Adoptive immunotherapy for the treatment of GBM: progress and possibilities

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Abstract

Patients with glioblastoma have a very poor prognosis. Adoptive cellular therapy (ACT) is defined as the collection of circulating or tumor-infiltrating lymphocytes, their selection, modification, expansion, and activation, and their re-administration to patients in order to induce antitumor activity. Although various ACTs have been attempted, most failed to improve the outcome. Immune checkpoint blockade antibodies and T cell engineering with tumor-specific chimeric antigen receptors suggest the emergence of a new era of immunotherapy.

Here, we summarize approaches with ACTs using genetically modified T cells, which have been improved by enhancing their antitumor activity, and discuss strategies to develop these therapies. The mechanisms by which gliomas modulate and evade the immune system are also discussed.

Keywords: glioblastoma, T cell receptor gene-modified T cells, chimeric antigen receptor T cells, immune modulators

Introduction

Glioblastoma (GBM) is the most common type of primary brain tumor in humans. Despite advances in surgery and chemoradiotherapy, patients with GBM have a very poor prognosis, with a 5-year overall survival rate of less than 10%. Therefore, new strategies are essential for improving the abysmal outlook of GBM patients. Adoptive cellular therapy (ACT) is defined as (1) the collection of circulating or tumor-infiltrating lymphocytes, (2) their selection, modification, expansion, and activation *ex vivo*, and (3) their re-administration to patients in order to induce antitumor activity [1]. Various ACTs exist for the treatment of malignant gliomas (MGs). These use nonspecific or specific effector cells such as lymphokine-activated killer (LAK) cells, tumor infiltrating lymphocytes (TILs), γδT cells, antigen-specific cytotoxic T lymphocytes (CTLs), and natural killer (NK) cells. However, all of these approaches have failed to improve the outcome of patients with MGs.

Immune checkpoint blockade antibodies against melanomas [2,3] and T-cell engineering with tumor specific chimeric antigen receptors (CARs) against hematological malignancies [4] have shown promising efficacy, indicating the emergence of a new era of immunotherapy [5].

Here, we summarize approaches with adoptive immunotherapies using genetically modified T cells, which have been improved by enhancing their antitumor activity, and discuss strategies to further develop these therapies.

Genetically modified adoptive cellular therapy for malignant gliomas

Gene transfer-based strategies have been developed to improve the therapeutic potential of ACT. Genetic engineering generates peripheral blood lymphocytes with improved secretory profiles, increased and sustained *in vivo* proliferative ability, an elevated tumor-infiltrating capacity, and superior cytotoxicity. Furthermore, genetic engineering can also be used to modify lymphocytes such that the immune evasion mechanisms of tumor cells are overcome [1]. The most common approaches to lymphocyte modification are T cell receptor (TCR) gene therapy and chimeric antigen receptor (CAR)-T cell therapy, as described below (Table 1).

TCR gene therapy

Tumor-reactive T cells that can recognize antigens with high avidity can be generated by transducing them with genes encoding tumor-specific TCRα and -β chains. This method is known as TCR gene therapy. The genes encoding TCR are cloned and inserted into self-inactivating (SIN) viral based vectors, which are then used to infect autologous T cells from the patient to be treated.

The advantage of this method is that genetically engineered TCRs can recognize all tumor-associated antigens, regardless of whether they are on the membrane or in cells. On the other hand, the disadvantages are the difficulty associated with the vulnerability to major histocompatibility complex (MHC) down-regulation and the impairment of antigen-presenting capabilities by tumor cells.

The first clinical trial of TCR gene therapy used MART-1 melanoma/melanocyte antigen and was reported in 2006. In this trial, two of 17 patients showed long-term persistence of injected engineered cells and objective shrinkage of metastatic melanoma lesions [6]. Subsequent to this trial, this approach has expanded the range of tumor histology to include not only melanoma but also synovial cell sarcoma (NY-ESO-1, MAGE-A3) [7, 8], colorectal cancer (CEA) [9], myeloma (MAGE-A3) [10], and esophageal cancer (MAGE-A3, A4) [8,11]. While some of these antigens are expressed in GBMs, this approach has not yet been applied to the treatment of brain tumors [12].

Despite the efficacy of TCR gene therapy, lethal graft-versus-host-disease (GvHD) has been reported in previous clinical trials. The major reason for this is cytokine-driven autoimmune pathology caused by the mispairing of transduced and endogenous TCR chains in TCR gene-modified T cells [13]. Furthermore, the existence of endogenous TCR decreases the expression of transduced TCR because of the competition for cell surface expression. To overcome these issues, a novel retroviral vector system was developed that can highly express target antigen-specific TCR, in which expression of endogenous TCR is suppressed by built-in short-interfering RNAs (siRNAs), namely the siTCR vector [14].

