Zika virus has oncolytic activity against glioblastoma stem cells

Zhe Zhu,1,2 Matthew J. Gorman,3 Lisa D. McKenzie,4,5 Jiani N. Chai,4 Christopher G. Hubert,2 Briana C. Prager,2 Estefanía Fernández,1 Justin M. Richner,4 Rong Zhang,4 Chao Shan,8,9 Eric Tycksen,10 Xiuxing Wang,1,2 Pei-Yong Shi,8,9 Michael S. Diamond,3,4,6,7 Jeremy N. Rich,1,2 and Milan G. Chheda4,5

INTRODUCTION

Glioblastoma is a highly lethal brain cancer that frequently recurs in proximity to the original resection cavity. We explored the use of oncolytic virus therapy against glioblastoma with Zika virus (ZIKV), a flavivirus that induces cell death and differentiation of neural precursor cells in the developing fetus. ZIKV preferentially infected and killed glioblastoma stem cells (GSCs) relative to differentiated tumor progeny or normal neuronal cells. The effects against GSCs were not a general property of neurotropic flaviviruses, as West Nile virus indiscriminately killed both tumor and normal neural cells. ZIKV potently depleted patient-derived GSCs grown in culture and in organoids. Moreover, mice with glioblastoma survived substantially longer and at greater rates when the tumor was inoculated with a mouse-adapted strain of ZIKV. Our results suggest that ZIKV is an oncolytic virus that can preferentially target GSCs; thus, genetically modified strains that further optimize safety could have therapeutic efficacy for adult glioblastoma patients.

1Department of Medicine, Division of Regenerative Medicine, University of California, San Diego, School of Medicine, La Jolla, CA
2Department of Stem Cell Biology and Regenerative Medicine, Lerner Research Institute, Cleveland Clinic, Cleveland, OH
3Department of Pathology and Immunology, 4Department of Medicine, 5Department of Neurology, 6Department of Molecular Microbiology, and 7The Andrew M. and Jane M. Bursky Center for Human Immunology and Immunotherapy Programs, Washington University School of Medicine, St. Louis, MO
8Department of Biochemistry and Molecular Biology and 9Department of Pharmacology and Toxicology, Sealy Center for Structural Biology and Molecular Biophysics, University of Texas Medical Branch, Galveston, TX
10Genome Technology Access Center, Department of Genetics, Washington University in St. Louis, St. Louis, MO

Correspondence to Michael S. Diamond: diamond@wustl.edu; Jeremy N. Rich: djeremyrich@gmail.com; Milan G. Chheda: mchheda@wustl.edu

Abbreviations used: CNS, central nervous system; DSC, differentiated glioma cell; FFA, focus-forming assay; FFU, focus-forming unit; GSC, glioblastoma stem cell; HuDNC, differentiated human neural stem cell; ISG, type I IFN–stimulated gene; MOI, multiplicity of infection; MS-DNC, mouse-differentiated neural cell; PFA, paraformaldehyde; qRT-PCR, quantitative RT-PCR; RNA-seq, RNA sequencing; TMZ, temozolomide; WNV, West Nile virus; ZIKV, Zika virus.

Supplemental material can be found at:
http://doi.org/10.1084/jem.20171093
ZIKV infects human GSCs and inhibits proliferation in vitro

To interrogate the effects of ZIKV on glioblastoma, we used patient-derived GSCs that express stem cell markers, self-renew, have differentiation potential, and form tumors upon xenotransplantation, as well as differentiated glioma cells (DGCs; Bao et al., 2006; Wang et al., 2017). We selected four GSC models representing the major transcriptional glioblastoma subtypes—proneural, classical, and mesenchymal—and induced cellular differentiation through serum exposure (Bao et al., 2006). We infected GSCs (Fig. 1 A; multiplicity of infection [MOI] of 5) with representative African (Dakar 1984) and American (Brazil 2015) ZIKV strains. 7 d later, spheres were obliterated (Fig. 1 B). Immunofluorescence microscopy demonstrated that greater than 60% of GSCs were infected by either strain at 48 h after infection (Fig. 1, C and D). We analyzed the fraction of ZIKV-infected cells that expressed a GSC marker (SOX2); greater than 90% of infected cells were SOX2+ (Fig. 1, E and F; and Fig. S1 A). Flow cytometry results were consistent with the microscopy data (Fig. S1, B–G) and demonstrated that the percentage of infected GSCs increased over time, consistent with virus spread. We next determined the impact of ZIKV on matched GSCs and DGCs. ZIKV could infect DGCs, but at a significantly lower rate than GSCs (Fig. S1, H and I). Infectious yield assays corroborated higher ZIKV levels from GSCs than from DGCs (Fig. S1, J and K). Whereas GSC proliferation was abolished by either ZIKV strain (Fig. 1, B and G), DGCs were nearly unaffected (Fig. 1 H). Sphere formation in serum-free conditions has been used as a surrogate for self-renewal (Reynolds and Weiss, 1992). Consistent with its preferential targeting of GSCs, ZIKV reduced GSC sphere formation (Fig. 1 I). These effects on cell number and sphere formation were also associated with reductions in SOX2 expression and proliferation (measured by Ki-67) and increases in apoptosis (measured by activated caspase-3 [AC3]; Fig. 1, J–N).

