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Zika virus oncolytic activity requires CD8+ T cells and is boosted by immune checkpoint blockade

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Abstract

Glioblastoma multiforme (GBM) is a fatal human cancer in part because GBM stem cells are resistant to therapy and recurrence is inevitable. Previously, we demonstrated Zika virus (ZIKV) targets GBM stem cells and prevents death of mice with gliomas. Here, we evaluated the immunological basis of ZIKV-mediated protection against GBM. Introduction of ZIKV into the brain tumor increases recruitment of CD8\(^+\) T and myeloid cells to the tumor microenvironment. CD8\(^+\) T cells are required for ZIKV-dependent tumor clearance, as survival benefits are lost with CD8\(^+\) T cell depletion. Moreover, while anti-PD1 antibody therapy alone moderately improves tumor survival, when co-administered with ZIKV, survival increases. ZIKV-mediated tumor clearance also results in durable protection against syngeneic tumor re-challenge, which also depends on CD8\(^+\) T cells. To address safety concerns, we generated an immune-sensitized ZIKV strain, which is effective alone or in combination with immunotherapy. Thus, oncolytic ZIKV treatment can be leveraged by immunotherapies, which may prompt combination treatment paradigms for adult GBM patients.
Introduction

GBM is the most aggressive primary brain tumor, as virtually all patients die within two years of diagnosis (1). Standard treatment includes surgery, radiation, temozolomide chemotherapy, and more recently, adjuvant use of tumor treating fields (2). Despite maximal treatment, most GBMs recur within 6 months, at which time no standard or curative treatment exists. Poor patient outcomes from GBM are multifactorial, including the presence of GBM stem cells (GSCs), which are resistant to radiation treatment and chemotherapy (3-5) and weak anti-tumor immunological responses (6-11).

We and others demonstrated that Zika virus (ZIKV), a flavivirus that emerged in 2015 as a cause of congenital brain anomalies, has specific lytic activity against GSCs (12-17). GSCs share properties with fetal neuronal progenitor cells (18-22). In vivo studies from independent groups validated that oncolytic ZIKV therapy extends survival of glioma bearing mice (13, 15, 17, 23). Although ZIKV is a neurotropic virus in fetuses, it rarely infects the brain or causes neurological disease in adults (24). Thus, oncolytic ZIKV could have therapeutic potential in adult patients with GBM.

Oncolytic viral therapies for treating GBM include the measles virus (25-28), poliovirus (29, 30), adenovirus (31, 32), herpesviruses (33-36), myxoma virus (37, 38) and vesicular stomatitis virus (39). Clinical trials with several of these oncolytic viruses have been reported or are under development for treatment of GBM. However, these therapies do not specifically target GSCs.

GBM is resistant to immunotherapy due, in part, to the immunosuppressive tumor microenvironment (40, 41), which is characterized by T cell dysfunction (42, 43), regulatory T cell–imposed tolerance (6, 44, 45), inadequate antigen presentation (46), and immunosuppressive activity of recruited myeloid suppressor cells (8, 9, 47). In other solid tumors, immune checkpoint blockade targeting inhibitory receptors expressed on T cells such as cytotoxic T lymphocyte-associated protein-4 (CTLA-4) or programmed death-1 (PD-1) elicits
clinical improvement and tumor regression (48-50). However, clinical trials of immune checkpoint blockade in GBM have largely failed (51-55). One small study in recurrent GBM using anti-PD1 immunotherapy prior to tumor resection extended median survival by five months (56), suggesting the timing of the therapy might be important.

Overcoming resistance to immune checkpoint blockade and augmenting immune responses that suppress tumor growth is a therapeutic priority in GBM. In this context, it is notable that ZIKV treatment reduces tumor size and extends survival in mice beyond that expected for its anti-GSC effects. We hypothesized that oncolytic ZIKV treatment of GBM reshapes the immunological microenvironment, which might be leveraged further by immunotherapy. Here, we evaluate the immunological basis of protection mediated by ZIKV therapy and establish ways to enhance its efficacy.
RESULTS

ZIKV increases CD8* T cell infiltration into the tumor bed. We implanted two syngeneic glioma cell models, GL261 and CT2A transduced with a luciferase reporter, by stereotactic injection into the right cerebral hemisphere of eight-week old C57BL6/J mice. After confirming tumor growth using bioluminescence, we randomized mice to intratumoral treatment with either mouse-adapted ZIKV-Dakar strain (10^5 focus-forming units [FFU]) or PBS (57) (Figure 1A). ZIKV treatment increased median survival, and the long-term survival rates increased from ~10% to 63% for GL261 bearing mice and 0% to 37% for CT2A bearing mice (Figure 1B and C).

Histological analysis revealed comparable tumor sizes between the ZIKV and PBS groups at day 14 post tumor implantation (7 days post ZIKV treatment) but a decrease in tumor size one week later at day 21 post tumor implantation (14 days post ZIKV treatment) in response to ZIKV treatment (Figure 1D and E). We also observed infiltration of immune cells in the tumor microenvironment at days 14 and 21 post tumor implantation in animals treated with ZIKV (7 and 14 days post ZIKV treatment) (Figure 1F and G). Analysis of the kinetics of viral replication in the brain revealed that ZIKV RNA was cleared by 14 days post-infection (Figure S1).

