Cover Page

The handle http://hdl.handle.net/1887/25830 holds various files of this Leiden University dissertation.

Authors: Bovenberg, Maria Sarah Sophie & Degeling, Marja Hannah
Title: Cancer and glioma : an integrated approach of gene therapy and bioluminescence imaging
Issue Date: 2014-05-27

CHAPTER XII

⌘

Cell-based immunotherapy against gliomas:
from bench to bedside

M. Sarah S. Bovenberg[1,2,3*], M. Hannah Degeling[1,2,3*], and Bakhos A. Tannous[1,2]

[1]Experimental Therapeutics and Molecular Imaging Laboratory, Neuroscience Center, Department of Neurology, [2]Program in Neuroscience, Harvard Medical School, Boston, MA 02114 USA. [3]Department of Neurosurgery, Leiden University Medical Center, Leiden, The Netherlands. * These authors contributed equally

ABSTRACT

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 an adjuvant experimental therapeutic approach to treat malignant brain tumors. In this review, we discuss advances in active, passive, and vaccine immunotherapeutic strategies for gliomas both at the bench and in the clinic.

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 years survival rate of 40% and a median survival of 18-21 months.[1, 2] Current standard of care includes surgical debulking of the tumor mass, followed by radiation and chemotherapy (temozolomide);[3] however, as the poor survival rate indicates, these treatments have not been effective in preventing disease progression. The location of malignant gliomas (the brain) and their invasive properties cause complete surgical resection of the tumor mass nearly impossible, while high doses of radiotherapy cannot be delivered due to potential damage to the normal brain. Chemotherapeutics often cannot cross the blood-brain barrier efficiently and gliomas are known to develop resistance along these treatment regimen.[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 application can be dated back into the fifties where it was used in the bone marrow transplantation field.[9, 10] Currently, a broader spectrum of cellular therapy application is pursued. Different cell types are used in replacement therapies, taking over the function of diseased cells in the target organ, as can be seen in diabetes where insulin producing cells are injected in order to replace their malfunctioning originals in the pancreas.[11, 12] Tissue engineering in

which *ex vivo* whole organs are recreated out of cells is in its early phase but holds a tremendous potential for the future.[13] An example that found its way to the clinic is artificial skin grown from collagen scaffolds seeded with the patient's own epidermal skin cells.[14] This technique is FDA-approved and has 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. myoblast for the generation of muscle tissue), other research is focusing on the application of cells for tasks outside of their preprogrammed function profile. For instance, stem cells or immune cells can be used for immunotherapy or as carriers of therapeutic genes or pro-drugs that gets activated at a specific location in the body. This cell-based therapy provides a new and interesting strategy for the treatment of cancer including brain tumors. Cellular Immunotherapy in particular has the potential to both specifically target brain tumor cells (and thereby limiting brain damage), and to establish a long term antitumor response by stimulating the immune system, and thus is being explored as an alternative therapy for gliomas. Currently, a wide range of strategies is investigated at the bench, while slowly but steadily a small portion of this research is 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-injected 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 as pseudopallisade 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 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 focusing 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 allows for long-term antitumor effect, which not only enhances the likelihood of the tumor being eradicated, but also decreases the risk of tumor recurrence. T-cells, dendritic cells, and macrophages are the cells of choice for this therapeutic strategy (Table1; Box1).

Cell type	Transgene/modification strategy	Application	References
T cells	Anti-HER2 receptor	Anti-glioma immunotherapy, evaluation of associated autoimmune pathology	16
	HER2-CAR	Anti-glioma immunotherapy; evaluation of enhanced CAR-mediated tumor cell recognition.	17
	IL13Ra2	Anti-GSC immunotherapy	18
Dendritic cells (DC)	IL13Ra2	Anti-GSC immunotherapy; more efficient expression of antigens at MHC level	19
	Ad-Flt3L/Ad-TK	Anti-glioma viral therapy; more efficient delivery and enhanced viral distribution at the tumor site.	20
	CSC antigen load	Anti-glioma immunotherapy; enhanced antitumor response	21
Macrophages	Nanoshell load	Photothermal-mediated glioma therapy; proof of concept of macrophage as delivery vehicles for NS	22

Table 1 Overview of experimental immuno-cell therapy against gliomas

Abbreviations: HER2, human epidermal growth factor receptor 2; CAR, chimeric antigen receptor; IL13Ra2, interleukin-13 zetakine 2; Ad-Flt3L/Ad-TK, adenovirus expressing Flt3L/TK; CSCs, cancer stem cells; NS, nanoshell

Box 1 Cells used for immune-cellular therapy
T Cells or T-lymphocytes are part of the white blood cell compartment and play an important role in the cell-mediated immunity. Hallmark of these cells is expression of the T Cell Receptor (TCR) 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 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. CLTs, or **CD8 T Lymphocytes** are responsible for direct cell mediated killing. They recognize their target by binding to antigens expressed by MCH 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** (NKTs). These cells are very similar to NK cells (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 cytotoxic T lymphocytes, 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 in 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 function as APCs, just like macrophages. Their hallmark is expression of the toll like receptor (TLR). They are present in skin, respiratory and gastro-intestinal 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. Additionally, these cells are capable of secreting cytokines that further enhance differentiation of surrounding immune cells. |

