Virally Mediated Immunotherapy for Brain Tumors

Pankaj K. Agarwalla, MSt[a], Zachary R. Barnard, MS[b], William T. Curry Jr, MD[b,c,*]

KEYWORDS

• Glioblastoma • Immunotherapy • Oncolytic virus
• Brain cancer • Immunosuppression • Gene therapy

Brain tumors are a leading cause of mortality and morbidity in the United States. Malignant brain tumors occur in approximately 80,000 adults.[1] Furthermore, the average 5-year survival rate for malignant brain tumors across all ages and races is approximately 30% and has remained relatively static over the past few decades, showing the need for continued research and progress in brain tumor therapy.[1] Improved techniques in molecular biology have allowed expansion in understanding of tumor genetics and have permitted viral engineering and the anticancer therapeutic use of viruses as directly cytotoxic agents and as gene vectors. Preclinical models have shown promising antitumor effects, and generation of clinical grade vectors is feasible. In parallel to these developments, better understanding of antitumor immunity has been accompanied by progress in cancer immunotherapy, the goal of which is to stimulate host rejection of a growing tumor. This article reviews the intersection between the use of viral therapy and immunotherapy in the treatment of malignant gliomas. Each approach shows great promise on its own and in combined or integrated forms.

This article initially reviews the fundamental concepts of viral therapy for brain tumors and traces how these have developed. Next, how viruses, wild-type and oncolytic, interact with the immune system is examined, followed by how immunomodulation, both positively and negatively, can augment antitumor effects of viruses. The final sections briefly review how the immune-modifying capacity of an oncolytic virus can be used to enhance a standard immunotherapy vaccine approach.

VIRAL THERAPY FOR BRAIN TUMORS

Viral therapy for brain tumors can be considered in two main categories: (1) the use of replication-defective viruses, which do not multiply or propagate progeny at the site of inoculation, and (2) the use of replication-selective viruses, which divide in tumor cells and lyse them (oncolysis), and whose progeny infect and kill neighboring cells, continuing the cycle.[2] Although these approaches have yielded promising data in preclinical studies, more recent work, particularly in combination with immunotherapy, has focused on the use of oncolytic (replicating) viruses. The therapeutic effect of replication-defective viral vectors is not produced through direct killing, but rather through the expression of transgenes.

The types of genes inserted and expressed can be divided into five categories based on mechanism of purpose[2,3]:

[a] Brain Tumor Immunotherapy Laboratory, Department of Neurosurgery, Massachusetts General Hospital/Harvard Medical School, Boston, MA, USA
[b] Department of Neurosurgery, Stephen E. and Catherine Pappas Center for Neuro-oncology, Massachusetts General Hospital, 55 Fruit Street/Y9E, Boston, MA 02114, USA
[c] Department of Neurosurgery, Harvard Medical School, Boston, MA, USA
* Corresponding author. Department of Neurosurgery, Stephen E. and Catherine Pappas Center for Neuro-oncology, Massachusetts General Hospital, 55 Fruit Street/Y9E, Boston, MA 02114, USA.
E-mail address: wcurry@partners.org (W. T. Curry).

Neurosurg Clin N Am 21 (2010) 167–179
doi:10.1016/j.nec.2009.08.013
1042-3680/09/$ – see front matter

1. Correction of genetic defects in cancer, such as replacement of wild-type *p53* in glioma, which often contains *p53* mutations.
2. Expression of antiangiogenic gene products, such as decoy vascular endothelial growth factor receptors.
3. Immune-modifying genes, particularly cytokines, designed to stimulate the immune system.
4. Drug resistance–modifying genes that help prevent the development of resistance to commonly used chemotherapeutics.
5. Prodrug-activating enzymes that enhance chemotherapeutic targeting of tumors.

Challenges in treating malignant brain tumors with replication-defective viruses have included delivering the agent to a sufficient number of cells within a tumor and in achieving adequate viral titers. For example, retrovirus was initially a commonly used vector in cancer gene therapy models because it infects actively mitotic cells (eg, tumor cells and tumor-associated vascular endothelium).

To increase the intratumoral retroviral load, vector-producing cells (VPCs), typically fibroblasts, have been infected with retrovirus and implanted intratumorally, with the goal of generating higher and effective titers in the tumor microenvironment. However, initial studies showed very little spread of the virus through the tumor, with activity limited to a small zone at the injection site.[4] More recent advances, particularly with alternative vectors such as adenovirus and lentivirus, have streamlined this approach, but significant limitations in transduction efficiency remain.[5] Contributing factors include a lack of expression on many tumors of the adenovirus receptor (eg, Coxsackie adenovirus receptor [CAR]) and the natural host immune response against the vectors, as is discussed later.[6]

In contrast, oncolytic viruses infect and kill tumor cells, selectively allowing spread of viral progeny into adjacent tumor cells.[2] In 1991, Martuza and colleagues[7] showed that a genetically engineered herpes simplex virus type 1(HSV-1) effectively and safely treated xenografted glioma tumors in mice. Since then, several oncolytic viruses have been studied and used in preclinical models and patients who have gliomas. These viruses are modified to replicate selectively in tumor cells, conferring specificity and safety.

Although their principal effect may be through direct oncolysis, oncolytic viruses may be designed to simultaneously deliver gene products that enhance antitumor activity. The most commonly used oncolytic virus backbones have been adenovirus and HSV-1. Clinical trials in malignant glioma have also been performed with reovirus.[8]

Human adenoviruses belong to a family of nonenveloped DNA viruses, of which serotypes Ad5 and Ad2 have been most commonly studied and used in oncolytic therapy.[9] Adenoviruses bind to cells through a fiber protein and penton base, which mediate cell-binding by way of specific receptors on cells, depending on the serotype.[10] A common receptor for many oncolytic adenoviruses is CAR, which is expressed on human glioma, thereby allowing efficient transduction of these tumor cells.[6] Onyx-015 is an early-developed oncolytic adenoviral vector. Onyx-015 carries a mutation in the *E1B* gene, the 55kD product of which inactivates normal p53 function in cells, thus resulting in a replication-selective oncolytic virus. Initial studies of this mutated virus suggested that it can replicate selectively in tumor cells deficient in p53 function, which is a common feature in many tumors, including gliomas. Further investigation has shown that Onyx-015 tumor selectivity may actually be conferred through tumor substitution of another *E1B* function; that is, late viral mRNA export.[5,11–14]

Delta-24 adenovirus is a well-developed vector currently in clinical trials for glioma (http://clinicaltrials.gov/ct2/show/NCT00805376, accessed June 3, 2009). Delta-24 adenovirus has a 24-bp deletion in the viral gene *E1A*.[11,15] The *E1A* gene product normally stabilizes the Rb protein so that E2F (a transcription factor) is released from E2F–Rb complexes and is free to activate the E2 promoter of the adenovirus, and several other cell-cycle genes.

