Multifaceted oncolytic virus therapy for glioblastoma in an immunocompetent cancer stem cell model

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Glioblastoma (World Health Organization grade IV) is an aggressive adult brain tumor that is inevitably fatal despite surgery, radiation, and chemotherapy. Treatment failures are attributed to combinations of cellular heterogeneity, including a subpopulation of often-resistant cancer stem cells, aberrant vasculature, and noteworthy immune suppression. Current preclinical models and treatment strategies do not incorporate or address all these features satisfactorily. Herein, we describe a murine glioblastoma stem cell (GSC) model that recapitulates tumor heterogeneity, invasiveness, vascularity, and immunosuppressive microenvironment in syngeneic immunocompetent mice and should prove useful for a range of therapeutic studies. Using this model, we tested a genetically engineered oncolytic herpes simplex virus that is armed with an immunomodulatory cytokine, interleukin 12 (G47Δ-mIL12). G47Δ-mIL12 infects and replicates similarly to its unarmed oncolytic herpes simplex virus counterpart in mouse 005 GSCs in vitro, whereas in vivo, it significantly enhances survival in syngeneic mice bearing intracerebral 005 tumors. Mechanistically, G47Δ-mIL12 targets not only GSCs but also increases IFN-γ release, inhibits angiogenesis, and reduces the number of regulatory T cells in the tumor. The increased efficacy is dependent upon T cells, but not natural killer cells. Taken together, our findings demonstrate that G47Δ-mIL12 provides a multifaceted approach to targeting GSCs, tumor microenvironment, and the immune system, with resultant therapeutically significant benefit in a stringent glioblastoma model.

antiangiogenesis | immunotherapy | virotherapy

Glioblastoma (GBM), the most common primary brain tumor in adults, is invariably fatal with a median survival of 12–16 mo (1). It is characterized by rapid and invasive growth, heterogeneous morphology, and a complex microenvironment, including local immunosuppression and high degrees of neovascularization (1). Recently, GBM stem cells (GSCs) were isolated from patient tumors and characterized as cells that sustain themselves through self-renewal, differentiate into multiple more mature lineages, and efficiently initiate tumors in mice (2), making them an important and previously unrecognized target for therapy in GBM patients. GBM exhibits multiple mechanisms for evading the immune system that facilitate tumor progression, including systemic as well as local immunosuppression that has been attributed to a preponderance of regulatory T cells (Tregs) (3, 4). The standard of care for GBM (maximal surgical resection, temozolomide administration, irradiation, and sometimes bevacizumab) has only minimally increased survival for patients in the past decades. Therapy typically fails to eradicate GSCs, which contribute to infiltrating and treatment-resistant tumor cells, or induce immune responses. Newer molecular targeted therapies to single pathways are also unlikely to be sufficient for such a complex disease. Thus, novel combination strategies are needed to target not only the bulk tumor cells, but also GSCs and the tumor microenvironment.

It remains unknown whether GSCs can be successfully targeted in vivo by antitumor immune responses, partially due to the prior lack of an immunocompetent syngeneic GSC tumor model. In vitro analysis has demonstrated human GSC immunosuppressive activity, while suggesting susceptibility to T- and natural killer (NK)-cell killing (5–7). In turn, adoptive lymphocyte transfer studies showed that engineered antigen-specific T cells could eliminate human GSC-derived tumors in SCID mice (8, 9), but such immunodeficient tumor models have not been useful for examining the effects of host immune responses. Here, we use a mouse GSC-derived intracerebral tumor model in syngeneic immunocompetent mice. Mouse 005 GSCs were derived from gliomas arising after lentivirus transduction of activated Harvey-Ras (H-Ras) and protein kinase B (Akt) in tumor suppressor gene p53 (T(p53)fl/fl) mice (10).

Oncolytic virotherapy is a distinct antitumor therapeutic modality where replication-competent viruses selectively kill cancer cells, amplifying and spreading throughout the tumor, as well as inducing antitumor immune responses (11). However, insufficient targeting of the complex GBM microenvironment limits its efficacy. In this study, we use a genetically engineered oncolytic herpes simplex virus (oHSV), G47Δ that is efficacious against human GSCs and has been safely administered to patients with recurrent malignant glioma (12, 13). To enhance oHSV efficacy, the virus can be “armed” to express therapeutic transgenes locally within the tumor. Given the need to target the tumor microenvironment, we generated G47Δ-mIL12, expressing murine interleukin 12 (IL-12). IL-12 is a heterodimeric proinflammatory cytokine, which bridges innate and adaptive immunity (14). It strongly promotes the proliferation of activated T and NK cells, stimulates T-helper 1 (Th1) differentiation, and induces IFN (IFN)–γ production, which exerts pleiotropic effects, facilitating T-cell–mediated killing and antiangiogenesis (14). Owing to these diverse effects, IL-12 has been regarded as a master regulator of antitumor immunity. Although we and others have previously tested IL-12-armed oHSV as a therapy in multiple tumor models (15–17), this study is unique in demonstrating the targeting of GSCs, as well as the complex tumor microenvironment in a stringent preclinical GBM model in immunocompetent mice. In this model, G47Δ-mIL12 activity was multifaceted; direct oncolysis, reduction of local Tregs, stimulation of Th1-type immunity, T-cell–mediated survival advantage, and inhibition of tumor angiogenesis. G47Δ-mIL12 thus represents a unique and potent therapy for GBM, with a broad antitumor repertoire.