The selective effects of ZIKV on GSCs are not observed with WNV

More than 60 yr ago, WNV was tested for its oncolytic efficacy but displayed substantial toxicity (Southam and Moore, 1952; Moore, 1954). We compared the effects of WNV (New York 1999) to those of ZIKV in our models. WNV infected both GSCs and DGCs to high levels (Fig. S2, A–G), inducing death in both cell types (Fig. S2, A and B). WNV also infected normal human neural cells in culture and brain slices from freshly resected epilepsy tissues and targeted NeuN+ neurons and GFAP+ astrocytes (Fig. S2, H–K). WNV continued to infect cells over time (Fig. S2 L), and this was associated with significant reductions in cell number (Fig. S2 M). Thus, the GSC specificity of ZIKV is not a general property of related neurotropic flaviviruses.

In human tissue specimens, ZIKV targets GSCs, with fewer effects on DGCs and normal neural cells

To confirm these results in the absence of culture, we collected glioblastoma specimens (Fig. 3, A–C) immediately after surgical resection and inoculated them with the two ZIKV strains. Over a 1-wk period, ZIKV progressively infected the tumors (Fig. 3, D–S; and Fig. S2 N). Costaining of sections revealed that the majority of ZIKV-infected cells expressed SOX2 (Fig. 3, D, G, J, M, P, and S). Unlike in organoids in patient specimens, which have different growth dynamics, ZIKV did infect proliferating cells (Fig. 3, E, H, K, N, Q, and S) but still rarely infected DGCs (Fig. 3, F, I, L, O, R, and S). These results support the hypothesis that ZIKV preferentially targets and kills GSCs.

To test the effects of ZIKV on normal adult human neural cells, we inoculated nonmalignant neural tissues from adult epilepsy specimens (Fig. 3, T–V). ZIKV did not infect normal adult human brain tissues, including NeuN+ neurons (Fig. 3, W and Y) and GFAP+ glial cells (Fig. 3, X and Z), as limited viral replication was detected compared with glioblastomas (Fig. S2, N and O). In addition, compared with GSCs and DGCs, the human brain neural cell cultures that were derived from epilepsy patients (NM55 and NM177) or from differentiated human neural stem cells (Hu-DNCs) demonstrated limited ZIKV infection (Fig. S2, P–U). Limited toxicity in these neural cell models was confirmed using a cell viability assay over a 1-wk time course with two ZIKV strains (Fig. S2 V), and ZIKV replicated poorly in normal neural cells (Fig. S2, W and X).
Figure 1. ZIKV causes loss of GSC self-renewal and proliferation. (A and B) GSCs were uninfected (A) or infected (B) with ZIKV-Dakar (7 dpi). (C–F) GSCs uninfected (C and E) or infected (D and F) with ZIKV-Dakar for 48 h underwent immunofluorescence staining for ZIKV envelope protein (green) and DAPI (blue; C–F) with Sox2 (red; E and F). Brightfield and immunofluorescence images in A–F are representative of three independent experiments.
ZIKV attenuates glioma growth, prolongs survival, and has marginal effects on normal neural cells

We recapitulated relevant conditions for human brain tumor therapy using mice. As mice are not natural hosts for ZIKV, pathogenesis studies have used animals with acquired or genetic deficiencies of type I IFN signaling (Lazear et al., 2016). To overcome this limitation, we used a mouse-adapted ZIKV-Dakar that had gained virulence through passage in a Rag1-/- host (Govero et al., 2016; Sapparapu et al., 2016). We first compared the efficacy of the parental and mouse-adapted ZIKV-Dakar strains against three mouse glioma models developed in the C57BL/6 background (GL261, GL26, and CT-2A; Wang et al., 2017) and two mouse CNS lines (BV2 and mouse-differentiated neural cells [MS-DNCs]) in vitro (Fig. 4, A and B). The mouse-adapted ZIKV-Dakar strain attenuated the growth of the mouse glioma cells, whereas the parental ZIKV strain was less effective (Fig. 4 A). In contrast, neither the parental nor the mouse-adapted ZIKV-Dakar inhibited the growth of either mouse CNS cells (BV2 and MS-DNCs; Fig. 4 A). These results were confirmed by virus titration at 1 wk with mouse glioma cells (GL26, GL261, and CT-2A) but not with BV2 cells and MS-DNCs (Fig. 4 B).