We quantified the infiltrating immune cell composition (lymphoid and myeloid cells) in the brain at 14 and 21 days post tumor implantation (7 and 14 days post ZIKV treatment) using flow cytometry (Figure S2). Analysis of cells at 14 days post GL261 tumor implantation revealed that ZIKV treatment promoted increased numbers of CD45* leukocytes (~6.5-fold), including CD4* T cells (~7.8-fold), CD8* T cells (~20.1-fold), CD3*NK1.1* natural killer (NK) cells (~8-fold), CD3*NK1.1* NK-T cells (~4.8-fold) and CD8*CD44*CD69*CD103* resident memory T cells (Trm) (~14.6-fold) compared to PBS-treated tumor bearing mice (Figure 2A). Similarly, in the CT2A tumor model, ZIKV treatment elicited an increase in numbers of CD45* leukocytes (~4-fold) including CD4* T cells (~7.5-fold), CD8* T cells (~8.9-fold), NK cells (~2-fold), NK-T cells (~2-fold) and Trm cells (~8-fold), compared to control-treated mice (Figure 2B). The numbers of FoxP3* regulatory T cells (Treg) were similar between ZIKV-treated and PBS-treated controls in both the
GL261 and CT2A models. By 21 days after GL261 or CT2A tumor implantation (14 days post ZIKV treatment), animals treated with ZIKV treatment had increased numbers of CD8\(^+\) T cells (~2 and 2.8-fold respectively) and CD8\(^+\) memory T cells (Trm) (~2 and 3.8-fold respectively) whereas we detected no differences in numbers of CD4\(^+\) T cells, NK-T cells and Tregs. We observed a reduction of NK cells (~4-fold) at this time point in ZIKV-treated animals (Figure 2C and D). Comparison of immune cells from GL261 tumor bearing mice to infection with ZIKV alone (no tumor) revealed that ZIKV generated a greater CD8\(^+\) T response than the tumor itself (~2.2-fold), whereas tumors were associated with greater numbers of NK cells than ZIKV alone (~4.5-fold) at day 21 post tumor implantation or 14 days post ZIKV treatment (Figure S3).

A similar analysis of lymphoid cells at 21 days post CT2A tumor implantation revealed no difference in numbers of NK cells, NK-T cells or Tregs but an increase in numbers of CD4\(^+\) T cells (~4.2-fold) (Figure 2D). Collectively, the data from the two glioma models suggest that ZIKV treatment in glioma-bearing mice results in enhanced infiltration of multiple lymphoid cell subsets. While the differences in the early recruitment of immune cells resolves for most cell types, by later time points ZIKV treated gliomas sustain increased numbers of CD8\(^+\) T cells and CD8\(^+\) Trm in the tumor bed.

Given that the immune suppressive tumor microenvironment in gliomas downregulates major histocompatibility complex (MHC) antigen expression and compromises the ability of myeloid cells to cross-present antigen to cytotoxic T cells (46, 58, 59), we hypothesized that ZIKV treatment of gliomas might trigger inflammatory responses that activate microglia and recruit antigen-presenting cells into the tumor region. To evaluate this idea, we analyzed myeloid cells and their activation state in the brain in response to ZIKV treatment in GL261 or CT2A glioma-bearing mice. At 14 days post GL261 tumor implantation (7 days post ZIKV treatment), ZIKV treatment was associated with a small increase in total numbers of microglia (~1.8 fold) but a more substantial increase in numbers of MHC-class II expressing microglia (~4-fold) (Figure 2A). ZIKV treatment of GL261 gliomas also resulted in increased recruitment of Ly6C\(^+\) monocytes (31-
fold), F4/80+ macrophages (~15-fold) and CD11b+ monocyte-derived DCs (~8-fold) (Figure 2A). Also seen were increased numbers of iNos-producing microglia (~4.3-fold), monocytes (~29-fold), and macrophages (~7.5-fold), suggesting an enhanced inflammatory potential of myeloid cells in the tumor bed (Figure 2A). ZIKV treatment of both GL261 and CT2A tumor models had limited effects on neutrophil recruitment to the tumor bed (Figure 2A and B). Whereas ZIKV treatment of CT2A glioma also led to rapid increases in the numbers of Ly6C+ monocytes (~16.5-fold) and iNos-producing Ly6C+ monocytes (~24-fold) in the brain by day 14, it was not associated with activation of microglia, F4/80+ macrophages, or CD11b+ monocyte-derived DCs (Figure 2B). At 21 days post GL261 tumor implantation (14 days post ZIKV treatment), we observed increased numbers of microglia (~3.8 fold) and MHC class II-expressing microglia (~5.2-fold) (Figure 2C). However, these differences at day 21 were not detected in the ZIKV-treated CT2A tumor bearing mice or tumor-naïve PBS-treated counterparts (Figure 2C and D). At 21 days post GL261 and CT2A tumor implantation (14 days post ZIKV treatment), continued increases in numbers of Ly6C+ monocytes (~2 and 6.8-fold, respectively) were observed, but not in numbers of neutrophils, F4/80+ macrophages, or CD11b+ monocyte-derived DCs (Figure 2C and D).