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morphologically heterogeneous with multinucleated giant cells, infiltrative along white matter tracts, and highly vascularized, as illustrated by CD31\textsuperscript{+} diluted blood vessels and VEGF expression, all hallmarks of GBM (Fig. 1B). The 005 GSCs maintain stem cell characteristics in vivo, as shown by immunohistochemical staining of Nestin and oligodendrocyte lineage transduction factor 2 (Olig2) and limited expression of GFAP (Fig. 1B).

It is well documented that tumors can escape T-cell–mediated elimination by down-regulating molecules essential for immune recognition (18). We observed a lack of cell-surface expression of MHC class I, CD40, and CD80, and CD86 (Fig. 1 C and D) on 005 GSCs, as assessed by flow cytometry, demonstrating their nonimmunogenic phenotype and making them a stringent model for immunotherapy. MHC class I (Kb and Db) expression could be up-regulated by treatment with recombinant mouse IFN-γ for 24 h (Fig. 1C). The 005 GSCs do express high levels of NK-cell receptor ligands, retinoic acid early inducible-1 (Rae-1) (Fig. 1C) and CD155 (Fig. S1E). Thus, 005 GSCs, like human GBMs (4), inherently lack the capacity for antigen presentation and costimulation necessary to stimulate T-cell activation or proliferation.

**G47Δ-mIL12 Can Replicate in and Kill Mouse 005 GSCs in Vitro While Expressing IL-12.** To ensure that G47ΔΔ-mCherry was capable of replicating and spreading in 005 GSCs, as mouse cells are typically not very permissive to oHSV replication (19), we constructed G47ΔΔ-mCherry, expressing the fluorescent reporter mCherry. G47ΔΔ-mCherry spread within and between 005 GSCs spheres after low multiplicity of infection (MOI) (Fig. 2A). To assess therapeutic efficacy of oHSV armed with IL-12, we constructed G47ΔΔ-mIL12 and control G47ΔΔ-E, with no transgene (Fig. S2). G47ΔΔ-E and G47ΔΔ-mIL12 replicated similarly, but not well, in 005 GSCs (Fig. 2B). Both were equally cytotoxic to 005 GSCs in vitro (Fig. 2C), with a similar EC\textsubscript{50} (~0.1 MOI) as that reported for G47Δ in human GSCs (20). IL-12 was released from G47ΔΔ-mIL12–infected 005 GSCs, with secretion increasing to about 60 ng/mL over 96 h, whereas cells infected with G47ΔΔ-E did not produce any detectable IL-12 (Fig. 2D). Furthermore, recombinant IL-12 had no direct cytotoxic effect on 005 GSCs (Fig. S3A). Therefore, expression of IL-12 did not alter the oncolytic activity of G47Δ in vitro (Fig. 2C).

**G47Δ-mIL12 Treatment Inhibits Intracranial Tumor Growth and Extends Survival.** To assess the effects of G47Δ-mIL12 on survival and tumor burden, mice bearing established intracranial 005 GSC-
derived tumors were treated with intratumoral injections of PBS, G47Δ-E, or G47Δ-mIL12 on days 8 and 12 after tumor implantation. G47Δ-E had a modest effect (P < 0.05 versus PBS; Fig. 3A), whereas G47Δ-mIL12 significantly enhanced survival of mice compared with either G47Δ-E (P < 0.005) or PBS (P < 0.001, >50% increase in median survival), with 10% mice surviving long term (Fig. 3A). Neither body weight loss nor other treatment-related toxicities were observed in any of the groups. To quantify tumor burden, cells were extracted from the right tumor-bearing quadrant of the brain 3 d after the second virus treatment (day 15) and GFP+005 cells were sorted by flow cytometry (Fig. 3B). Treatment with G47Δ-E did not alter the percentage of GFP+ cells (7.7% ± 1.9 vs. 8% ± 0.74 in PBS), whereas treatment with G47Δ-mIL12 significantly decreased the percentage obtained (3.3% ± 0.69; P < 0.05; Fig. 3B). After sorting, the GFP+005 cells were tested for their ability to form spheres in vitro (self-renewal).

At limiting dilution, both G47Δ-E and G47Δ-mIL12 treatment significantly decreased the number of spheres per well (36% decrease) as well as the percentage of wells with spheres (25% decrease), indicating a similar effectiveness in reducing the stem cell subpopulation with self-renewal capability (Fig. 3C). Neither G47Δ-E nor G47Δ-mIL12 treatment changed the CD133+ fraction among GFP+ tumor cells compared with PBS, but G47Δ-mIL12 significantly reduced the percentage compared with G47-E (Fig. 3D). The significant decrease in total GFP+ glioma cells with relative maintenance of the CD133+/CD133− ratio in tumors by G47Δ-mIL12 (Fig. 3B and D) indicates that this treatment targets both the CD133−positive and -negative cell populations in vivo. This is important as recent publications have shown that tumorigenic and stem-cell-like cells exist in the CD133−subpopulation (21, 22). Because G47Δ-E did not replicate well in 005 cells in vitro, we examined whether virus replication was occurring in vivo using bioluminescent imaging and G47Δ-U511fluc (Fig. S2), with luciferase expression driven by the HSV-1 U511 late promoter, so that bioluminescence is dependent upon virus replication and the intensity correlates with virus yield (23). Luciferase expression in 005 intracerebral tumors was elevated from days 1–5 and then decreased at day 6 (Fig. 3E), demonstrating virus replication in the established tumors.