To assess the oncolytic effects of ZIKV in vivo, we generated mouse gliomas from two different mouse cell lines (GL261 and CT-2A) grown in syngeneic hosts. Glioma cells were transduced with a luciferase reporter and permitted to form tumors, which were validated by bioluminescence imaging and histopathology (Fig. 4, C–E). Mice with tumors were randomized into two groups and treated 2 wk after implantation with either PBS control or mouse-adapted ZIKV-Dakar (105 focus-forming units [FFU]). Histological examination at 1 wk after tumor treatment demonstrated that the ZIKV-treated tumors were smaller in size compared with PBS-treated tumors (Fig. 4, F–I). Notably, ZIKV infection extended the life spans of tumor-bearing mice (Fig. 4 J). To test whether tumor-bearing mice could benefit from a higher dose of virus, we inoculated 106 FFU of the mouse-adapted ZIKV-Dakar at 1 wk after implantation with the GL261 model. The survival time of tumor-bearing mice was greater compared with that of the control or the 105-FFU dose (Fig. 4 K). To determine the specificity of cell targeting, we stained for ZIKV antigen and markers of stem cell proliferation and differentiation (Fig. 4, L–T). ZIKV infected ∼6% of glioma cells at the endpoint (Fig. 4 T), with the majority of these cells expressing the precursor marker SOX2 (Fig. 4, L, M, and T). In contrast, GFAP+ tumor cells were less infected (Fig. 4, N and O). Effects on proliferating cell populations were measured by Ki-67 staining and BrdU treatment and staining. The majority of ZIKV+ cells were negative for Ki-67 (>70%; Fig. 4, P, Q, and T) or BrdU (>80%; Fig. 4, R–T). These results support the efficacy of ZIKV in vivo against nonproliferating, stem-like cells (Li et al., 2016; Qian et al., 2016). 2 wk after treatment, viral RNA remained localized to the tumor, and ZIKV-treated tumors had extensive cell death as assessed by caspase-3 staining (Fig. 4 U).

IFN signaling is one determinant of differential sensitivity of GSCs to ZIKV

Although the mechanism by which ZIKV preferentially targets GSCs for infection and killing remains unknown, GSCs can suppress antitumor immune responses (Sarkar et al., 2014; Alvarado et al., 2017). To address the possible target specificity of ZIKV, we performed RNA sequencing (RNA-seq) comparing uninfected GSCs and DGCs and defined a group of differentially expressed immune genes, including type I IFN–stimulated genes (ISGs; Fig. S3). Gene set enrichment analysis revealed that many ISGs were up-regulated in DGCs (Fig. S3, A and B). To further elucidate the signaling pathways that regulate ZIKV targeting of GSCs, we infected three GSC models (387, 3565, and 4121) with the ZIKV-Dakar strain for 36–48 h and then performed RNA-seq. IFN signaling was the top gene ontology pathway activated by ZIKV infection (Fig. S3, C and D). The RNA-seq data were validated by qPCR (Fig. S3 E). As ZIKV cannot fully antagonize ISGs, IFN responses may contribute to the specificity of ZIKV inhibition of GSC growth but more limited killing of DGCs or normal brain neurons and glial cells. Consistent with this hypothesis, treatment of DGCs with a blocking antibody against IFN-α/β receptor 2 (IFNAR2) resulted in increased ZIKV infection (Fig. S3 F) and decreased relative cell number (Fig. S3 G).

