

# Interferon- $\beta$ Sensitivity of Tumor Cells Correlates With Poor Response to VA7 Virotherapy in Mouse Glioma Models

Janne Ruotsalainen<sup>1</sup>, Miika Martikainen<sup>1</sup>, Minna Niittykoski<sup>1</sup>, Tuulia Huhtala<sup>1</sup>, Tytti Aaltonen<sup>2</sup>, Jari Heikkilä<sup>2</sup>, John Bell<sup>3</sup>, Markus Vähä-Koskela<sup>3</sup> and Ari Hinkkanen<sup>1,4</sup>

<sup>1</sup>A.I. Virtanen Institute for Molecular Sciences, Department of Biotechnology and Molecular Medicine, University of Eastern Finland, Kuopio, Finland; <sup>2</sup>Department of Biochemistry, Åbo Akademi University, Turku, Finland; <sup>3</sup>Center for Innovative Cancer Therapeutics, Ottawa Hospital Research Institute, Ottawa, Ontario, Canada; <sup>4</sup>Cancer Center of Eastern Finland, Kuopio, Finland

In our recent study, replicative alphaviral vector VA7 was found to be effective against orthotopic human U87-glioma xenografts in an athymic mouse model eradicating the tumors with single intravenous (i.v.) injection. Here, we tested the efficacy of VA7 in immunocompetent orthotopic GL261 and CT-2A glioma models of C57BL/6 mouse *in vivo*. The cell lines were susceptible to VA7 infection *in vitro*, but GL261 infection was highly restricted in confluent cell cultures, and mouse interferon- $\beta$  (IFN $\beta$ ) pretreatment prevented the replication of VA7 in both cell lines. When mice bearing orthotopic GL261 or CT-2A tumors were administered neurotropic VA7, either i.v. or intracranially (i.c.), the vector was unable to infect the tumor and no survival benefit was achieved. Pretreatments with immunosuppressive cyclophosphamide (CPA) and rapamycin markedly lowered serum-neutralizing antibodies (NAbs) but had no effect on tumor infection or survival. Intracranial GL261 tumors were refractory also in athymic C57BL/6 mice, which have serious defects in their adaptive immunity. Implanted VA7-infected GL261 cells formed tumors with only slightly delayed kinetics and without improving survival thus excluding the participation of physical barriers and indicating robust host IFN action. Mouse and human IFN $\beta$  do not seem to be species cross-reactive, which might limit the translational relevance of xenograft models in oncolytic virotherapy.

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## INTRODUCTION

The most common primary brain tumors such as glioblastoma multiforme are often inoperable and relatively resistant both to radiation and chemotherapy. DNA-alkylating agent temozolomide has demonstrated some efficacy in combination with standard therapy and with adenovirus-thymidine kinase/ganciclovir suicide gene therapy. Nevertheless, patients cannot be cured and

they rarely live beyond 2 years after diagnosis, the median survival of patients with glioblastoma multiforme being 12–15 months.<sup>1,2</sup> Oncolytic virotherapy is a new promising treatment strategy to tackle this devastating disease. Viruses that have entered phase 1 and phase 1/2 clinical trials in treatment of malignant glioma include herpes simplex virus-1, adenovirus, reovirus, Newcastle disease virus, and measles virus.<sup>3</sup> Semliki Forest virus (SFV) is a novel candidate in the field of oncolytic virotherapy, and in addition to its ability to infect and kill a number of tumor cell lines *in vitro*, it has also proven safe and efficacious in preclinical lung adenocarcinoma, melanoma, osteosarcoma, and glioma models.<sup>4–9</sup> SFV is an enveloped positive-strand RNA virus of the family *Togaviridae* with broad host range and high replication efficiency. Infection with SFV A7(74) strain and the derived vector VA7 (ref. 10) leads to transient brain viremia in mice.<sup>11</sup> Recently, we demonstrated that intravenously (i.v.) administered VA7-EGFP virus effectively homes to the brains of athymic mice eradicating implanted human U87-Fluc glioma tumors, while sparing the healthy tissue.<sup>4</sup> However, testing of vector safety and efficacy in immunocompetent animal models is necessary, as immunocompromised xenograft models do not feature the inflamed, yet immunosuppressive environment of human gliomas.<sup>2</sup> Innate [complement, interferons (IFNs), tumor necrosis factor] and humoral (antibodies) immune responses can seriously impede virotherapy by preventing the infection of tumor tissue or by eradicating the therapy virus at an early stage.<sup>12</sup> Previously, we detected IFN $\alpha$ / $\beta$  response against VA7 in subcutaneous (s.c.) A549 human lung cancer model.<sup>6</sup>

