Cell-based Immunotherapy Against Gliomas: From Bench to Bedside

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Glioblastoma (GBM) comprises 51% of all gliomas and is the most malignant form of brain tumors with a median survival of 18–21 months. Standard-of-care treatment includes maximal surgical resection of the tumor mass in combination with radiation and chemotherapy. However, as the poor survival rate indicates, these treatments have not been effective in preventing disease progression. Cellular immunotherapy is currently being explored as therapeutic approach to treat malignant brain tumors. In this review, we discuss advances in active, passive, and vaccine-based immunotherapeutic strategies for gliomas both at the bench and in the clinic.

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INTRODUCTION

Gliomas account for about 60% of all primary central nervous system tumors. Glioblastoma (GBM), which comprises 51.2% of all gliomas, is the most malignant form with a 2-year survival rate of 40% and a median survival of 18–21 months.[1,2] The current standard-of-care includes surgical debulking of the tumor mass followed by radiation and chemotherapy using temozolomide.[3] However, as indicated by the poor survival rate, these treatments have not been effective in preventing disease progression. Complete surgical resection of a tumor mass is nearly impossible due to the location and invasive properties of malignant gliomas. High doses of radiation therapy cannot be delivered due to potential damage to the normal brain. Likewise, chemotherapeutics often cannot penetrate the blood–brain barrier efficiently; and the resistances that gliomas are known to develop further compound the issues involved with these treatments.[4–8]

Cellular therapy is based on the idea of introducing a specific cell type into a particular tissue to treat the disease. Its earliest applications can be dated back to the 1950's where it was used in the bone marrow transplantation field.[9,10] Currently, a broader spectrum for the application of cellular therapy is being pursued. Different cell types are used in replacement therapies to take over the function of diseased cells in the target organ. This is exhibited in cases of diabetes where insulin-producing cells can be injected to replace the malfunctioning diseased cells in the pancreas.[11,12] Tissue engineering, in which *ex vivo* whole organs are recreated out of cells, is in early phases of development but holds a tremendous potential for the future.[13] One example that has reached the clinic is the seeding of artificial skin, grown from collagen scaffolds, with the patient's own epidermal skin cells.[14] This technique is Food and Drug Administration-approved and has been shown to drastically improve the life of patients with burn injuries. While both replacement therapy and tissue engineering focus on the use of cells for their inherent function (e.g., myoblasts for the generation of muscle tissue), other research is focusing on the application of cells for tasks outside of their pre-programmed function. For instance, stem cells and immune cells can be used for immunotherapy and as carriers of therapeutic genes or prodrugs that get activated at a specific location in the body. This cell-based therapy provides a new and interesting strategy for the treatment of cancers including brain tumors. Cellular immunotherapy in particular has the potential to both specifically target brain tumor cells, thereby limiting brain damage, and to establish a long-term antitumor response by stimulating the immune system. Thus, cellular immunotherapy is being explored as a new alternative therapy for gliomas. Currently, a wide range of strategies are being investigated at the bench, with a slow but steady portion of this research getting transitioned to the clinic. In this review, we cover recent advances in the field of immuno-cellular therapy for malignant gliomas both in the early experimental phase, as well as in the clinical setting.

EXPERIMENTAL IMMUNO-CELL THERAPY

Over the last decade, extensive studies have been performed evaluating the use of modified immune cells as a potential therapeutic approach for gliomas. *In vivo* glioma xenografts of intracranial or subcutaneously implanted cells, as well as spontaneously induced gliomas, are widely used and commonly accepted models that depict an accurate and reproducible tumor environment in rodents. Histopathological changes such as pseudopalisade necrosis, glomeruloid vascular hyperplasia, and infiltrating cells mimic those found in human gliomas.[15] In this section, we discuss experimental approaches using a variety of cells to boost the immune

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system and to establish a potent immune response against malignant brain tumors.

Overview of immuno-cell therapy

Cellular immunotherapy is based on the use of cells from the innate and adaptive immune system to elicit an antitumor response. Either passive or active immunotherapy can be pursued. Passive immunotherapy involves the *ex vivo* activation of immune cells, which are subsequently injected back into the patient to attack the tumor directly. Often, these cells are not only activated, but also genetically modified to have enhanced antitumor properties. This approach has proven to be successful, but has the limitation of lacking prolonged or continuous antitumor response. Recent studies are focused towards active immunotherapy or a combination of both. Active immunotherapy relies on the activation of the endogenous immune system either by vaccines or *ex vivo*-activated cells. This approach elicits long-term antitumor effects, which not only enhance the likelihood of the tumor being eradicated, but also decrease the risk of tumor recurrence. T cells, dendritic cells (DCs), and macrophages are the cells of choice for this therapeutic strategy (**Table 1**; **Box 1** and **Box 2**).

