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The Long and Winding Road: from the high affinity choline uptake site to clinical trials for malignant brain tumors

Pedro R. Lowenstein, MD, PhD and Maria G. Castro, PhD

Department of Neurosurgery, and Department of Cell and Developmental Biology, The University of Michigan, The Medical School, Ann Arbor

Abstract

Malignant brain tumors are one of the most lethal cancers. They originate from glial cells and invade throughout the brain. Current standard of care involves surgical resection, radiotherapy and chemotherapy, and median survival is currently ~14–20 months post-diagnosis. Glioma tumors are highly infiltrative. Given that the brain immune system is deficient in priming systemic immune responses to glioma antigens present within the brain, we proposed to reconstitute the brain immune system to achieve immunological priming from within the brain. Two adenoviral vectors are injected into the resection cavity or remaining tumor. One adenoviral vector expresses the HSV-1 derived thymidine kinase which converts ganciclovir into a compound only cytotoxic to dividing glioma cells. The second adenovirus expresses the cytokine *fms*-like tyrosine kinase 3 ligand (Flt3L). Flt3L differentiates precursors into dendritic cells and acts as a chemokine for dendritic cells. HSV-1/ganciclovir killing of tumor cells releases tumor antigens that are taken up by dendritic cells within the brain tumor microenvironment. Tumor killing also releases HMGB1, a TLR2 agonist that activates dendritic cells. HMGB1 activated dendritic cells, loaded with glioma antigens, migrate to cervical lymph nodes to stimulate a systemic CD8⁺ T cells cytotoxic immune response against glioma. This immune response is specific to glioma tumors, induces immunological memory, and does neither cause brain toxicity nor autoimmune responses. An IND was granted by the FDA on 4/7/2011. A Phase I, first in person, to test whether re-engineering the brain immune system is potentially therapeutic is ongoing.

Keywords

immunotherapy; cancer; gene therapy; brain tumors; Flt3L

To Joe Coyle, “Ask and it will be given; seek and you will find; knock and the door will open.”

Matthew 7:7-8

Baltimore, Hopkins, The Coyle Lab, and all that Jazz...

The son of German Jewish Holocaust survivors, I grew up in Buenos Aires, Argentina, where I completed my M.D. in 1981 and my PhD 1984 (with D. Cardinali). Both degrees were granted by the University of Buenos Aires Medical School. My mentor Dr. Jaime- Etcheverry knew Joe Coyle and convinced him to give me a chance. In October the adventure began.

Arriving in Baltimore was a culture shock. I had only seen glimpses of New York City through the eyes of television series and movies. Dinner invitations for 5pm were confusing as I was used to appetizers at 10pm. Luckily, generous colleagues from The Coyle Lab eased my way into the secrets and delights of the American way of life. Upon arriving one of my biggest challenges turned out to be the English language. I could read and write scientific English, but not communicate.

Additionally, I was not the only one suffering a culture shock. “They” did too! Once my English got better, communication did not improve immediately. Colleagues were baffled by someone who would actually answer lengthily to “How are you doing?” Yet, Randy Blakely, Christine Hohmann, Laure Ory-Lavollee, Mike Robinson, Robert Zaczek’s, Mario Saltarelli, and many others kindly taught me about baseball, football, Stephen Jay Gould, the space program, and become an organized lab citizen.

Coming from Argentina, at the time still a ‘Third World Country’ (now a ‘developing country’), certain items related to scientific work were in short supply. Computers had not yet reached the laboratories by 1984, though they were already being utilized in the USA. Thus, the first time I did an experiment calculating the affinity and pharmacology of hemicholinium binding sites, I carefully drew my results by hand on nice graph paper. When my turn came to show my results I candidly raised my hand drawn graph so that everyone could see it. Following a brief silence, subdued chuckling turned into laughter, as my audience wondered why I drew a graph by hand. I am still thankful to Joe that he did not kick me out—though he must have wondered whether I would ever make it further-, and kindly continued to training me! After this I quickly learned how to present experimental results the American way.

A second anecdote also occurred during a lab meeting. Joe shared with us an unexpectedly large bill from The Welch Medical Library. He thought there had been a mistake, but nevertheless wanted to confirm that it was an error. The lab had a photocopier key was being kept at the library and we would borrow it when needed. In Argentina photocopies were inordinately expensive, and journals were difficult to come by especially before the Internet. Finding a particular paper meant a one hour bus ride only to discover that the particular issue one needed was unavailable, or that one did not have the proper identification to enter the library, or one lacked sufficient hierarchy to do photocopies. Getting a handle on literature was a major challenge. Coming to Hopkins, and obtaining access to The Welch Medical Library containing all volumes of all journals was overwhelming. Being told about the photocopier key was a dream come true. After Joe’s question everyone looked as baffled as Joe, while I suddenly felt a wave of uncomfortable embarrassment rising to my face.