An attenuated ZIKV maintains effectiveness against GSCs and has additive effects with temozolomide chemotherapy

We previously reported a mutation of the flavivirus NS3 gene (E218A) that sensitizes the virus to translational inhibition by type I IFN and IFIT1 (Daffis et al., 2010) resulting in attenuation in cells responsive to type I IFNs. To begin to enhance the safety features of a potential oncolytic ZIKV, we compared the tumoricidal effects of a wild-type ZIKV (FSS13025,
Figure 2. ZIKV infection causes loss of self-renewal and proliferation of human glioblastoma-derived organoids. (A–F) Brightfield images of GSC organoids after infection with ZIKV. GSCs were incubated in Matrigel for 3 d (A) or 3 wk (B). Organoids were infected with ZIKV-Brazil (C and E) or ZIKV-Dakar (D and F) 2 (C and D) or 4 (E and F) wk after infection. Images in A–F are representative of two independent experiments. (G) Organoid areas

H

I

P

Figure 2. ZIKV infection causes loss of self-renewal and proliferation of human glioblastoma-derived organoids. (A–F) Brightfield images of GSC organoids after infection with ZIKV. GSCs were incubated in Matrigel for 3 d (A) or 3 wk (B). Organoids were infected with ZIKV-Brazil (C and E) or ZIKV-Dakar (D and F) 2 (C and D) or 4 (E and F) wk after infection. Images in A–F are representative of two independent experiments. (G) Organoid areas.
Zika virus has oncolytic activity against GSCs | Zhu et al.

Cambodia 2010; Shan et al., 2016) and its recombinant derivative ZIKV-E218A against three GSC models (Fig. 5). Both the parental and E218A mutant ZIKV strains displayed anti-GSC activity, as measured by cell viability and sphere formation (Fig. 5, A and B). Although the parental ZIKV strain was more potent in reducing GSC growth, both strains were effective. Both the parental strain and the E218A-attenuated strain preferentially infected SOX2+ tumor cells and induced apoptosis (Fig. 5, C–H). Although tumor cells have the potential to activate the IFN response pathway, we still observed significant death of GSCs treated with ZIKV-E218A. As GSCs often display resistance to chemotherapy, including the standard-of-care temozolomide (TMZ; Stupp et al., 2009; Chen et al., 2012), we evaluated the combinatorial efficacy of TMZ and ZIKV E218A. Whereas TMZ alone had limited effects on GSCs, ZIKV-E218A combined with TMZ for 1 wk showed greater antitumor efficacy (Fig. 5, A and B) and induction of apoptosis (Fig. 5, G–I). We tested ZIKV-E218A infection capacity over a 1-wk time course. ZIKV-E218A had self-limited replication capacity in three GSC models (387, 3565, and 4121) relative to that of the parental ZIKV strain (Fig. 5 J). These data suggest that engineered mutant ZIKV strains may promote infection and lysis of GSCs with less toxicity to surrounding differentiated neural cells.

Engineering of ZIKV may provide a therapeutic modality against GSCs

Our findings suggest that because of its tropism for neuroprogenitor cells, ZIKV may offer a tailored therapy that could be used in combination with conventional therapies (e.g., cytotoxic chemotherapy) that target bulk tumor cell populations. The reason for its tropism for specific cells is uncertain, as no definitive ZIKV receptor has been established. Our study is a first step in the development of engineered ZIKV as a glioblastoma therapy. Safety remains a paramount concern. The E218A mutant virus has two nucleotide changes in the same codon that abrogate the 2'-O methyltransferase activity in the NS5 protein (Zhou et al., 2007) and attenuates its replication in cells responsive to type I IFNs (Daffis et al., 2010). Regressor to pathogenic virus requires a low-probability event of concurrent nucleotide changes causing the exact amino acid reversion. Notwithstanding this, the E218A mutant virus represents a framework for further genetic modification to ensure safety and maintain efficacy. For example, second-site mutations in the 3' untranslated region that affect production of a subgenomic RNA (Akiyama et al., 2016; Donald et al., 2016) could further sensitize the virus to type I IFN.

Public health concerns must be addressed with additional preclinical testing, including assays that evaluate for dissemination and genetic reversion.

Although we observed significant effects on mouse high-grade glioma models in vivo and on patient-derived GSCs in vitro, it remains to be determined how ZIKV strains perform in patient-derived GSCs in vivo. This will require overcoming the technical challenges of creating patient-derived tumor models in more immunocompetent mice. We envision possible therapeutic use of modified ZIKV strains to target GSCs through orthotopic injection into the tumor beds of patients. Other modified viruses are under development to treat glioblastoma, including measles (Bach et al., 2013; Hardcastle et al., 2017), polio (Dobrikova et al., 2008), and herpes viruses (Cheema et al., 2013). Our work serves as a foundation for further mechanistic studies and the genetic engineering of a safe and effective ZIKV, which could become an important tool in neuro-oncology.

**MATERIALS AND METHODS**

**Ethics statement**

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.