**Intratumoral Release of IL12 and IFN-γ in Vivo After G47Δ-mIL12 Treatment.** To understand the mechanisms by which G47Δ-mIL12 enhances survival, we first examined the local release of IL-12 by performing ELISAs on brain tumor homogenates (Fig. 4A). There was a significant increase in IL-12 on the day following the second injection of G47Δ-mIL12, although this was absent by day 6 posttreatment (Fig. 4B). Importantly, this IL-12 production was accompanied by marked release of downstream IFN-γ in the brain tumors on day 1 and, to a lesser extent, day 6 following treatment with G47Δ-mIL12 (Fig. 4C). No systemic IL-12 was detected in the serum, whereas systemic IFN-γ was detected at day 1 posttreatment only with G47Δ-mIL12 (Fig. 4D).

**Antiangiogenic Effect of G47Δ-mIL12 in 005 GSC Brain Tumors.** To determine the vascularity of treated tumors, endothelial cells were stained with anti-CD31 antibody 6 d posttreatment (after the second injection). We observed a prominent reduction in CD31+ blood vessels only in brain tumors treated with G47Δ-mIL12 (Fig. 5A). The antiangiogenic activity of IL-12 has been attributed to IFN-inducible protein 10 [IP-10 or CXCL9/MIP1α] (24), an IFN-γ-activated chemokine that mediates chemotaxis of lymphocytes and angiostatic effects (14). Western blotting of protein lysates from brain tumor homogenates on day 6 posttreatment demonstrated a strong induction of IP-10 in all three tumors treated with G47Δ-mIL12 (Fig. 5B). We also observed a reduction in VEGF expression after G47Δ-mIL12 treatment, another likely contributor to decreased angiogenesis in the brain tumor, although one mouse in the G47Δ-E group also had reduced expression (Fig. 5B). We previously reported that G47Δ inhibited VEGF secretion from U87 glioma cells to a limited extent in vitro (24). In vitro, supernatants from G47Δ-mIL12–infected 005 GSCs inhibited tube formation of human umbilical vein endothelial cells (HUVECs) (Fig. S3B), an indicator of antiangiogenic activity. Taken together, this suggests that arming G47Δ with IL-12 can elicit potent antiangiogenic effects.

**Survival Advantage of G47Δ-mIL12 Is T-Cell Mediated.** IL-12 acts as a “switch” that skews T cells toward Th1-type immune function, which is the desired mode for antitumor activity, and stimulates innate immunity, mediated by NK cells, which can also serve a role in antitumor immune responses. With regards to NK cells, the low expression of MHC I and high expression of NK ligand Rae-1 on 005 GSCs (Fig. 1C) makes them potentially susceptible to NK activity. We also observed an increase in NK tumor infiltration in G47Δ-mIL12–treated mice (Fig. S4). Given these observations, the enhanced efficacy conferred by G47Δ-mIL12 treatment and
The strong immune effects of IL-12, we determined whether NK or T cells might be contributing to the G47Δ-mIL12-mediated survival advantage. For these purposes, C57BL/6 mice bearing 005 GSC intracranial tumors were depleted of NK cells using anti-NK1.1 antibody injected on day 6 after tumor implantation and every 3 d thereafter. Depletion of NK cells in vivo was confirmed by flow cytometry (Fig. S5). However, this had no effect on G47Δ-mIL12’s ability to enhance mouse survival, indicating that NK cells are not critical for the antitumor activity observed with G47Δ-mIL12 (Fig. 6A). Next, to investigate the role of T cells, 005 GSCs were implanted in athymic/nude mice. In the absence of T cells, G47Δ-mIL12 was unable to significantly enhance survival over G47Δ-E (Fig. 6B), indicating a critical role of T cells in the IL-12-mediated antitumor activity.

**G47Δ-mIL12 Decreases Tregs in 005 GSC Brain Tumors.** Given the demonstrated importance of T cells in mediating the survival advantage seen with G47Δ-mIL12, we further characterized the effects of G47Δ-mIL12 on T-cell responses, both locally and systematically. To quantitatively assess T-cell numbers, we performed CD3+ and CD8+ T-cell depletion in vivo was confirmed by flow cytometry (Fig. S5). However, this had no effect on G47Δ-mIL12’s ability to enhance mouse survival, indicating that NK cells are not critical for the antitumor activity observed with G47Δ-mIL12 (Fig. 6A). Next, to investigate the role of T cells, 005 GSCs were implanted in athymic/nude mice. In the absence of T cells, G47Δ-mIL12 was unable to significantly enhance survival over G47Δ-E (Fig. 6B), indicating a critical role of T cells in the IL-12-mediated antitumor activity.