Looking forward while trying to avoid everyone's gaze, I slowly raised my hand and took responsibility for the mountain of photocopies. Everyone laughed and luckily Joe forgave me. All my grant applications used to carry an additional request for photocopying; with the Internet I now just request extra toner.

Whether 'tis nobler in the mind to suffer the slings and arrows of outrageous fortune, or to take arms against a sea of troubles, and by opposing end them?

Shakespeare (Hamlet)

Towards Gene Therapy for Brain Tumors: re-engineering the brain immune system

Malignant Brain Tumors: Glioblastoma Multiforme (GBM), WHO Grade IV

Malignant brain tumors are universally fatal (Omuro & DeAngelis, 2013). Statistics of patient survival go back to the 1930's, where survival was ~6 months post-diagnosis (Molinaro, Wensch, Jenkins, & Eckel-Passow, 2015). In the absence of high quality tumor imaging surgical resection was challenging as tumors were located by their neurological symptoms. Whether complete tumor resection would lead to long term patient survival was challenged by high rates of tumor recurrence and mortality within two years of surgery. In the 40's and 50's survival continued to remain at 6–12 months post-surgery. The idea that resecting brain tissue well beyond the extent of tumors would cure tumors was again tested when Bailey published results of hemispherectomies in glioma patients. Tumors still progressed. It was concluded that malignant glioma tumors invade regions removed from the primary tumor from where they reestablish lethal tumor growth. Introduction of high resolution magnetic resonance imaging (MRI) in the 1970's permitted precise glioma location, radiotherapeutic treatment, and patient follow-up. Earlier detection did not cause significant life extension.

After 2005 the current standard of care was established: surgery (Pannullo, Fraser, Moliterno, Cobb, & Stieg, 2011; Sanai & Berger, 2008), chemotherapy (Pitz, Desai, Grossman, & Blakeley, 2011), and radiotherapy (Rock et al., 2012; Stadlbauer, Buchfelder, Salomonowitz, & Ganslandt, 2010). Temozolomide is now considered the standard chemotherapeutic agent (Field, Jordan, Wen, Rosenthal, & Reardon, 2015; Hart, Garside, Rogers, Stein, & Grant, 2013; Olson, Nayak, Ormond, Wen, & Kalkanis, 2014; Stupp et al., 2005). Radiotherapy also improves survival, and its administration at short times post-surgery is crucial. Surgery is needed to reduce tumor mass and brain swelling to avoid patients' death through brain herniation (Kotsarini, Griffiths, Wilkinson, & Hoggard, 2010). Brain edema is mostly treated with dexamethasone. Median survival in large cohorts of patients is considered to be 14–18 months post-surgery, and in the largest academic centers ~18–24 months. Patients participating in clinical trials also appear to survive longer. This indicates that in the last 80 years survival improved by ~12 months, i.e., less than 5 days per year (deSouza et al., 2016).

In addition there exists strong evidence concerning the extent of tumor resection v. survival. The most comprehensive series of glioma surgery discovered a survival threshold at ~70%

tumor resection; below this threshold there is no survival benefit, and above the threshold survival is proportional to the extent of resection (Mitchell, Ellison, & Mendelow, 2005; Sanai & Berger, 2008). As these data concern a retrospective analysis, a prospective phase III double blinded and randomized clinical trial would further support the strength of these data.

The desperate situation to obtain new treatments for patients suffering from GBM is illustrated by the number of actual clinical trials/patient (trials taken from www.clinicaltrials.gov): for **high grade glioma** it is **143**; ovarian cancer, 111; pancreatic cancer, 42; breast cancer, 31; lung cancer, 28; melanoma, 25; prostate cancer, 15, while the actual frequency of these cancers is: breast cancer > lung cancer > prostate cancer > melanoma > pancreatic cancer > ovarian cancer > GBM, highlighting the zeal to develop new treatments.