T cell-based immunotherapy

T cells are often used as cellular vehicles for the treatment of different types of cancers typically resistant to conventional therapy. These cells can be easily modified with tumor-specific antigens to target the tumor. An interesting strategy in T cell-based immunotherapy is the use of chimeric antigen receptors (CARs). CARs combine the antigen-binding domain of antibodies with the ζ chain of T cell receptors, creating an increased binding between the T cell and the tumor antigen. In a proof-of-concept study, Wang *et al.*[16] showed that $CD8^+$ T cells can become tumor-specific when directed towards a tumor epitope such as the human epidermal growth factor receptor 2 (HER2), overexpressed in tumors versus normal tissues. Genetically engineered T cells expressing an anti-HER2 chimeric receptor were intravenously transferred in combination with lymphoablation and interleukin-2 (IL-2). The autoimmune effect had no significant toxicity on normal mammary and brain tissues expressing HER2, whereas an antitumor effect could be demonstrated, indicating that the modified T cells were specific to the tumor. Nakazawa *et al.*[17] similarly combined HER2 and CARs to modify a T cell population, creating a functional HER2-CAR. Although HER2 is overexpressed by many different tumors, including malignant gliomas, the expression is often too low to be recognized by T cells. Intratumoral injection of CARs demonstrated efficient killing of tumor cells *in vivo*, including the CD133-positive glioma stem-like cells (GSC; also known as tumor-initiating cells resistant to chemo- and radiotherapy), and resulted in increased survival rate.[18] The challenge of this therapeutic strategy is to establish stable expression of the activated antigen while achieving enough expansion with nonspecific activated T cells (known to be intolerant to transfection) as well as a persistent antitumor effect. To overcome these limitations, the same group used Epstein-Barr virus (EBV) to stimulate T cells (EBV-cytotoxic T lymphocytes (CTLs)), a method known to elicit an enhanced immune response.[19] EBV-CTLs outperformed the activated T cells in both expansion and antitumor persistence.[20–22] Further, the nonviral piggybac (PB) transposon system was evaluated for HER2-CAR gene delivery to T cells. While this model has been successfully applied in mouse primary cells, human cell lines, and inducible pluripotent stem cells, PB has not been evaluated for *in vivo* immunotherapeutic models. No preferential integration near or into oncogenes was observed, as typically is the case with retrovirus and lentivirus-based transduction methods. PB gene transfer was highly effective and the transduced cells could be maintained for >100 days in culture, while retaining stable transgene expression and T cell properties. Since transposons are less expensive and easier to produce than viral vectors, this method provides an interesting new approach for gene delivery.

DC and macrophage-based therapy

DCs are the most potent antigen-presenting cells of the human body, sensitizing T cells to all acquired antigens. In contrast to activation of T cells *ex vivo*, activation and stimulation of DCs induces a long-term immune response and is therefore considered active immunotherapy. DCs can be loaded with tumor antigens *ex vivo*, which can subsequently activate the endogenous immune system upon injection. Although this technique is safe, clinical efficiency still needs to be improved to achieve high expression of the antitumor antigen at the major histocompatibility complex (MHC) on the DC surface, and to expand the subgroup of these cells that primes naive T cells. Saka *et al.*[23]

Table 1 Overview of experimental immuno-cell therapy against gliomas

Cell type	Transgene/ modification strategy	Application	References
T cells	Anti-HER2 receptor	Antiglioma immunotherapy, evaluation of associated autoimmune pathology	16
	HER2-CAR	Antiglioma immunotherapy; evaluation of enhanced CAR-mediated tumor cell recognition	17
	IL13Ra2	Anti-GSC immunotherapy	35
Dendritic cells (DCs)	IL13Ra2	Anti-GSC immunotherapy; more efficient expression of antigens at MHC level	23
	Ad-Flt3L/Ad-TK	Antiglioma viral therapy; more efficient delivery and enhanced viral distribution at the tumor site	24
	CSC antigen load	Antiglioma immunotherapy; enhanced antitumor response	27
Macrophages	NS (Nanoshell) load	Photothermal-mediated glioma therapy; proof-of-concept of macrophages as delivery vehicles for NS	29

Abbreviations: Ad-Flt3L/Ad-TK, adenovirus expressing Flt3L/TK; CAR, chimeric antigen receptor; CSC, cancer stem cell; GSC, glioma stem-like cell; HER2, human epidermal growth factor receptor 2; IL13Ra2, interleukin-13 zetakine 2; MHC, major histocompatibility complex.

Box 1 Cells used for immune-cellular therapy

T cells or T lymphocytes are part of the WBC compartment and play an important role in the cell-mediated immunity. Hallmark of these cells is expression of the T cell receptor on their surface. Several subtypes of T cells do exist, all with a different role in the adaptive immune response. CD4 lymphocytes, or T helper lymphocytes are the mediators of the immune system. Once activated by encounter of antigen-presenting cells (APCs) expressing antigens in the major histocompatibility complex (MHC) class II, they start secreting cytokines that in turn activate cytotoxic T lymphocytes (CTL) and macrophages, helping differentiation of B cells into plasma cells, initiating a humoral immune response. CTLs, or CD8 T lymphocytes are responsible for direct cell-mediated killing. They recognize their target by binding to antigens expressed by MHC class I complex, found on the surface of virtually every cell in the body. Their main targets are cells infected with virus, transplants, and tumor cells. The last group of T lymphocytes are the natural killer T cells. These cells are very similar to natural killer cells of the innate immune system. Their job is to recognize glycolipid antigen expressed by CD1d. Once activated, they can differentiate into either T helper lymphocytes or CTL, initiating both a cytokine-mediated and direct cytolytic immune response

Macrophages play an important role in the immune response. They can be recognized by surface expression of CD14, CD40, CD11b, lysozyme M, Mac1/3, and CD68. They originate from monocytes, which, once activated through local inflammatory factors, starts differentiating. Macrophages play a role in both innate and adaptive immune system, in which they have three distinct roles. They phagocytose pathogens and cellular debris, cleaning the inflammation site; these cells present the digested pathogens in MHC class II, thereby stimulating CD4 lymphocytes, and they secrete various local monokines and interleukins, creating a strong chemotactic environment for T cells.