**Discussion**

The capabilities of GBM for GSC-mediated self-renewal, aggressive neoangiogenesis, and marked local and systemic immunosuppression all contribute to current treatment inadequacy and tumor recurrence (25). GSCs, in addition to their key roles in GBM growth, heterogeneity, and resistance to therapy, also contribute directly to the tumor’s immunosuppressive functions (5, 6, 26). Substantial evidence suggests that serum-cultured GBM cell lines do not recapitulate the genotype or phenotype of GBM and therefore have limitations in regards to translating therapies to the clinic (27, 28). Thus, the curative potential of anti-GBM therapies hinges on eradicating GSC in addition to countering beneficial features of the GBM microenvironment. Unfortunately, there has not been an immunocompetent GSC tumor model to evaluate the multitude of therapies being developed for GBM, especially those involving immunotherapy. Here we describe a GSC transplantable model in syngeneic mice. Murtine 005 GSCs retain the important biological and pathophysiological characteristics of human GBM tumors; aggressive, infiltrative, angiogenic, heterogeneously, poorly immunogenic, and immunosuppressive. Therefore, this preclinical model provides a stringent test for investigating a given therapy’s impact on the multiple features of GBM. Using this model, we demonstrate that oHSV G47Δ armed with mIL12 is highly efficacious in treating GBM, and its activity is multifaceted: (i) replicating and killing GSCs, (ii) inducing IFN-γ production, (iii) reducing abnormal vasculature, (iv) ameliorating the immunosuppressed microenvironment, and (v) prolonging survival that is T-cell mediated.

Oncolytic viruses have great potential for the treatment of tumors, using both direct cytotoxic and immune-stimulating mechanisms, and have progressed to phase III clinical trials in patients (11). A number of oHSVs have been developed that have proved safe for use in the brain, including G47Δ (19, 29). G47 has a number of advantages over other oHSVs examined in GBM clinical trials; it replicates in human GSCs (12) and lacks infected cell protein 47 (ICP47), which blocks MHC class I presentation in human cells (19). Thus, G47Δ-infected tumor cells should be more visible to T cells, as we showed with human melanoma (19), particularly in the context of increased local IFN-γ production. It is also possible that this “heightened visibility” could decrease virus replication and efficacy, although OncoVexGM-CSF, with a similar ICP47 deletion, exhibited efficacy and induction of antitumor immune responses in patients (30). Arming oHSV with cytokines, including IL-12, has been shown to enhance their efficacy in mouse models (15–17, 31). However, none of these studies used cancer stem cells or mechanistically characterized the multiple contributions of IL-12.

IL-12 is a 70-kDa heterodimer composed of p35 and p40 subunits, and is one of the most effective immune-stimulatory cytokines, serving as a switch that initiates a path toward Th1-type immunity (14). IL-12 and its downstream Th1 mediator, IFN-γ, have multiple antitumor effects; unmasking the immunogenicity of cancer cells by up-regulating surface MHC class I, as illustrated for 005 GSCs (Fig. I4); inducing Th1 differentiation; stimulating proliferation of NK, NKT, and T cells with induction of cytotoxic effectors; and antiangiogenesis (14). IFN-γ stimulates the production of angiostatic chemokines, such as MIG and IP-10, which
have direct and CXC chemokine receptor (CXCR)3-independent effects on endothelial cells, in addition to their inflammatory effects on lymphocytes (32, 33). Systemic administration of IL-12, however, has been associated with serious toxicity as well as marginal clinical responses in most patients. Recent studies with engineered tumor-targeted T cells have highlighted the role of the tumor microenvironment and being key to mediating antitumor immune effects (34, 35).

Oncolytic HSVs are an excellent platform for localized IL-12 delivery, expression, and immunomodulatory activity, because they are tumor specific and activate a limited antitumor adaptive immune response that IL-12 augments (31). Typically mouse cells are much less permissive for oHSV replication and cytotoxicity than human cells (36), as we found for 005 GSCs; however, G47Δ replication was detected for at least 6 d in vivo. Whereas the in vitro replication and cytotoxicity of G47Δ-mIL12 was similar to the nontransgene vector G47Δ-E, IL-12 expression significantly improved in vivo efficacy in immunocompetent mice. In G47Δ-mIL12–treated tumors, there was a strong induction of IP-10 and down-regulation of proangiogenic VEGF. Accordingly, the high microvessel density (CD31+ blood vessels) in the 005 tumors, part of the histological criteria of World Health Organization grades III and IV gliomas, was significantly reduced after treatment with G47Δ-mIL12. Although oHSV has exhibited antiangiogenic effects in human U87 glioma models (37–40), G47Δ-E did not reduce blood vessel density in the 005 tumor model. It is important to bear in mind that the mechanisms underlying the antitumor activity of antiangiogenic agents are multifaceted and not fully understood (41).