**CD8+ T cells are required for ZIKV-mediated glioma clearance.** As we observed consistent increases in CD8+ T cells in the brain following ZIKV treatment of glioma, we hypothesized that these cells contribute to tumor clearance. To investigate this idea, we performed CD8+ T cell depletion studies. Beginning 14 days after GL261 or CT2A tumor implantation (7 days post ZIKV treatment), we administered a CD8+ T cell depleting or isotype control antibody. Depletion of CD8+ T cells abrogated the therapeutic effect of ZIKV in both glioma models (Figure 2E and F). The loss of efficacy was not due to inadequate control of ZIKV, as non-tumor bearing mice treated with ZIKV survived the CD8+ T cell depletion regimen (Figure 2E and F, dashed green line). Exposure to ZIKV alone is not sufficient to protect mice from subsequent glioma, as ZIKV infection prior to tumor implantation does not confer any survival
benefit against tumor (Figure S4). Collectively, these data demonstrate the potential importance of ZIKV-instructed CD8$^+$ T cells for protection against primary tumor pathogenesis.

**Long-term survivors of glioma after ZIKV treatment are protected against secondary syngeneic glioma in a CD8$^+$ T cell dependent manner.** To model the tumor recurrence that occurs inevitably in patients, we performed re-challenge experiments in mice that were long term survivors of GL261 gliomas after ZIKV-treatment. We implanted syngeneic GL261 cells into the contralateral side of the brains of ZIKV treated tumor survivors, 3 months or even 1.5 years after primary tumor implantation (Figure 3A). Whereas age-matched tumor-naïve mice succumbed to the GL261 tumor as expected, ZIKV-treated tumor survivors were protected against syngeneic tumor rechallenge and survived for at least 150 days (Figure 3B and C). We next evaluated whether memory CD8$^+$ T cell responses after ZIKV treatment prevent growth of the secondary syngeneic tumor. When we depleted CD8$^+$ T cells before GL261 tumor re-challenge, protective phenotype was reversed (Figure 3D). To understand the temporal dynamics of tumor formation following re-challenge, we performed serial bioluminescent imaging of mice that were rechallenged 18 months after their treatment with ZIKV. Age matched control mice had luciferase signals at day 7 and they succumbed to tumor by day 21. While two of the rechallenged mice had luciferase signals at day 7 post-re-challenge, they had little to no luciferase signals by day 58, suggesting tumors either did not engraft or grow in these mice (Figure 3E). Histological analysis of survivors at 150 days after tumor re-challenge revealed no evidence of tumor (Figure 3F). In contrast, the one mouse that did not survive the rechallenge had luciferase signal above the limit of detection at days 7 and 21, and had extensive tumor at day 63 post re-challenge (Figure 3E and 3F). Analysis of re-challenged mice that died beyond day 100 did not show any signs of tumor formation (data not shown), suggesting they likely died from non-tumor causes, as they were 2.5 years old and near the end of their natural lifespan. Collectively, these data demonstrate the potential importance of ZIKV-instructed memory CD8$^+$ T cells for protection against secondary tumor development.
ZIKV treatment improves the response to immune checkpoint blockade. T cell deficits that occur during GBM pathogenesis are characterized by increased expression of immune checkpoint molecules (e.g., PD1, Tim3, and Lag3) that negatively regulate tumor immune responses (11, 60-63). In fact, GBM infiltrating lymphocytes upregulate PD1 expression on up to 95% of CD8+ T cells (60). We investigated whether ZIKV infection changed the expression of immune checkpoint molecules. Flow cytometry analysis revealed that ZIKV treatment did not alter the expression of PD1, Tim3 or Lag3 or the numbers of PD1+, Lag3+ or Tim3+ CD8+ T cells (Figure 4A, S5A and B). However, the numbers of activated PD1+CD44+CD8+ T cells were higher (~10-fold) in the brains of ZIKV-treated gliomas compared to those treated with PBS control (Figure 3A).

The presence of spontaneous tumor infiltrating lymphocytes correlates with better prognosis, especially for tumor immunotherapies (64). As the GBM tumor microenvironment has few numbers of T cells, augmenting CD8+ T cells numbers is one way to alleviate resistance to immune checkpoint blockade therapy. Since ZIKV treatment increases lymphocyte number in the tumor bed, we evaluated whether the combination of ZIKV-treatment with checkpoint blockade immunotherapy enhances glioma clearance and promotes survival. We used the CT2A model, which is resistant to immunotherapy (65), and treated tumor-bearing mice with either ZIKV or PBS, with or without anti-PD-1 and its respective isotype control. Combined blockade of PD-1 and ZIKV treatment was superior to either treatment alone (Figure 4B). Serial bioluminescent imaging every 4 days demonstrated that ZIKV and anti-PD1 treated tumors regressed at approximately day 18 post tumor implantation (11 days post virus treatment) (Figure 4C and D).