Dendritic cells (DCs) function as APCs, just like macrophages. Their hallmark is expression of the toll-like receptor. They are present in skin, respiratory, and gastrointestinal tract, patrolling all tissues in contact with the external environment. Once a pathogen is encountered, migration towards the lymph nodes occurs, where they present their pathogen to B and T lymphocytes. DCs are the only APCs capable of presenting antigens both through the MHC class I and class II pathways, thereby stimulating B cells, CD4 T lymphocytes, and CD8 lymphocytes. In addition, these cells are capable of secreting cytokines that further enhance differentiation of surrounding immune cells.

Box 2 Cell isolation and preparation for immunotherapy

T lymphocytes can be obtained from several sources, including thymus, lymphnodes, speen, and peripheral blood, the latter being the most accessible. Cells are separated from the whole blood samples by Ficoll-Isopaque–based density gradient separation. Since lymphocytes are less dense than erythrocytes, they can be easily extracted after centrifugation. Distinction between B and T lymphocytes can then be made based on differences in growth patterns, with T lymphocytes forming rosettes in the presence of sheep erythrocytes and B lymphocytes being non-rosette forming. Nylon fiber column separation is an alternative approach, allowing to specifically select for adherent T lymphocytes. Several commercial kits are available to specifically purify T lymphocyte subtypes (CD4, CD8, natural killer T cell) based on monoclonal antibody reactions. To generate cytotoxic T lymphocytes (CTLs) against specific antigens (for instance expressed on tumor cell surface), the CTLs can be cultured in the presence of antigen-presenting cells loaded with the desired antigen. Cells are cultured in basal medium containing RPMI 1640, 10% fetal calf serum, penicillin/streptomycin, L-glutamate, phytohemagglutinin, and a buffer solution. New studies are focusing on the development of serum-free medium, in order to standardize T cell populations and eliminate confounders.

Macrophages can be isolated from various tissues. One strategy involves isolation of these cells from peripheral blood. Blood-derived macrophages are isolated based on the very same Ficoll gradient centrifugation protocol described for T lymphocytes. Antibody-based cell separation kits selecting the CD14 monocyte fraction are available. Subsequent culture of these cells in the presence of macrophage colony-stimulating factor-1 (M-CSF-1) will result in macrophage differentiation. The same RPMI culture media is used as described for T lymphocytes.

Dendritic cells (DCs) can be generated through various protocols. One technique involves DCs separation from the whole blood samples by Ficoll gradient centrifugation. B lymphocytes and monocytes are then subtracted from the cell suspension using monoclonal antibodies directed towards CD19 and CD14. DCs are then isolated from the remaining (B and monocyte depleted) mixture by CD304-, CD141-, and CD1c-directed antibodies. Selected cells are cultured in RPMI media containing M-CSF-1 yielding to DCs differentiation.

designed a DC vaccine-based strategy aimed at targeting the IL-13 zetakine (IL13Ra2), which is overexpressed in gliomas. Since proper expression of this antitumor antigen at the DC MHC was problematic, a late endosomal/lysosomal sorting signal was added to the IL13Ra2 plasmid. DCs were transduced with this plasmid and injected intraperitoneally in glioma-bearing mice on days 3 and 10 of post-tumor implantation. A significant increase in the number of $CD4^+$ and $CD8^+$ T lymphocytes in the IL13Ra2-DC–treated tumor environment was observed, resulting in an increased survival rate.[23] In a model in which gene therapy is effective but DC vaccination is not effective, Mineharu *et al.*[24] demonstrated that combining *in situ* Ad-Flt3L/Ad-TK–mediated gene therapy (an Food and Drug Administration-approved adenoviral vector expressing either fms-like tyrosine kinase 3 ligand or thymidine kinase) with DC vaccination increased therapeutic efficacy and antitumor immunity as compared with *in situ* Ad-Flt3L/Ad-TK–mediated gene therapy alone. Ad-Flt3L and Ad-TK were intratumorally injected, followed by systemic administration of the prodrug ganciclovir. Flt3L causes DCs to migrate, differentiate, and expand within the tumor microenvironment of mice and rats. Thymidine kinase at the tumor site converts ganciclovir into a highly toxic phosphorylated drug causing the death of dividing tumor cells. Further, tumors treated with ganciclovir released high mobility group 1 protein, which serves as an adjuvant of the innate immune system by stimulating toll-like receptor 2 in signaling bone marrow-derived DCs thus confirming previous studies.[25,26] The DCs were conditioned *ex vivo* with Fl3t and IL-6 to achieve enhanced proliferation and antitumor effects.[24]