There is a dynamic cross-talk between tumors and the immune system that can regulate tumor growth, especially in GBM, which is highly immunosuppressive (4, 18). GBM employs multiple mechanisms to evade immune recognition, including impairment of MHC class I antigen presentation, down-regulation of costimulatory molecules (CD80 and CD86), and elaboration of immunosuppressive factors (i.e., TGF-β, IL-10, and STAT3) that recruit and expand Tregs, which in turn inhibit Th1 immunity and promote counterproductive Th2 responses (4, 18). Therefore, Tregs serve as an immune threshold that a successful antitumor immune response must overcome. These immunosuppressive features are present in the 005 GSC model. Interestingly, the percentage of Tregs among CD4+ T cells in patient GBMs is 25–40% (42, 43), similar to what we detected in 005 tumors. In keeping with its role as a promoter of Th1 immunity, IL-12 was recently shown to inhibit Treg expansion, in a fashion dependent upon IL12Rβ2 and IFN-γ (44). In this study, G47Δ-mIL12 injection resulted in a significant reduction in intratumoral Tregs, possibly via similar mechanisms given the local increases in IL-12 and IFN-γ. This presumably produced a more permissive environment for antitumor immunity, as evidenced by a T-cell–mediated survival advantage of G47Δ-mIL12 treatment. G47Δ-E also elicited a decline in Tregs (Fig. 6D), an effect seen in melanoma xenografts treated with oHSV expressing GM-CSCF (30), but opposite that seen during peripheral HSV infection of mice, where Tregs were increased, activated, and protective (45). We did not observe any significant changes in T-cell numbers in the brains, spleens, or lymph nodes of mice after treatment, suggesting that the antitumor immune effects were local to the tumor.

The role of NK cells in oncolytic virotherapy is complex, with tumor cytotoxicity balanced by innate antiviral responses (46). Murine 005 GSCs expressed high levels of the NK group 2 member D (NKG2D) ligand Rae-1 and DNAX accessory molecule-1 (DNAM-1) ligand CD155, with no detectable MHC class I, which should make them sensitive to NK recognition and killing. Mouse breast cancer stem cells have also been shown to express Rae-1 and CD155 (47). Furthermore, G47Δ-mIL12 treatment increased NK-cell tumor infiltration (Fig. S5). Therefore, we examined whether NK cell depletion would alter G47Δ-mIL12–mediated efficacy. It did not, indicating that NK cells do not play a critical role in the ability of G47Δ-mIL12 to enhance survival in this model. Rather, the IL-12–mediated benefit was dependent upon T-cell activity as it was completely abrogated in T-cell–deficient athymic mice. This is in contrast to the inhibitory effect of NK cells on oHSV rQNestin34.5 activity in a human glioma cell line xenograft model (48). The poor immunogenicity of 005 cells was likely counteracted by the observed increases in IFN-γ, which could up-regulate MHC I expression on 005 GSCs (Fig. 1A).

Cancer clinical trials using systemic delivery of IL-12 were suspended a decade ago because of dose- and regimen-related toxicities and limited clinical efficacy that was poorly understood (14). This study provides valuable information concerning the potential benefits and mechanisms when IL-12 is instead expressed locally in the context of oncolytic virus infection. In particular, it illustrates the multifaceted activities of G47Δ-mIL12; antiangiogenesis, successful shifts toward Th1-type antitumor immunity, and viral oncolysis. These results are especially salient given the use of a stringent model that was representative of the diversity of subterfuges used by GBM. These promising data and the preclinical safety studies of another oHSV expressing IL-12 (M002) (49) provide a compelling rationale for the clinical translation of IL-12–armed oHSV for the treatment of GBM in patients.

Materials and Methods

A detailed description of the reagents and protocols used in this study is provided in SI Materials and Methods.

Cells and Viruses. Mouse 005 GSCs, GFP-positive, p53−/−, with activated Ras and Akt (10), were cultured in neurosphere media with EGF and FGF2. Armed G47Δ vectors, G47Δ–IL12, G47Δ–E, G47Δ–mCherry, and G47Δ–Us11flu (Fig. S2) were constructed using the flip-flop HSV BAC system (50).
Cell Viability and Virus Replication Assays. Cells were treated as described previously (20). More details are provided in SI Materials and Methods.

Self-Renewal Neurosphere Formation Assay. Three days after second virus infection, brain tumor quadrants were harvested and sorted for GFP+ cells by FACS.Aria. Viable cell (trypan blue excluding) counts were determined and cells resuspended in serum-free media and seeded into 96-well plates at 10 or 30 cells per well. Fifteen days later, the number of neurospheres (diameter, >60 μm) and wells containing neurospheres were counted. Six mice per group and counts were performed in a blinded manner.

In Vivo Studies. Mice bearing intracerebral tumors were intratumorally treated with virus (~5 × 10^6 pfu/μl). In some cases, brain tumor quadrants were excised and cells isolated or tissue was homogenized. NK-cell depletion was performed with anti-NK1.1 antibody (BioXcell). All mouse procedures were approved by the subcommittee on research animal care at Massachusetts General Hospital.