In cells with normal Rb function, the viral gene *E1A* allows wild-type adenoviral infection and replication. Normal cells are, however, resistant to infection by Delta-24 adenovirus. The *E1A* mutation prevents binding of Rb and the subsequent transcription of viral genes by way of E2F in normal cells. In tumor cells that are defective in Rb and its upstream tumor suppressor, p16, Delta-24 is able to replicate, which is the basis for its tumor cell selectivity. Enhancements related to this basic premise have been made to increase selectivity for glioma cells.[16,17]

HSV-1 is a particularly well-developed asset as an oncolytic virus against brain tumors. HSV-1 is an enveloped, double-stranded DNA virus with several advantages for use in gene and oncolytic therapy: (1) it is a large genome suitable for insertion of foreign genes; (2) its tropism for neural cells; (3) it has a safety mechanism in its sensitivity to agents such as ganciclovir; (4) high titers can be generated; and (5) it does not integrate into the host genome, so it is unlikely to be oncogenic.[2]

The first engineered HSV-1 oncolytic virus had a mutation in the viral thymidine kinase (TK) gene, and showed killing of glioma cells in vitro and in in vivo models of glioma.[7,18] Tumor specificity of this mutant is achieved through the virus' dependence for replication on up-regulated human TK, present in rapidly dividing tumor cells. Viral TK is the substrate for the antiviral efficacy of nucleoside analogs, such as ganciclovir, which interrupts DNA synthesis when incorporated as ganciclovir triphosphate.

HSV-TK is much more efficient than human nucleoside kinases at monophosphorylating ganciclovir, which is subsequently converted by other cellular kinases into the toxic triphosphate form.[2] Ganciclovir, therefore, has specificity against the HSV, which serves as a potential ''shut-off'' mechanism. Transduction of tumor cells with the HSV-TK gene (either through HSV itself or other vectors such as adenovirus) in conjunction with systemic ganciclovir administration is a potent mechanism for tumor killing, with important immune consequences.[2]

The inability to use nucleoside analogs as a safety mechanism for this first-generation viral TK–mutated vector raised significant safety concerns, and, in fact, neurotoxicity was seen at high doses.[18] In an alternative HSV-1 vector, a mutation in the γ_1*34.5* gene was introduced. The γ_1*34.5* gene and its product, ICP34.5, allow normal HSV to subvert the host's shut-off response against infection.

Once infected with HSV, a normal cell will activate protein kinase R (PKR), which in turn phosphorylates and inactivates eukaryotic initiation factor-2α (eIF-2α), thereby shutting down protein synthesis in the normal host cell. ICP34.5 restores protein synthesis through activating protein phosphatase-1α which dephosphorylates and restores eIF-2α function.[18] Mutations in this gene, γ_1*34.5*, result in an HSV that cannot replicate in normal cells, which abrogate protein-synthesis machinery. In malignant cells, however, the activation of PKR is less pronounced, likely because of other mutations, and therefore the ICP34.5-mutant HSV can selectively replicate.

A second mutation was added that confers tumor selectivity—the interruption of the gene for the large subunit of ribonucleotide reductase (ICP6). Double-mutant viruses are theoretically safer because the chances of in vivo recombination and restoration of wild-type HSV phenotype are decreased.[18,19] This second-generation virus, termed *G207*, harbors an insertion of the *Escherichia Coli lacZ* gene into the *ICP6* gene, which also allows immunohistochemical detection of the virus in tumor cells.[19] Using the G207 backbone, a third-generation oncolytic HSV-1, G47Δ, was developed through deleting the viral α*47* gene. α*47* encodes a protein that inhibits transporter associated with antigen presentation (TAP). Wild-type HSV-1 evades immune detection partially through inhibiting TAP in infected cells, thereby interfering with peptide assembly with major histocompatibility complex class I (MHC-I) molecules in the endoplasmic reticulum and preventing antigen presentation.[20]

Recently, bacterial artificial chromosome (BAC)–mediated systems have improved the efficiency of inserting transgenes into the HSV-1 backbones, including with G47Δ, which may have particular relevance for immunotherapy.[21,22]

IMMUNE COMPARTMENTS, BRAIN TUMORS, AND VIRUSES

Recently, understanding of cancer immunology has advanced dramatically. A model illustrating the dynamic and complex relationship between tumor cells and the immune system is that of cancer immunoediting. In this framework, newly transformed cancer cells are initially subject to immunosurveillance, which may result in their elimination. In response, developing tumor cells may acquire mutations that allow them to avoid being fully eliminated, but they remain quiescent, existing in a tenuous equilibrium state, because the immune response prevents uncontrolled proliferation and spread.

Eventually, this balance is tipped in favor of the malignant cells, because they secrete cytokines or cause proliferation of cellular subsets that result in immunosuppression and ultimately immune escape.[23–25] For instance, mechanisms of escape may include secretion of soluble natural killer (NK) cell ligands, recruitment and activation of regulatory T cells, and release of immunosuppressive cytokines.

Human immunity can be broadly divided into innate and adaptive arms. Innate immunity is antigen-nonspecific, provides a crucial barrier against many foreign antigens, and stimulates the development of adaptive immunity.[26] Adaptive immunity is specific to certain antigens and includes direct cell killing through specifically activated cytotoxic lymphocytes, and activation of lymphocytes that generate specific antibody responses. Antigen-presenting cells (APCs) link the innate and adaptive immune systems; the most efficient APC in the dendritic cell (DC). Precursors in the bone marrow give rise to immature DCs, which circulate throughout the body, or are resident in tissues, and act as immunologic sensors for foreign antigen. These immature DCs

express specific Pattern-Recognition Receptors (PRRs) that are capable of recognizing pathogenic epitopes.

The most well-studied group of PRRs are the toll-like receptors (TLRs), which identify and bind to evolutionarily conserved patterns from microbes, including viruses.[27] For example, TLR9 is expressed on the nuclear membrane of APCs and binds unmethylated CpG motifs in DNA, which are often derived from viral infection.[27] TLRs are also found on NK cells, which are important effectors of the innate immune system that can help inhibit viral replication. Once a TLR-bearing immature DC is activated by an immune danger signal, such as mediators released after viral or bacterial infection, it migrates to lymphoid tissue as it matures and activates effectors of the adaptive immune system, including T cells and B cells.[28]

Efficient antigen presentation requires high expression of MHC class I and II molecules. Stimulation of T lymphocytes by APCs also requires surface expression of costimulatory molecules, such as CD80 and CD86, and ligation of cognate receptors (eg, CD28) on the lymphocyte side of the immune synapse. Activated DCs up-regulate expression of MHC and costimulatory molecules and also secrete immunostimulatory cytokines, such as interleukin (IL)-12. Antigen presentation without concomitant costimulation can actually result in immune anergy.[26,28]

The expression of immunostimulatory cytokines in the immune environment is critical for activating the relevant cellular entities, and many immunotherapeutic and oncolytic viral approaches drive cytokine overexpression, creating a more stimulatory milieu to shift the immune system toward an antitumor immune response. Although many cytokines and chemokines are involved, some of the most prominent with respect to the response to oncolytic viruses include granulocyte macrophage colony-stimulating factor (GM-CSF), IL-1, IL-2, IL-4, IL-12, IL-17, IL-18, interferon (IFN)-γ, and FMS-like tyrosine kinase 3 ligand (FLT3L).[26]

IFN-γ, FLT3L, and GM-CSF are important mediators for improving tumor cell antigen presentation and DC function. IL-2 and IL-4 activate T cells, whereas IL-12 and IL-18 shift the T-cell response to a type 1 helper T cell (T_H1) rather than a type 2 helper T cell (T_H2) response.[26] Although the array of cytokines involved in the immune response can be complex, this is one of the primary mechanisms through which oncolytic viral therapy and immunotherapy are intertwined. Genetically modifying oncolytic viruses to carry genes for immune-modulating cytokines may enhance stimulation of antitumor immunity. Viral infection itself results in the cellular elaboration of cytokines, some of which are involved in DC maturation and activation.

