of CTs, including herpes simplex virus (HSV), adenovirus (AdV), measles virus (MV), poliovirus (PV), reovirus, H1 parvovirus and Newcastle disease virus (NDV), after preclinical researches showing anti-tumor activity, and many of the remaining OVs are in advanced preclinical stages [63].

HSV is a neurotropic human pathogen that provides tumor selectivity with safety and the most intensively studied examples of genetically modified mutant HSV are G207 and HSV1716 [51]. Phase I and Ib trials performed to evaluate the safety of G207 inoculation into the brain resection cavity for recurrent GBM showed no treatment-related toxicity neither serious adverse effects, with no patients developing HSV encephalitis, and encouraging therapeutic efficacy was reported [2]. Another phase I trial that combines G207 oncolytic HSV therapy with radiotherapy also demonstrated safety and potential antitumor activity in the treatment of recurrent GBM patients [64]. HSV1716 is also reported in phase I trials, where no toxicity ascribed to HSV1716 was demonstrated [65, 66]. Another example of a genetically modified mutant HSV that has been investigated (HSV-1 M032) is presented in **Table 2**. Relatively to genetically engineered AdVs, which are non-enveloped virus with a double-stranded linear DNA genome, the most commonly investigated in GBM treatment are DNX-2401, ONYX-015 and AdV-tK [2, 49–51]. In the case of DNX-2401, the tumor targeting is achieved and increased due to the presence of cyclic arginine/glycine/aspartic acid (cRGD) peptide that permits the attachment to host's immune cells [50]. In a phase I trial of DNX-2401 oncolytic AdV, patients with recurrent high-grade glioma were subjected to an intratumoral injection in a dose escalation, demonstrating that this AdV is able to infect, replicate in and kill human glioma cells, without a harming toxicity profile. Furthermore, despite the early stage of the trial, a promising efficacy was expected, where 12% of the patients demonstrate durable complete therapeutic responses [67]. Other phase I CTs of DNX-2401 are displayed in **Table 2**. On the other hand, ONYX-015 oncolytic AdV was also tested in a phase I trial, through intracerebral injection of various doses in patients with recurrent glioma that was resected, where no serious related-treatment adverse events were identified, even at the highest administered dose [2]. For AdV-tK, an AdV mutant thymidine kinase, a randomized phase II trial (NCT00870181) was carried out with the parallel administration of ganciclovir, showing an improvement of PFS and OS in the treatment of patients with recurrent high grade glioma. Other CTs of AdV-tK are presented in **Table 2**. MV is an RNA virus and the genetically modified derivatives of MVs, such as modified Edmonston (MV-Edm) vaccine, have an affinity for cellular CD46 receptor abundantly expressed on tumor cells [2]. In addition, with the aim of facilitating *in vivo* monitoring of viral gene expression and replication, this MV has been engineered to express the human carcinoembryonic antigen (CEA; MV-Edm-CEA), which is expressed by various types of cancers, but not by gliomas [51]. However, MV-Edm-CEA has exhibited a great potential as an oncolytic therapy for GBM in preclinical studies. A phase I CTs with this MV (NCT00390299) was initiated to evaluate the MV-Edm-CEA-associated adverse effects and its best dose in patients with recurrent GBM, however, this study was recently suspended by reasons not yet reported. PV is an enterovirus that encompasses a protein capsid and RNA, expressing high-affinity for neoplastic cells and tropism to motor neurons [51]. A genetically recombinant polio/rhinovirus chimera, PVSRIPO, was developed to reduce the neurotoxicity frequently observed in cases of human poliomyelitis, avoiding neurovirulence tendency. This PV recognize the CD155 cell surface polio receptor, a tumor antigen widely expressed in tumor cells, including GBM [51, 58]. A phase I trial is underway to evaluate the safety and the occurrence of potential antitumor responses of intratumoral administration of PVSRIPO in patients with recurrent GBM (NCT01491893). Preliminary reported results of this clinical trial described that the infusion

of PVSRIPO was well-tolerated and revealed a promising efficacy, as well as provided a survival advantage when compared to historical control group of patients that was also analyzed [51]. Reovirus is a double-stranded RNA virus that is isolated from the respiratory and gastrointestinal tracts of humans. The safety of intratumoral administration in a dose-escalation of this genetically unmodified OV was also assessed in a phase I trial in patients with recurrent GBM that received prior radiotherapy with or without chemotherapy [68]. More information about a phase I trial of reolysin, a genetically engineered reovirus, is depicted in **Table 2**.

The use of OVs has been shown through some CTs as a harmless strategy with promising results; however, no OV has yet been approved by the FDA for GBM [58]. On the other hand, their therapeutic efficacy needs to be thoroughly assessed and proven with convincing clinical success in advanced phase trials. This task is hampered by the high genetic heterogeneity of GBM, and the ability of BBB to inhibit migration of OVs to the tumor site, thus compromising the success of the treatment [6].

4. Conclusions

The current, first line, GBM standard therapy has not provided the necessary and expected improvements in overall patient survival rates. Immunotherapy is being explored as an alternative strategy, revealing to be a promising field, associated with good outcomes and fewer adverse events.

The advances in technology, as well as in scientific knowledge regarding gene expression and signaling pathways analysis, have provided data to translate into new perspectives on personalized therapeutic approaches. In this context, immunotherapy has gained importance insofar as the identification of specific biomarkers in each patient could be useful for prognosis of personal immune responses. Several immunotherapeutic CTs are currently ongoing for GBM, predominantly using vaccines, and the preliminary results attained so far yielded satisfactory clinical responses associated with antitumor activity. However, some challenges have been reported, which are associated to finding therapeutic agents capable to penetrate the BBB, the identification of suitable, specific and immunogenic tumor antigens and appropriate pre- and post-therapeutic markers in order to develop immune-targeted agents. Other limitations comprise the reduced number of GBM patients skilled to incorporate particular clinical studies, and the insufficient understanding of the immune system as well as the GBM microenvironment. Given the molecular heterogeneity and immunosuppression that so well depict the GBM, combinatorial therapies targeting multiple pathways now become a need and so they have already been explored. A variety of regimens have been equated, combining immunotherapeutic strategies (vaccines, cell therapies, multiple checkpoint inhibitors, and antibodies) along with molecules targeting either tumor cells or their microenvironment, as well the current standard therapy. Consequently, more advanced CTs need to be underway to deeply explore the fundamental insights of these therapies. In addition, the discovery of new targets and mechanisms is essential in order to help widespread this field of research, and develop optimized therapeutic strategies for GBM.

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Conflict of interest

The authors declare they have no conflict of interest.

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