Supporting Information

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SI Materials and Methods

Cell Culture. Mouse 005 glioblastoma stem cells (GSCs) were obtained from I. Verma (Salk Institute, San Diego). These cells are GFP-positive, p53<sup>−/−</sup>, with activated Harvey-Ras V12 (Ras) and protein kinase B (Akt) (1). Cultures of 005 GSCs were maintained as spheres in serum-free media, composed of Advanced DMEM/F12 medium (Life Technologies) with t-glutamine (2 mM; Cellgro), 1% N2 supplement (Life Technologies), heparin (2 μg/mL; Sigma), penicillin-streptomycin (Cellgro), recombinant EGF (20 ng/mL; R&D Systems), and recombinant FGF2 (20 ng/mL; Peprotech). Cells were passaged after dissociating neurospheres with TrypLE Express (Life Technologies). Vero cells, obtained from ATCC, were grown in DMEM with 10% (vol/vol) calf serum.

Viruses. Armed G47Δ vectors were constructed using the flip-flop HSV BAC system (2), Mouse IL-12 cDNA (p35 and p40 separated by two bovine elastin motifs) (3) cloned into the shuttle vector plasmid pVvec92-fmILL2 was inserted into pG47Δ-BAC using Cre recombinase (New England Biolabs). The BAC sequences were removed by cotransfection with a FLP recombinase expressing plasmid into Vero cells, and infectious G47Δ-mILL2 (Fig. S2) isolated and plaque purified (4). G47Δ-E (Fig. S2) and G47Δ-mCherry (Fig. S2) were constructed in the same fashion, except with pVvec91 and pVvec91-mCherry [HSV IE4/5 immediate-early promoter driving mCherry cDNA (provided by Sena Esteses, University of Massachusetts, Worcester, MA)] respectively. G47Δ-mCherry was similarly constructed using pFLS-U511fluc (HSV U511 late promoter driving firefly luciferase), as described in ref. 5.

Cell Viability Assays. Cells were dissociated and seeded into 96-well plates (4,000 cells per well), treated the next day with either G47Δ-E or G47Δ-mILL2 at the indicated multiplicity of infection (MOI), incubated at 37 °C for up to 96 h, and CellTiter96 AQueous One Solution Cell Viability (MTS) Assays (Promega) performed according to the manufacturer’s instructions. Values for virus-infected cells were normalized to those for mock-infected cells (percent cell viability). The experiments were performed in triplicate and repeated at least three times.

Viral Replication Assays. Dissociated GSCs were plated at 8 × 10<sup>4</sup> cells/250 μL in 24-well plates, incubated overnight at 37 °C, and oncolytic HSV (OHSV) was added to the media at a MOI = 0.1. After 1.5 h for virus adsorption, 250 μL per well of media was added, cells were incubated at 37 °C, and harvested with supernatant at indicated time points. After three freeze–thaw cycles and sonication, the titers of infectious virus were determined by plaque assay on Vero cells.

In Vivo Studies. Immunocompetent C57BL/6 (aged 7–9 wk) and athymic mice were obtained from National Cancer Institute, Frederick, MD. All mouse procedures were approved by the subcommittee on research animal care at Massachusetts General Hospital. Dissociated 005 GSCs (2–5 × 10<sup>5</sup> cells) in 3 μL PBS were implanted stereotaxically into the striatum (2.5-mm lateral from Bregma and 3-mm deep) to generate orthotopic intracranial tumors. On days 8 and 12 after tumor implantation, mice were randomly divided into three groups and intratumorally injected (same stereotaxic coordinates) with G47Δ-E or G47Δ-mILL2 (~5 × 10<sup>5</sup> pfu/3 μL) or PBS. Mice were then followed for signs of discomfort or neurological symptoms and euthanized before becoming moribund (survival, n = 10 mice per group).

For quantitative analysis, mice were intratumorally injected with PBS, G47Δ-E, or G47Δ-mILL2 on days 19 and 23 after 005 GSC implantation and then killed 1 and 6 d posttreatment (days 24 and 29). Brain tumor quadrants were excised, minced, and homogenized in 10 units/mL DNAse I for 10 min at 37 °C, triturated, and passed through a 40-μm cell screen, washed, and resuspended in FACS buffer (PBS + 2 mmol/L of EDTA + 0.5% BSA). Spleens and cervical lymph nodes were harvested, minced, and pushed through 70-μm cell screens to create single cell suspensions. Blood lysis was done as needed with mouse RBC lysis buffer (Boston Bioproducts). Excised brain tumor quadrants were homogenized in PBS for ELISA or with RIPA buffer containing a mixture of protease and phosphatase inhibitors (Boston Bioproducts) for protein lysates.

For bioluminescent imaging (BLI) of virus replication, 1 × 10<sup>5</sup> 005 GSCs were intracerebrally implanted in SCID mice and 13 d later G47Δ-U511fluc (1 × 10<sup>9</sup> pfu in 2 μL) was injected into the tumor. At 24, 48, 72, and 144 h postvirus injection mice were anesthetized with 2% isoflurane and BLI was performed after i.p. injection of 4.5 mg β-luciferin (Gold Biotech). Under imaging conditions used (binning, 16 s and exposure, 60 s), the background was below 100 photons/s.