As an alternative of loading DCs with regular tumor antigens, Xu *et al.*[27] explored the use of cancer stem cell (CSC) antigens as a source for DC antiglioma vaccination. CSCs are thought to play an important role in the onset, progression, and recurrence of malignant gliomas and are known to express high levels of MHCs and tumor-associated antigens. A sufficient T cell response against CSCs and an increase in survival of mice bearing 9L gliosarcoma CSC tumors were observed as compared to DCs loaded with daughter or conventionally cultured 9L cells after intradermal injection of the vaccine. Albeit conventional loading and CSC antigen loading of DCs require further comparison in DC maturation and memory T cell generation *in vivo*, the authors speculate that CSC antigens might indeed be more suited for DC loading as compared with conventional tumor antigens. A clinical trial evaluating DC vaccines using CSCs is being considered and will start shortly.[27]

Table 2 Current immuno-cell therapy in clinical trials

Therapy	Cell/vaccine type	Transgene/modification strategy	Application	Phase	Clinicaltrial.gov identifier number	References
Cellular immunotherapy	$CD8^+$ T lymphocytes	Expression of IL-13 zetakine chimeric immunoreceptor, Hy/TK selection/suicide fusion protein	Assessment of the feasibility and safety of *ex vivo* expanded and genetically modified autologous $CD8^+$ T lymphocytes in patients with recurrent or refractory high-grade malignant glioma	Pilot	NCT00730613 (completed)	34,35
	T cells	Expression of EGFRvIII CAR (PG13-139-CD8-CD28BBZ (F10))	Evaluation of the safety and feasibility of administering T cells expressing anti-EGFRvIII chimeric antigen receptor to patients with malignant gliomas expressing EGFRvIII	Phase I/II	NCT01454596	58,59
Vaccine cell therapy	CTL	BTIC antigen load	Evaluation of the feasibility of administering imiquimod/BTIC lysate-based therapy for diffuse intrinsic pontine glioma in children and young adults	Pilot	NCT01400672	60
	Dendritic cells (DCs)	ICT-107	Evaluation of the safety and efficacy of ICT-107 in newly diagnosed patients with GBM following resection and chemoradiation	Phase II	NCT01280552	38
		205-NY-ESO-1 fusion protein	Evaluation of the side effects and best schedule of dendritic vaccine therapy with/without sirolimus in treating patients with NY-ESO-1 expressing solid tumors	Phase I	NCT01522820	39,61
		WT1 protein	Evaluation of the immunogenicity and clinical efficacy of WT1-specific $CD8^+$ T cell antitumor response after intradermal vaccination with autologous WT1 mRNA-transfected DC	Phase I/II	NCT01291420	40,41
		$A2B5^+$ antigen load	Evaluation of the efficacy of vaccination with DCs loaded with glioma stem-like cells-associated antigens against GBM	Phase II	NCT01567202	62
		Autogenic GBM cell lysate	Evaluation of the adverse and therapeutic effects of a postoperative autologous DC tumor vaccine in patients with malignant glioma	Phase I/II	Published	42
			Evaluation of the immunologic response to cervical intranodal vaccination with autologous tumor lysate-loaded DCs in patients with GBM after radiation therapy and TEM	Phase I	Published	43
	IMA 950	TUMAPs: multipeptide vaccine (IMA 950) containing 11 TUMAPs found in a majority of GBMs designed to activate TUMAP-specific T cells	Evaluation of the safety and tolerability of IMA 950 when given with cyclophosphamide, granulocyte-macrophage colony-stimulating factor (GM-CSF) and imiquimod in patients with GBM	Phase I	NCT01403285	63
		TUMAPs	Evaluation of the side effects of IMA 950 vaccine therapy when given together with temozolomide and radiation therapy in treating patients with newly diagnosed GBM	Phase I	NCT01222221	64,65

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Table 2 Continued

Therapy	Cell/vaccine type	Transgene/modification strategy	Application	Phase	Clinicaltrial.gov identifier number	References
	GBM cells	Autologous tumor cells treated with ILGFR1 antisense oligodeoxynucleotide *ex vivo* and re-implanted in diffusion chambers to stimulate the native immune system	Evaluation of the safety of rectus sheath implantation of diffusion chambers encapsulating autologous malignant glioma cells treated with ILGFR1 antisense oligodeoxynucleotide in patients with recurrent GBM	Phase I	NCT01550523	45
Cellular vaccine and immunotherapy combined	TVI-Brain-I glioma cells; killer T cells	• Autologous glioma cells *ex vivo* neutralized to elicit a killer T cell response *in vivo* • Autologous DC-stimulated killer T cell precursors cultured and stimulated *ex vivo* to reach a higher activity level	Evaluation of the safety and efficacy of TVI-Brain-1 as a treatment for recurrent GBM	Phase II	NCT01290692	66
	Glioma vaccine; DC; DCIK	• DCs pulsed with tumor lysateCIK cells activated by • DCs stimulation (DCIKs)	Evaluation of DCIK combined with DC treatment for glioma	Phase I/II	NCT01235845	54
	DCs; CTL	• CMV presenting DCs • Cytotoxic T lymphocytes stimulated by CMV and EBV	Evaluation of the safety and persistence of escalating doses of autologous CMV-specific CTL in patients with CMV-positive GBM	Phase I	NCT01205334	52,67
		• CMV presenting DCs • Cytotoxic T lymphocytes stimulated by CMV and modified to express CARs targeting the HER2 molecule (FRP5.CD28.CAR)	Evaluation of the safety, persistence, and antitumor efficacy of escalating doses of autologous CMV-specific CTL expressing FRP5.CD28.CAR in patients with HER2-positive recurrent GBM	Phase I/II	NCT01109095	18,52