Although immunostimulatory activity may be productive for strengthening antitumor immunity, the normal immune response to viruses can significantly inhibit viral spread and subsequent tumor cell lysis. Wild-type HSV, for example, activates strong innate and then adaptive antiviral immune responses. Players in the innate immune system, including complement, NK cells, and type 1 IFNs, are activated early in the infection.[29] The complement cascade activates the immune system against oncolytic viruses; complement depletion, in fact, allows better oncolytic viral replication in animal hosts.[30]

Type 1 IFNs play an important role in activating innate immune responses. Although all cell types can express type I IFNs, plasmacytoid DCs, also activated through toll-like receptors, are the heaviest producers of type 1 IFNs. Oncolytic viruses, including HSV-1 and vesicular stomatitis virus (VSV), clearly induce strong type 1 IFN responses.[31,32] NK cells, stimulated by the postinfectious inflammatory cytokine environment, bind to up-regulated receptors on infected cells and kill them.[33]

Shortly after this innate response to oncolytic viral infection, the adaptive immune system is activated and the developing T_H1 immune response causes activation of CD4+ T lymphocytes and release of IFN-γ and IL-12. T_H1 responses are more likely effective against tumors themselves, although usually the response to viral infection is skewed toward T_H2, with expression of IL-4 and IL-10.[29]

In summary, viral infection causes a strong innate immune response, largely characterized by a type I IFN response, followed by adaptive (T_H1 and T_H2) antiviral responses, which are associated with cell-mediated humoral immunity, and then the virus may be eradicated or driven into latency (**Fig. 1**).

IMMUNITY AND THERAPEUTIC ANTICANCER VIRUSES

Direct viral oncolysis and the virally mediated delivery of immune-modifying transgenes impact the host immune system in separate, related, and complementary ways. Although antiviral immunity is provoked after infection, oncolysis also initiates a cascade through which danger signals are released within the tumor microenvironment at the same time as lethal damage occurs to tumor cells, with exposure of previously hidden tumor-associated antigens in the midst of this inflammatory milieu. Therefore, antitumor immunity accompanies antiviral immunity.

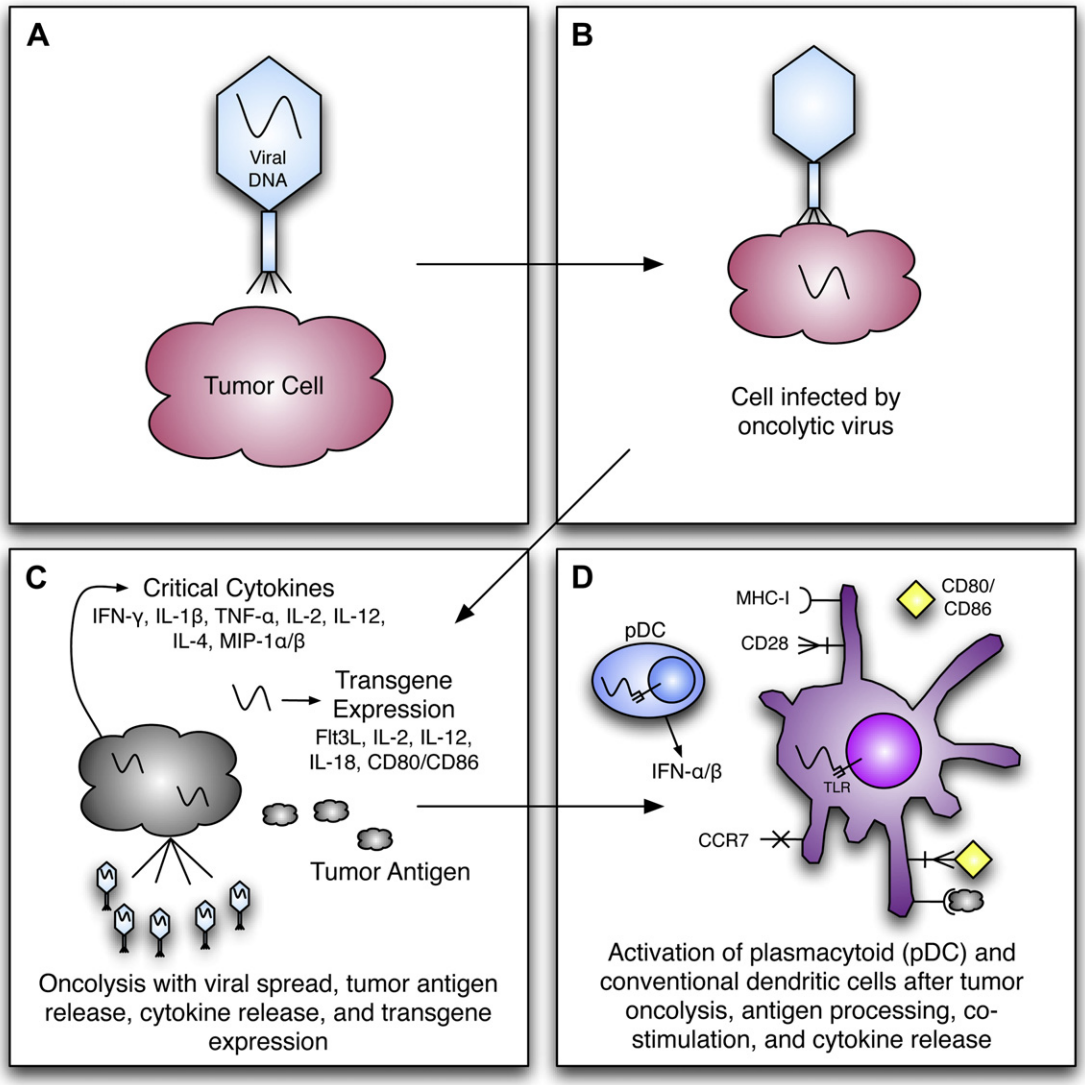

Fig. 1. Mechanism of therapeutic anticancer virus and immunity shows viral oncolysis and subsequent stimulation of dendritic cells. (*A*) Anticancer virus introduced to tumor cell. (*B*) Infection of tumor cell with viral DNA. (*C*) Viral infection of tumor cell permits viral replication and spread. Simultaneously, tumor antigens, immunostimulatory type I interferons, and other cytokines critical to mount an antiviral and antitumor immune response are released. If transgenes are inserted into viral DNA, these products will be expressed, including immunostimulatory molecules. (*D*) Viral infection creates inflammatory milieu of "danger signals" with cytokines, costimulatory molecules (CD80/CD86 with CD28), and viral activation of nuclear toll-like receptors, facilitating major histocompatibility complex I tumor antigen presentation and dendritic cell activation with CCR7 chemokine receptor up-regulation, for example.

Although this vaccine effect of oncolytic viral infection of tumors was a somewhat serendipitous finding, it may be invaluable in extending the therapeutic effect beyond tumor cells that are directly infected and may allow for durability of response.