New trials explore chemotherapy based on the dissection of the genomic landscape of GBM, chemotherapy active in other cancers that share signaling pathways active in GBM, new delivery methods to cover unresectable tumors, such as the Diffuse Intrinsic Pontine Glioma, an inoperable and very serious glioma in children (Buczkowicz, Bartels, Bouffet, Becher, & Hawkins, 2014; Buczkowicz & Hawkins, 2015; Grimm & Chamberlain, 2013; Jansen, van Vuurden, Vandertop, & Kaspers, 2012), anti-angiogenic agents (i.e., antagonists of VEGF) (Chinot et al., 2014; Fine, 2014; Gilbert et al., 2014; Gilbert, Sulman, & Mehta, 2014), the testing of novel surgical techniques to increase tumor resection with the aid of new imaging technologies (e.g., MRI (Bohman et al., 2010; Kubben et al., 2011), 5-Aminolevulinic acid (5-ALA (Hauser, Kockro, Actor, Sarnthein, & Bernays, 2015; Jaber et al., 2015; Lau et al., 2015)), Raman spectroscopy (Ji et al., 2013), as well as immunization (e.g., dendritic cells primed with unknown or known tumor antigens; with TLR agonists; with heat shock proteins) (Batich, Swartz, & Sampson, 2015; Finocchiaro & Pellegatta, 2014; Reardon et al., 2013; See et al., 2011; Weiss, Weller, & Roth, 2015). In summary, GBM remains one of the most lethal cancers, and the search for effective treatments needs to carry on.

Endogenous Immunotherapy': re-engineering the brain immune system to treat malignant brain tumors

In the treatment of breast, colon, lung or even pancreatic cancer, recent clinical trials have reduced morbidity and increased longevity. In some cases improvements have been small for a single trial, but cumulative over several trials. This has not been the case for GBM, where in spite of reported benefits in early Phase I and II clinical trials, Phase III randomized double-blind trials have almost universally failed to show consistent increased survival (exception being radiotherapy, and temozolomide). Vaccination trials, gene therapy trials, chemotherapy trials, and anti-angiogenic trials have performed poorly in Phase III tests (Khasraw, Ameratunga, Grant, Wheeler, & Pavlakis, 2014).

The brain immune system

The brain displays a set of particular immune responses that differ from those seen in most other organs. The main differences are structural and physiological. At the structural level, it has been known for a long time that the brain parenchyma lacks proper lymphatic channels,

even if the choroid plexus located within the brain ventricles and the meninges of the spinal cord and brain, contain lymphatics and all immune cells. Eventually the brain can drain extracellular fluid through well characterized perivascular channels that eventually lead to lymphatics near the olfactory bulb, and then drain through the cribiform plate to the cervical lymph nodes (Carare, Hawkes, & Weller, 2014; Iliff et al., 2012; Kida, Pantazis, & Weller, 1993; Laman & Weller, 2013; Weller, Engelhardt, & Phillips, 1996; Weller, Kida, & Zhang, 1992; Yang et al., 2013).

At the physiological level, one main difference is that the brain itself can be a target of an immune response (i.e., in multiple sclerosis, or paraneoplastic syndromes), but it is virtually impossible to stimulate a systemic immune response by carefully delivering a particulate insoluble antigen directly into the brain parenchyma. Another important characteristic of the brain immune system is that it actually consists of two immune systems. One immune system—which can be referred to as the ‘ventricular immune system’ - is located within the ventricles, choroid plexi, meninges, and contains all cellular, vascular, and functional elements of the systemic immune system. Injection of any type of antigen into the ‘ventricular immune system’ will prime a systemic immune response.

The second immune system—the ‘parenchymal immune system’ - is located within the brain parenchyma proper. It contains several types of microglia (macrophage like cells), lacks proper lymphatic channels, and upon careful delivery of a non-infectious, particulate antigen a systemic immune response against such antigen will not be primed. Striking results were described by the group of Charles Bangham in London, UK, who showed that careful injection of live influenza virus into the brain parenchyma—influenza virus replicates in the CNS-, caused a major local inflammation, but no systemic immune response was seen until the replicating virus penetrated the ‘ventricular immune system’ (Matyszak & Perry, 1996, 1998; Perry, 2000; Stevenson, Freeman, Bangham, & Hawke, 1997; Stevenson, Hawke, Sloan, & Bangham, 1997).

Lymphocytes that enter the brain will only persist within the CNS if their cognate antigen is engaged; otherwise they leave the brain (Hawke, Stevenson, Freeman, & Bangham, 1998). This demonstrates the existence of immune monitoring of the CNS (e.g., the T cells that enter the brain in search of antigen), and the long term persistence –and potential inflammation and toxicity- if the target antigen is encountered (Hawke, et al., 1998; van Den Pol, Mocarski, Saederup, Vieira, & Meier, 1999). NK cells, which are not antigen specific, are also able to contribute to neuropathology in both stroke (Gan et al., 2014), and in brain tumors (Baker et al., 2014). Immune responses to non-replicating viral vectors can also be induced, yet only following a systemic immunization against the vector, because these vectors are unable to diffuse to the ventricles or exit the CNS. Nevertheless, diffuse brain autoimmunity is never encountered in these experimental paradigms This effect has been described for RAdV, AAV, and lentivirus, and can either block transgene expression or even cause overt neuropathology depending on vector dose and experimental design. (Abordo-Adesida et al., 2005; Byrnes, MacLaren, & Charlton, 1996; Byrnes, Rusby, Wood, & Charlton, 1995; Dewey et al., 1999; Larocque et al., 2010; Peden et al., 2009; Thomas, Birkett, Anozie, Castro, & Lowenstein, 2001; Thomas, Schiedner, Kochanek, Castro, & Lowenstein, 2000, 2001; Zirger et al., 2012).