**Isolation and culture of GSCs, differentiated tumor cells, and nonmalignant brain cultures**

Glioblastoma or nonmalignant epilepsy resection tissues were obtained from excess surgical materials from patients at the Cleveland Clinic after neuropathology review with appropriate informed consent in accordance with a Cleveland Clinic Foundation Institutional Review Board–approved protocol (2559). To prevent culture-induced drift in glioblastoma models, patient-derived subcutaneous xenografts were generated in NOD-scid IL2Rγnull mice (Jackson Laboratory) and maintained as a recurrent source of tumor cells for study. Upon xenograft removal, a papain dissociation system (Worthington Biochemical) was used to dissociate tumors according to the manufacturer’s instructions. Cells were then cultured in Neurobasal complete media (Neurobasal Medium; Life Technologies) supplemented with 1× B27 without vitamin A at 2 or 4 wk after ZIKV infection were determined for three GSC organoid models (387, 3565, and 4121). Data represent two independent experiments. (H–O) Representative images of uninfected control and ZIKV-Dakar-infected GSC organoids 2 wk after infection stained for ZIKV (green) and DAPI (blue; H–O) with Sox2 (red; H and I), AC3 (red; J and K), Ki-67 (red; L and M), or GFAP (red; N and O). Images are representative of three independent experiments. (P) Quantification of Sox2+, Ki-67+, AC3+, and GFAP+ subpopulations of DAPI+ cells; n = 6 organoids for each condition from two independent experiments. Values represent mean ± SD. Significance was analyzed with one-way ANOVA with Tukey’s multiple comparison test (**, P < 0.01; ***, P < 0.001; ****, P < 0.0001). Bars: (A–F) 725 µm; (H–O) 200 µm.
Figure 3. ZIKV infects isolated human glioblastoma but not normal brain tissue slices. (A–C) Representative images from three independent experiments showing freshly resected glioblastoma after staining with H&E (A) or for Ki-67 (B) or GFAP (C). (D–R) Immunofluorescence staining of glioblastoma tissue uninfected (D–F) or infected with ZIKV-Dakar (G–L) or ZIKV-Brazil (M–R) after 7 d for ZIKV E protein (green) and DAPI (blue) with Sox2 (red; D, G, J, M, and P), Ki-67 (red; E, H, K, N, and Q), or GFAP (red; F, I, L, O, and R). D–R are representative images from three independent experiments. (S) Quantification of tumor cells infected with ZIKV (left) and ZIKV-infected cells that co-stain for Sox2, Ki-67, or GFAP (right). Values represent mean ± SD and are pooled from three independent experiments. (T–Z) Representative images showing freshly resected normal brain after staining with H&E (T) or for Ki-67 (U) or GFAP (V).
Zika virus has oncolytic activity against GSCs | Zhu et al.
Figure 4. Mouse-adapted ZIKV-Dakar attenuates growth of mouse glioma cells and prolongs survival of mice with glioma in vivo. (A) Mouse glioma cells (C57BL/6 background: GL26, GL261, and CT-2A), microglial cells (BV2), and MS-DNCs were infected with the parental or mouse-adapted ZIKV-Dakar, and relative cell number was assessed over 1 wk, normalized to day 0. (B) Viral titer from supernatants of
Immunofluorescence staining and microscopy

8-µm-thick cryosections were air dried and fixed in 4% PFA for 15 min before being washed twice with PBS. Tissues were permeabilized by incubating the slides with 1% Triton X-100 in PBS for 15 min at room temperature and peroxidase quenched by incubating them in 1% H2O2 (InVitrogen) for 10 min at room temperature. After blocking for 1 h at room temperature in blocking buffer (5% goat serum, 2.5% BSA in 1× PBS), slides were incubated overnight in a humidified chamber at 4°C with primary antibodies ZIKV (Millipore; AB10216; working dilution 1:1,000), Sox2 (Millipore; AB5603; stock: 1 mg/ml; working dilution 1:400), Ki-67 (Millipore; AB9620; working dilution 1:400), GFAP (Sigma-Aldrich; G9269; working dilution 1:1,000), PAX6 (Abcam; AB5790; stock: 1 mg/ml; working dilution 1:200), NeuN (Abcam; AB177487; working dilution 1:500), Stat1 (Abcam; AB31369; stock: 1 mg/ml; working dilution 1:1,000), BrdU (Abcam; AB6326; stock: 1 mg/ml; working dilution 1:1,000), and Iffar1 (Sino Biological; 50469; stock: 1 mg/ml; working dilution 1:1,000). After PBST (1× PBS with 0.05% Tween 20) washes, slides were incubated with Alexa Fluor 488–, 594–, or 647–conjugated anti–mouse, anti–rat, or anti–rabbit secondary antibodies (Thermo Fisher). Slides were subsequently washed and mounted using Vectashield with DAPI (Vector Labs). For cell immunofluorescence staining, 105 cells were seeded into a 12-well chamber slide (Thermo Fisher) and cultured overnight. Slides were then processed as described previously for tissue staining. 10×, 20×, and 40× images were collected at room temperature on an Eclipse 80i epifluorescence microscope (Nikon; Jiang et al., 2016). The cells were identified based on DAPI. Image analysis was performed by thresholding for positive staining and normalizing to total tissue area using ImageJ and MetaMorph v7.7.0.0 software. Quantitation was initially performed in an unblinded manner. However, many of the key results were requantitated by a second individual in a blinded manner to eliminate bias.