The following discussion delineates the understanding of how an oncolytic virus can stimulate sustained antitumor immunity and antivector immunity that constrains oncolytic viral efficacy.

Early studies on the model of virally delivered HSV-TK in brain tumors described an interesting phenomenon of enhanced tumor killing beyond the area of infection.[34] Culver and colleagues[34] first showed this enhanced tumor killing in a rat model of glioma and termed it a *bystander effect*, wherein tumor cells that were not transduced with HSV-TK were still killed, perhaps because of contact with neighboring transduced cells.

In a similar rat model of intracranial glioma, Barba and colleagues[35] later showed that long-term survivors after HSV-TK/ganciclovir gene therapy developed long-lasting antitumor immunity, confirmed through protection from subsequent tumor challenge and intratumoral infiltration of inflammatory cells, including macrophages/microglia and CD8+ T cells. This bystander effect, which was previously believed to require cell-to-cell contact,[2] was thereby shown to be at least partly caused by development of antitumor immunity.

Okada and colleagues[36] conducted a series of experiments in which this theory was confirmed. Adenoviral and TK-mediated killing of subcutaneous tumors led to antitumor immune responses, again shown through protection against secondary intracranial tumor challenge. The HSV-TK–mediated killing of subcutaneous glioma cells provided a mechanism for improved antigen exposure, presentation, and processing, and the development of sustainable antitumor immunity.

The generation of antitumor immunity from oncolytic viral infection of tumors is not restricted to the HSV-TK model. Todo and colleagues[37] showed this vaccination effect through infecting subcutaneous N18 murine neuroblastoma tumors with G207 oncolytic HSV-1. G207 infection of a flank tumor resulted in reduced growth in a contralateral uninfected tumor, in a manner that was dependent on CD8+ T lymphocytes. In addition, growth of intracranial tumors was reduced after infection of subcutaneous tumors, showing that this immunity extended to tumors within the central nervous system. In a subsequent study, simultaneous treatment with corticosteroids had no impact on oncolysis but abrogated the antitumor immune effects.[37,38]

To confirm that these immune effects with G207 were tumor-specific, Toda and colleagues[39] used a syngeneic colorectal carcinoma model (CT26 cells in BALB/c mice), which has poor immunogenicity but a known MHC-I restricted antigenic peptide. G207 again was effective in inhibiting growth in injected flank and contralateral tumors. Not only was this effect dependent on CD8+ T lymphocytes but also specificity for the CT26 cell line and its immunodominant peptide was clarified. Similar results were seen in the same study using the M3 syngeneic melanoma model.

In multiple syngeneic, immunocompetent models, G207 infection of tumors drives a specific vaccination effect, strongly linking oncolytic therapy with immunotherapy. This vaccine effect of G207 in these models is comparable to the efficacy of active cellular immunotherapeutic approaches. More recent work shows that this antigen-specific immunity can be generated by G207 infection of intracranial glioma tumors, even when the cells are poorly susceptible to direct oncolysis.[40]

SUPPRESSING ANTIVIRAL IMMUNITY DURING ONCOLYTIC VIRAL THERAPY OF GLIOMA

Suppressing innate immunity allows better oncolytic viral replication and tumor cell killing (**Table 1**). Although blood–brain barrier disruption allows oncolytic HSV-1 to reach brain tumors when

Table 1
Oncolytic viruses and immunosuppressive immunotherapy

Oncolytic Virus Type	Oncolytic Virus Variant	Immunosuppressive Agent	Action/Mechanism
HSV	hrR3	Cyclophosphamide	Reduction in microglia/macrophages and intratumoral IFN-γ[44,45] Depletion of complement and pre-immune IgM[42,43]
HSV	hrR3 and MGH-1	Cobra venom factor and cyclophosphamide	Depletion of complement[30]
VSV	VSV-ΔM51	HDIs and carrier cells	HDIs inhibit IFN-mediated antiviral response in normal cells and carrier cells shield the virus from the immune system for improved local delivery[49,50,52]

Abbreviations: HDI, histone deacetylase inhibitors; HSV, herpes simplex virus; IFN, interferon; VSV, vesicular stomatitis virus.

administered intravascularly, its effect is attenuated by innate immunity, shown in the rat to be related to preimmune IgM.[41] Cyclophosphamide, a strongly immunosuppressive alkylating agent, substantially increases viral efficacy and limits the inactivation of virus by IgM.[42] The initial hypothesis was that preimmune IgM causes aggregation of viral particles, allowing activation of the classical pathway of complement. Cyclophosphamide, however, affects B cells primarily in their production of preimmune IgM and IgG, while leaving the complement system intact.[42]

Ikeda and colleagues[43] examined the effects of complement depletion on the efficacies of oncolytic adenovirus and HSV. After exposing these viruses to rat plasma pretreated with complement-depleting cobra venom factor, they showed improved virus activity, which was further augmented by addition of cyclophosphamide.[43]

Although innate immunoglobulins such as IgM and their resulting activation of the complement cascade might provide an initial immune response to oncolytic viruses, other effectors of the innate immune system, including natural killer cells and cells of monocyte lineage (macrophages/microglia), play a significant role, particularly in the brain.

To investigate the mechanism of cyclophosphamide and its enhancement of oncolytic viral therapy, Fulci and colleagues[44] studied a rat model of glioma with intratumoral injection of oncolytic HSV-1 and showed a rapid increase in the number of intratumoral NK cells, intratumoral IFN-γ, and intratumoral microglia and macrophages. Pretreatment with cyclophosphamide significantly reduced infiltration of these innate immune effectors, allowing for increased oncolytic virus replication and spread. In later work, both peripheral macrophages and brain-resident microglia were depleted from rodents to confirm that these innate immune effectors significantly decreased the titer of oncolytic virus in the brain (**Fig. 2**).[45]

VSV is another oncolytic virus whose efficacy is impacted by innate immunity. Although primarily studied in tumor models other than glioma, oncolytic VSV shows the importance of innate immunity in shaping the activity of therapeutic viruses. VSV is a single-stranded, negative-sense RNA virus that is neurotropic and very sensitive to the host type-1 IFN response.[46] In fact, type-1 interferons are primarily responsible for preventing central nervous system VSV infection from progressing to encephalitis.[47] Cancer cell selectivity is conferred, therefore, by the fact that several malignances are deficient in their interferon response to viral infection.[46] An attenuated version of wild-type VSV is safer and more selective, because normal cells fully arrest viral replication, whereas malignant cells are unable to do so.[48] One attenuated VSV has an insertion in the gene for the viral matrix (M) protein and is denoted *VSV-ΔM51*.[49] One of the M protein's functions is to block intracellular interferon mRNA; a mutated M protein allows restoration of the normal type-1 IFN response of infected cells.

Using this attenuated virus, Lun and colleagues[49] showed significant tumor inhibition against 14 human glioma cell lines in vivo. Systemic delivery of VSV-ΔM51 prolonged survival against orthotopic U87 invasive glioma in nude mice.[49] Significant neurotoxicity from inflammation was seen when the virus was delivered intracranially, suggesting that further engineering may be necessary to control the innate immune response for safer and more selective VSV replication. In response, the investigators treated colon and breast cancer cell lines in nude mice with VSV-ΔM51 and histone deacetylase inhibitors (HDIs). HDIs are known to depress the IFN-based antiviral immune response, suppressing innate immunity.[50]

Power and colleagues[51,52] explored another method to escape antivector immunity with VSV. Nonimmunogenic carrier cells were designed to convey VSV to the tumor site and release the virus there, extending the period of immune evasion in a syngeneic mouse model.