The sum of the specific immune responses detected in the brain, when compared to immune responses elsewhere, is described as ‘the brain immune privilege’ (Bechmann, Galea, & Perry, 2007; Galea, Bechmann, & Perry, 2007). In summary, the brain can be targeted by a systemic immune response—provided the immunization is done systemically but not within the brain parenchyma-. In addition, antigens injected directly into the brain parenchyma will elicit a transitory innate immune response including release of cytokines and possible local mobilization of microglia, but no systemic immune response. Particulate infectious agents, e.g. an adenoviral vector, injected exclusively into the brain parenchyma will remain there unless a systemic immunization is elicited (Barcia et al., 2007; Byrnes, et al., 1996; Byrnes, et al., 1995; Kajiwar, Byrnes, Charlton, Wood, & Wood, 1997; Thomas, et al., 2000; Thomas, Schiedner, et al., 2001).

Injection of any type of antigen into the ventricular system or the meninges will elicit a systemic immune response against the antigen. Immune characteristics of the brain have important consequences for virally mediated gene therapy. Careful direct injection of viral vectors into the brain parenchyma is predicted to cause a transitory innate immune inflammation, but no systemic immune reaction. Therefore, potentially therapeutic replication-incompetent viral vectors remain within the brain and express encoded therapeutic proteins long term, as would be required for strategies aiming to treat Parkinson’s Disease. For the treatment of malignant brain cancer shorter expression are sufficient given glioma’s faster progression.

Gene immunotherapy for brain tumors

Our approach to eliminate experimental brain tumors was based on the use of HSV1-TK delivered to the brain using Adv, though other vectors had been used (Kaufmann & Chiocca, 2014; Shand et al., 1999). These vectors have a high transduction efficacy in brain tumors, and the combination of HSV1-TK and ganciclovir is accepted to be very powerful. The efficiency of HSV1-TK/ganciclovir was tested in a syngeneic rat model of glioma (Dewey, et al., 1999). CNS1 glioma cells were injected into the striatum of Lewis rats and studied both their survival, and toxicity of the treatment. If the therapy was delivered a short time after implantation of the tumor cells, all animals survive. However, if therapy was delivered at 12 days post implantation when tumors occupied the whole rat striatum, Adv-TK/ganciclovir only protected 20% of treated animals. Thus, depending on tumor size the therapy’s efficiency decreased from 100% to 20% (Ali et al., 2004; Ali et al., 2005). Thus, directly cytotoxic therapies that necessitate the transduction of ~100% of target cells would be difficult to achieve in human clinical trials. Later clinical trials utilizing strategies that need to transduce a vast majority of tumor cells to be effective therapeutically failed in clinical trials. Direct tumor cytotoxic killing had to be combined with some type of approach that would either increase virus replication within the tumor (Parker, Bauer, Cody, & Markert, 2009), or stimulate the immune system, namely by avoiding the limitations imposed by the BBB, the particular physiology of brain immunity (i.e., brain immune privilege), and the immunosuppressive nature of the brain tumor microenvironment (Curtin et al., 2009).

Many explanations have been given for the brain's immune privilege. The BBB evolved very early in evolution and is thought to have facilitated the development and growth of neuronal assemblies by maintaining the level of ions within levels permissive to neuronal function. Though the BBB was thought to be responsible for the brain's immune privilege, this is now discredited as the main function of the BBB is to limit the entry of ions, small molecules and antibodies into the brain. Immune cells, and especially activated immune cells, can enter the brain even if no disruption of the BBB is induced. This explains why in multiple sclerosis or paraneoplastic syndromes the priming of immune responses occurs outside the brain. Immune surveillance of the brain further suggests that the low number of immune cells necessary for immune surveillance can also enter the brain. Finally, perivascular macrophages have a slow turnover and are replaced throughout the life of the organism by macrophages from the bone marrow. Activated lymphocytes, including pathogenic lymphocytes, can enter the brain demonstrating that the failure to mount therapeutic immune responses against brain tumors must lie in a failure for brain tumors to stimulate an active and systemic immune response (Stevenson, Freeman, et al., 1997). Further, if an immune response against brain tumors could be induced, activated cytotoxic lymphocytes ought to be able to target glioma cells. A number of mechanisms could be invoked to explain the failure to activate the systemic anti-tumor immune response. A lack of priming from within the brain parenchyma could be explained by a downregulation of immune responses, the inability of antigen containing cells to exit the brain, or, the absence of afferent antigen presenting dendritic cells from the brain parenchyma proper. Therefore, a systemic immunization against brain tumors could be active in inducing a therapeutic anti-glioma immune response (Iliff, et al., 2012; Jessen, Munk, Lundgaard, & Nedergaard, 2015; Kress et al., 2014; Weller, Djuanda, Yow, & Carare, 2009; Weller, et al., 1992).