Immunohistochemistry

Tissues were fixed in 10% formalin, embedded in paraffin, and incubated with antibodies as previously described (Jiang et al., 2016). In brief, 6-µm-thick sections were deparaffinized in xylene, rehydrated in graded ethanol, and subjected to antigen retrieval by steam heating in Citra antigen retrieval solution (BioGenex). After blocking for 1 h at room temperature in blocking buffer (5% goat serum, 2.5% BSA in 1× PBS), slides were incubated overnight in a humidified chamber at 4°C with primary antibodies Ki-67 (Millipore; AB9620; working dilution 1:400), GFAP (Sigma-Aldrich; G9269; working dilution 1:1,000), and cleaved caspase-3 (Cell Signaling; 9661; working dilution 1:100). Slides were then incubated at room temperature for 30 min with anti–rabbit (EnVision+ System HRP Labeled Polymer; Dako) or anti–mouse (VECTASTAIN ABC Rabbit IgG detection kit; Vector Laboratories) secondary antibodies. Staining was detected using 3,3-diaminobenzidine (DAB). Image acquisition and analysis were similar to those of immunofluorescence imaging.

RNA in situ hybridization (ISH) was performed using RNAscope 2.5 HD (Brown; Advanced Cell Diagnostics) according to the manufacturer’s instructions and as previously described (Govero et al., 2016). PFA-fixed, paraffin–embedded tissue sections (from ZIKV-infected or uninfected tumors) were deparaffinized by incubating them for 60 min at 60°C, and endogenous peroxidases were quenched with H2O2 for 10 min at room temperature. Slides were then boiled for 15 min in RNAscope Target Retrieval reagents and incubated for 30 min in RNAscope Protease Plus reagent before ZIKV RNA probe (Advanced Cell Diagnostics; 467771). Sections were counterstained with Gill’s hematoxylin and visualized by brightfield microscopy.

Organoids

Organoids were formed by suspending tumor cells in Matrigel and forming 20-µl pearls on Parafilm molds before culture (Hubert et al., 2016). Organoids were cultured in 6-well or 10-cm plates, shaking in Neurobasal complete media. Images of growing organoids were acquired using an EVOS FL Cell Imaging System (Invitrogen) for microscopic imaging.
Figure 5. ZIKV-E218A inhibits the growth of GSCs and has additive effects with TMZ. (A and B) GSCs were mock treated or incubated with parental ZIKV (MOI of 5), ZIKV-E218A (MOI of 5), TMZ (250 µM), or ZIKV-E218A (MOI of 5) and TMZ (250 µM) combined (E218AT). After 1 wk, GSC lines (387, 3565, and 4121) were assayed for relative cell number normalized to day 0 (A) and sphere formation (B). (C–F) Immunofluorescence staining of uninfected control (C and E) and ZIKV-E218A treated (D and F) GSCs on day 7 for Sox2 (red), DAPI (blue), and ZIKV-E218A (green). (G and H) Immunofluorescence staining of ZIKV-E218A-infected GSCs without (G) and with TMZ (250 µM; H) on day 7 for AC3 (red), DAPI (blue), and ZIKV-E218A (green). (I) Quantification of AC3+ apoptotic cells in three GSC lines treated with TMZ, ZIKV-E218A, or ZIKV-E218A combined with TMZ (E218AT). (J) Viral titer from supernatants of parental ZIKV-infected and ZIKV-E218A-infected GSCs over 1 wk measured by FFA. For every panel, data were pooled from three independent experiments performed in duplicate. Values represent mean ± SD. Significance was analyzed with one-way ANOVA with Tukey’s multiple comparison test (A, B, and I) and two-way ANOVA with the Bonferroni multiple comparison test (J; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001). Bars: (C–H) 100 µm.
Organoids were grown until 35 d under these conditions. Organoids were infected with ZIKV-Dakar or ZIKV-Brazil at 10^6 FFU for 2 h, and then the media was removed. Organoids were subsequently washed three times with PBS, and fresh Neurobasal complete media was added. Images were acquired using EVOS Cell Imaging System 3 (Thermo Fisher). Areas of individual organoids were measured with ImageJ software.