Neurobehavioral disease assessments (0, no disease; 1, ruffled fur, piloerection, weight loss or slow movements; 2, lethargy, unsteady gait or hunched back; 3, decreased strength in either fore and/or hind limbs; 4, restricted movement, extreme body weight loss (over 20%), or convulsions; and 5, moribund or death) revealed less central nervous system deficits in animals receiving the combination of ZIKV and anti-PD1 than those with either individual treatment (Figure 4E).
However, approximately 15% of mice were resistant to combination therapy and succumbed to
tumor burden (Figure 4B).

**An attenuated ZIKV strain with therapeutic potential.** Although there are few reports
of ZIKV-induced induced encephalitis in adults (66, 67), safety is paramount in any potential
clinical application. To further develop ZIKV as an oncolytic therapy, we designed a safer strain
by making it more sensitive to the host innate immune response without compromising its ability
to replicate in and kill GSCs. We engineered a deletion of 10 nucleotides in the 3’ untranslated
region (Δ10 3’-UTR ZIKV) of the ZIKV-Dakar cDNA clone (Figure 5A). This deletion abrogates
production of a short subgenomic flaviviral RNA species that antagonizes cell-intrinsic innate
immune responses (15, 68). The Δ10 3’-UTR ZIKV is attenuated in immunocompromised mice,
and this mutation also is the basis of a candidate ZIKV vaccine (68). We compared the tumoricidal
effects of the parental virus and Δ10 3’-UTR ZIKV against mouse glioma cell lines (CT2A, GL261,
SB28) and human GSCs (0308, 667, 3565, 387) (Figure. S6). The Δ10 3’-UTR ZIKV strain
displayed anti-GSC oncolytic activity that was similar to the parental virus strain. In vivo, this
immune-sensitized Δ10 3’-UTR ZIKV also retained efficacy, as treatment increased the survival
rate of GL261 tumor-bearing mice (Figure 5B). We also determined whether treatment of Δ10 3’-
UTR ZIKV in combination with anti-PD1 immunotherapy enhanced survival, as it did for the parent
WT-ZIKV strain. We treated GL261 and CT2A tumor-bearing mice, with either Δ10 3’-UTR ZIKV
or PBS, with or without anti-PD-1 or isotype control mAbs. Whereas Δ10 3’-UTR ZIKV or anti-PD-
1 therapy individual treatments improved long term survival rates in GL261 tumor bearing mice
from 0% to 32% (anti-PD-1 alone) and 33% (Δ10 3’-UTR ZIKV alone), combination therapy
improved long term survival to approximately 80%. (Figure 5C). In the CT2A tumor model, the
median survival times of Δ10 3’-UTR ZIKV, anti-PD-1, and PBS treatment with isotype controls
were 24 days, 25 days and 22 days respectively (Figure 5D), suggesting little benefit of immune-
sensitized virus treatment alone on survival of these glioma bearing mice. However, the
combination of Δ10 3′-UTR ZIKV with anti-PD-1 prolonged median survival to 33.5 days post tumor implantation and the survivor rate increased from 0% to ~40% in the combination treatment group (Figure 5D). CD8 depletion of the Δ10 3′-UTR ZIKV, and anti-PD1 combination treatment group reversed the phenotype, suggesting that the efficacy was driven by CD8+ T cells (Figure 5E). Thus, our data demonstrates that combined Δ10 3′-UTR ZIKV treatment and PD-1 blockade had efficacy, and this was better than either regimen alone.
**DISCUSSION**

GBM remains a clinical challenge. Despite the advances in cataloging tumor genomic alterations through large scale projects like The Cancer Genome Atlas (TCGA), precision medicine has not yet changed dismal patient outcomes (15). A major barrier has been intratumoral heterogeneity, and immunotherapy and oncolytic treatment provide the opportunity to destroy transformed cells across a diverse tumor genetic landscape (15, 69). Despite the success of immunotherapies for solid tumors such as melanoma (48, 70) and non-small cell lung cancer (71), the treatments have largely failed in GBM (50-54). GBM harbors a low mutational burden and exerts a potent immunosuppressive effect on the microenvironment (72). Also relevant is the correlation between the cancer stem cell frequency in tumors and weakness of the anti-tumor immune response (73). Thus, a central problem in GBM remains finding ways to induce a robust immunological response against the tumor. Here we show that ZIKV treatment remolds the GBM microenvironment and supports a CD8$^+$ T cell infiltrative response in the tumor environment, and this is crucial for therapeutic efficacy against both primary and secondary tumors. Moreover, treatment using a parental or immune-sensitized, attenuated ZIKV strain converts the poorly inflamed tumor environment into an immunostimulatory one that overcomes resistance to anti-PD-1 treatment. Further studies are needed to clarify whether ZIKV treatment improves functional anti-tumor CD8$^+$ T cell responses against GSCs or common glioma antigens by promoting antigen cross-presentation or other immunomodulatory mechanisms.