ENHANCING ANTIGLIOMA IMMUNITY THROUGH ONCOLYTIC VIRAL TREATMENT

The potential downside of using immunosuppression to improve viral load and replication is the possible depression of antitumor immune effects. Rather than immunosuppressive modification of the tumor microenvironment, an alternative approach to glioma therapy is to bolster the antitumor immune effects seen with oncolytic viral infection (**Table 2**). Several variations of this have been used, with the most common approach being insertion of immunostimulatory transgenes or cotreatment with helper viruses engineered to express immune-active agents.

A rat glioma model showed that intratumoral adenoviral (nonlytic) delivery of the immune-stimulatory gene Flt3L was ineffective by itself but caused significantly prolonged survival when combined with an oncolytic viral system (ie, with expression of HSV-TK and systemic administration of ganciclovir).[53] This effect was lost with depletion of CD4+ T lymphocytes or macrophages, but depletion of CD8+ cells or NK cells had no effect.

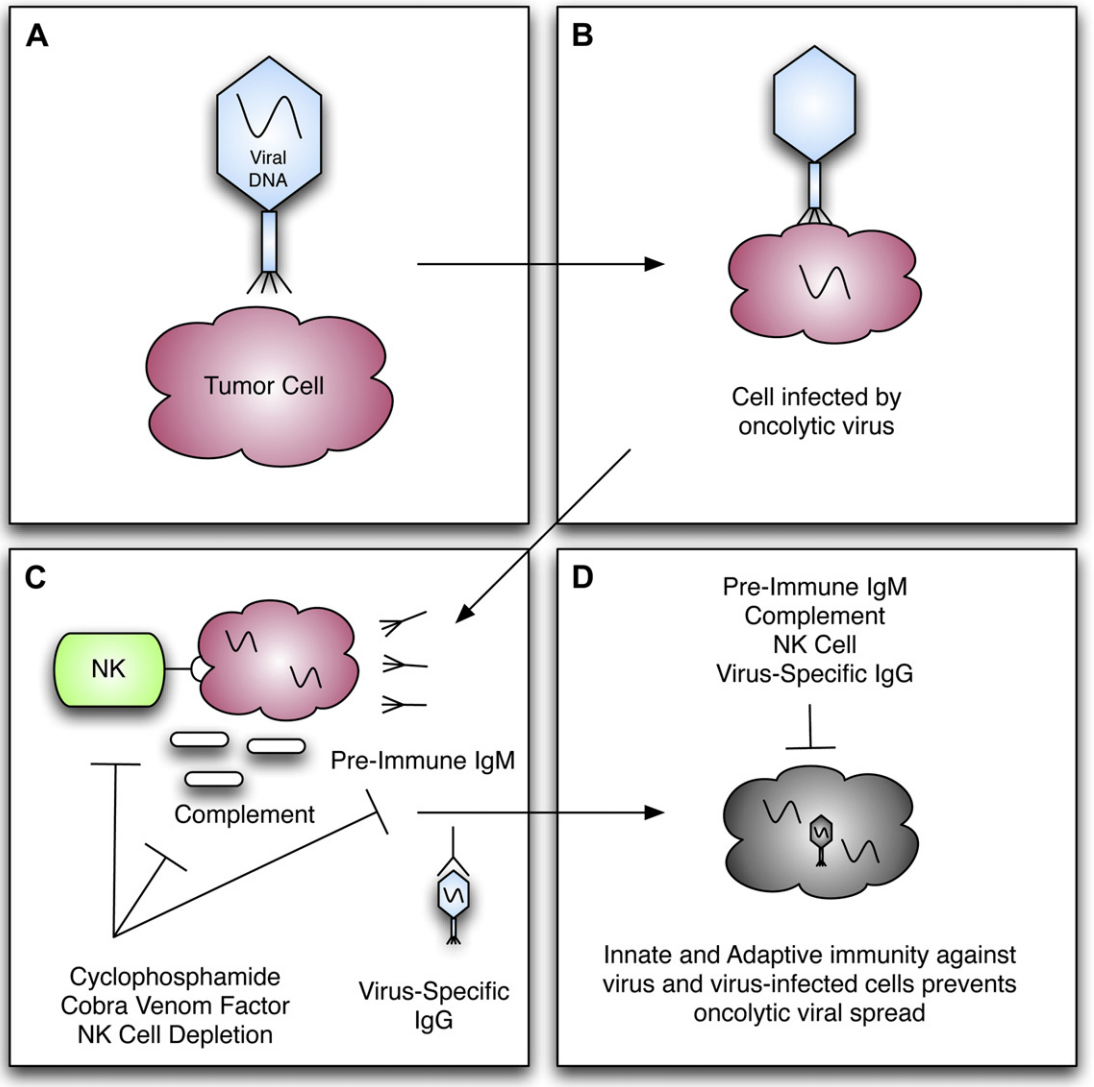

Fig. 2. Innate and adaptive immunity act to suppress therapeutic anticancer viral infection and replication. (*A*) Anticancer virus introduced to tumor cell. (*B*) Infection of tumor cell with viral DNA. (*C*) Strategies to limit antiviral immune response include cyclophosphamide, cobra venom factor, and natural killer cell depletion. (*D*) Without specific immunosuppressive strategies to allow viral replication and oncolysis, natural killer cells, complement, preimmune IgM, and virus-specific IgG act in concert to limit anticancer viral infection and replication.

A likely mechanism for these effects is Flt3L stimulation of antigen-presenting cells, which may be attracted to the environment by chemokines and other danger signals associated with viral infection and HSV-TK–mediated cell death. DCs are the most important link between the introduction of tumor-associated antigens and the activation of the adaptive immune system.[28] DCs express several receptors for pathogen-associated molecular patterns, including toll-like receptors, which can activate a DC to up-regulate costimulatory molecules, such as CD80 and CD86 (B7-1 and B7-2, respectively), that assist in activating an immune response.