Abbreviations: 06BG, 06-Benzylguanine; BTIC, brain tumor-initiating cell; CAR, chimeric antigen receptor; CMV, cytomegalovirus; CTL, cytotoxic T lymphocyte; DCIK, dendritic cell (DC)-activated cytokine-induced killer cell (CIK); EBV, Epstein-Barr virus; EGFR, epidermal growth factor receptor; GBM, glioblastoma; HER2, human epidermal growth factor receptor 2; IL-13, interleukin-13; ILGFR1, insulin-like growth factor receptor-1; TEM, temozolomide; TUMAP, tumor-associated peptides.

Another antigen-presenting cell used for immuno-cell therapy are macrophages. The advantage of these cells is their ability to easily travel across the blood–brain barrier, which often remains a great limitation for effective brain tumor therapy. Tumor-associated macrophages are often observed in the glioma microenvironment, and intravenously injected macrophages target the brain tumor site.[28] In a recent study, Baek *et al.*,[29] used macrophages loaded with gold-coated nanoshells for the treatment of human multicellular glioma spheroids. This *in vitro* model has similar characteristics in both resistance to radio- and chemotherapy, as well as growth and metabolic rates of glioma tumors *in vivo*, while simulating the tumor before vascularization. Nanoshells are spherical nanoparticles consisting of a dielectric core (called the silica), and an outer layer coated with a thin metallic shell (often made of gold) that converts the absorbed light to heat with great efficiency. Nanoshells are relatively small and can thereby easily be taken up by macrophages. In this study, using the glioma spheroid model, the authors compared macrophages loaded with empty nanoshells to macrophages with gold-coated nanoshells and found that the latter were able to inhibit tumor growth by photothermal therapy, whereas no response was observed with the empty control group.

IMMUNO-CELL THERAPY IN THE CLINIC

Despite an abundance of experimental research, only a small number of clinical trials are currently in progress focusing on the safety, efficacy, and feasibility of immuno-cellular therapeutic approaches in a phase I/II setting. While experimental therapies show a wide variety of strategic approaches, the clinic reflects a somewhat more conservative approach with DC vaccines and modified T lymphocytes dominating the picture (**Table 2**). Recently, two clinical trials showed the potential of immuno-cell therapy for the treatment of cancer. A study led by Professor Carl June at the University of Pennsylvania showed the success of adoptive CAR T cell therapy in treating chronic lymphocytic leukemia (CLL), where two out of three patients saw complete remission after CAR-CD19 therapy and had remained so for >1 year after treatment.[30,31] Another trial led by Drs Renier Brentjens and Michel Sadelain at the Memorial Sloan Kettering Cancer Institute showed that the same CAR strategy can successfully treat patients with CLL and B cell acute lymphoblastic leukemia forms of blood cancer.[32,33] These successful trials lend support to the significance of immuno-cellular therapeutic strategies for treating different tumors such as gliomas.

T cell immunotherapy in the clinic

It is well known that malignant gliomas evade provocation of an immune response. However, immune cells could be of tremendous value in the fight against brain tumors. These cells can provide a very efficient elimination mechanism of the tumor bulk and metastatic/invasive cells without the need to impair patient quality-of-life, as is the case with chemo- and radiotherapeutic paradigms. Many of the current strategies are exploring methods to overcome the lack of an immune response to glioma cells by

artificially stimulating the immune system by either passive or active immunization. The use of T cells is one of the most popular strategies in the clinic, and is often used in combination with DC vaccines. Two clinical trials focused on *ex vivo* stimulation of T cells to boost a passive immune response are currently in progress.

In one trial, by Forsman *et al.* at the City of Hope Medical Center, autologous peripheral blood mononuclear cells are collected and genetically modified to express the membrane-tethered IL-13 cytokine chimeric T cell receptor targeting the IL-13 receptor α2 (IL13Rα2) present in over 80% of malignant gliomas. This IL-13 zetakine has an E13Y mutation, which enhances its specificity for the IL13Rα2 receptor by >50-fold, as compared with the normal IL-13 receptor expressed by healthy brain tissue.[34,35] In addition to the IL-13 zetakine, the CTL were further modified to express the thymidine kinase suicide gene (Hy/TK) under the control of the constitutively active cytomegalovirus (CMV) promoter incase immediate ablation of CTL activity is required. Repeated CTL infusion was performed over 2 weeks (three times/week), followed by an injection every 3 weeks in the absence of disease progression and signs of autoimmunity. Recently, an experimental study from the same group was published discussing the use of the IL-13 zetakine in an orthotopic mouse tumor model.[34,35] The authors showed that IL13Rα2 is expressed by both GSCs and the more differentiated tumor cell population, and that IL13Rα2 zetakine therapy ablates the tumor-initiating activity of IL13Rα2-positive GSCs. At time of writing, the pilot study had been completed though the results had not been published yet.