For example, the antitumor effects of Wilms tumor gene product 1 (one of the zinc finger transcriptional regulators that is expressed in various types of tumors; WT1)-specific siTCR gene-transduced T cells (WT1-specific/HLA-A*2402-restricted T cells) against

acute leukemia *in vivo* were markedly enhanced when compared with conventional TCR gene-transduced T cells [15]. We demonstrated high expression of WT1 in malignant meningiomas and a cytotoxic effect of WT1-siTCR transduced T cells in human meningioma cells in an HLA-class I-restricted manner. Moreover, retardation of tumor growth and prolonged overall survival was seen *in vivo* following a single injection of WT1-siTCR-transduced T cells [16].

Furthermore, siTCR gene-transduced T cells have the potential to be used for allogeneic cell therapy as an 'off-the-shelf' product following allogeneic hematopoietic stem cell transplantation (HSCT) for the treatment of patients with hematological malignancies, as siTCR vectors may reduce the capacity of allogeneic T cells to induce GvHD [17]. Thus, further study of TCR gene therapy with allogeneic lymphocytes may lead to the development of treatments for other types of cancer, such as MGs.

CAR-T cell therapy (Figure 1)

CARs are genetically constructed from a single-chain variable fragment (scFv) from a monoclonal antibody (mAb) connected by a transmembrane hinge region, CD3ζ, and costimulatory signaling domains (e.g., CD28, 4-1BB, OX-40, and ICOS). CARs have theoretical and technical advantages such as (1) the ability to recognize specific antigens without the need for MHC-restricted presentation, which is often down-regulated in tumor cells, (2) the availability of an adequate quantity of tumor-specific effector cells in a short period, and (3) high sensitivity and specificity. Furthermore, CARs can be used to transduce not only CD8+ cells but also CD4+ T cells and non-T cells such as NK cells, and they have high affinity compared to normal TCRs. However, this method has the disadvantage that the target antigens are limited to only cell surface molecules of tumor cells, unlike TCR. In addition, although rarely, cytokine release syndrome (CRS) has led to serious adverse effects in some cases [18].

In the 1980s and early 1990s, initial CAR was comprised of CD4 or CD8 linked to CD3ζ, and the development of single-chain CAR and its cytotoxicity *in vitro* were first reported [19]. Subsequently, modular single-chain CAR was designed, which included either a single costimulatory domain (CD28 or 4-1BB; 2nd generation CAR) or double costimulatory domains (CD28 + OX40 or 4-1BB; 3rd generation CAR) to provide the

signals necessary for sustaining T cell cytotoxicity, proliferation, cytokine secretion, and *in vivo* persistence. The first trial of CAR-T cell therapy was conducted in patients with colon cancer [20] , and a number of subsequent trials have been reported.

In particular, the treatment of B cell hematological malignancies with CAR-T cells targeting CD19 showed not only a remarkable objective response, but also long-term durable remissions [21], [4]. These trials prompted great excitement in the field of cancer immunotherapy, and were described as a major breakthrough [5].

Epidermal growth factor variant III (EGFRvIII), HER2 (HER2/neu, ERBB2), interleukin-13 receptor α2 subunit (IL-13Rα2), and erythropoietin-producing hepatocellular carcinoma A2 (EphA2) have been reported as representative targets of CAR-T cell therapy for glioma [22,23,24,25]. These targets are discussed in more detail in the following sections.

EGFRvIII (epidermal growth factor variant III)

EGFRvIII is the most common variant of EGFR, and is expressed in many different cancers. As a result of the fusion of exon 1 to exon 8 of the ligand-binding portion of EGFR gene, EGFRvIII constitutively delivers tumorigenic prosurvival signals through the RTK/RAS/PI3K pathway and induces increased proliferation and reduced apoptosis in cancer cells. The type III variant is characterized by an 801-base pair in-frame deletion, which creates a unique sequence with a glycine residue at the fusion junction. EGFRvIII has attractive characteristics for immunotherapy because it is frequently expressed in GBM cells (24–67%) but not in normal tissue, and is related to survival, invasion, and angiogenesis. So far, therefore, many immunotherapeutic approaches for MGs have targeted EGFR and EGFRvIII.