IFN $\alpha$  and IFN $\beta$  belong to type I IFNs and play a crucial role in controlling the replication of SFV and other alphaviruses, and mice lacking functional IFN $\alpha$ / $\beta$  receptor die within 48 hours post infection (p.i.).<sup>13–15</sup> IFN $\alpha$ / $\beta$  functions in preventing the widespread and lethal infection of avirulent SFV strain A7(74) in peripheral organs, but A7(74) does not display virulent pattern of neural infection even in the IFN $\alpha$ / $\beta$  receptor-deficient mice.<sup>14,15</sup> Nevertheless, the meningeal cells, ependymal cells, and oligodendrocytes were reported to be infected as a result of impaired type I IFN response, suggesting that separate antiviral cytokines may protect neurons and glial cells.

The first two authors contributed equally to this work.

Correspondence: Ari Hinkkanen, A.I. Virtanen Institute for Molecular Sciences, University of Eastern Finland, P.O. Box 1627, Neulaniementie 2, Kuopio 70211, Finland. E-mail: ari.hinkkanen@uef.fi

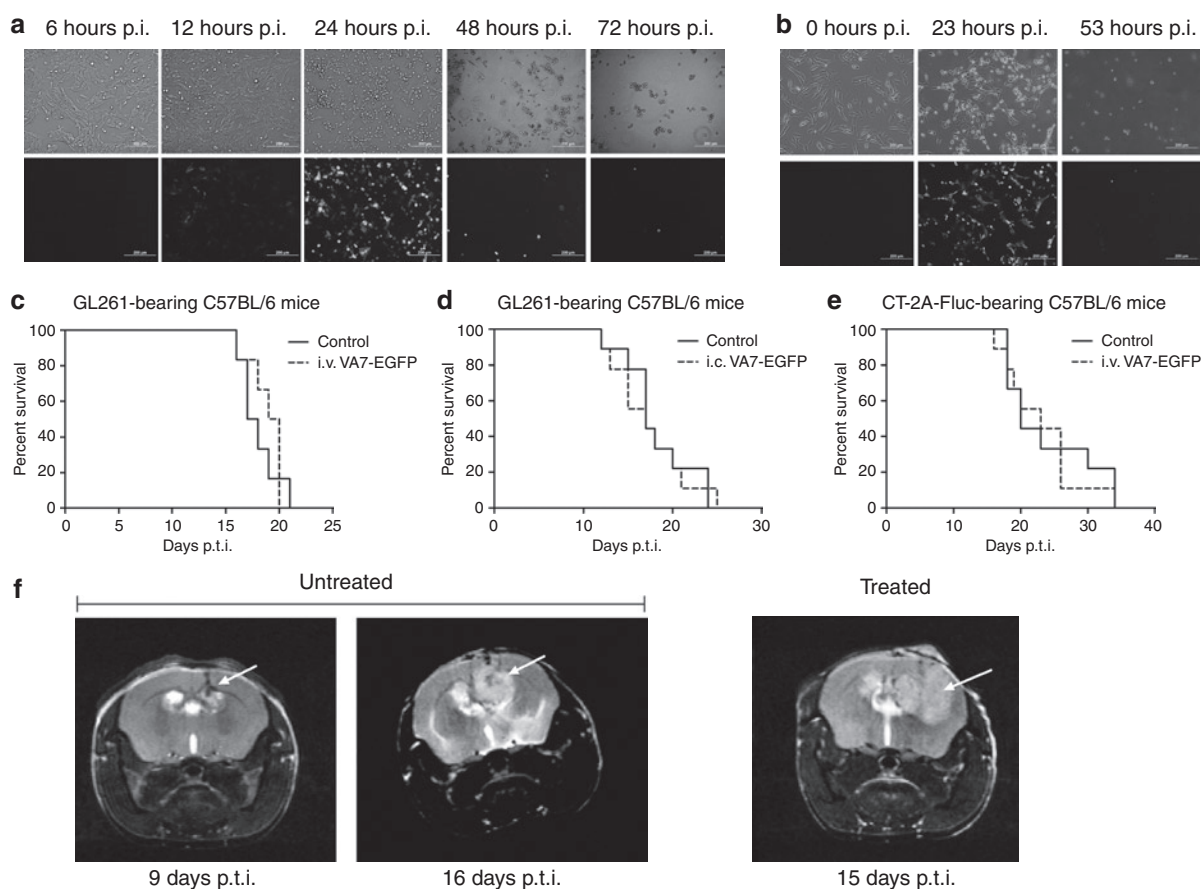
Some tumors have acquired mutations that render them unable to produce and/or respond to IFN $\alpha/\beta$  thus enabling tumor-specific replication of IFN-sensitive viruses.<sup>2,16</sup> On the other hand, immunosuppressive agents such as cyclophosphamide (CPA)<sup>17–21</sup> and rapamycin<sup>21–23</sup> have been used to inhibit virus-neutralizing immunity in order to improve oncolysis and vector efficacy.