Self-Renewal Neurosphere Formation Assay. Three days after second virus infection, brain tumor quadrants were harvested and sorted for GFP<sup>+</sup> cells by FacsAria. Viable cell (trypan blue excluding) counts were determined, cells resuspended in serum-free media, and seeded into 96-well plates at 10 or 30 cells per well. Fifteen days later, the number of neurospheres (diameter, >60 μm) and wells containing neurospheres were counted. Six mice per group and counts were performed in a blinded manner.

In Vivo NK-Cell Depletion. Mice were intraperitoneally injected with 200 μg anti-NK1.1 antibody (BioXcell) on days 6, 8, and thereafter every 3 d after tumor implantation. NK-cell depletion was confirmed by staining with antibodies to NK1.1 (BD Biosciences), Dx5 (Biolegend), and NKp46 (BD Biosciences), and FACS analysis.

ELISA and Western Blots. Mouse IL-12 and IFN-γ were quantified from cell culture (infected at MOI = 0.1) or brain supernatants using the Quantikine IL-12 and IFN-γ kits (R&D Systems) as per manufacturer’s instructions. Protein concentration in lysates was measured by Bradford assay, and 40 μg of protein loaded onto Pharmingen), VEGF (AbD Serotec), GFAP (Sigma), oligoden- drocyte lineage transcription factor 2 (Olig2) (Abcam), or Nestin (Millipore) followed by incubation with appropriate secondary

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antibodies (Jackson ImmunoResearch). Microvessel density (CD31+ pixels as a percentage of total pixels from five random fields, n = 3 mice per group) was assessed using National Institutes of Health Image J. For immunocytochemistry, neurophores were plated on coverslips coated with fibronectin overnight, fixed with 4% paraformaldehyde, permeabilized with 0.05% Triton, followed by primary antibodies against Nestin, βIII-tubulin (Millipore), GFAP (Sigma), appropriate fluorescently conjugated secondary antibodies, and DAPI.

Immune Cell Isolation and Flow Cytometry. Fluorescently conjugated antibodies to mouse MHC class I molecules H2-Kb and -Db, MHC class II, CD40, CD86, CD80, CD3e (145-2C11), CD4 (L3T4), forkhead box P3 (Foxp3, FJK-16s), CD45, as well as appropriate isotype controls, were obtained from BD Biosciences, and to CD155 and retinoic acid early inducible-1 [Rae-1 (pan-specific)] from R&D Systems, and to mProminen-1 (mCD133 homolog) from Miltenyi Biotec. Cells were spun, counted, and resuspended in FACS buffer, and incubated with antibodies against surface markers for 30 min at 4 °C in the dark. For intracellular Foxp3 staining, cells were then fixed and permeabilized using 1xFix/Perm solution (eBioscience), washed in 1x permeabilization buffer (eBioscience), and stained with antibody for 30 min at 4 °C in the dark. Cells were then washed, resuspended in 1x PBS + 2% FCS + 1% buffered neutral formalin, and analyzed on a LSRII flow cytometer (BD Biosciences). Data were analyzed with BD FlowJo software (Tree Star).

Statistical Analysis. Comparisons of data in cell survival and virus yield assays were performed using a two-tailed Student t test. Survival data were analyzed by Kaplan Meier survival curves, and comparisons determined by log rank test. P values of less than 0.05 were considered significant. Statistical analysis was conducted using Prism software.


Fig. S1. (A) Western blot of SRY-box containing gene 2 (Sox2) and VEGF protein from 005 GSCs and CT2A mouse glioma cell line, both syngeneic to C57BL/6 mice. (B) The sensitivity of 005 GSCs to temozolomide was examined after 4 d using an MTS assay. The EC50 was ~300 μM. (C) 005 GSCs were implanted intracranially in the striatum (St; 2 × 10^4 and 5 × 10^5 cells) and hippocampus (Hippo; 2 × 10^5 cells) (1.5-mm lateral and 2-mm posterior from Bregma and 2.3-mm deep) of C57BL/6 mice. Glioblastoma (GBM) tumors formed in both locations with a median survival of 26 and 31 d in St and 40 d in Hippo. (D) Brains from mice with tumors in St (Left) and Hippo (Right), note hemorrhage (arrows). (E) Expression of CD155 on 005 GSCs by FACS.
Fig. S2. Construction G47Δ-mIL12. Murine IL-12 was inserted into the backbone of G47Δ using the flip-flop HSV-BAC system (2). G47Δ is a third-generation oHSV with deletions in the γ34.5 and α47 genes and an inactivating LacZ insertion into infected cell protein 6 (ICP6) (6). G47Δ-mIL12 contains an IL-12 fusion protein driven by the CMV immediate-early (IE) promoter. G47Δ-E was constructed at the same time using a shuttle plasmid lacking a transgene. G47Δ-mCherry is similar to G47Δ-mIL12 except the transgene is mCherry, which is driven by HSV-1 IE4/5 promoter. G47Δ-Us11fluc contains firefly luciferase driven by the HSV-1 Us11 late promoter.