Various types of systemic anti-glioma immunization have been attempted. Disappointingly the results have not demonstrated consistent anti-glioma immune responses and extension of patient's survival beyond individual cases. Several of these approaches are now in Phase III clinical trials. Though some of these have presented data suggesting an increase in progression free survival, none have been able to demonstrate an unequivocal increase in overall patient survival in Phase III randomized, double blinded, clinical trials, the gold standard in medical treatment. Given the excitement generated by these approaches, reasons for the underperformance of these trials are not yet fully understood (Anguille, Smits, Lion, van Tendeloo, & Berneman, 2014).

Another clinically relevant characteristic of malignant brain tumors, and which is beyond clinical treatment at this time, is the high mutation rate of these tumors (Ceccarelli et al., 2016). This means that the molecular makeup of these tumors at first resection differs significantly from those tumors re-resected at tumor recurrence. A capacity to recognize tumor neo-antigens is a central determinant of tumor immune rejection (Kreiter et al., 2015; Linnemann et al., 2015; Yadav et al., 2014). However immunization performed with pre-identified antigens is likely to allow tumor antigenic escape, as has been detected in clinical trials. Checkpoint inhibitors are now being exploited to re-stimulate endogenous pre-existing anti-tumor immune responses, that have been rendered ineffective due to T cell exhaustion (Larkin et al., 2015; Robert et al., 2015; Romano et al., 2015; Weber et al., 2015).

Re-engineering the brain immune system to treat malignant brain tumors

Many studies including evolutionary comparisons of the evolution of the brain and the immune system suggest that “the brain’s immune privilege” is the consequence of the lack of systemic priming following the injection of particulate, non-infectious antigens into the brain parenchyma (Bechmann, et al., 2007; Galea, et al., 2007; Lowenstein, 2002). At the cellular level the incapacity of the brain to mount a systemic immune response to particulate antigens can be simply explained by the lack of functionally competent afferent antigen presenting dendritic cells. This suggests that the brain lacks a cell that is able to take up antigens, process them and transport them to lymph nodes where antigen is presented to naïve T-cells; microglial cells, astrocytes and other brain cells are unable to fulfill such a role, even if there is still controversy regarding the presence of dendritic cells in the CNS (Bulloch et al., 2008; D’Agostino, Gottfried-Blackmore, Anandasabapathy, & Bulloch, 2012; Lowenstein, 2002).

To overcome these physiological characteristics of brain immune responses we decided to re-engineer the brain immune system. We proposed to recruit dendritic cells to the brain so that immune cells entering the brain tumor microenvironment could sample glioma antigens present therein, in such a way as to reconstitute the immune loops that serve in all other tissues to mount immune responses against infectious agents and tumors (Curtin et al., 2006). We also expected this strategy to induce immunological memory (King, Muhammad, et al., 2008). In this case memory T-cells should identify tumor neo-antigens and mount immune responses against the evolving genomic and antigenic landscape of gliomas (King et al., 2011).

We thus proposed to re-engineer the brain tumor microenvironment to recognize brain glioma antigens to mount a systemic cytotoxic immune response, induce immunological memory, and continue sampling the brain tumor microenvironment to recognize glioma neo-antigens that appear during tumor evolution. To do so, based on the pathophysiological understanding of the physiology and evolution of the brain immune system, we concluded that recruiting dendritic cells, and especially plasmacytoid dendritic cells (pDCs) to the brain could serve to simulate an anti-glioma immune response, and thus be of therapeutic benefit (Curtin, et al., 2006).

Dendritic cells sample tumor antigens and transport them to lymph nodes in order to present these antigens to naïve T cells. To attract pDCs to the brain we constructed an Adv expressing Flt3L, the most powerful inducer of DCs, and a cytokine for pDCs in our experiments. To promote the capacity of pDCs to brain tumor antigens we constructed an Adv expressing HSV1-TK. Upon the systemic administration of ganciclovir glioma cells are killed, releasing antigens in the context of a pro-inflammatory cell death. Antigens are then picked up by dendritic cells which go on to stimulate a systemic immune response (Anguille et al., 2015).