Rosenberg *et al.*, at the National Institutes of Health Clinical Center, took on a similar approach by genetically modifying peripheral blood lymphocytes to express the anti-EGFRvIII chimeric antigen receptor. As in the case for IL13Rα2, the mutant EGFRvIII receptor is overexpressed in 30–70% of glioblastomas, whereas no expression is seen in the normal brain.[36] After *ex vivo* preparation, the autologous-modified cells would be intravenously injected and safety, feasibility, and progression-free interval would be monitored.

Vaccine therapy in the clinic

With nine clinical trials either in progress or recently completed, vaccine therapy is the most popular clinical immuno-cellular therapeutic approach for malignant gliomas. Vaccine therapy is based on active immunization of the body against glioma, resulting in a permanent and sustained attack on the tumor by the immune system. In five out of the nine trials, autologous DCs are used to stimulate the patient immune system to evoke an antitumor immune response. DCs are the most potent antigen-presenting cells with the capability of presenting antigenic material not only by the MHC II pathway (stimulating $CD4^+$ T lymphocytes), but also by MHC I pathway (stimulating a $CD8^+$ lymphocyte response) through a process called "cross presenting" which results in a diversification of the immune response.[37,38] ImmunoCellular Therapeutics (Woodland Hills, CA) recently initiated a Phase II study using the immunotherapeutic vaccine ICT-107 composed of synthetically purified antitumor antigens corresponding to epitopes found on GBM cells. Autologous DCs are *ex vivo* pulsed with ICT-107 and injected intradermally upon completion of tumor removal and after 6 weeks of temozolomide therapy. An earlier Phase I study demonstrated safety and efficacy of this therapeutic strategy. Earlier this year Odumsi *et al.*, at the Roswell Park Cancer Institute, initiated a large Phase I study evaluating the safety and feasibility of a new vaccine aimed at NY-ESO-1 expressing solid tumors in combination with the mTOR inhibitor sirolimus.[39] Autologous DCs are *ex vivo* pulsed with the 205-NY-ESO-1 fusion protein and intranodally injected. The investigators hope that this strategy will elicit a stronger immune response yielding to enhanced tumor killing. At the same time, Berneman *et al.*, at the University Hospital (Antwerp, Belgium), are evaluating immunogenicity and efficacy of intradermal vaccination with autologous DCs genetically modified to express WT1 protein which is overexpressed in a variety of solid tumors. A previous Phase I study in patients with acute myeloid leukemia demonstrated the vaccine is well tolerated and elicits a $CD8^+$ T lymphocyte response.[40,41] In China, Zhou *et al.*, at the Huashan Hospital, initiated a Phase II study evaluating the overall survival (OS) of patients with primary and/or secondary GBM after treatment with autologous DCs loaded with autogenic GSCs ($A2B5^+$). A study investigating the adverse and therapeutic effect of a postoperative DC-derived tumor vaccine was recently published by Chang *et al.*,[42] in the Journal of Clinical Neuroscience, reporting an increase in the median survival to 525 days and a 5-year survival rate to 18.8% as compared with the historical control group (380 days and 0%). Patients underwent surgery to debulk the tumor mass and the vaccine was prepared using cells from the surgical specimen. Autologous DCs were administered with 10 injections over the course of 6 months. The authors report that 47% of the enrolled patients developed a transient elevation in both alanine aminotransferase and aspartate aminotransferase levels, which correlated with the vaccination schedule and high doses of the DC vaccine. At lower levels of DC vaccine, no increase in serum alanine aminotransferase/aspartate aminotransferase was observed, suggesting a safe upper limit of 2×10^7 DCs/dose.

Another recently completed study by Fadul *et al.*[43] was reported in the Journal of Immunotherapy which focused on the immune response, progression-free survival, and OS of GBM patients treated with an intranodal autologous tumor lysate DC vaccination. CTL tumor-specific activation was measured and correlated with both progression-free survival and OS. All patients survived past 6 months post-diagnosis and a progression-free survival of 9.5 months was reported. Median OS was 28 months, which is significantly higher than the OS of 18–21 months in GBM patients receiving standard therapy.[2]

As an alternative to the standard DC approach, Andrews et al., at the Thomas Jefferson University (Philadelphia, PA), initiated a pilot study evaluating the possibility of stimulating the DC population *in vivo*. *In vivo* stimulation is thought to be more effective and is expected to elicit a stronger and longer immune response as compared with *ex vivo* stimulation.[44] Diffusion chambers containing autologous tumor cells treated *ex vivo* with insulin-like growth factor receptor-1 antisense oligodeoxynucleotide were re-implanted in the rectus sheet to stimulate the native immune system. Loss of insulin-like growth factor receptor-1 is expected to result in apoptosis with subsequent release of tumor antigens containing exosomes (microvesicles), which will allow the diffusion chamber to act as a slow-release antigen depot.[45] Since a wound containing a foreign body is created upon implantation of the diffusion chamber, high levels of DCs are expected to be

present in the immediate surroundings which enhances the efficacy of antitumor activation of the immune system.