At the heart of combination oncolytic viral therapy and immunotherapy is the goal of augmenting this natural DC activation and function, which can be achieved not only using viral particles, which activate toll-like receptors, but also using tumor cell necrotic and apoptotic death, associated with release of antigens for processing by APCs. Oncolytic viruses that express cytokines

Table 2
Oncolytic virus and immunostimulatory immunotherapy

Oncolytic Virus Type	Oncolytic Virus Variant	Immunostimulatory Agent	Action/Mechanism
Adeno	Ad5	FLT3L and HSV-TK	FLT3L stimulates antigen presentation by DCs and HSV-TK with systemic ganciclovir eradicates tumor with additional bystander effect[53,54]
Adeno	Ad5	IFN-alpha and DCs	IFN-alpha and DCs were injected intratumorally, which helps present antigen derives from oncolytic virus killing[71]
HSV	MGH-1-BAC	Soluble B7-1 (CD80), IL-12, IL-18	B7-1 improves costimulation, whereas IL-12 and IL-18 stimulate T-cell–based adaptive immunity[22]
HSV	G47Δ-BAC	Soluble B7-1 (CD80) and IL-18	Improved costimulation and T-cell adaptive immunity[21]
HSV	G47Δ	Intratumoral DC injection	Improved presentation of antigen by DCs after tumor cell lysis by oncolytic virus[56]
Vaccinia	rVV	IL-2 and IL-12	IL-2 stimulates expansion of cytotoxic T cells and IL-12 enhances NK cells and T-cell–based adaptive immunity[72,73]
VSV	VSV-ΔM51	DCs infected by VSV-ΔM51	DCs are infected with VSV-ΔM51 and the virus itself is used as an immunostimulatory adjuvant for costimulation with antigen presentation[49,74]
NDV	NDV	Autologous glioma vaccine infected with NDV	NDV infection of glioma vaccine functions as immune adjuvant to elicit interferon response[58]

Abbreviations: DC, dendritic cell; FLT3L, FMS-like tyrosine kinase 3 ligand; HSV, herpes simplex virus; IFN, interferon; IL, interleukin; NDV, Newcastle disease virus; NK, natural killer; TK, thymidine kinase; VSV, vesicular stomatitis virus.

such as Flt3L, which helps maturation of DCs, further augment the stimulation of antitumor immunity against tumors. Ultimately, this therapy can generate a powerful and effective antitumor response against intracranial glioma.[53]

To simulate invasive and diffusely infiltrating human malignant glioma, the same group used this Flt3L/TK treatment system in a multifocal glioma model.[54] Brain tumors that were not directly inoculated with virus were controlled, confirming that distant, infiltrating glioma cells can be tracked by the effectors of oncolytic gene therapy.

Consistent with this approach, other transgenes whose products enhance antigen presentation have been used to arm oncolytic viruses. In an immunocompetent mouse model of neuroblastoma, oncolytic HSV-1 was modified through a BAC system to express IL-18 and soluble B7-1 (CD80). IL-18 is an important cytokine that induces

the release of IFN-γ, which helps stimulate T-cell immunity. Soluble B7-1 is an important costimulatory molecule that binds to CD28 on T lymphocytes to activate the immune response.[21] Combined expression of IL-18 and release of this soluble B7-1 showed significant efficacy against prostate cancer and neuroblastoma tumors.[21] The capacity for engineering viral vectors with multiple complementary immunostimulatory genes was shown through the triple arming of oncolytic HSV-1 with genes for IL-12, IL-18, and soluble B7-1.[22]

The intra- or peritumoral presence of DCs is associated with improved survival in some human cancers. Oncolytic virus infection itself may increase the number of DCs in the tumor environment.[55] Likewise, viral expression of chemokines or Flt3L further increases the number of intratumoral DCs. We have shown that combining intratumoral injection of oncolytic HSV-1 with direct injection of immature DCs establishes powerful curative immunity in mice with subcutaneous tumors.[56] This model has a specific requirement for immature DCs, because injecting mature cells does not augment the effects of virus alone. This research suggests that oncolytic HSV-1 infection of tumors provides the antigenic material for processing by immature DCs and the activating signals required for DC maturation and subsequent migration and antigen presentation in lymph nodes.

Newcastle disease virus (NDV) has been shown to be safe and feasible for use in patients who have recurrent malignant glioma.[57] NDV is an oncolytic paramyxovirus that replicates selectively in tumor cells. Cellular response to infection by NDV is characterized by up-regulated expression of immune danger signals, such as double-stranded RNA, IFNs, and chemokines. This property has been exploited in an autologous tumor cell vaccination clinical trial. Glioma cells were harvested at surgery and infected with NDV, before being injected intradermally into patients.[58]

Results of this phase I trial were promising, because biologic activity was seen in essentially every patient, partly shown through positive delayed-type hypersensitivity reactions. Later preclinical work in a rat model used active specific immunization with NDV–glioma vaccine plus transforming growth factor (TGF)-β antisense oligonucleotides in the form of nanoparticles to show increased survival in treated rats and an increase in effector immune cells.[59]

In something of a twist, the immunostimulatory properties of an oncolytic virus were used for ex vivo modification of a tumor vaccine, promoting better in situ immune recognition and activation.

As oncolytic viral therapy and immunotherapy become confluent, one can envision scenarios for specific targeting of various aspects of the immune system. For instance, the neuroblastoma microenvironment is particularly suppressive to DC maturation.[60] The combination of intratumoral oncolytic HSV-1 and intratumoral injection of ex vivo–generated immature DCs may be particularly well suited for these tumors. In the case of glioma, the proportion of regulatory T cells (expressing the transcription factor *Foxp3*) and high expression of TGF-β contribute to tumor-derived immunosuppression, and, therefore, combining oncolytic viral infection with T-regulatory cell depletion or targeting would be rational.[61] These areas are being actively studied, and this type of work is already being translated into clinical applications. In a small trial of patients who had recurrent glioblastoma, gene therapy with HSV-TK and IL-2 was shown to be safe and feasible, and achieved objective tumor responses.[62]

IMMUNOTHERAPEUTIC TARGETING OF VIRAL ANTIGENS AS THERAPY

This article has focused on the use of viruses as vectors for gene therapy or as oncolytic agents. Human cytomegalovirus (HCMV) is a herpes virus that seems to be highly expressed in gliomas, perhaps because of a natural tropism for glial cells.[63] In addition, HCMV gene products have been implicated in the disruption of cellular pathways that could lead to cellular transformation.[64–66] HCMV viral DNA was also noted to be shed in the blood of patients who have malignant glioma.[67]

In one study involving vaccinations of DCs pulsed with autologous tumor lysate, a patient who had glioblastoma developed a strong CD8+ T-cell response to the pp65 epitope of HCMV, which was found to be expressed in the patient's tumor.[68] Ongoing clinical studies involve vaccinating malignant glioma patients with DCs pulsed with tumor antigens (http://clinicaltrials.gov/ct2/show/NCT00639639).

SUMMARY

Although much remains to be learned about the mechanisms, virus-mediated death of tumor cells seems to be associated with development of real antitumor immunity. From an immunotherapy point of view, viral vectors were initially considered carriers of immunostimulatory cytokines, with the goal of activating better tumor cell expression of MHC, activating T lymphocytes in the tumor milieu, or attracting circulating immune cells into

the tumor. Through immunoediting, cancer cells have effectively hidden themselves from immune detection.

Effective immunotherapy requires two achievements: (1) tumor-associated antigens must be exposed or unmasked and (2) negative immune regulation must be overcome. Oncolytic viral infection has promise as cancer therapy based on viral killing alone; furthermore, virus-associated cell death may provide the charge to the system required for initiating an antitumor immune response that has specificity and durability. In a clinical scenario, this immune activity itself is unlikely to cure advanced malignancies; however, if intelligently integrated among other therapies that may be synergistic (eg, low-dose chemotherapy, VEGF blockade, other immunotherapies), clinical benefit may be realized. Experts continue to explore combining oncolytic virus infection of tumors with other therapies in preclinical glioma models, with the specific intent of maximizing antitumor immunity.