Animal experiments
Mice and tumor implantation. Mouse glioblastoma cells (GL261 and CT2A) transduced with luciferase were grown in DMEM supplemented with 10% serum. Cells were harvested by trypsinization and then washed and resuspended in DMEM supplemented with 10% serum. Cells were harvested by trypsinization and then washed and resuspended in 0.1% (wt/vol) saponin (Sigma-Aldrich), and 0.025% NaN3 in PBS; Gold Biotechnology) and imaged using an IVIS50 imaging system (PerkinElmer). Exposure times were 10 or 60 s, and software-defined contour regions of interest were used to measure total photon flux (photons/s) using Living Image 2.6 (PerkinElmer).

Quantitative RT-PCR (qRT-PCR) for ZIKV RNA
ZIKV RNA levels were determined by one-step qRT-PCR (Thermo Fisher) on an ABI 7500 Fast Instrument (Applied Biosystems) using standard cycling conditions. Viral burden was expressed on a log10 scale as viral RNA equivalents per g after comparison with a standard curve produced using serial 10-fold dilutions of ZIKV RNA. For ZIKV, the following primer sets were used: For, 5′-CCACCAATGGTTCTCTTGAGCATATTG-3′; Rev, 5′-TTCGGACACGGGTGTCCAACACAAG-3′; and Probe, 5′-56-FAM/AGCTTACCTGGACAGTCG/3′-TGR

qRT-PCR for GSCs and DGCS
Total cellular RNA was isolated using TRIzol reagent (Sigma-Aldrich), followed by RT into cDNA using the qScript cDNA Synthesis kit (Quanta BioSciences). Real-time PCR was performed using an Applied Biosystems 7900HT cycler using SYBR Green PCR Master Mix (Thermo Fisher). Sequences for gene-specific primer sets were as follows: human IFNAR1 forward 5′-AACAGGAGCGATGAGTCTGTC-3′ and reverse 5′-TGGCGAAATGGTGTAATATGAGTCA-3′; human STAT1 forward 5′-CAGCTGGACTCAAAATTCTGGA-3′ and reverse 5′-TGAAGATACGGTTTGCTTTCCCTG-3′; human IRF1 forward 5′-ATGGCCCCTACACTCGGATGC-3′ and reverse 5′-CCCTCTGTTTCTGATATCTCAGACC-3′; human OAS2 forward 5′-CTGAGGCGTTTGGTTTTAT-3′ and reverse 5′-ACCATCTCTGCATGCTTGG-3′; human IFIH1 forward 5′-TCAATAGGGTATTCCACACAG-3′ and reverse 5′-GTGGCGACTGCTTCTGAA-3′; 18S RNA forward 5′-AACCCGTGGTAACACCATCTG-3′ and reverse 5′-CCCAATCGTGTAGTACCC-3′; and GAPDH forward 5′-CCTGGTTCCGACAGTCAAGCG-3′ and reverse 5′-CCGAAAATCGGCTTGACTCC-3′.

RNA-seq data acquisition, quality control, and processing
RNA was obtained from GSCs infected with ZIKV-Dakar for 36–48 h. Total cellular RNA was isolated using the RNeasy kit (Qiagen). RNA-seq reads were aligned to the Ensembl release 76 assembly with STAR v. 2.5.1a. Gene counts were derived from the number of uniquely aligned unambiguous reads by Subread:feature Count v. 1.4.6p5. Transcript counts were produced by Sailfish v. 0.6.3. Sequencing performance was assessed for total number of aligned reads, total number of uniquely aligned reads, genes and transcripts detected, ribosomal fraction, known junction saturation, and read distribution over known gene models with RSeQC v. 2.6.2.
All gene-level and transcript counts were imported into the R/Bioconductor package EdgeR, and TMM normalization size factors were calculated to adjust for samples for differences in library size. Genes or transcripts not expressed in any sample were excluded from further analysis. The TMM size factors and the matrix of counts were imported into R/Bioconductor package Limma, and weighted likelihoods based on the observed mean–variance relationship of every gene/transcript were calculated for all samples with the Voom function. Performance of the samples was assessed with a Spearman correlation matrix and multidimensional scaling plots. Gene/transcript performance was assessed with plots of residual standard deviation of every gene to their mean log count with a robustly fitted trend line of the residuals. Generalized linear models were created to test for gene- or transcript-level differential expression. Differentially expressed genes and transcripts were then filtered for FDR-adjusted P values less than or equal to 0.05.