Fig. S3. (A) Recombinant IL-12 had no cytotoxic effect on 005 mGSCs over a 4-d period (MTS assay). (B) Matrigel-based tube formation of human umbilical vein endothelial cells (HUVECs). HUVECs (Lonza) were grown in endothelial cell growth media (EGM)-2 media supplemented with the bullet kit (Lonza) and maintained in culture for no more than 10 passages. 005 GSCs were infected with oHSV at MOI = 0.8, incubated in EGM-2 media for 24 h and media supernatants collected followed by addition of 1% pooled human gamma globulin (GamaSTAN; Grifols Therapeutics) to neutralize infectious virus. HUVECs (8 × 10^4 cells) were resuspended in the conditioned media, plated into 24-well plates coated with 250 μL of Matrigel (BD Bioscience) for 20–24 h at 37 °C, and tube formation quantified (counting branches in five random fields per well) and imaged at 20×. Experiment was performed in triplicate. Images of representative wells (Left). **P < 0.01 (Right).
Fig. S4. Immunohistochemical staining of brain tumor sections for NK cells, using anti-NK-CD49d (DX5 clone, 1:100; BioLegend), followed by HR-conjugated secondary antibodies (Vector), and DAB straining (Dako) (arrows) on day 6 posttreatment with PBS, G47Δ-E, or G47Δ-mIL12. Histogram of DX5+ cell counts from five random fields, n = 2 per group. G47Δ-E had a slight increase in DX5+ cells over PBS (*P < 0.05), whereas G47Δ-mIL12 treatment significantly increased the number of DX5+ cells over G47Δ-E (**P < 0.01).

Fig. S5. Spleens from mice treated with (Right) or without NK-cell (Left) depletion were processed and (A) stained with anti-NK cell antibodies (Dx5 and NKp46) to ensure depletion. A significant decrease in NK cells (DX5+NKp46+) was observed, as shown by the representative flow cytometry graphs (black bordered quadrants). (B) Staining with anti-T-cell antibodies (CD3, CD4, and CD8). No differences were observed in CD4+ or CD8+ cells (expressed as a percentage of CD3+ cells) in spleens from mice treated either with (Right) or without anti-NK-cell antibody (Left).
Table S1. T-cell populations in the brain tumor, spleen, and cervical lymph nodes at 6 d posttreatment, expressed as an average percentage from three animals per group ± SEM

<table>
<thead>
<tr>
<th>Brain tumor</th>
<th>PBS</th>
<th>G47Δ-E</th>
<th>G47Δ-mIL12</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3⁺</td>
<td>2.8 ± 0.45</td>
<td>4.4 ± 0.81</td>
<td>3.5 ± 0.4</td>
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<tr>
<td>CD4⁺ (% of CD3)</td>
<td>22.03 ± 1.4</td>
<td>26.4 ± 1.94</td>
<td>31.5 ± 3.37</td>
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<tr>
<td>CD8⁺ (% of CD3)</td>
<td>18.77 ± 0.77</td>
<td>19.67 ± 1.65</td>
<td>16.33 ± 1.97</td>
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<tr>
<td>CD4⁺/CD8⁺</td>
<td>1.17 ± 0.05</td>
<td>1.34 ± 0.11</td>
<td>1.93 ± 0.4</td>
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<tr>
<td>CD4⁺CD69⁺</td>
<td>70.6 ± 0.75</td>
<td>71.5 ± 2.57</td>
<td>68.5 ± 2.75</td>
</tr>
<tr>
<td>CD8⁺CD69⁺</td>
<td>45.3 ± 3.78</td>
<td>48.3 ± 5.8</td>
<td>41 ± 4.8</td>
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<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3⁺</td>
<td>33.1 ± 1.1</td>
<td>34.4 ± 0.4</td>
<td>34.7 ± 0.9</td>
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<tr>
<td>CD4⁺ (% of CD3)</td>
<td>44.8 ± 0.6</td>
<td>45.1 ± 0.8</td>
<td>42.9 ± 1.2</td>
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<tr>
<td>CD8⁺ (% of CD3)</td>
<td>36.3 ± 0.5</td>
<td>37.2 ± 0.9</td>
<td>38.3 ± 0.6</td>
</tr>
<tr>
<td>CD4⁺/CD8⁺</td>
<td>1.2 ± 0.03</td>
<td>1.2 ± 0.05</td>
<td>1.1 ± 0.04</td>
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<tr>
<td>CD4⁺CD69⁺</td>
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<td>15.2 ± 0.7</td>
<td>15.6 ± 1.4</td>
</tr>
<tr>
<td>CD8⁺CD69⁺</td>
<td>19.1 ± 1.0</td>
<td>19.4 ± 1.1</td>
<td>15.6 ± 1.4</td>
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<tr>
<td>Cervical lymph nodes</td>
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<td></td>
</tr>
<tr>
<td>CD3⁺</td>
<td>53 ± 2.74</td>
<td>51.8 ± 1.3</td>
<td>46.9 ± 1.5</td>
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<tr>
<td>CD4⁺ (% of CD3)</td>
<td>48.4 ± 1.8</td>
<td>46.1 ± 1.2</td>
<td>43.7 ± 1.8</td>
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<tr>
<td>CD8⁺ (% of CD3)</td>
<td>41.4 ± 1.3</td>
<td>40.9 ± 0.7</td>
<td>42.3 ± 0.8</td>
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<td>CD8⁺CD69⁺</td>
<td>15.7 ± 2.8</td>
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