Rindopepimut (CDX-110) is a peptide vaccine that consists of a unique 13 amino acid sequence and specifically targets EGFRvIII. Three phase II trials (ACTIVATE, ACTII, ACTIII) of rindopepimut in newly-diagnosed GBM resulted in a median progression-free survival (PFS) of 12.3 to 15.3 months from diagnosis, and a median overall survival (OS) of 24 months from diagnosis [26]. However, the suspension of a pivotal double-blind, randomized phase III trial (ACT IV) was announced at the ASCO 2016

meeting, as it was judged unlikely to meet primary OS endpoint in patients with minimal residual disease. The median OS with rindopepimut arm was 20.4 months compared with 21.1 months in the control arm (hazard ratio = 0.99), nevertheless this trial had performed satisfactorily at the prior phase II stage.

Anti-EGFR mAbs can also be used to inhibit EGFR activity. Cetuximab is a chimeric antibody that binds to EGFR with high affinity, and is clinically approved for use against several malignancies such as colorectal and head and neck cancers. This agent also exhibited antitumor activity against MG cells overexpressing EGFRvIII in a preclinical study [27]. The antitumor activity of cetuximab is because of its inhibition of ligand binding to the EGFR extracellular domain, which induces antibody-dependent cell-mediated cytotoxicity (ADCC). However, compared with antibody-mediated approaches, CAR-T cells have a stronger and more direct cytotoxicity.

To exploit the advantages of CAR-T approach, we previously generated a mouse mAb, namely 3C10 [28], and its scFv antibody that specifically recognizes the glycine residue of EGFRvIII [29]. Furthermore, we successfully constructed human T cells that expressed CAR targeting the EGFRvIII antigen using 3C10-scFv (3C10-CAR) [22]. Several CAR constructs targeting EGFRvIII, including 3C10-CAR, showed biological activities such as induction of IFN-γ production when they were added to EGFRvIII-expressing target cells[30], and stimulation of efficient tumor lysis *in vitro* and *in vivo* [22], [31]. Humanized 3C10-CAR was subsequently generated to avoid a human anti-mouse antibody response, which restricts the persistence of 3C10-CAR-T cells and causes anaphylaxis [32]. Phase I clinical trials of EGFRvIII-targeting CAR-T cell therapy for recurrent GBMs are currently underway (NCT01454596, NCT02209376).

HER2 (HER2/neu, ERBB2)

HER2 encodes a 185-kDa transmembrane glycoprotein with tyrosine-specific kinase activity and extended homology in structure and sequence to EGFR. The HER2 antigen is overexpressed in approximately 30% of breast cancer patients as well as in several cancers including GBM, and is associated with more aggressive disease [33]. HER2 overexpression results in increased HER2 heterodimerization with EGFR and HER3. EGFR/HER2 and HER2/HER3 heterodimers drive proliferation and invasion of cancer

cells [34]. Furthermore, overexpression of this receptor correlates with the grade of malignancy in MGs, and *HER2* is mutated at a high frequency in GBM [35]. Together, these data indicate that HER2 is an attractive therapeutic target in MG.

Subsequently, the antitumor activity of HER2-CAR clinical study against MGs was demonstrated in a pioneering clinical study [23]. Stimulation of HER2-CAR-T cells with HER2-positive autologous GBM cells induced T cell proliferation and secretion of IFN-γ and IL-2 in a HER2-dependent manner. Importantly, HER2-CAR-T cells effectively killed CD133-positive tumor stem cells in GBMs; this is significant since cells with this marker are generally resistant to current chemo- and radiotherapies.

A phase I clinical trial using autologous HER2 CMV-bispecific CAR-T-cells demonstrated their clinical benefit and safety (NCT01109095, [36]). Seventeen CMV-positive patients with progressive HER2-positive recurrent GBM were treated, and no severe adverse events occurred. The median survival was 11.6 months from infusion and 24.8 months from diagnosis. However, the issue of off-tumor/on-target toxicity remained, because HER2 is also expressed at some extents of levels in normal tissues, including the lungs. Indeed, several reports have shown fatal adverse effects associated with CRS after infusion of HER2-CAR-T cells [37].

IL-13Rα2 (interleukin-13 receptor α2 subunit)

IL-13 binds to two receptors: IL-13Rα1 and IL-13Rα2. IL-13Rα1 forms a heterodimer with IL-4 receptor (IL-4R) that binds IL-13, whereas IL-13Rα2 is a decoy monomeric molecule that lacks the signaling chain present on the IL-13α1, and interferes with any IL-13-mediated signaling pathway. In addition, the high affinity of IL-13 for IL-13Rα2 effectively removes ligand from IL-13Rα1. Therefore, the ratio of IL-13Rα1 to IL-13Rα2 expression, and the abundance of IL-13 are critical factors that regulate downstream signaling pathways. IL-13Rα2 is abundantly expressed in the majority of MGs, and its expression level correlates with malignancy grade [38], tumor migration, an aggressive mesenchymal gene expression signature, and a poorer patient prognosis [39]. Since IL-13Rα2 is expressed in tumor cells but not in normal tissue (except for testis), this receptor could be a target for glioma immunotherapy [40] [41].