Injections of RAdv/Flt3L on its own into large experimental brain tumors induced an anti-tumor response that was smaller compared to that elicited by RAdv/HSV1-TK (Ali, et al., 2004; Ali, et al., 2005). Importantly, expression of Flt3L by itself within the brain tumor microenvironment attracted cells whose morphology was compatible with that of cDCs

and/or pDCs (Curtin, et al., 2006). These experiments demonstrated that even if Flt3L by itself was able to induce entry of dendritic cells into the brain this was not sufficient to induce tumor rejection. We concluded that there was a factor missing which was necessary for either therapy to succeed and eliminate experimental brain tumors.

To test this hypothesis the combination of the cytotoxic and immune-therapeutic engineering approach was carried out. Only 15–20% of animals implanted with large tumors and treated with RAdv/HSV1-TK showed a beneficial effect. The addition of RAdv/Flt3L increased survival of animals to 70–80%. Thus, RAdv/Flt3L (recruitment of dendritic cells to the tumor) adds a major synergistic effect to RAdv/HSV1-TK (cytotoxicity of glioma cells).

Two further challenges of brain tumors are that (i) they can be multifocal at diagnosis, and (ii) they always recur. We designed experiments to test whether our therapy would treat gliomas both in a model of recurrence and in a model of a multifocal tumor. Multifocal tumors were modeled by injecting tumor cells into both hemispheres, while the combination of therapeutic adenoviruses was injected into only one hemisphere. The treatment protected a high percentage of animals (70%) demonstrating that the combined Adv induced a systemic immune response capable of recognizing, attacking and destroying a second tumor site not targeted by the initial treatment injection (King, Muhammad, et al., 2008).

To model tumor recurrence animals that survive gliomas 60 days following the first treatment were challenged with a second tumor injected into the contralateral brain hemisphere. All animals that survived the initial tumor also survived the second glioma challenge. This strongly suggests that a systemic immune response had indeed been induced following the initial treatment. This was tested in further experiments. Immune contribution to the efficiency of the anti-glioma RAdv/HSV1-TK and RAdv/Flt3L induced response was established in the model of glioma recurrence. In a large set of experiments performed both in rat and mouse models of glioma animals that survived the first tumor and its treatment, were challenged with a contralateral tumor implantation and, were simultaneously injected systemically with antibodies to deplete various populations of immune cells. Depletion of CD4+ and CD8+ T cells abolished the efficiency of the anti-tumor immune response. Depletion of macrophages and other immune cells only had very minor effects on the treatment (King, Muhammad, et al., 2008).

Therefore, the efficiency of combined RAdv/HSV1-TK and RAdv/Flt3L in eliminating large experimental gliomas, multifocal gliomas, and a model of recurrent glioma, in the absence of long time behavioral (King, Kroeger, et al., 2008) or inflammatory side effects (Barcia et al., 2006; Barcia, et al., 2007; Gerdes, Castro, & Lowenstein, 2000; Larocque, et al., 2010; Thomas, Birkett, et al., 2001; Thomas, et al., 2000; Thomas, Schiedner, et al., 2001) strongly indicates the induction of a potentially therapeutic systemic cytotoxic anti-glioma immune response. This treatment thus eliminates primary tumors, multifocal tumors, and upon tumor recurrence—in the absence of any further treatment—secondary tumors are also rejected via a systemic cytotoxic CD4+ and CD8+ T cells

In subsequent experiments we also tested whether the hypothesis concerning the proposed mechanism of action was supported. Indeed, we were able to demonstrate that upon the

intratumoral injection of RAdv/HSV1-TK and RAdv/Flt3l pDCs were recruited to the tumoral microenvironment. Recruited pDCs took up fluorescent microbeads as surrogates for the uptake of tumoral antigens, and were detectable within draining cervical lymph nodes. Subsequently, there is also an increase in the number of circulating antigen-specific CD8⁺ cytotoxic T cells, whose depletion blocked the anti-tumoral effect of the gene/immunotherapy (Candolfi et al., 2012; Curtin, et al., 2006; Curtin, et al., 2009).