Oncolytic viral therapy for solid tumors has been successful in cancer; the recent FDA approval of talimogene laherparepvec (T-VEC), a genetically engineered herpesvirus to treat melanoma, was a milestone. Investigating viral agents in brain tumors is not new; over the last three decades, there have been a number of attempts to use viruses as either gene therapy delivery systems or as oncolytic agents, In the early 1990s, Martuza and colleagues engineered HSV capable of selective replication and killing of GBM (36). Since that time, oncolytic viruses have been shown to target GBM in multiple ways: direct tumor killing combined with activation of
innate and anti-tumor T cell responses. Oncolytic strains of herpesvirus (G47Δ) (34), measles virus (MV-141.7/MV-AC133) (26), adenovirus virus (DNX2401 [NCT03714334 NCT02197169, NCT01956734] or AdFlt3L/AdTK) (74-76), myxoma virus (37), vaccinia virus (77) (NCT03294486) and poliovirus (PVSRIP0) (29) (NCT04479241) are now under evaluation in GBM. The differential properties and relative advantages of each of these viruses, and even ZIKV, remain poorly understood. One potential advantage of ZIKV is its specificity against GSCs, a highly treatment-resistant subpopulation of GBM cells that may drive recurrence. Our results suggest that the efficacy of ZIKV stems from GSC targeting and its ability to induce immune responses that facilitate CD8+ T cell dependent clearance of tumor components not directly killed by ZIKV.

Solid tumors with low amounts of T cell infiltration generally do not benefit from immune checkpoint blockade therapy (78-80). In these cases, oncolytic virotherapy is an attractive treatment option, as virus-induced inflammation can enhance efficacy of checkpoint blockade therapy. A study in humans showed that T-VEC with anti-PD1 immunotherapy in melanoma had a tolerable safety profile, and the combination appeared to have greater efficacy against melanoma than T-VEC or checkpoint blockade monotherapy (81). Due to the success of immune checkpoint inhibitors in other cancers and their possible additive effects with oncolytic viruses, many virus/antibody combinations are currently being investigated in clinical trials (82, 83). This includes an ongoing phase 2 clinical trial with an oncolytic adenovirus (DNX-2401) combined with pembrolizumab (anti-PD1) for patients with recurrent GBM (NCT02798406). Our data suggests an analogous combination with ZIKV may also be worth pursuing. In addition, future studies in non-responders to ZIKV and PD1 blockade combination therapy might identify mechanisms of resistance, such as loss of tumor antigens, reduction of immune infiltration surrounding the tumor, or other mechanisms of T cell anergy or exhaustion.

We observed that ZIKV treatment also increased the tumor-associated myeloid cell response in the tumor bed, particularly the monocyte and microglia populations. Given that tumor-associated macrophage subsets may contribute to antigen presentation and the anti-tumor
immune cycle, or promote tumor cell growth and suppress an immune response (84-86), further studies must clarify what re-balancing and myeloid cell skewing ZIKV treatment initiates.

While previous work has demonstrated that ZIKV replication is largely self-limited to GSCs, partially because of their inherently attenuated innate immune response (13) and expression of key integrin signaling molecules that facilitate infection (14), safety remains a paramount concern. The safer, immune-sensitized strain is less potent in the CT2A glioma model, but has significant additive effect with immune checkpoint blockade. However, further histological analysis of the subventricular zone and hippocampus of long-term survivors is required to ensure that normal stem cell niches remain intact from ZIKV treatment. Genetic modifications of Δ10 3'-UTR ZIKV to express cytokines or chemokines, for example, IL-6 (87), IL-12 (88, 89), and/or TNF-α (90, 91), to help manipulate the tumor microenvironment may boost its efficacy (92, 93). Optimization of the timing of ZIKV administration with respect to radiation and chemotherapy, both of which immunosuppress patients, will be important considerations for evaluating its possible clinical use and benefit. Nonetheless, given its unique tropism for GSCs and its combinatorial effects with immune checkpoint blockade, ZIKV offers a potential therapeutic opportunity for adult GBM patients.
METHODS

Tumor implantations. Single cell suspensions of GL261 or CT2A cells (4 × 10⁴ cells in 4 µl) were implanted into the right cerebral hemisphere of 8 to 9-week old C57BL/6J female mice (000664, Jackson Laboratory) after mice were anesthetized with ketamine (10 mg/kg), xylazine (100 mg/kg) and Buprenorphine SR (1 µg/g). Mice were mounted onto a stereotactic apparatus (Stoelting) and an incision was made over the cranial midline. A burr hole was made 2.5 mm lateral and 1.5 mm anterior to lambda. A 29.5-gauge Hamilton syringe was inserted to a depth of 3 mm and withdrawn 0.5 mm to a depth of 2.5 mm. The cell suspension was injected over the course of 5 min, and the syringe was slowly withdrawn. The incision site was closed by surgical sutures.

Bioluminescence imaging. Animals were monitored for tumor development via bioluminescence imaging. Beginning at day 6 post tumor implantation, mice were anesthetized by isoflurane (2% vaporized in oxygen) and were injected intraperitoneally with D-Luciferin (150 mg/kg; Gold Bio) and imaged using an IVS50 imaging system (Perkin Elmer). Total photon flux (photons/sec) from the tumor was measured using Living Image 2.6 software (Perkin Elmer).