Although pretreatment with immunosuppressive agents such as cyclophosphamide would seem to impair postviral inoculation antitumor immunity, this may not true, and increasing intratumoral viral spread is a rational goal. Low-dose cyclophosphamide may act as an immune adjuvant, perhaps because of selective suppression of regulatory T lymphocytes.[69] If timed properly, vaccination efforts may benefit from some degree of myeloablation, because effector cells reconstitute at a different pace than do regulatory cells.[70] Clearly, details of dose, schedule, and agent must be worked out, but a strategy that will optimize viral replication without compromising postinfectious antiglioma immunity is envisioned. Clinical trials designed to address these issues are critical to maintaining momentum toward these goals.

REFERENCES

1. CBTRUS. Statistical report: primary brain tumors in the United States 2000–2004. Chicago: Central Brain Tumor Registry of the United States; 2008.
2. Chiocca EA, Aghi M, Fulci G. Viral therapy for glioblastoma. Cancer J 2003;9:167–79.
3. Selznick LA, Shamji MF, Fecci P, et al. Molecular strategies for the treatment of malignant glioma–genes, viruses, and vaccines. Neurosurg Rev 2008;31:141–55.
4. Rainov NG, Ren H. Gene therapy for human malignant brain tumors. Cancer J 2003;9:180–8.
5. Bischoff JR, Kirn DH, Williams A, et al. An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. Science 1996;274:373–6.
6. Asaoka K, Tada M, Sawamura Y, et al. Dependence of efficient adenoviral gene delivery in malignant glioma cells on the expression levels of the Coxsackievirus and adenovirus receptor. J Neurosurg 2000; 92:1002–8.
7. Martuza RL, Malick A, Markert JM, et al. Experimental therapy of human glioma by means of a genetically engineered virus mutant. Science 1991;252:854–6.
8. Forsyth P, Roldan G, George D, et al. A phase I trial of intratumoral administration of reovirus in patients with histologically confirmed recurrent malignant gliomas. Mol Ther 2008;16:627–32.
9. Vecil GG, Lang FF. Clinical trials of adenoviruses in brain tumors: a review of Ad-p53 and oncolytic adenoviruses. J Neurooncol 2003;65:237–46.
10. Cattaneo R, Miest T, Shashkova EV, et al. Reprogrammed viruses as cancer therapeutics: targeted, armed and shielded. Nat Rev Microbiol 2008;6: 529–40.
11. Chiocca EA. Oncolytic viruses. Nat Rev Cancer 2002;2:938–50.
12. Geoerger B, Grill J, Opolon P, et al. Oncolytic activity of the E1B-55 kDa-deleted adenovirus ONYX-015 is independent of cellular p53 status in human malignant glioma xenografts. Cancer Res 2002;62: 764–72.
13. O'Shea CC, Johnson L, Bagus B, et al. Late viral RNA export, rather than p53 inactivation, determines ONYX-015 tumor selectivity. Cancer Cell 2004;6: 611–23.
14. O'Shea CC, Soria C, Bagus B, et al. Heat shock phenocopies E1B-55K late functions and selectively sensitizes refractory tumor cells to ONYX-015 oncolytic viral therapy. Cancer Cell 2005;8:61–74.
15. Fueyo J, Gomez-Manzano C, Alemany R, et al. A mutant oncolytic adenovirus targeting the Rb pathway produces anti-glioma effect in vivo. Oncogene 2000;19:2–12.
16. Heise C, Hermiston T, Johnson L, et al. An adenovirus E1A mutant that demonstrates potent and selective systemic anti-tumoral efficacy. Nat Med 2000;6:1134–9.
17. Johnson L, Shen A, Boyle L, et al. Selectively replicating adenoviruses targeting deregulated E2F activity are potent, systemic antitumor agents. Cancer Cell 2002;1:325–37.
18. Markert JM, Parker JN, Buchsbaum DJ, et al. Oncolytic HSV-1 for the treatment of brain tumours. Herpes 2006;13:66–71.
19. Mineta T, Rabkin SD, Yazaki T, et al. Attenuated multi-mutated herpes simplex virus-1 for the treatment of malignant gliomas. Nat Med 1995;1:938–43.
20. Todo T, Martuza RL, Rabkin SD, et al. Oncolytic herpes simplex virus vector with enhanced MHC class I presentation and tumor cell killing. Proc Natl Acad Sci U S A 2001;98:6396–401.

21. Fukuhara H, Ino Y, Kuroda T, et al. Triple gene-deleted oncolytic herpes simplex virus vector double-armed with interleukin 18 and soluble B7-1 constructed by bacterial artificial chromosome-mediated system. Cancer Res 2005;65:10663–8.
22. Ino Y, Saeki Y, Fukuhara H, et al. Triple combination of oncolytic herpes simplex virus-1 vectors armed with interleukin-12, interleukin-18, or soluble B7-1 results in enhanced antitumor efficacy. Clin Cancer Res 2006;12:643–52.
23. Dunn GP, Bruce AT, Ikeda H, et al. Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol 2002;3:991–8.
24. Dunn GP, Old LJ, Schreiber RD. The immunobiology of cancer immunosurveillance and immunoediting. Immunity 2004;21:137–48.
25. Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. Annu Rev Immunol 2004; 22:329–60.
26. Dranoff G. Cytokines in cancer pathogenesis and cancer therapy. Nat Rev Cancer 2004;4:11–22.
27. Rakoff-Nahoum S, Medzhitov R. Toll-like receptors and cancer. Nat Rev Cancer 2009;9:57–63.
28. Pulendran B. Modulating vaccine responses with dendritic cells and Toll-like receptors. Immunol Rev 2004;199:227–50.
29. Broberg EK, Hukkanen V. Immune response to herpes simplex virus and gamma134.5 deleted HSV vectors. Curr Gene Ther 2005;5:523–30.
30. Wakimoto H, Ikeda K, Abe T, et al. The complement response against an oncolytic virus is species-specific in its activation pathways. Mol Ther 2002; 5:275–82.
31. Balachandran S, Barber GN. Defective translational control facilitates vesicular stomatitis virus oncolysis. Cancer Cell 2004;5:51–65.
32. Balachandran S, Thomas E, Barber GN. A FADD-dependent innate immune mechanism in mammalian cells. Nature 2004;432:401–5.
33. Lanier LL. Evolutionary struggles between NK cells and viruses. Nat Rev Immunol 2008;8:259–68.
34. Culver KW, Ram Z, Wallbridge S, et al. In vivo gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. Science 1992; 256:1550–2.
35. Barba D, Hardin J, Sadelain M, et al. Development of anti-tumor immunity following thymidine kinase-mediated killing of experimental brain tumors. Proc Natl Acad Sci U S A 1994;91:4348–52.
36. Okada T, Shah M, Higginbotham JN, et al. AV.TK-mediated killing of subcutaneous tumors in situ results in effective immunization against established secondary intracranial tumor deposits. Gene Ther 2001;8:1315–22.
37. Todo T, Rabkin SD, Sundaresan P, et al. Systemic antitumor immunity in experimental brain tumor therapy using a multimutated, replication-competent herpes simplex virus. Hum Gene Ther 1999;10: 2741–55.
38. Todo T, Rabkin SD, Chahlavi A, et al. Corticosteroid administration does not affect viral oncolytic activity, but inhibits antitumor immunity in replication-competent herpes simplex virus tumor therapy. Hum Gene Ther 1999;10:2869–78.
39. Toda M, Rabkin SD, Kojima H, et al. Herpes simplex virus as an in situ cancer vaccine for the induction of specific anti-tumor immunity. Hum Gene Ther 1999; 10:385–93.
40. Iizuka Y, Kojima H, Kobata T, et al. Identification of a glioma antigen, GARC-1, using cytotoxic T lymphocytes induced by HSV cancer vaccine. Int J Cancer 2006;118:942–9.
41. Rainov NG, Zimmer C, Chase M, et al. Selective uptake of viral and monocrystalline particles delivered intra-arterially to experimental brain neoplasms. Hum Gene Ther 1995;6:1543–52.
42. Ikeda K, Ichikawa T, Wakimoto H, et al. Oncolytic virus therapy of multiple tumors in the brain requires suppression of innate and elicited antiviral responses. Nat Med 1999;5:881–7.
43. Ikeda K, Wakimoto H, Ichikawa T, et al. Complement depletion facilitates the infection of multiple brain tumors by an intravascular, replication-conditional herpes simplex virus mutant. J Virol 2000;74: 4765–75.
44. Fulci G, Breymann L, Gianni D, et al. Cyclophosphamide enhances glioma virotherapy by inhibiting innate immune responses. Proc Natl Acad Sci U S A 2006;103:12873–8.
45. Fulci G, Dmitrieva N, Gianni D, et al. Depletion of peripheral macrophages and brain microglia increases brain tumor titers of oncolytic viruses. Cancer Res 2007;67:9398–406.
46. Stojdl DF, Lichty B, Knowles S, et al. Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus. Nat Med 2000;6:821–5.
47. Detje CN, Meyer T, Schmidt H, et al. Local type I IFN receptor signaling protects against virus spread within the central nervous system. J Immunol 2009; 182:2297–304.
48. Stojdl DF, Lichty BD, tenOever BR, et al. VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents. Cancer Cell 2003;4:263–75.
49. Lun X, Senger DL, Alain T, et al. Effects of intravenously administered recombinant vesicular stomatitis virus (VSV(deltaM51)) on multifocal and invasive gliomas. J Natl Cancer Inst 2006;98:1546–57.
50. Nguyen TL, Abdelbary H, Arguello M, et al. Chemical targeting of the innate antiviral response by histone deacetylase inhibitors renders refractory cancers sensitive to viral oncolysis. Proc Natl Acad Sci U S A 2008;105:14981–6.