T cell-based immunotherapy. T cells are often used as cellular vehicles for the treatment of different types of cancer, typically resistant to conventional therapy. These cells can be easily modified with tumor-specific antigens to target tumor cells. 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. Gene-engineered T-cells to express anti-HER2 chimeric receptor were intravenously transferred in combination with lymph ablation and interleukin-2 (IL-2). The autoimmune effect had no significant toxicity on normal mammary and brain tissues expressing HER2, while 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 chimeric antigen receptor (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 increased survival rate.[23] The challenge of this therapeutic strategy is to establish stable expression of the activated antigen while achieving enough expansion with nonspecific activated T cells (ATC; known to be intolerant to transfection) as well as persistent antitumor effect. To overcome these limitations, the same group used Epstein-Bar virus (EBV) to stimulate T cells (EBV-CTLs), a method known to elicit an enhanced immune response.[24] EBV-CTLs outperformed the ATCs in both expansion and antitumor persistence.[25-27] 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 (iPSC), PB has not been evaluated for *in vivo* immunotherapeutic model. No preferential integration near or into oncogenes was observed, as typically the case with retrovirus and lentivirus-based transduction method. 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 than viral vectors, and easier to produce, this method provides an interesting new approach for gene delivery.

Dendritic cell and macrophage-based therapy. Dendritic Cells (DCs) are the most potent antigen-presenting cells (APCs) of the human body, sensitizing T cells to all acquired antigens. In contrast to activation of T cells *ex vivo*, dendritic cells activation and stimulation induce a long-term immune response and is therefore considered active immunotherapy. Dendritic cells can be loaded with tumor antigens *ex vivo* that can subsequently activate the endogenous immune system upon injection. Although this technique is safe, clinical efficiency is still to be improved to achieve high expression of the antitumor antigen at the major histocompatibility complex (MHC) on the dendritic cell surface and to expand the subgroup of these cells that primes naïve T cells. Saka et al.,[19] designed a DC vaccine-based strategy aimed at targeting the interleukin-13 zetakine (IL13Ra2), 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 day 3 and 10 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.[19] In a model in which gene therapy is effective but dendritic cell vaccination is not-effective, Mineharu et al.,[20] demonstrated that combining *in situ* Ad-Flt3L/Ad-TK–mediated gene therapy (an FDA-approved adenoviral vector expressing either fms-like tyrosine kinase 3 ligand or thymidine kinase) with dendritic cell vaccination increases therapeutic efficacy and antitumor immunity as compared to 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 (GCV). Flt3L causes dendritic cells to migrate, differentiate, and expand within the tumor microenvironment of mice and rats. TK at the tumor site converts GCV into a highly toxic phosphorylated drug causing the death of dividing tumor cells. Further, tumor treated with GCV releases high mobility group 1 protein (HMGB1), which serves as an adjuvant of the innate immune system by stimulating Toll like receptor 2 in signaling bone marrow-derived DCs (BMCDs) confirming previous studies.[28, 29] To achieve enhanced proliferation

and antitumor effect, the dendritic cells were conditioned *ex vivo* with Fl3t and interleukin-6 (IL-6).[20]

As an alternative of loading DCs with regular tumor antigens, Xu et al.,[21] explored the use of cancer stem cells (CSC) antigens as a source for DC anti-glioma 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 (TAA). A sufficient T cell response against CSCs and an increase in survival of mice bearing 9L gliosarcoma CSCs tumors was observed as compared to DCs loaded with daughter or conventionally cultured 9L cells after intradermal injection of the vaccine. Albeit conventional loading and CSCs 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 DCs loading as compared to conventional tumor antigens. A clinical trial evaluating DCs vaccines using CSCs is being considered and will start shortly.[21]

Another antigen-presenting cells 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 tumors therapy. Tumor-associated macrophages are often observed in glioma microenvironment, and intravenously injected macrophages targets the brain tumor site.[30] In a recent study, Baek et al.,[22] used macrophages loaded with gold-coated nanoshells for the treatment of human multicellular glioma spheroids, an *in vitro* model with similar characteristics in both resistance to radio- and chemotherapy, and in growth and metabolic rates of glioma tumors *in vivo*, while simulating the tumor before vascularization. Nanoshells are spherical nanoparticles consisting of a di-electric 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 spheroids 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 photo-thermal therapy, while 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 is currently in progress focusing on the safety, efficacy, and feasibility of immuno-cellular therapeutic approach 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 dendritic cell 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 2 out of 3 patients underwent complete remission after CAR-CD19 therapy and are remaining so for >1 year after treatment.[31, 32] 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 (ALL), two forms of blood cancer.[33, 34] These successful trials prove the significance of immuno-cellular therapeutic strategies for treating different tumors, including gliomas.