Treatment and animal monitoring. At day 7 post tumor implantation, mice with similar flux were randomized between groups. Using the same coordinates as for tumor implantation, mice were inoculated intratumorally with either mouse-adapted ZIKV (10⁵ FFU), Δ10 3'-UTR-ZIKV (10⁶ FFU) or PBS, each in 10 µl. CD8-depleting antibodies (Clone 2.43, Bio X Cell) or isotype control IgG2b (Clone LTF-2, Bio X Cell) were injected intraperitoneally starting at day 14 post tumor implantation with an initial dose of 25 mg/kg and followed with booster doses of 12.5 mg/kg every 5 days until day 26. Representative mice were bled to confirm depletion. Checkpoint blockade antibodies against PD1 (clone 29F.1A12, Bio X Cell) or corresponding IgG2a control (2A3, Bio X Cell), was injected intraperitoneally on days 8, 10, 12, and 14 with a dose 10 mg/kg.
Mice were monitored daily for signs of neurological impairment and were euthanized when moribund. Animal caretakers were blinded to treatments.

**Neurobehavioral score.** Tumor bearing animals were scored from 0 to 5 based on the following scale: 0, no disease; 1, ruffled fur, piloerection, weight loss or slow movements; 2, lethargy, unsteady gait or hunched back; 3, decreased strength in either fore and/or hind limbs; 4, restricted movement, extreme body weight loss (over 20%), or convulsions; and 5, moribund or death (94, 95).

**Cells.** Murine glioma cell lines (GL261 and CT2A, H-2b) (13) and SB28 cells (96) transduced with luciferase were cultured in DMEM (Invitrogen) supplemented with 10% FBS (Sigma) and 1% penicillin G-streptomycin sulfate amphotericin B complex (Corning) at 37°C in an incubator with 5% humidified CO2. Cells were dissociated with 0.25% trypsin and 0.53 mM EDTA (Corning). Cells passaged less than 5 times were used for all experiments after ensuring their mycoplasma-free status by PCR (Genome Technology Access Center, Washington University).

Human GSCs (0308, 667, 387 and 3565) (13, 14, 97, 98) were grown as neurospheres in NBE medium comprised of Neurobasal-A medium (Thermo Fisher), Glutamax 100X (Thermo Fisher), N2 (100X) (Thermo Fisher), B27 (50X) supplement without vitamin A (Thermo Fisher), and recombinant human basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) (200 ug/ml each; Peprotech), and maintained at 37°C with 5% CO2. For dissociation, cells were harvested by Accumax cell dissociation reagent (Innovative Cell Technologies).

**In vitro viral infection and GSC proliferation assay.** Cells were plated at 10^3 cells per well in 96-well plates and allowed to attach and grow overnight. Relative cell number was approximated using CellTiter-Glo (Promega). Cells were inoculated with mouse-adapted ZIKV or Δ10 3'-UTR-ZIKV at a multiplicity of infection (MOI) of 5, and luminescence was measured at 0, 3, 5 and 7 days post infection (Biotek).
**Generation of Δ10 3'-UTR-ZIKV.** Using a recombinant NS4B(G18R) mouse-adapted infectious Dakar 41525 ZIKV cDNA clone (Gen-Bank: KU955591.1 (Senegal, 1984)), we engineered a 10-nucleotide deletion in the 3' untranslated region (Δ3'-UTR) as described (68). Constructs were verified by DNA sequencing. Δ10 3'-UTR-ZIKV was propagated as described (68). In brief, 10-nucleotide deletion ZIKV RNA were in vitro transcribed using a T7 mMessage mMachine kit (Ambion) from cDNA plasmids pre-linearized by ClaI. The RNA was precipitated with lithium chloride, washed with 70% ethanol, re-suspended in RNase-free water, quantitated by spectrophotometry, and stored at −80°C in aliquots. The RNA transcripts (10 μg) were electroporated into Vero cells following a previously described protocol (99). The virus derived from RNA transfection, defined as the P0 stock, was propagated in Vero cells as described (57) after inoculating at an MOI of 0.01 and incubating for 72 h. Viral titers were quantified by plaque assay (100), and the viral genome was confirmed with sequencing.

**Generation of viral stocks.** Both mouse-adapted ZIKV (Dakar strain) (57) and Δ10 3'-UTR-ZIKV viral stocks were propagated in Vero cells after inoculating at an MOI of 0.01 and incubating for 72 h. Viral titers were quantified by plaque assay (100, 101) and stored at −80°C.