IL-13-zetakine is a chimeric immunoreceptor using membrane-tethered IL-13 E13Y mutein for selective binding to IL-13Rα2. IL-13-zetakine T cells induced T cell proliferation and secretion of IFN-γ and TNF-α, and demonstrated antitumor activity when cocultured with IL-13Rα2-expressing GBM cells *in vitro*. Moreover, IL-13-zetakine T cells showed prolonged survival of mice bearing human glioma xenografts [42]. In clinical studies, IL-13-zetakine was importantly used in the first human pilot trial to evaluate CAR-engineered autologous T cells for the treatment of recurrent GBM. The study demonstrated the feasibility and safety of using IL-13-zetakine-CAR T cells, as a transient antitumor response was observed in two out of three patients who received these cells in the resection cavity [43]. Recently, second-generation CAR targeting IL-13Rα2 (IL-13Rα□-CAR)-transduced T cells were shown higher anti-tumor effect than IL-13-zetakine [24], and HER2/IL-13Rα2-bispecific CAR in single T cells were demonstrated the improvement of tumor control owing to offset antigen escape *in vivo* [44]. Subsequently, clinical trial using second-generation IL-13Rα2-CAR transduced T cells is ongoing (NCT02208362).

However, the issues are that as IL-13Rα2 is predominantly located intracellularly, and that the antibody, B-D13 mAb, which is commonly based in anti-IL-13Rα2 CAR-T cells, seems to also recognize VCAM-1[45]. Therefore, reinvestigation of cell surface expression of IL-13Rα2 would be required.

EphA2 (Erythropoietin-producing hepatocellular carcinoma A2)

EphA2 is a member of the Eph family of receptor tyrosine kinases, and primarily binds to the glycosylphosphatidylinositol-anchored ephrin-A ligand. The majority of GBMs exhibit amplification or overexpression of ephrin (~90%). Overexpression of EphA2 is found in many different cancers, including MGs [46]. Its overexpression in GBM correlates with pathological grade, poor prognosis, neovascularization [47], and tumorigenesis. EphA2 has alternative functions, as it exhibits ligand-dependent inhibition and ligand-independent promotion of cell migration and invasion. The Akt kinase can phosphorylate EphA2 at Ser-897 and promotes cell migration in a ligand-independent manner; conversely, EphA2 stimulation by Ephirin-A1 ligand negatively regulates Akt activation and inhibits cell polarization and migration. However,

phosphorylation of EphA2 at Ser-897 is also regulated by ERK-RSK signaling, which regulates malignant phenotypes such as migration and invasion [48, 49]. These data suggest that EphA2 is a potential target for glioma immune therapy.

A novel CAR specific for EphA2 has also been developed, and exhibit potent antitumor activity [25]. Specifically, EphA2-CAR-transduced T cells induced the secretion of IFN-γ and IL-2 in an antigen-dependent manner when co-cultured with EphA2-positive target cells. Together with the finding that EphA2-CAR-T cells can disrupt neurosphere formation, this implies that glioma-initiating cells are sensitive to CAR-T cell therapy. Furthermore, EphA2-CAR-T cells induce the regression of established xenografts *in vivo*. Based on this evidence, a phase I/II clinical trial of EphA2-CAR-T cell therapy for EphA2-positive MGs was initiated in China in 2015 (NCT02575261).

CAR-NK cell therapy

In contrast to many preclinical and clinical studies of CAR-T therapy, reports related to CAR-transduced NK cells are relatively limited. However, these cells have several properties that are beneficial in the antitumor response, and thus they have received increasing attention potential CAR effectors. Importantly, NK cells contribute to the graft-versus-leukemia/tumor (GvL/GvT) effect, but are not responsible for GvHD [50]. Although the antitumor effects of CAR-T cells are dependent on their persistence in patients, the risks, such as the off-tumor/on-target toxicity against normal tissue, would also increase as the persistence increases.

CAR-NK cells have two activities that trigger target cell death. These involve a CAR-specific mechanism and a spontaneous cytotoxic effect in a tumor associated-antigen (TAA)-independent manner that is mediated by specific natural cytotoxicity receptors (NCRs). Additionally, NK cells have IgG Fc receptors. These receptors recognize and bind to the Fc fragment of antibodies, and induce antibody-dependent cell-mediated cytotoxicity (ADCC). These multiple activities are significantly different from those of CAR-T cells, since the latter cell type induces tumor cell death via a CAR-specific mechanism.