To enhance the biological interpretation of the large set of transcripts, grouping of genes/transcripts based on functional similarity was achieved using the R/Bioconductor packages GAGE and Pathview. GAGE and Pathview were also used to generate pathway maps on known signaling and metabolism pathways curated by KEGG.

For the matched GSC and DGC lines, RNA-seq data were evaluated for type I and II IFN signatures between GSCs and DGCs using gene set enrichment analysis and were validated using RNA-seq data on additional matched GSCs and DGCs using gene set enrichment analysis and permutation-based or SEM as indicated in the figure legends. Student’s t tests, one-way ANOVA with multiple comparison correction, and two-way ANOVA with multiple comparison correction were used to assess the significance of differences between groups. These tests were performed when the sample size was large enough to assume that the means were normally distributed or that the distribution of residuals was normal, respectively. For groups being statistically compared, variances in data were similar. For animal survival analysis, Kaplan–Meier curves were generated and the log-rank test was performed to assess statistical significance between groups.

Online supplemental material

Fig. S1 demonstrates that ZIKV infection efficiency is lower in DGCs compared with GSCs. Fig. S2 demonstrates that WNV has global toxicity on normal cells, GSCs, and DGCs, whereas ZIKV has minimal impact on the normal adult brain compared with GSCs and DGCs. Fig. S3 demonstrates that IFN signaling is one determinant of the differential effects of ZIKV on GSCs compared with DGCs.

ACKNOWLEDGMENTS

We thank Julie Prior, Samuel Achilefu, and the Molecular Imaging Center and Oncologic Imaging Program of the Siteman Cancer Center for animal imaging; Jennifer Govero for preparation of virus stocks; Edward Oates for help with animal experiments; David G. DeNardo for use of his microscope; and Q. Xie for providing RNA sequencing data. We thank the Genome Technology Access Center in the Department of Genetics at the Washington University School of Medicine for assistance with RNA sequencing and analysis.

National Institutes of Health (NIH) grants (R01 AI073755 and R01 AI104972 awarded to M.S. Diamond and CA139718, CA154130, CA189117, CA171652, NS087913, and NS109272 awarded to J.N. Rich) and grants from the Elsa U. Pardee Foundation, the Concern Foundation, the Cancer Research Foundation, and the McDonnell Center for Cellular and Molecular Neurobiology of Washington University (awarded to M.G. Chheda) supported this work. The Genome Technology Access Center of Washington University in St. Louis is supported by National Center for Advancing Translational Sciences (NCATS) ICTS/CTSA grant UL1 TR002345 and NCII Cancer Center support grant P30 CA91842. The Imaging Program is supported by NCII Cancer Center support grant P50 CA094056. The Center is partially supported by NCII Cancer Center support grant P30 CA91842 to the Siteman Cancer Center and by National Center for Research Resources (NCRR) ICTS/CTSA grant UL1TR000448, a component of the NIH, and the NIH Roadmap for Medical Research.

M.S. Diamond is a consultant for Inbios and Visterra, is on the Scientific Advisory Boards of Moderna and OvaGene, and is a recipient of grants from Moderna and Visterra. The authors declare no additional competing conflicts of interest. This publication is solely the responsibility of the authors and does not necessarily represent the official views of the NCRR or NIH.

Author contributions: Z. Zhu, M.J. Gorman, L.D. McKenzie, J.N. Chai, C.G. Hubert, B.C. Frager, E. Fernandez, J.M. Richner, R. Zhang, X. Wang, and M.G. Chheda performed the experiments. C. Shan and P.-Y. Shi provided key reagents. E. Tycksen provided RNA-seq analysis and methods. Z. Zhu, J.N. Chai, M.S. Diamond, M.G. Chheda, and J.N. Rich wrote the initial draft of the manuscript, with the other authors contributing to editing the manuscript into its final form.

Submitted: 17 June 2017
Revised: 30 July 2017
Accepted: 9 August 2017

REFERENCES


human antibodies prevent Zika virus replication and fetal disease in mice. *Nature.* 540:443–447. [dx.doi.org/10.1038/nature20564]