**Plaque Assay.** ZIKV-treated tumor bearing mice were euthanized on day 7 or day 14 post viral treatment (day 14 or day 21 post tumor implantation). Tissues were stored in −80°C until virus titration. Samples were thawed, weighed and homogenized with zirconia beads in a MagNA Lyser instrument (Roche Life Science) in 1ml of infection media, DMEM (Invitrogen) supplemented with 2% FBS (Sigma) and 1% penicillin G-streptomycin sulfate amphotericin B complex (Corning). Samples were clarified by centrifugation (2,000 × g at 4°C for 10 min), and then diluted serially prior to infection of Vero cells. Plaque assays were overlaid with 1% methylcellulose and 5 days later were fixed with 10% formaldehyde and stained with crystal violet (101).
**Flow cytometry.** Mice were anesthetized with ketamine (10 mg/kg) and xylazine (100 mg/kg), perfused with 20 ml of 1X PBS (Gibco). Brains were excised, treated with digestion buffer containing HBSS (Cellgro 21-022-CM), 0.05% Collagenase D (50mg/ml; Sigma C-0130), 10 ug/ml DNase I (Sigma D5025 150KU), 0.1 ug/ml TLCK trypsin inhibitor (Sigma T-7254) and 10 mM of HEPES (1M; Cellgro 25-060-Cl) at room temperature for 25 min, minced and strained through a 70-um strainer (EASYstrainer). Cell suspensions were washed and subjected to gradient centrifugation in freshly prepared 30% isotonic Percoll (GE Heathcare 17-5445-02) gradient in RPMI (Gibco). After discarding myelin and debris, the cell pellets were stained with fluorochrome conjugated anti-mouse antibodies at a dilution of 1:200. Single cell suspensions were preincubated with Fc Block antibody (BD PharMingen) in PBS + 2% heat-inactivated FBS for 10 min at RT before staining. Cells were incubated for 30 min at 4°C with the following antibodies: BUV395 anti-CD8 (clone 53-6.7, BD), PE anti-CD44 (clone 1M7, Biolegend), anti-NK1.1 (clone PK136, Biolegend), APC anti-CD103 (clone 2E7; eBioscience), BV711 anti-CD3 (clone 145-2C11, Biolegend), BV421 anti-CD69 (clone H1.2F3, Biolegend), AF700 anti-CD45 (clone 30F-11, Biolegend), BV605 anti-CD4 (clone RM4-4, Biolegend), AF488 anti-F4/80 (clone BM8, Biolegend), APC anti-P2RY12 (clone S16007D, Biolegend), PE-Cy7 anti-Ly6G (clone 1A8, Biolegend), APC-Cy7 anti-CD11c (clone N418, Biolegend), BV711 CD11b (clone M1/70, Biolegend), BV421 anti-I-A/II-E (clone M5/114.15.2, Biolegend), BV605 anti-Ly6C (clone HK1.4, Biolegend), BV750 anti-CD223/Lag3 (clone C9B7W, BD), BV421 anti-CD279/PD1 (clone RMP1-30, BD), APC anti-Tim3 (clone RMT3-23, Biolegend). Dead cells were identified with Fixable Viability Dye eFluor™506 (eBioscience). Cells were stained for 30 min at 4°C, washed, and fixed and permeabilized with Foxp3/Transcription Factor Staining Buffer Set (eBiosciences, 00-5523-00), followed by intracellular staining with PE-Cy5 anti-FoxP3 (clone FJK-16s, eBioscience) and PE anti-Nos2 (clone CXNFT; eBioscience). Our gating strategy is shown in Figure S2. Absolute cell counts were determined using TruCount beads (BD). Flow cytometry data were acquired on a cytometer (BDX-20; BD Biosciences) and analyzed using FlowJo software (Tree Star).
Histology. Brain tissues were fixed in 10% buffered formalin (Thermo Fisher), embedded in paraffin, cut into 5-µm thick sections, and stained with hematoxylin and eosin (Thermo Fisher). Whole-tissue scans at 20X magnification were obtained on a Zeiss Axio Scan Z1 brightfield/fluorescence Slide Scanner and images were post-processed using the Zeiss Zen Blue 3.1 software.

Statistics. All data are from at least two independent biological experiments (unless mentioned otherwise) with multiple mice in each group. Only animals that survived tumor and/or virus implantation procedures were used for the study. Cohort size and number of technical replicates are specified in each figure legend. Statistical differences were calculated with Prism 8 (GraphPad) using log-rank Mantel-Cox tests (survival), unpaired two-tailed Mann-Whitney tests (to compare two groups with nonparametric data distribution), or two-way ANOVA with Dunnett’s multiple comparison test (to compare more than two groups with parametric distribution). Differences with a p-value of <0.05 were defined as significant.

Study approval. This study was performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH). The protocols were approved by the Institutional Animal Care and Use Committee at the Washington University School of Medicine (assurance no. A338101). Inoculations were performed under anesthesia induced and maintained with ketamine hydrochloride and xylazine, and all efforts were made to minimize animal suffering.
Author contributions