However, there are obstacles to obtaining a sufficient number of autologous NK cells from patients, as they represent only 10% of the lymphocyte population, and are often dysfunctional, and autologous NK cells disappear from circulation relatively rapidly. Moreover, the transduction efficiency of NK cells is lower than that of T cells, even when viral methods are used.

Allogeneic NK cells may be able to overcome the disadvantages associated with these blood-derived NK cells. Few clonal NK cell lines have been established from patients with NK cell lymphoma in the past 20 years. Indeed, NK-92 is the only cell line that has consistent and highly cytotoxic effects on targets, and that can be easily transfected with constructs expressing CAR. Although NK-92 cells should be irradiated when they are applied for clinical applications to prevent permanent engraftment, CAR modified NK-92 cells have undergone preclinical studies and clinical trials for several cancers [51]. These studies suggest that NK cell lines, and in particular NK-92, have potential as 'off-the-shelf' products for treatment of various cancers, including MGs, and that they will not be associated with GvHD. In MGs, NK-92 cells exhibited potent and specific activity against ErbB2-positive GBM in preclinical models, and were associated with induction of prospective endogenous antitumor immunity following therapy with CAR-NK cells targeting ErbB2 [52]. Additionally, CAR-NK cells targeting both wild type EGFR and EGFRvIII has been demonstrated in GBM using primary NK cells and NK cell lines, including NK-92 [53]. EGFR-targeting CAR-NK cells enhanced antitumor activity and secretion of IFN-γ when co-cultured with EGFR-expressing GBM cells. Furthermore, administration of EGFR-targeting CAR-NK-92 cells suppressed tumor growth and prolonged survival of GBM xenograft mice. Consequently, clinical studies with CAR-NK cells targeting CD19 are ongoing (NCT00995137, NCT01974479).

Mechanisms of glioma-mediated immune evasion and immune modulators

In the cancer microenvironment (including that associated with GBM), a plethora of mechanisms facilitate evasion of the host's immune system. These mechanisms can be

divided into the following two types: immune evasion originating from glioma cells themselves, and activation of the host's intrinsic immune evasion system (Figure 2).

In the former mechanism, glioma cells secrete a variety of immune inhibitory cytokines (e.g., IL-10, transforming growth factor-β or TGF-β) and immunomodulatory factors (e.g., prostaglandin E2, PGE2), which suppress the activation of T cells, inhibit T cell proliferation, and mediate the induction of regulatory T cells (Tregs). The most prototypical T cell-inhibiting molecule is programmed death-ligand 1 (PD-L1; B7-H1), which is a cell surface protein of the B7 family and a ligand for PD-1. PD-L1 expression is observed in 88.0% of newly diagnosed GBM specimens and in 72.2% of recurrent GBM specimens while being undetectable in normal tissue, and is more highly expressed in the mesenchymal subtype of GBM [54]. Although a correlation between PD-L1 and survival has been demonstrated in several cancers, this has been controversial in GBM [54], [55]. PD-L1 blocks T cell activation because it suppresses production of cytokines such as IFN-γ and IL-2 and thereby prevents tumor-specific T cells from inducing apoptosis.

Antibodies against cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed cell death protein 1 (PD-1) co-inhibitory molecules found on activated T cells and Tregs, and the study of their inhibitors has resulted in significant progress in the field of cancer immunotherapy.

CTLA-4 is the receptor for the CD80 and CD86 ligands, a property that it shares with CD28. Characteristically, CTLA-4 translocates to the cell surface where it binds its ligands only once the immunological synapse between TCR and MHC/peptide complex is formed; in contrast CD28 is constitutively expressed at the cell surface [56]. Since CTLA-4 has greater affinity for the same ligands, it can compete with CD28 for ligand binding in spite of its low expression in many cell types. CTLA-4 thus plays a pivotal role in terminating T cell activation. On the other hand, Tregs constitutively express CTLA-4 on their surface, and suppress dendritic cells (DCs) upon binding to CD80 and CD86 [57]. Anti-CTLA-4 mAbs can act as 'immune modulators', which interfere with CTLA-4 ligation and prevent the immunosuppressive function of Tregs in a tumor context. The development of an anti-CTLA-4 mAb, ipilimumab, revealed that an immune checkpoint inhibitor can improve survival in patients with advanced cancer. On the basis of the results, ipilimumab was approved by the Food and Drug Administration (FDA) in 2011 for advanced melanomas [2], and has shown efficacy against brain metastases

originating from melanomas [58]. Remarkably, the survival plot derived from patients in these studies plateaus at approximately two years. This implies that immune checkpoint inhibitors generate immunological memory that contributes to their prevention of tumor recurrence and to their ability to block development of therapy-resistant malignant cancer clones [59]. The efficacy of anti-CTLA4 mAbs for the treatment of glioma has been demonstrated in several studies [60]. Moreover, a glioma vaccine in combination with CTLA-4 blockage resulted in significantly improved survival in mice with established intracranial gliomas [61].