At this stage it remained to determine whether any innate immune mechanisms were also necessary for the combined gene/immunotherapy to be effective. Adenoviruses and RAdv are known to be able to stimulate innate immune responses which in the brain include the release of IL8, IL1 α / β , and TNF α , amongst other cytokines. In the brain, these innate immune responses are short lived and are accompanied by cellular activation of microglia and astrocytes. To test the hypothesis that TLR signaling was necessary for our treatment's effectiveness experiments identical to those described above were performed in animals knocked out for various TLRs. Animals lacking TLR2 succumbed to tumor growth, indicating that TLR2 signaling was necessary for the effectiveness of our treatment. The surprising aspect of this finding was the fact that TLRs usually recognize exogenous antigens, rather than tumor antigens. We eventually identified an endogenous ligand of TLR2, HMGB1, that was being released from dying glioma cells. The role of HMGB1 was further elucidated by inhibiting HMGB1 through the administration of polyclonal anti-HMGB1 antibodies, or through the injection of glycyrrhizin, an inhibitor of HMGB1 used in Chinese medicine. Both approaches blunted the therapeutic effect of RAdv/HSV1-TK and RAdv/Flt3l pDCs indicating that release of HMGB1 from dying glioma cells is necessary for the combined therapy to eliminate glioma tumors. TLR2 were then identified on pDCs originating from the bone marrow. The absence of TLR2 from pDCs also eliminated the efficiency of RAdv/HSV1-TK and RAdv/Flt3l pDCs therapy (Candolfi, et al., 2012; Candolfi et al., 2009; Curtin, et al., 2006; Curtin, et al., 2009).

In summary, endogenous re-engineering of the brain immune response as a therapy for deadly malignant brain tumors involves the induction of glioma cell death and the release of HMGB1 in response to RAdv/HSV1-TK/ganciclovir, and the recruitment of pDCs to the glioma microenvironment by RAdv/Flt3L. pDCs then sample the antigens within tumor microenvironment, take up tumor antigens, and upon their activation by HMGB1 acting on TLR2, pDCs migrate to the draining lymph nodes where they induce a systemic CD8⁺ cytotoxic immune response which eliminates glioma growth from the brain.

The holy grail: endogenous immunotherapy trials in human patients suffering from glioblastoma multiforme

It was then decided to move forward with a clinical trial of RAdv/HSV1-TK/ganciclovir and RAdv/Flt3l in patients suffering from malignant brain tumors, namely glioblastoma grade IV (WHO). Following the preparation of clinical grade recombinant adenoviruses, their toxicity testing, and other groundwork, we submitted our application for an IND to the FDA. The letter from the FDA allowing us to proceed with the Phase I Clinical Trial (IND #14574) was received in 2011. The first patient to the trial was recruited at the beginning of 2014. The trial can be located by its identifier, NCT# NCT01811992, at <https://>

clinicaltrials.gov/ct2/show/NCT01811992, an NIH supported website describing all ongoing clinical trials.

So far patients in the first and second cohort have been treated. The trial involves 6 cohorts of 3 patients each, for a total of 18 patients. The trial will hopefully be completed by end of 2017.

Coda

In summary, the checkered flag on the long and winding road from bench to trial may be reached soon, and then we will be closer to knowing whether our new treatment for brain tumors will provide a significant benefit to patient survival.

And where did I spend all the time between Joe's lab and The University of Michigan. Following productive and fun years in Joe's lab I learned neuronal cell culture at NIH (with Hal Gainer, NINDS), electron microscopy of the neocortex at Oxford University, England (with Peter Somogyi, MRC). After my postdoctoral training I moved to independent positions at The University of Dundee, Scotland (as Lecturer in the Department of Anatomy), The University of Wales, Cardiff (as Senior Lecturer in Neuroscience in the Department of Physiology). At the University of Manchester, England, I became Lister Institute Professor of Molecular Medicine and Gene Therapy. In 2001 we were recruited to Cedars-Sinai Medical Center and UCLA, Los Angeles, CA, USA, to establish and direct a Gene Therapeutics Research Institute. During our stay in Los Angeles development and toxicity testing of the RAdv was finalized and the IND eventually submitted to FDA. In 2011 we were recruited to the Department of Neurosurgery at The University of Michigan in Ann Arbor, and in 2013 we opened the clinical trial "Combined Cytotoxic and Immune-Stimulatory Therapy for Glioma", NCT01811992 on *clinicaltrials.gov*.

But it all started with Joseph T. Coyle by opening a door and providing the unique opportunity to get started. For this and for everything else he provided over the many years I will be forever grateful.

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Figure 1.

This T1-weighted axial gadolinium-enhanced magnetic resonance image demonstrates an enhancing tumor situated in the left fronto-parietal region. This image is compatible with a high grade malignant glioma (WHO IV). The tumor can be seen as the white amorphous structure to the right of the figure. The darker center represents the tumor proper, with substantial areas of necrosis, while the whitish shadow indicates the enhancement on MRI. Enhancement on MRI suggests impaired blood brain barrier with leakage of contrast agents into the extravascular spaces, i.e. brain edema.