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Conflicts of Interest

Washington University has filed a patent application entitled “Zika virus strains for the treatment of glioblastoma,” and C.S., J.N.R, P.Y.S, M.S.D., and M.G.C. are inventors. M.S.D. is a consultant for Inbios, Vir Biotechnology, NGM Biopharmaceuticals, and on the Scientific Advisory Board of Moderna and Immunome. The Diamond laboratory has received unrelated funding support from Moderna, Vir Biotechnology and Emergent BioSolutions. The Chheda laboratory has received unrelated funding support from NeoimmuneTech, Inc. and M.G.C. receives royalties from UpToDate.
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Figure 1. ZIKV extends survival of glioma-bearing mice. (A) Scheme of experiments. C57BL6/J mice were implanted with $4 \times 10^4$ GL261 or CT2A glioma cells transduced with luciferase and treated intratumorally with $10^5$ FFU mouse-adapted ZIKV-Dakar or PBS control on day 7. (B–C) Survival analysis of mice bearing GL261 ($n = 19-21$ mice) (B) or CT2A ($n = 16-17$) (C). (D–G) Images of hematoxylin and eosin staining of coronal brain sections at 7 and 14 days post ZIKV treatment. Scale bars represent 1000 $\mu$m (top) and 50 $\mu$m (bottom). Arrows indicate immune cells. Statistical differences were determined by (B–C) log-rank test: $***$, $P < 0.001$. All data are pooled from at least 2 to 3 independent experiments.
Figure 2. CD8+ T cells are required for ZIKV efficacy in mice bearing primary tumors. (A–D) Absolute numbers of immune cells in the brain at 14 and 21 days post tumor implantation (7 and 14 days after ZIKV treatment). Bars indicate median values. (E–F) Survival analysis of mice bearing GL261 (n=17-19) (E) or CT2A (n=14-17) (F) glioma cells, treated with ZIKV or PBS on day 7 and anti-CD8 or isotype control antibody as described in the Methods. Mice without tumor (green lines) (n=9) were similarly treated. Statistical differences were determined by (A–D) Mann-Whitney test *, P < 0.05; **, P < 0.01; ****, P < 0.0001; ns, not significant and log-rank test: ***, P < 0.001; ns, not significant. All data are pooled from at least 2 to 3 independent experiments.
Figure 3. CD8+ T cells are required for ZIKV efficacy in mice during rechallenge. (A) Scheme of tumor re-challenge experiments. (B–C) Surviving mice from GL261 studies with ZIKV were re-challenged 3 months (B) (n=9-10) or 18 months later (C) (n=8) with 4 x 10⁴ GL261 cells on the contralateral side. Age matched (20 months old; n=10 and 26 months old; n=8) naive mice served as controls. (D) Surviving mice from GL261 studies with ZIKV were and re-challenged 3 months later with 4 x 10⁴ GL261 cells and treated with antibodies against CD8 or isotype control as described in the Methods. Age matched (20 months old; n=7) mice served as controls. (E) Photon flux (photons/sec) of bioluminescent images of brains of mice described from (C) at indicated times after re-challenge. (F) Representative images from (C) of hematoxylin and eosin staining of coronal brain sections from a mouse that did not survive re-challenge (Mouse #1) and those surviving up to day 150 post rechallenge (Mice #2-4). Scale bars represent 1000 μm. Horizontal lines indicate median values. The dotted line denotes the limit of detection (E). Statistical differences were determined by (B–D) log-rank test: ***, P < 0.001, ****, P < 0.0001; ns, not significant; and (E) Mann-Whitney test ***, P < 0.001. Data are pooled from at least 2 independent experiments.
Figure 4. ZIKV and anti-PD1 protect against glioma in mice. (A) Mean fluorescent intensity (MFI) of PD1 expression on CD8+ T cells and total numbers of PD1+ CD8+ T cells and PD1+ CD8+ CD44+ T cells from PBS- or ZIKV-treated glioma bearing mice at day 21 after tumor implantation (14 days after ZIKV treatment). (B) Survival analysis of mice bearing CT2A tumors, treated with ZIKV or PBS on day 7 and anti-PD1 or isotype control antibody as described in the Methods (n=34-37). (C) Representative images from (B) at day 6 and day 18 post tumor implantation (11 days post ZIKV treatment). (D) Photon flux of bioluminescence images from CT2A tumor bearing mice treated as in (B). (E) Neurobehavioral score (0 to 5) as described in methods in CT2A tumor-bearing mice treated with antibody against PD1 or isotype control. Bars/horizontal lines indicate median values. The dotted line denotes the limit of detection (D). Data are from two independent experiments. Statistical differences were determined by (A) Mann-Whitney test (***, P < 0.001; ns - not-significant), (B) log-rank test (**, P < 0.01; ***, P < 0.001; ****, P < 0.0001; ns- not-significant), and (D-E) two-way ANOVA test with Dunnett’s post-test (*, P < 0.05; **, P<0.01).
Figure 5. Immune-sensitized Δ10 3'-UTR ZIKV is effective alone or in combination with anti-PD1 therapy. (A) Schematic of Δ10 3'-UTR ZIKV. (B) Mice were implanted with GL261 (n=14-16) and treated with 10^6 FFU of Δ10 3'-UTR ZIKV, or PBS on day 7 (downward arrow). (C-D) Treatment included Δ10 3'-UTR ZIKV, or PBS as in (B), combined with anti-PD1 or isotype control antibodies administered days 8, 10, 12, and 14, in mice bearing GL261 (n=9-10) (C) or CT2A (n=15-16) (D). (E) Survival analysis of mice bearing CT2A glioma cells, treated with Δ10 3'-UTR ZIKV and anti-PD1 or isotype control antibody as well anti-CD8 or isotype control antibody as described in the Methods (n=13-15). Data are pooled from two independent experiments. Statistical differences were determined by the log-rank test (**, P < 0.01; *** , P < 0.001).