A phase I trial testing the safety of combination therapy of anti-PD-1 mAb (nivolumab) and anti-CTLA-4 mAb (ipilimumab) in patients with recurrent GBM is ongoing (NCT02017717).

PD-1 is a negative mediator of the immune response, and therefore its activity triggers an inhibitory checkpoint in terms of tumor surveillance. PD-1 induction leads to suppression of IFN-γ and IL-2 secretion. Additionally, the interaction of PD-1 with its ligand, PD-L1, leads to anergy and apoptosis of antigen-specific lymphocytes. Furthermore, PD-1 expression down-regulates immunoglobulin production in B cells, reduces cytotoxicity of NK cells, and leads to improper activation of macrophages [62]. Pembrolizumab and nivolumab are humanized mAbs that bind to the PD-1 receptor and block its interaction with PD-L1. The FDA approved both agents for patients with advanced melanoma in 2014, based on clinical trials that demonstrated their safety, activity, and clinical advantages. Currently, these anti-PD-1 mAbs are at the head of clinical oncology, because pembrolizumab demonstrated a longer median PFS and OS for patients with advanced melanoma than did ipilimumab [63]. Nivolumab was subsequently approved for treatment of non-small cell lung cancer (NSCLC) in 2015. Additional investigations are ongoing to expand the application to other malignancies. Several clinical trials are enrolling patients with GBM. At the ASCO 2015, the primary results of a randomized phase III trial comparing nivolumab with bevacizumab in combination with ipilimumab in GBM patients showed that nivolumab was safe and well tolerated. Moreover, a phase III trial comparing nivolumab and temozolomide (TMZ) with radiation therapy for newly diagnosed GBM (NCT02617589), a phase II trial testing durvalumab, an anti-PD-L1 mAb (NCT02336165), a phase II study of pembrolizumab with or without bevacizumab for recurrent GBM (NCT021337491), and a phase I trial of nivolumab with or without a DC vaccine are underway (NCT02529072).

As a note of caution, immune checkpoint inhibitors, in some cases, have been associated with immune-related adverse events. Toxicities due to immune checkpoint inhibitors have been reported in the skin (rush and pruritus), gastrointestinal tract (diarrhea and colitis), liver (hepatotoxicity), endocrine system (hypophysitis and hypothyroidism), and other organ systems [64]. The incidence of grade 3/4 adverse events is generally higher with inhibitors of CTLA-4 when compared with agents that modulate other immune checkpoints.

Aside from immune checkpoint inhibitors, other agents that modulate the immune system of the cancer microenvironment are in development. These include agonist mAbs directed against 4-1BB, OX40, and glucocorticoid-induced TNFR-related protein (GITR), all of which are co-stimulatory molecules involved in T cell activation. Besides these mAbs, we focused on lenalidomide, which is a synthetic derivative of thalidomide and has a broad range of immunomodulatory abilities. Lenalidomide inhibits the production of pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, and IL-12, and elevates the production of IL-10 [65]. This agent has the capacity to down-regulate the expression of immune checkpoint modulators, such as CTLA-4 and PD-1, on T cells [66].

In addition, lenalidomide inhibits the proliferation and suppresses the function of Tregs and MDSCs [67]. The immunomodulatory activity of lenalidomide makes it a potential candidate for use in combination with ACT. We demonstrated that lenalidomide enhances the antitumor activity of EGFRvIII-targeting CAR (3C10-CAR)-T cell therapy in mice bearing EGFRvIII-expressing GBM xenografts. Furthermore, we have found that lenalidomide enhanced immune synapse formation between 3C10-CAR-T cells and GBM cells expressing EGFRvIII [68]. Although immune checkpoint inhibitors are a promising approach for treating malignancies, lenalidomide has the potential to augment other immunotherapies, including ACT.