A pilot study by Moertel *et al.*, at the Masonic Cancer Center (University of Minnesota) developed a cell-based cancer vaccine composed of glioma stem-like associated antigens found in the brain tumor-initiating cell line GBM6.[46,47] Upon administration, the brain tumor-initiating cell vaccine is thought to stimulate an antitumor CTL response against both GSCs and the more proliferated tumor bulk. Since GSCs have the ability of self-renewal and seem to drive tumor growth and initiation, elimination of this specific group of glioma cells would be of tremendous benefit. Vaccine administration will start following radiation therapy and will be given every 2 weeks for 4 weeks in combination with the drug imiquimod, which acts as an immune response modifier.

Two separate groups are conducting a Phase I study to test the safety and feasibility of IMA 950, which is a therapeutic multipeptide vaccine containing 11 tumor-associated peptides found in a majority of GBMs designed to activate tumor-associated peptide-specific T cells. Rumpling *et al.*, (Cancer Research UK) are testing the vaccine in combination with granulocyte-macrophage colony-stimulating factor, radiation, and chemotherapy (temozolomide) for patients with newly diagnosed gliomas.[48] Sul *et al.* (Immatics Biotechnologies (Tuebingen, Germany) in collaboration with the National Cancer Institute) follow a similar approach to test IMA 950 with granulocyte-macrophage colony-stimulating factor and locally applied imiquimod 20 minutes after each vaccination. Patients will further be treated with one dose of cyclophosphamide before the first vaccination.

Vaccine and cellular therapy combined

Two clinical trials, using a combined approach of vaccines and immunotherapy, are being performed by Ahmed *et al.* at the Baylor College of Medicine. In the first trial, autologous CTLs are stimulated *ex vivo* with human β-herpes CMV presenting DCs. CMV-specific antigens can be detected in 70–90% of malignant glioma cells, but not in the normal brain.[49,50] The CMV-specific CTLs are then cultured in the presence of EBV-infected cells to elicit a stronger immune response upon intravenous administration.[19] The second trial furthers this through the genetic modification of the CMV-specific CTLs to express the CAR targeting HER2, which is associated with 70% of GBM malignancies.[51] Both trials are still in their initial Phase I stage; however, a recently published pilot study by the same group evaluated the use of CMV-specific T cells and demonstrated that autologous T cells could successfully be activated and expanded, are able to recognize the CMV antigens pp65 and IEI, and are capable of killing CMV-infected autologous GBM cells.[52] Concurrently, Wood *et al.* (TVAX Biomedical, Lenexa, KS) are testing in a Phase II trial (supported by positive safety and efficacy studies in a Phase I trial) a brain cancer vaccine called TVI-Brain I that consists of neutralized autologous tumor cells. An immune response of killer T cells is expected upon vaccination, yielding highly effective antitumor activities. Yao *et al.*, at Quindao University, used a somewhat similar approach in a Phase I/II trial combining intranodal DC vaccination with subsequent *ex vivo* expansion of activated T cells in patients with recurrent glioma. The study is specifically aimed at a group of T cells, named cytokine-induced killer cells, which are known to express a very potent antitumor activity.[53,54] These cells will be selected by expression of the cell markers CD3 and CD56.

LIMITATIONS AND FUTURE PROSPECTS OF IMMUNO-CELL THERAPY

Although a wide range of potential targets and immuno-cellular therapeutic strategies have been investigated experimentally, only the most successful ones are transitioned to the clinic. The translation from bench to bedside remains a difficult path, with the DC vaccine strategy being the most successful example. DC therapy has been proven safe with some therapeutic success; however, no breakthrough has been achieved using this therapeutic strategy for gliomas. The clinical outcome did not reflect the expected results on the bench, showing perhaps a limitation in the existing glioma models. Vaccination is mostly given before tumor implantation for DC therapy to be effective in animal models. This, of course, is impossible in human patients. While many pathophysiological similarities between the rodent glioma models and the human tumors can be observed, many models are performed in immunocompromised mice. Therefore, tumor-associated immunosuppression and immune-modulating events are not likely to be reflected accurately and their usefulness as models for evaluating immuno-cellular therapy might be limited. Furthermore, tumor xenografts will not mimic the process of tumorigenesis *de novo*, resulting in a slightly different tumor microenvironment. The use of rodents with intact immune systems, and the development of genetically induced glioma models, could help optimize preclinical studies and lead to a more predictable transition to the clinic.