Table 2. Current immuno-cell therapy in clinical Trials

Therapy	Cell/vaccine type	Transgene/modification strategy	Application	Phase	Clinicaltrial.gov Identifier #	Ref
Cellular immunotherapy	CD 8+ 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)	18, 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	36, 37
Vaccine cell therapy	CTL	BTIC antigen load	Evaluation of the feasibility of administering of Imiquimod/BTIC Lysate-Based therapy for Diffuse Intrinsic Pontine Glioma in children and young adults	Pilot	NCT01400672	38
	Dendritic cells (DCs)	ICT-107	Evaluation of the safety and efficacy of ICT-107 in newly diagnosed patients with GBM following resection and chemo-radiation.	Phase II	NCT01280552	39
		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	40, 41
		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	42, 43
		A2B5+ antigen load	Evaluation of the efficacy of vaccination with DCs loaded with Glioma Stem-like Cells Associated Antigens against GBM	Phase II	NCT01567202	44
		Autogenic GBM cell lysate	Evaluation of the adverse and therapeutic effects of a postoperative autologous dendritic cell tumor vaccine in patients with malignant glioma	Phase I/II	Published	45
			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	46
	IMA 950	TUMAPs: multi-peptide 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 IMA950 when given with cyclophosphamide, granulocyte macrophage-colony stimulating factor (GM-CSF) and imiquimod in patients with GBM	Phase I	NCT01403285	47
		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	48, 49
	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	50

Cellular vaccine and immunoth erapy 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	NCT01290 692	51
	Glioma vaccine, DC; DCIK	• Dendritic cells pulsed with tumor lysate • CIK cells activated by DCs stimulation (DCIKs)	Evaluation of DCIK Combined With DC Treatment for Glioma	Phase I/II	NCT01235 845	52
	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	NCT01205 334	53, 54
		• 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	NCT01109 095	23, 53

Abbreviations: BTIC, brain tumor initiating cell; CTL, cytotoxic T lymphocyte; DCIK, dendritic cell (DC) activated cytokine induced killer cell (CIK); 06BG, 06-Benzylguanine; EGFR, epidermal growth factor receptor; CAR, chimeric antigen receptor; HER2, human epidermal growth factor receptor 2; TUMAP, tumor-associated peptides; ILGFR1, Insulin-like Growth Factor Receptor-1

T cell immunotherapy in the clinic. With malignant gliomas being known for their immune evasive strategies, barely any immune response is provoked. However, the immune cells could be of tremendous value in the fight against brain tumors; they could provide a very efficient elimination mechanism of the tumor bulk and metastatic/invasive cells, without the need to undergo quality of life impairing as in the case for chemo- or radiotherapeutic paradigms. Many of the current strategies are exploring methods to overcome the lack of immune response to glioma cells by artificially stimulating the immune system either by passive or active immunization. The use of T cells is one of the most popular strategies in the clinic, often used in combination with dendritic cell vaccines. Two clinical trials focus 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 (PBMC) are collected and genetically modified to express the membrane-tethered IL13 cytokine chimeric T cell receptor (TCR) targeting the IL13 receptor α2 (IL13Rα2) present in over 80% of malignant gliomas. This IL13 zetakine has an E13Y mutation, which enhances its specificity for the IL13α2 receptor by >50-fold, as compared to the normal IL13 receptor expressed by

the healthy brain tissue.[18, 35] in addition to the IL13 zetakine, the cytotoxic T lymphocytes (CTL) were further modified to express the thymidine kinase suicide gene (Hy/TK) under the control of the constitutively active cytomegalovirus (CMV) promoter in case immediate ablation of CTL activity is required. Repeated CTL infusion was performed over 2 weeks (3 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 IL13 zetakine in an orthotopic mouse tumor model.[18, 35] The authors showed that IL13Rα2 is expressed by both glioma stem cells 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 has been completed, however, the results have not been published yet.

Rosenberg et al., at the National Institutes of Health Clinical Centre takes 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 glioblastoma, while no expression is seen in the normal brain.[55] After *ex vivo* preparation, the autologous modified cells will be intravenously injected and safety, feasibility, and progression-free interval will be monitored.

Vaccine therapy in the clinic. With 9 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 of the tumor by the immune system. In five out of the nine trials, autologous dendritic cells are used to stimulate the patient immune system to evoke an antitumor immune response. Dendritic cells 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.[39, 56] ImmunoCellular therapeutics, LTD., 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 dendritic cells are *ex vivo* pulsed with ICT-107 and

injected intradermally upon completion of tumor removal and 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 Sirolimus, an mTOR inhibitor.[40] Autologous dendritic cells 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 dendritic cells genetically modified to express WT1 protein, 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.[42, 43] In China, Zhou et al., at the Huashan Hospital initiated a Phase II study evaluating the overall survival of patients with primary and/or secondary GBM after treatment with autologous dendritic cells loaded with autogenic glioma stem cells (A2B5+). A study investigating the adverse and therapeutic effect of post-operative dendritic cells-derived tumor vaccine was recently published by Chang et al.,[45] 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 to 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 dendritic cells were administered using a 6 months-10 injections course. The authors report that 47% of the enrolled patients developed a transient elevation in alanine aminotransferase (ALT)/aspartate aminotransferase (AST) levels, which correlated with the vaccination schedule and high doses of dendritic cells vaccine. At lower levels of dendritic cells vaccine, no increase in serum ALT/AST was observed suggesting the safe upper limit of 2×10^7 dendritic cells/dose.