Future perspective

Adoptive immunotherapies for MGs using genetically modified T cells have shown great potential and impressive efficacy *in vitro*, and a first-in-human pilot study reported their

safety and feasibility [43]. Moreover, clinical trials of CAR-T cell therapy for other solid cancers are ongoing. Representative therapeutic targets for the treatment of metastatic adenocarcinoma and neuroblastoma are CEA and GD2, respectively (NCT01723306, 21984804). The recent development of ACTs, especially CAR-T cell therapy, is based on concepts such as ‘activating (accelerating) immune cells’, ’blocking multiple immunologic brakes’, and ‘minimizing off-tumor/on-target toxicity’. While effective in hematologic malignancies, the use of CAR-T cell therapy for treatment of solid tumors has many limitations, mainly because of the heterogeneity of these tumors, the lack of tumor-specific antigens, and the immunosuppressive microenvironment. However, several countermeasures have been implemented to improve the efficacy of CAR-T cell therapy in solid malignancies.

A reasonable approach is combining ACT with existing therapies. For example, TMZ does not inhibit, but rather enhances antitumor immunity. This is since TMZ increases tumor-specific immune responses via the cross-priming of apoptotic tumor cell death and the suppression of Tregs [69]. Radiation also enhances antigen presentation to the immune system by increasing the expression of MHC-I, Fas, intracellular adhesion molecule 1 (ICAM-1), and natural killer group 2 member D (NKG2D) ligand on GBM cells [70]. The synergistic effect of immune checkpoint inhibitors and CAR-T cells is a promising method for augmenting ACT [71]. The first trial of this method is currently underway and will evaluate the efficacy of ipilimumab combined with CD19-CAR-T cells against B cell non-Hodgkin lymphoma, acute lymphocytic leukemia, and chronic lymphocytic leukemia (NCT00586391). Furthermore, CAR-T cells engineered to secrete anti-PD-1 antibodies have recently been developed; these cells induced regression of renal cell carcinoma in an *in vivo* model [72]. These innovations provide renewed potential for CAR-T cell immunotherapy to successfully treat solid cancers.

Immunotherapies become ineffective once tumors develop ‘escape variants’ that lack the original target antigens; this happens because of the high mutation rate in GBM. Therefore, it is important to find new suitable antigens that are overexpressed on cancer cells and minimally expressed on normal cells in order to minimize off-tumor/on-target toxicity. In this regard, tumor-specific carbohydrates and glycolipids are reasonable novel candidate targets, because CARs (unlike classical TCRs) can recognize structures other than protein epitopes [73]. Moreover, a new approach to antigen discovery has focused on the recognition of somatic mutations present in tumor

antigens. Indeed, mutant peptides have been demonstrated to serve as T cell epitopes [74], and tumor epitopes identified by using whole-exome sequencing analysis with mass spectrometry have revealed immunogenic mutant peptides [75]. These methods may thus be vital for finding new TAAs in the future.

The functional and structural improvements of CAR have occurred rapidly. Bispecific CAR-T cells were produced by modifying individual T cells to express two distinct CAR molecules, in order to offset antigen escape and enhance activity of the cells. For example, bispecific CAR-T cells targeting HER2 and IL-13R□2 have enhanced functionality against GBM cells, and provide increased tumor control *in vivo* [44]. Tandem CAR (TanCAR), in which a single transgenic receptor has a tandem element designed to recognize two distinct antigens, kills only dual-antigen-expressing tumors [76]. On the other hand, bispecific CARs, which include a second binding domain on the T cell that can lead to either an inhibitory or amplifying signal (iCAR), can increase the specificity for cancer cells and provide a dynamic self-regulating safety switch to prevent the consequences of inadequate T cell specificity [77]. However, these methods are associated with manufacturing difficulties, and the behavior of these CARs is extremely dependent on the delicate balance of different signaling receptor chains.

A promising new strategy, named synthetic Notch (synNotch) AND-gated CAR, has recently been reported [78]. This approach exploits combinatorial antigen recognition T cell circuits, in which a synNotch receptor for one antigen drives the inducible expression of a CAR for a second antigen. These dual receptor AND-gated T cells are only activated in the presence of tumor cells that express both antigens. The synNotch receptor first recognizes a tumor-localized antigen, thereby driving the expression of a CAR that recognizes a second tumor antigen. The molecular mechanism is independent from CAR/TCR signaling, because the synNotch receptor does not directly trigger T cell activation. Therefore, it simply results in the priming response of inducing CAR expression. The AND-gated T cells demonstrated robust therapeutic discrimination *in vivo*, and overcame the problem off-tumor/on-target cross reaction in normal tissue.