Figure 2.

Different brain immune compartments determine systemic immune responses to a viral particulate antigen, influenza hemagglutinin. An adenovirus vector expressing influenza hemagglutinin, RAD-CMV-HA, was injected either into the brain parenchyma (upper left box, blue), into the brain ventricle (upper right box, red), or subcutaneously (lower left box, green). ELISPOT was used to quantify the responses to the influenza hemagglutinin. It can be seen that injections into the brain parenchyma (upper left) did not cause detectable systemic immune responses, while these were seen (as in increased number of spots) when virus was injected into the brain ventricles (upper right). Systemic immune responses were also detected when virus was injected subcutaneously (lower left). As a control, a virus expressing an unrelated protein (β -galactosidase) did not induce an immune response to influenza hemagglutinin.

Figure 3.

Schematic illustration of first generation adenoviral vectors (shown above the dotted line), in comparison with the high-capacity helper-dependent adenoviral vectors. Notice that first generation adenoviral vectors retain substantial amounts of viral sequences. Consequently, the packaging limit of the first generation vectors is ~10Kbp, while the high-capacity helper-dependent vectors could carry up to a theoretical value of ~35Kbp. The total size of the adenoviral genome is 36Kbp.

Figure 4.

This illustration demonstrates how glioma tumor size determines the response to the therapeutic vector RADTK + ganciclovir. Syngeneic CNS1 glioma cells were injected into the brains of Lewis rats. A1 shows the size of the tumor at 3 days post-implantation, and B1 shows that if vector therapy is administered at that time 100% of animals are protected from tumor growth. The saline and RAD β gal labels indicate that saline injection or a control vector expressing β -galactosidase protein have no therapeutic effect. A3 indicates that if the same treatments are administered to rats at 10 days post tumor implantation, RADTK + ganciclovir only protect ~15–20% of animals. (Modified from Ali et al., *Cancer Research*, 65:7194–7204, 2005).

Figure 5.

This figure illustrates the effects on tumor progression and animal survival following the addition of RAd-Flt3L. Note that just adding RAd-Flt3L to RAdTK + ganciclovir (as shown in detail in Figure 4) improves long term animal survival from 20% to 77%. The controls for this experiment include saline, RAd0 (an adenovirus vector devoid of transgene), RAd-Flt3L administered alone, and RAd-TK + ganciclovir administered alone. (Modified from Ali et al., *Cancer Research*, 65:7194–7204, 2005, and, Ali et al., *Molecular Therapy*, 10:1071–1081–2004)

Figure 6.

Treatment of multifocal gliomas with RAdTK + ganciclovir and RAd-Flt3L. **A** illustrates the experiments schematically. Tumors were implanted first into a single brain hemisphere. Ten days later implanted tumors were treated with gene therapy, and a new tumor was implanted into the contralateral hemisphere, and then followed by ganciclovir. **B** shows that only treated animals implanted with either a unilateral, or a bilateral tumor survive long term.

• **Clinical trial design: combined dose escalation**

Experimental Cohorts

- .Cohort A= Ad-Flt3L: 1×10^9 iu and Ad-TK: 1×10^{10} iu
- .Cohort B= Ad-Flt3L: 1×10^9 iu and Ad-TK: 1×10^{11} iu
- .Cohort C= Ad-Flt3L: 1×10^{10} iu and Ad-TK: 1×10^{10} iu
- .Cohort D= Ad-Flt3L: 1×10^{10} iu and Ad-TK: 1×10^{11} iu
- .Cohort E= Ad-Flt3L: 1×10^{11} iu and Ad-TK: 1×10^{10} iu
- .Cohort F= Ad-Flt3L: 1×10^{11} iu and Ad-TK: 1×10^{11} iu

		1×10^9 iu	1×10^{10} iu	1×10^{11} iu
		Ad-Flt3L	Ad-Flt3L	Ad-Flt3L
1×10^{10} iu	Ad-TK	Cohort A; n=3	Cohort C; n=3	Cohort E; n=3
1×10^{11} iu	Ad-TK	Cohort B; n=3	Cohort D; n=3	Cohort F; n=3

Figure 7.

Dose-finding Phase I Clinical Trial for the treatment of glioblastoma multiforme. The figure illustrates each of the six experimental cohorts and the amount of each vector which will be injected into the resection cavity of each patient. Below, indicates the combinations of both vectors, and specifically that only two doses of RAd-HSV1-TK will be tested, and three of RAd-Flt3L.

Table 1

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