Another recently completed study by Fadul et al.,[46] was reported in the Journal of Immunotherapy focusing on the immune response, progression free survival (PFS), and overall survival (OS) of GBM patients treated with intranodal autologous tumor lysate dendritic cells vaccination. CTL tumor specific activation was measured and correlated with both PFS and OS. All patients were still alive 6 months after diagnosis and a PFS 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 dendritic cells approach, Andrews et al., at the Thomas Jefferson University (Philadelphia) initiated a pilot study evaluating the possibility of stimulating the dendritic cells 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 to *ex vivo* stimulation.[57] Diffusion chambers containing autologous tumor cells treated *ex vivo* with insulin-like growth factor receptor-1 (IGFR-1) antisense oligodeoxynucleotide were re-implanted in the rectus sheet to stimulate the native immune system. Loss of IGFR-1 is expected to result in apoptosis with subsequent release of tumor antigen containing exosomes (microvesicles), which will allow the diffusion chamber to act as a slow-release antigen depot.[50] Since a wound containing a foreign body is created upon implantation of the diffusion chamber, high levels of dendritic cells are expected to be present in the immediate surroundings, enhancing the efficacy of antitumor activation of the immune system.

Three studies demonstrated that the use of dendritic cells for vaccine therapy is not the only way to go. A pilot study by Moertel et al., at the Masonic Cancer Centre (University of Minessota) developed a cell-based cancer vaccine composed of glioma stem-like associated antigens found in the brain tumor initiating cell line GBM6 (BTICs).[58, 59] Upon administration, the BTIC 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, a therapeutic multi-peptide vaccine containing 11 tumor-associated peptides (TUMAPs) found in a majority of GBMs designed to activate TUMAP-specific T cells. Rumpling et al., (Cancer Research UK) are testing the vaccine in combination with granulocyte-macrophage colony-stimulating factor (GM-CSF) and radiation and chemotherapy (temozolomide) for patients with newly diagnosed gliomas.[60] Sul et al., (Immatics Biotechnologies GmbH in collaboration

with the National Cancer Institute) follow a similar approach to test IMA 950 with GM-CSF and locally-applied imiquimod, 20 min after each vaccination. Patients will further be treated with one dose of cyclophosphamide prior to the first vaccination.

Vaccine and cellular therapy combined. Two clinical trials using the combined approach of vaccine and immunotherapy are being performed by Ahmed et al., at the Baylor College of Medicine. In the first trial, autologous CTLs are *ex vivo* stimulated with human β herpes cytomegalovirus (h-CMV) presenting dendritic cells. CMV-specific antigens can be detected in 70-90% of malignant glioma cells, but not the normal brain.[61, 62] The CMV-specific CTLs are then cultured in the presence of EBV-infected cells, to boost a stronger immune response upon intravenous administration.[24] The second trial goes a step further and genetically modifies the CMV-specific CTLs to express the chimeric antigen receptor targeting HER2, which is associated with 70% of GBM malignancies.[63] 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.[53] In the meantime, Wood et al., (TVAX Biomedical) are testing in a phase II trial (supported by positive safety and efficacy study in a phase I trial) a brain cancer vaccine called TVI-Brain I, consisting of neutralized autologous tumor cells. Upon vaccination, an immune response of killer T cells is expected yielding a highly effective antitumor activity. Yao et al., at Quindao University use a somewhat similar approach in a phase I/II trial combining intranodal dendritic cells 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, called cytokine-induced killer cells (CIK), which are known to express a very potent antitumor activity.[52, 64] 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 are investigated experimentally, only the most successful are transitioned to the

clinic. The translation from the laboratory to the clinic remains a difficult phase, with dendritic cells vaccine strategy being the most successful example. Dendritic cell therapy has 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. For dendritic cell therapy to be effective in animal models, vaccination is mostly given before tumor implantation. 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. Further, 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 system, and the development of genetically-induced glioma models could help optimizing preclinical studies, leading to a more predictable transition to the clinic.