51. Power AT, Bell JC. Taming the Trojan horse: optimizing dynamic carrier cell/oncolytic virus systems for cancer biotherapy. Gene Ther 2008;15:772–9.
52. Power AT, Wang J, Falls TJ, et al. Carrier cell-based delivery of an oncolytic virus circumvents antiviral immunity. Mol Ther 2007;15:123–30.
53. Ali S, King GD, Curtin JF, et al. Combined immunostimulation and conditional cytotoxic gene therapy provide long-term survival in a large glioma model. Cancer Res 2005;65:7194–204.
54. King GD, Muhammad AK, Curtin JF, et al. Flt3L and TK gene therapy eradicate multifocal glioma in a syngeneic glioblastoma model. Neuro Oncol 2008;10:19–31.
55. Benencia F, Courreges MC, Conejo-Garcia JR, et al. HSV oncolytic therapy upregulates interferon-inducible chemokines and recruits immune effector cells in ovarian cancer. Mol Ther 2005;12:789–802.
56. Farrell CJ, Zaupa C, Barnard Z, et al. Combination immunotherapy for tumors via sequential intratumoral injections of oncolytic herpes simplex virus 1 and immature dendritic cells. Clin Cancer Res 2008;14: 7711–6.
57. Freeman AI, Zakay-Rones Z, Gomori JM, et al. Phase I/II trial of intravenous NDV-HUJ oncolytic virus in recurrent glioblastoma multiforme. Mol Ther 2006;13:221–8.
58. Schneider T, Gerhards R, Kirches E, et al. Preliminary results of active specific immunization with modified tumor cell vaccine in glioblastoma multiforme. J Neurooncol 2001;53:39–46.
59. Schneider T, Becker A, Ringe K, et al. Brain tumor therapy by combined vaccination and antisense oligonucleotide delivery with nanoparticles. J Neuroimmunol 2008;195:21–7.
60. Shurin GV, Shurin MR, Bykovskaia S, et al. Neuroblastoma-derived gangliosides inhibit dendritic cell generation and function. Cancer Res 2001;61:363–9.
61. Mitchell DA, Fecci PE, Sampson JH. Immunotherapy of malignant brain tumors. Immunol Rev 2008;222: 70–100.
62. Colombo F, Barzon L, Franchin E, et al. Combined HSV-TK/IL-2 gene therapy in patients with recurrent glioblastoma multiforme: biological and clinical results. Cancer Gene Ther 2005;12:835–48.
63. Cobbs CS, Harkins L, Samanta M, et al. Human cytomegalovirus infection and expression in human malignant glioma. Cancer Res 2002;62:3347–50.
64. Loenen WA, Bruggeman CA, Wiertz EJ. Immune evasion by human cytomegalovirus: lessons in immunology and cell biology. Semin Immunol 2001;13:41–9.
65. Lokensgard JR, Cheeran MC, Gekker G, et al. Human cytomegalovirus replication and modulation of apoptosis in astrocytes. J Hum Virol 1999; 2:91–101.
66. Salvant BS, Fortunato EA, Spector DH. Cell cycle dysregulation by human cytomegalovirus: influence of the cell cycle phase at the time of infection and effects on cyclin transcription. J Virol 1998;72:3729–41.
67. Mitchell DA, Xie W, Schmittling R, et al. Sensitive detection of human cytomegalovirus in tumors and peripheral blood of patients diagnosed with glioblastoma. Neuro Oncol 2008;10:10–8.
68. Prins RM, Cloughesy TF, Liau LM. Cytomegalovirus immunity after vaccination with autologous glioblastoma lysate. N Engl J Med 2008;359: 539–41.
69. Liu J-Y, Wu Y, Zhang X-S, et al. Single administration of low dose cyclophosphamide augments the antitumor effect of dendritic cell vaccine. Cancer Immunol Immunother 2007;56:1597–604.
70. Heimberger AB, Sun W, Hussain SF, et al. Immunological responses in a patient with glioblastoma multiforme treated with sequential courses of temozolomide and immunotherapy: case study. Neuro Oncol 2008;10:98–103.
71. Tsugawa T, Kuwashima N, Sato H, et al. Sequential delivery of interferon-alpha gene and DCs to intracranial gliomas promotes an effective antitumor response. Gene Ther 2004;11:1551–8.
72. Chen B, Timiryasova TM, Andres ML, et al. Evaluation of combined vaccinia virus-mediated antitumor gene therapy with p53, IL-2, and IL-12 in a glioma model. Cancer Gene Ther 2000;7:1437–47.
73. Chen B, Timiryasova TM, Gridley DS, et al. Evaluation of cytokine toxicity induced by vaccinia virus-mediated IL-2 and IL-12 antitumour immunotherapy. Cytokine 2001;15:305–14.
74. Boudreau JE, Bridle BW, Stephenson KB, et al. Recombinant vesicular stomatitis virus transduction of dendritic cells enhances their ability to prime innate and adaptive antitumor immunity. Mol Ther 2009;17: 1465–72.