Finally, another possible strategy is to create universal donor CAR-T cells. Human T cells in which HLA class I has been genetically deleted in order to evade the immune response provides a source of cells from a single donor can be administered to multiple recipients [79]. In addition, antigen-specific T cell-derived induced pluripotent stem cells

(iPSCs) have been generated; the iPSCs can be re-differentiated into CD8+ T cells that have antigen-specific killing activity. These cells also have the same pattern of TCR gene arrangement as the original T cells from which they were derived [80], [81]. Together, these strategies have potential to fundamentally transform the ACT field.

In the next 5 to 10 years, the essential requisites for genetically modified T cell therapy to become a standard cancer treatment are not only the structural and functional sophistication of CAR itself as described above but also the establishment of a robust manufacturing process that can produce reliable genetically modified T cells. With this in place, commercial success and subsequent cost reduction efforts will be possible. Furthermore, the production of off-the-shelf and mass-produced genetically modified T cells will be a powerful addition to the ACT toolbox. Confidence that such improvements will become reality in the near future is high, as dramatic progress in ACT research (and in particular genetically modified T cell therapy) has been made by many research groups.

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Figure legends

Figure 1

Strategy of engineering CAR T cells and their efficiency in treating both human glioblastoma (GBM) patients and mice bearing GBM xenografts.

Figure 2

Interactions of immune players with glioma cell and genetically modified T cells.

Executive summary

TCR gene therapy for GBMs

- Genetically engineered TCR can recognize all tumor-associated antigens, regardless of whether the antigens are on the membrane or in cells.
- Short-interfering TCR (siTCR) gene-transduced T cells have the potential to be used for allogeneic cell therapy as an ‘off-the-shelf’ product.

CAR-T cell therapy for GBMs

- CAR-T cells can recognize target antigens with high sensitivity and specificity without the need for MHC-restricted presentation.
- EGFRvIII is a promising target antigen for CAR-T cell therapy because it is not expressed in normal tissues.
- A phase I clinical trial of humanized EGFRvIII-targeting CAR-T cell therapy for recurrent GBMs is underway.
- HER2-CAR-T cells or HER2 CMV-bispecific CAR-T cells demonstrated the clinical benefit and safety.
- Several reports have been shown off-tumor/on-target toxicity associated with CRS after HER2-CAR-T cells infusion because HER2 is expressed at low levels in a variety of normal tissues.
- IL-13Rα2 is the most authentic CAR targeting glioma-associated antigen, because it was used for the first human pilot trial evaluating CAR-engineered autologous T cells for the treatment of recurrent GBM and has provided valuable proof-of-concept.
- Use of EphA2-targeting CAR-T cells demonstrated that glioma-initiating cells are susceptible to CAR-T cell therapy.

CAR-NK cell therapy for GBMs

- CAR-NK cells have dual activity for killing the targets: a CAR-specific mechanism and a spontaneous cytotoxic effect that occurs in a tumor associated-antigen (TAA)-independent manner via specific natural cytotoxicity receptors (NCRs).
- CAR-NK cell therapies using NK cell lines such as NK-92 have potential as ‘off-the-shelf’ products that will not elicit GvHD, and can be used in various cancers, including MGs.

- Multimodal therapy combinations that include CAR-T cells will be a promising future approach.

Immune modulators

- GBMs evade the immune system by two mechanisms. First, as they originate from glioma cells themselves, they secrete immune inhibitory cytokines and immune modulatory factors. Second, they can modulate the host's intrinsic immune evasion system (Tregs, MDSCs, TAMs).
- Immune checkpoint inhibitors such as anti-PD-1 mAb (nivolumab) and anti-CTLA-4 mAb (ipilimumab) represent a promising approach for treating GBMs, especially when combined with other immune therapies.
- Lenalidomide, which has a broad range of immunomodulatory abilities, has the potential to argument other immune therapies.

Future perspective

- TMZ does not inhibit, but enhances antitumor immunity through increased tumor-specific immune responses.
- Radiation enhances antigen presentation to the immune system.
- The synergistic effect of immune checkpoint inhibitors and CAR T cells is a promising method for augmenting ACT.
- The discovery of novel antigens that are overexpressed on cancer cells and minimally expressed on normal cells will be critical to minimize off-tumor/on-target toxicity.
- Identification of tumor epitopes using whole-exome sequencing analysis with mass spectrometry will be a useful approach for finding new TAAs in the future.
- The structural improvement of CAR (TanCAR, iCAR, and AND-gated CAR) has yielded promising strategies to enhance antitumor efficacy and to overcome the problem of the off-tumor/on-target toxicity.
- Antigen-specific T cell-derived induced pluripotent stem cells (iPSCs) have potential to revolutionize the ACT field.