Another difficulty in assessing the efficacy and success of dendritic cells vaccination (or any other strategy) in the clinic is the relatively low number of glioma patients per trial group often leading to a weak statistical significance. Further, it is difficult to compare study outcomes from different trials, since inclusion criteria and injection route differ from one group to another, which 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 is halted. In the case of vaccination, improvements have only been seen when compared to historical controls, which are improper controls to use for glioma studies. When compared to standard of care, no clinical significant benefits have been reported. Furthermore, caution has to be exerted when interpreting effects on immune function following vaccination. In most if not all clinical trials of vaccination, brain inflammation has never been detected. Although this is usually (wrongly) interpreted as the vaccines being safe, the absence of any adverse effects in hundreds of immunized patients, most likely speaks of the ineffective vaccination. Thus, at this stage, we remain unable to differentiate the

absence of side effects as a result of non-effective vaccination, or, actual safety. So far, only a single study combining gene/vaccine therapy in dogs showed physiologically effective immune activation associated with brain inflammation, which resulted in clinical benefits.[65] 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 undermine 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 uttermost important. The ability to track immune cells and to determine their fate, tropism, migration, interaction with the surroundings, and mechanism of action will answer important questions regarding safety and efficacy. Several imaging tools are currently available in the preclinical setting (e.g. 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 femuroxide, which allows them to be tracked *in vivo* by magnetic resonance imaging (MRI) has been successfully reported to monitor in real-time migration and distribution of these cells at the tumor site.[66] 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 suited for *in 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. Similar problems can be seen in the clinic where 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 ALL 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 co-stimulatory domain was added to the CAR construct, resulting in >1000-fold higher proliferation rate of T cells once injected *in vivo*, with each T cell killing approximately 1000 CLL cells. Three out of three CLL patients showed clinical activity lasting for over 6 months, with 2 out of 3 patients reaching complete remission.[31, 32] Kloss et al., demonstrated a similar successful approach in a prostate cancer model using a chimeric co-stimulator receptor (CCR) together with CARs, with increased selectivity of the modified T cells for prostate cancer cells.[67] 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 cells glioma therapy (similar to the Nakazawa[17] and Wang[16] studies), potentially in combination with EBV-CLT.

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, or systemic injection, in an attempt to enhance the delivery success. Direct comparison of these delivery strategies should be performed in order 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 CAR-CD19 trial showed that 2 out of the 3 CLL patients are in remission for over a year, which is extraordinary, one must realizes that with a median survival of 8 to 10 years, the CLL population is not comparable to glioma patients. We advocate that in a patient population where the 2 years 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] Further, the 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 against increased survival, but also for better "quality of life". Hopefully, optimizing some of the strategies discussed here will eventually turn the wheel and allow us to increase our goals and expectations.

For now, it is difficult to conclude the role and effect of immuno-cellular therapy on malignant gliomas. If some of the discussed issues can be addressed, and current clinical trials show promising results, this therapeutic strategy has the potential to give a tremendous value in the search for a cure for tumors as heterogeneous as GBM, complementing current standard therapy.

Box 2 Cell isolation and preparation for immunotherapy

T lymphocytes can be obtained from several sources, including thymus, lymphnodes, spleen and peripheral blood, the latter being the most accessible. Cells are separated from the whole blood samples by Ficol isopaque-based density gradient separation. Since lymphocytes are less dense than erythrocytes, they can easily be 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, NKT) based on monoclonal antibody reactions. To generate CLTs against specific antigens (for instance expressed on tumor cell surface), the CTLs can be cultured in the presence of APCs loaded with the desired antigen. Cells are cultured in basal medium containing RPMI 1640, 10% fetal calf serum, penicillin/streptomycin, L-glutamate, phytohaemagglutin 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 Ficol-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 can be generated through various protocols. One technique involves DCs separation from the whole blood samples by Ficol gradient centrifugation. B-lymphocytes and monocytes are then subtracted from the cell suspension using monoclonal antibodies directed towards CD19 and CD14. Dendritic cells are then isolated from the remaining (B and monocyte depleted) mixture by CD304, CD141 andCD1c directed antibodies. Selected cells are cultured in RPMI media containing M-CSF-1 yielding to DCs differentiation.

REFERENCES

1. Johnson DR, Ma DJ, Buckner JC, Hammack JE. (2012). Conditional probability of long-term survival in glioblastoma: A population-based analysis. *Cancer.*

2. Grossman SA, Ye X, Piantadosi S, Desideri S, Nabors LB, Rosenfeld M, et al. (2010). Survival of patients with newly diagnosed glioblastoma treated with radiation and temozolomide in research studies in the United States. *Clin Cancer Res.*16(8):2443-2449.

3. Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJ, Janzer RC, et al. (2009). Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. *Lancet Oncol.*10(5):459-466.

4. Albesiano E, Han JE, Lim M. (2010). Mechanisms of local immunoresistance in glioma. *Neurosurg Clin N Am.*21(1):17-29.

5. Haar CP, Hebbar P, Wallace GCt, Das A, Vandergrift WA, 3rd, Smith JA, et al. (2012). Drug Resistance in Glioblastoma: A Mini Review. *Neurochem Res.*

6. Wu A, Wei J, Kong LY, Wang Y, Priebe W, Qiao W, et al. (2010). Glioma cancer stem cells induce immunosuppressive macrophages/microglia. *Neuro Oncol.*12(11):1113-1125.