Another difficulty in assessing the efficacy and success of DC vaccination in the clinic is the relatively low number of glioma patients per trial group, which often leads to a weak statistical significance. Further, it is difficult to compare study outcomes from different trials because inclusion criteria and injection route differs from one group to another. This can have a substantial effect on patient survival. The use of corticosteroids and other co-medication, as often seen in malignant glioma patients such as GBM, impairs objective assessment even further as efficacy of treatment might be limited, side effects might get masked, and differentiation of immune cells are halted. In the case of vaccination, improvements have only been seen when compared with historical controls, which are improper controls to use for glioma studies. When compared with standard-of-care, no clinically significant benefits have been reported. Furthermore, caution has to be exerted when interpreting effects on immune function following vaccination. Brain inflammation has never been detected inmost, if not all, clinical trials of vaccination. Although this is usually incorrectly interpreted as the vaccines being safe, the absence of any adverse effects in hundreds of immunized patients most likely speaks to the vaccination being ineffective. Thus, at this stage, we remain unable to differentiate the absence of side effects as a result of non-effective vaccination or actual safety. To date, only a single study combining gene/vaccine therapy in dogs showed physiologically effective immune activation associated with brain inflammation which resulted in clinical benefits.[55] This study is the only objective description supporting the idea that, under the right conditions, it is possible to stimulate a systemic immune response that can attack the brain and brain tumors.

To underline some of these problems, and to get a true understanding of the working mechanism and antitumor effect of immuno-cellular therapies, the development of adequate imaging tools is of the utmost importance. The ability to track immune cells and to determine their fate, tropism, migration, interaction with surroundings, and mechanism of action will answer important questions regarding safety and efficacy. Several imaging tools are currently available in the preclinical setting such as bioluminescence and fluorescence. However, these techniques are not yet translatable for use in humans due to several concerns including substrate toxicity and sensitivity. Labeling of stem cells with ferumoxide, which allows them to be tracked *in vivo* by magnetic resonance imaging, has been successfully reported to monitor real-time migration and distribution of these cells at the tumor site.[56] Similar approaches might be translated to the clinic to track immune cells, however, additional studies are required to fine tune this technique and increase its sensitivity to make it suitable for human use. While new imaging tools are a necessity to further develop the immuno-cell therapy field, another issue that needs to be addressed is the availability and efficacy of the cells themselves. High passage number of effector cells *in vitro*, in order to reach adequate levels, could lead to differentiation and change of phenotype, limiting their therapeutic potential. New techniques that allow rapid growth and expansion of these cells while maintaining their characteristics will be of extreme importance for the cellular immunotherapy field (**Box 2**). Similar problems can be seen in the clinic where a lack of *in vivo* expansion and inability to maintain high expression levels over a sufficient period of time could limit treatment efficacy. This may result not only in unsuccessful clinical trials, but also in the abandonment of a potentially successful strategy. The success of the CAR-CD19 adoptive T cell therapy study for CLL and acute lymphoblastic leukemia shows that, once the immune cells are manipulated, extensive *in vivo* expansion and high levels of gene expression could be maintained over time, therefore, immunotherapy can indeed be an effective strategy in the battle against cancer. In order to stimulate cell survival and proliferation, a 4-1 BB costimulatory domain was added to the CAR construct, resulting in >1,000-fold higher proliferation rate of T cells once injected *in vivo*, with each T cell killing ~1,000 CLL cells. Three out of three CLL patients showed clinical activity lasting for over 6 months, with two out of three patients reaching complete remission.[30,31] Kloss *et al.* demonstrated a similar successful approach in a prostate cancer model using a chimeric costimulator receptor together with CARs, with increased selectivity of the modified T cells for prostate cancer cells.[57] Although still at the experimental level, this strategy may greatly increase efficacy and safety of T cell adaptive immunotherapy. Both approaches could easily be adapted to T cell glioma therapy (similar to the Nakazawa[17] and Wang[16] studies), potentially in combination with EBV-CTL.

Several studies are exploring different strategies to deliver immune cells to the tumor. While many choose a direct injection route, others are exploring intranodal, intradermal, and systemic injections in an attempt to enhance the delivery success. Direct comparison of these delivery strategies should be performed to reach the optimal injection route for effective glioma therapy. Other research groups argue that *ex vivo* cell manipulation is time consuming and may result in cellular differentiation and an increased risk of infection. Thus, the focus should not be on "how to deliver the manipulated cells", but on "how to manipulate the cells *in vivo*". The studies being performed by Andrews *et al.*, at the Thomas Jefferson University, will shed new light on these possibilities.

Finally, when discussing treatment efficacy and success of new clinical strategies, it is important to bear in mind the current prognosis and treatment options available for glioma patients. While the results of the CAR-CD19 trial showed that two out of the three CLL patients are in remission for over a year, which is extraordinary, one must realize that with a median survival of 8–10 years, the CLL population is not comparable to glioma patients. We advocate that in a patient population where the 2-year survival rate is only 40%, and in the past 25 years the median survival rate has only increased by 3 months, our expectation on efficacy should be as equally moderate.[1,2] Furthermore, a gain of months rather than years should be valued as well as the decrease in side effects and/or increase in patient's well-being. The aim of trials should therefore not only be directed towards increased survival, but also for better quality-of-life. It is the hope that optimization of some of the strategies discussed here would increase both potential goals and expectations. For now, it is difficult to conclude what role and effect immuno-cellular therapy has on malignant gliomas. If some of the discussed issues can be addressed, and current clinical trials show promising results, this therapeutic strategy has potentially a tremendous value in the search for a cure for tumors as heterogeneous as GBM while complementing current standard therapy.

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