7. Stupp R, Pica A, Mirimanoff RO, Michielin O. (2007). [A practical guide for the management of gliomas]. *Bull Cancer.*94(9):817-822.

8. Stupp R, Roila F. (2009). Malignant glioma: ESMO clinical recommendations for diagnosis, treatment and follow-up. *Ann Oncol.*20 Suppl 4:126-128.

9. Gale RP. (1979). Bone marrow transplantation in acute leukemia: current status and future directions. *Haematol Blood Transfus.*23:71-78.

10. Santos GW, Elfenbein GJ, Tutschka PJ. (1979). Bone marrow transplantation--present status. *Transplant Proc.*11(1):182-188.

11. Joglekar MV, Hardikar AA. (2012). Isolation, expansion, and characterization of human islet-derived progenitor cells. *Methods Mol Biol.*879:351-366.

12. Efrat S, Russ HA. (2012). Making beta cells from adult tissues. *Trends Endocrinol Metab.*23(6):278-285.

13. Vacanti JP. (2012). Tissue engineering and the road to whole organs. *Br J Surg.*99(4):451-453.

14. Wain RA, Shah SH, Senarath-Yapa K, Laitung JK. (2012). Dermal substitutes do well on dura: comparison of split skin grafting +/- artificial dermis for reconstruction of full-thickness calvarial defects. *Clin Plast Surg.*39(1):65-67.

15. Jones TS, Holland EC. (2011). Animal models for glioma drug discovery. *Expert Opin Drug Discov.*6(12):1271-1283.

16. Wang LX, Westwood JA, Moeller M, Duong CP, Wei WZ, Malaterre J, et al. (2010). Tumor ablation by gene-modified T cells in the absence of autoimmunity. *Cancer Res.*70(23):9591-9598.

17. Nakazawa Y, Huye LE, Salsman VS, Leen AM, Ahmed N, Rollins L, et al. (2011). PiggyBac-mediated cancer immunotherapy using EBV-specific cytotoxic T-cells expressing HER2-specific chimeric antigen receptor. *Mol Ther.*19(12):2133-2143.

18. Brown CE, Starr R, Aguilar B, Shami A, Martinez C, D'Apuzzo M, et al. (2012). Stem-like tumor initiating cells isolated from IL13Ralpha2-expressing

gliomas are targeted and killed by IL13-zetakine redirected T cells. *Clin Cancer Res.*

19. Saka M, Amano T, Kajiwara K, Yoshikawa K, Ideguchi M, Nomura S, et al. (2010). Vaccine therapy with dendritic cells transfected with Il13ra2 mRNA for glioma in mice. *J Neurosurg.*113(2):270-279.

20. Mineharu Y, King GD, Muhammad AK, Bannykh S, Kroeger KM, Liu C, et al. (2011). Engineering the brain tumor microenvironment enhances the efficacy of dendritic cell vaccination: implications for clinical trial design. *Clin Cancer Res.*17(14):4705-4718.

21. Xu Q, Liu G, Yuan X, Xu M, Wang H, Ji J, et al. (2009). Antigen-specific T-cell response from dendritic cell vaccination using cancer stem-like cell-associated antigens. *Stem Cells.*27(8):1734-1740.

22. Baek SK, Makkouk AR, Krasieva T, Sun CH, Madsen SJ, Hirschberg H. (2011). Photothermal treatment of glioma; an in vitro study of macrophage-mediated delivery of gold nanoshells. *J Neurooncol.*104(2):439-448.

23. Ahmed N, Salsman VS, Kew Y, Shaffer D, Powell S, Zhang YJ, et al. (2010). HER2-specific T cells target primary glioblastoma stem cells and induce regression of autologous experimental tumors. *Clin Cancer Res.*16(2):474-485.

24. Quintarelli C, Savoldo B, Dotti G. (2010). Gene therapy to improve function of T cells for adoptive immunotherapy. *Methods Mol Biol.*651:119-130.

25. Pule MA, Savoldo B, Myers GD, Rossig C, Russell HV, Dotti G, et al. (2008). Virus-specific T cells engineered to coexpress tumor-specific receptors: persistence and antitumor activity in individuals with neuroblastoma. *Nat Med.*14(11):1264-1270.

26. Woltjen K, Hamalainen R, Kibschull M, Mileikovsky M, Nagy A. (2011). Transgene-free production of pluripotent stem cells using piggyBac transposons. *Methods Mol Biol.*767:87-103.

27. Woltjen K, Michael IP, Mohseni P, Desai R, Mileikovsky M, Hamalainen R, et al. (2009). piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. *Nature.*458(7239):766-770.

28. Ali S, King GD, Curtin JF, Candolfi M, Xiong W, Liu C, et al. (2005). Combined immunostimulation and conditional cytotoxic gene therapy provide long-term survival in a large glioma model. *Cancer Res.*65(16):7194-7204.

29. Curtin JF, Liu N, Candolfi M, Xiong W, Assi H, Yagiz K, et al. (2009). HMGB1 mediates endogenous TLR2 activation and brain tumor regression. *PLoS Med.*6(1):e10.

30. Shinonaga M, Chang CC, Suzuki N, Sato M, Kuwabara T. (1988). Immunohistological evaluation of macrophage infiltrates in brain tumors. Correlation with peritumoral edema. *J Neurosurg.*68(2):259-265.

31. Porter DL, Levine BL, Kalos M, Bagg A, June CH. (2011). Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. *N Engl J Med.*365(8):725-733.

32. Kalos M, Levine BL, Porter DL, Katz S, Grupp SA, Bagg A, et al. (2011). T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. *Sci Transl Med.*3(95):95ra73.

33. Brentjens RJ, Santos E, Nikhamin Y, Yeh R, Matsushita M, La Perle K, et al. (2007). Genetically targeted T cells eradicate systemic acute lymphoblastic leukemia xenografts. *Clin Cancer Res.*13(18 Pt 1):5426-5435.

34. Brentjens RJ, Riviere I, Park JH, Davila ML, Wang X, Stefanski J, et al. (2011). Safety and persistence of adoptively transferred autologous CD19-targeted T cells in patients with relapsed or chemotherapy refractory B-cell leukemias. *Blood.*118(18):4817-4828.

35. Kahlon KS, Brown C, Cooper LJ, Raubitschek A, Forman SJ, Jensen MC. (2004). Specific recognition and killing of glioblastoma multiforme by interleukin 13-zetakine redirected cytolytic T cells. *Cancer Res.*64(24):9160-9166.

36. Stroncek DF, Berger C, Cheever MA, Childs RW, Dudley ME, Flynn P, et al. (2012). New directions in cellular therapy of cancer: a summary of the summit on cellular therapy for cancer. *J Transl Med.*10(1):48.

37. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, et al. (2005). Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med.*352(10):987-996.

38. Finocchiaro G, Pellegatta S. (2011). Immunotherapy for glioma: getting closer to the clinical arena? *Curr Opin Neurol.*24(6):641-647.

39. Kim W, Liau LM. (2010). Dendritic cell vaccines for brain tumors. *Neurosurg Clin N Am.*21(1):139-157.

40. Tsuji T, Matsuzaki J, Kelly MP, Ramakrishna V, Vitale L, He LZ, et al. (2011). Antibody-targeted NY-ESO-1 to mannose receptor or DEC-205 in vitro elicits dual human CD8+ and CD4+ T cell responses with broad antigen specificity. *J Immunol.*186(2):1218-1227.

41. Wadle A, Mischo A, Strahl S, Nishikawa H, Held G, Neumann F, et al. (2010). NY-ESO-1 protein glycosylated by yeast induces enhanced immune responses. *Yeast.*27(11):919-931.

42. Van Driessche A, Van de Velde AL, Nijs G, Braeckman T, Stein B, De Vries JM, et al. (2009). Clinical-grade manufacturing of autologous mature mRNA-electroporated dendritic cells and safety testing in acute myeloid leukemia patients in a phase I dose-escalation clinical trial. *Cytotherapy.*11(5):653-668.

43. Van Tendeloo VF, Van de Velde A, Van Driessche A, Cools N, Anguille S, Ladell K, et al. (2010). Induction of complete and molecular remissions in acute myeloid leukemia by Wilms' tumor 1 antigen-targeted dendritic cell vaccination. *Proc Natl Acad Sci U S A.*107(31):13824-13829.

44. Hua W, Yao Y, Chu Y, Zhong P, Sheng X, Xiao B, et al. (2011). The CD133+ tumor stem-like cell-associated antigen may elicit highly intense immune responses against human malignant glioma. *J Neurooncol.*105(2):149-157.

45. Chang CN, Huang YC, Yang DM, Kikuta K, Wei KJ, Kubota T, et al. (2011). A phase I/II clinical trial investigating the adverse and therapeutic effects of a postoperative autologous dendritic cell tumor vaccine in patients with malignant glioma. *J Clin Neurosci.*18(8):1048-1054.

46. Fadul CE, Fisher JL, Hampton TH, Lallana EC, Li Z, Gui J, et al. (2011). Immune response in patients with newly diagnosed glioblastoma multiforme treated with intranodal autologous tumor lysate-dendritic cell vaccination after radiation chemotherapy. *J Immunother.*34(4):382-389.

47. Sul J, Fine HA. (2010). Malignant gliomas: new translational therapies. *Mt Sinai J Med.*77(6):655-666.

48. Brada M, Stenning S, Gabe R, Thompson LC, Levy D, Rampling R, et al. (2010). Temozolomide versus procarbazine, lomustine, and vincristine in recurrent high-grade glioma. *J Clin Oncol.*28(30):4601-4608.

49. Gorlia T, Stupp R, Brandes AA, Rampling RR, Fumoleau P, Dittrich C, et al. (2012). New prognostic factors and calculators for outcome prediction in patients with recurrent glioblastoma: A pooled analysis of EORTC Brain Tumour Group phase I and II clinical trials. *Eur J Cancer.*

50. Andrews DW, Resnicoff M, Flanders AE, Kenyon L, Curtis M, Merli G, et al. (2001). Results of a pilot study involving the use of an antisense oligodeoxynucleotide directed against the insulin-like growth factor type I receptor in malignant astrocytomas. *J Clin Oncol.*19(8):2189-2200.

51. Sloan AE, Dansey R, Zamorano L, Barger G, Hamm C, Diaz F, et al. (2000). Adoptive immunotherapy in patients with recurrent malignant glioma: preliminary results of using autologous whole-tumor vaccine plus granulocyte-macrophage colony-stimulating factor and adoptive transfer of anti-CD3-activated lymphocytes. *Neurosurg Focus.*9(6):e9.

52. Li H, Wang C, Yu J, Cao S, Wei F, Zhang W, et al. (2009). Dendritic cell-activated cytokine-induced killer cells enhance the anti-tumor effect of chemotherapy on non-small cell lung cancer in patients after surgery. *Cytotherapy.*11(8):1076-1083.

53. Ghazi A, Ashoori A, Hanley PJ, Brawley VS, Shaffer DR, Kew Y, et al. (2012). Generation of polyclonal CMV-specific T cells for the adoptive immunotherapy of glioblastoma. *J Immunother.*35(2):159-168.

54. Hoa N, Ge L, Kuznetsov Y, McPherson A, Cornforth AN, Pham JT, et al. (2010). Glioma cells display complex cell surface topographies that resist the actions of cytolytic effector lymphocytes. *J Immunol.*185(8):4793-4803.

55. Hatanpaa KJ, Burma S, Zhao D, Habib AA. (2010). Epidermal growth factor receptor in glioma: signal transduction, neuropathology, imaging, and radioresistance. *Neoplasia.*12(9):675-684.

56. Rodriguez A, Regnault A, Kleijmeer M, Ricciardi-Castagnoli P, Amigorena S. (1999). Selective transport of internalized antigens to the cytosol for MHC class I presentation in dendritic cells. *Nat Cell Biol.*1(6):362-368.

57. Tacken PJ, de Vries IJ, Torensma R, Figdor CG. (2007). Dendritic-cell immunotherapy: from ex vivo loading to in vivo targeting. *Nat Rev Immunol.*7(10):790-802.

58. Hu Y, Fu L. (2012). Targeting cancer stem cells: a new therapy to cure cancer patients. *Am J Cancer Res.*2(3):340-356.

59. Selvan SR, Carbonell DJ, Fowler AW, Beatty AR, Ravindranath MH, Dillman RO. (2010). Establishment of stable cell lines for personalized melanoma cell vaccine. *Melanoma Res.*20(4):280-292.

60. Zhan Y, Xu Y, Lew AM. (2012). The regulation of the development and function of dendritic cell subsets by GM-CSF: More than a hematopoietic growth factor. *Mol Immunol.*52(1):30-37.

61. Dziurzynski K, Chang SM, Heimberger AB, Kalejta RF, McGregor Dallas SR, Smit M, et al. (2012). Consensus on the role of human cytomegalovirus in glioblastoma. *Neuro Oncol.*14(3):246-255.

62. Lucas KG, Bao L, Bruggeman R, Dunham K, Specht C. (2011). The detection of CMV pp65 and IE1 in glioblastoma multiforme. *J Neurooncol.*103(2):231-238.

63. Potti A, Forseen SE, Koka VK, Pervez H, Koch M, Fraiman G, et al. (2004). Determination of HER-2/neu overexpression and clinical predictors of survival in a cohort of 347 patients with primary malignant brain tumors. *Cancer Invest.*22(4):537-544.

64. Mesiano G, Todorovic M, Gammaitoni L, Leuci V, Giraudo Diego L, Carnevale-Schianca F, et al. (2012). Cytokine-induced killer (CIK) cells as feasible and effective adoptive immunotherapy for the treatment of solid tumors. *Expert Opin Biol Ther.*12(6):673-684.

65. Pluhar GE, Grogan PT, Seiler C, Goulart M, Santacruz KS, Carlson C, et al. (2010). Anti-tumor immune response correlates with neurological symptoms in a dog with spontaneous astrocytoma treated by gene and vaccine therapy. *Vaccine.*28(19):3371-3378.

66. Thu MS, Najbauer J, Kendall SE, Harutyunyan I, Sangalang N, Gutova M, et al. (2009). Iron labeling and pre-clinical MRI visualization of therapeutic human neural stem cells in a murine glioma model. *PLoS One.*4(9):e7218.

67. Kloss CC, Condomines M, Cartellieri M, Bachmann M, Sadelain M. (2012). Combinatorial antigen recognition with balanced signaling promotes selective tumor eradication by engineered T cells. *Nat Biotechnol.*31(1):71-75.