In this study, we have studied viral oncolysis in GL261, CT-2A, and U87-Fluc glioma cell lines *in vitro* as well as in CT-2A and GL261 mouse glioma models *in vivo*. The data show that despite oncolytic efficacy *in vitro*, VA7-EGFP vector therapy failed to infect orthotopic tumors *in vivo*. Our results together with the parallel study by M. Vähä-Koskela, F. Le Boeuf, C. Lemay, N. De Silva, J.-S. Diallo, J. Cox *et al.* (submitted) indicate intact type I IFN response as one of the major barriers against the oncolytic efficacy of VA7-EGFP in some, but not all, syngeneic mouse tumor models. As IFN $\beta$  does not seem to feature cross-species reactivity, the immunocompromised tumor xenograft models may be of limited translational value for studies of oncolytic efficacy.

## RESULTS

### Orthotopic GL261 and CT-2A gliomas are refractory to VA7-EGFP infection

VA7-EGFP completely lysed GL261 [multiplicity of infection (MOI) = 1] and CT-2A (MOI = 0.01) cells seeded on 12-well plate at 48–72 hours p.i. as seen in fluorescence microscopy (Figure 1 a,b). In order to test the efficacy of VA7 virotherapy *in vivo*, we implanted GL261 and CT-2A-Fluc tumors intracranially (i.c.) into the *caudate putamen* of adult C57BL/6 mice. Solid tumors developed in majority of the mice, whereas some had more disseminated malignant formations. In some cases, tumor cell injection resulted in multiple tumors around the ventricles, indicating leakage of cells. Gliomas became visible in magnetic resonance imaging latest at day 9 post-tumor induction (p.t.i.) in VA7-EGFP-treated and untreated groups (Figure 1f). GL261 tumor-bearing mice were treated with VA7-EGFP either i.v. (Figure 1c) or i.c. (Figure 1d) but no survival benefit was seen. Similarly, CT-2A-Fluc tumors grew vigorously and despite the better infectivity



**Figure 1** VA7-EGFP infects and kills GL261 cells *in vitro* but not *in vivo*. **(a)** A volume of  $2 \times 10^5$  GL261 cells were seeded on 12-well plate and infected the next day with VA7-EGFP, MOI = 1. Pictures were taken with fluorescence microscope at 6, 12, 24, 48, and 72 hours p.i. Upper row: phase contrast, lower row: fluorescein isothiocyanate (FITC). Bar = 200  $\mu$ m. **(b)** A volume of  $1 \times 10^5$  CT-2A seeded on 12-well plate and infected the next day with VA7-EGFP, MOI = 0.01. Pictures taken with fluorescence microscope 0, 23, and 53 hours p.i. Upper row: phase contrast, lower row: FITC. Bar = 200  $\mu$ m. **(c)** Survival of GL261 tumor-bearing C57BL/6 mice ( $n = 8$ ) treated with intravenous injections of VA7-EGFP virus on days 10, 13, and 16 p.t.i. ( $1 \times 10^8$  PFU/dose). Nontreated mice ( $n = 8$ ) are the control group. **(d)** Survival of GL261 tumor-bearing C57BL/6 mice ( $n = 9$ ) treated with intracranial injection of VA7-EGFP on days 6, 12, and 18 p.t.i. ( $1 \times 10^6$  PFU/dose). Mice ( $n = 9$ ) mock treated with intracranial saline injections (at same time points) were used as a control group. **(e)** Survival of CT-2A-Fluc bearing C57BL/6 mice treated with intravenous injection of VA7-EGFP ( $1 \times 10^8$  PFU/dose, 12 days p.t.i.;  $n = 9$ ). Nontreated mice ( $n = 9$ ) were as a control group. **(f)** Representative magnetic resonance images of untreated GL261 glioma-bearing mice at 9 and 16 days p.t.i. and intravenously treated (VA7-EGFP injected at days 10 and 13 p.t.i.) mouse at day 15 p.t.i. i.c., intracranially, i.v., intravenously; MOI, multiplicity of infection; PFU, plaque-forming unit; p.i., post infection; p.t.i., post-tumor induction.

*in vitro*, no survival benefit was gained by i.v. VA7-EGFP treatment (Figure 1e).

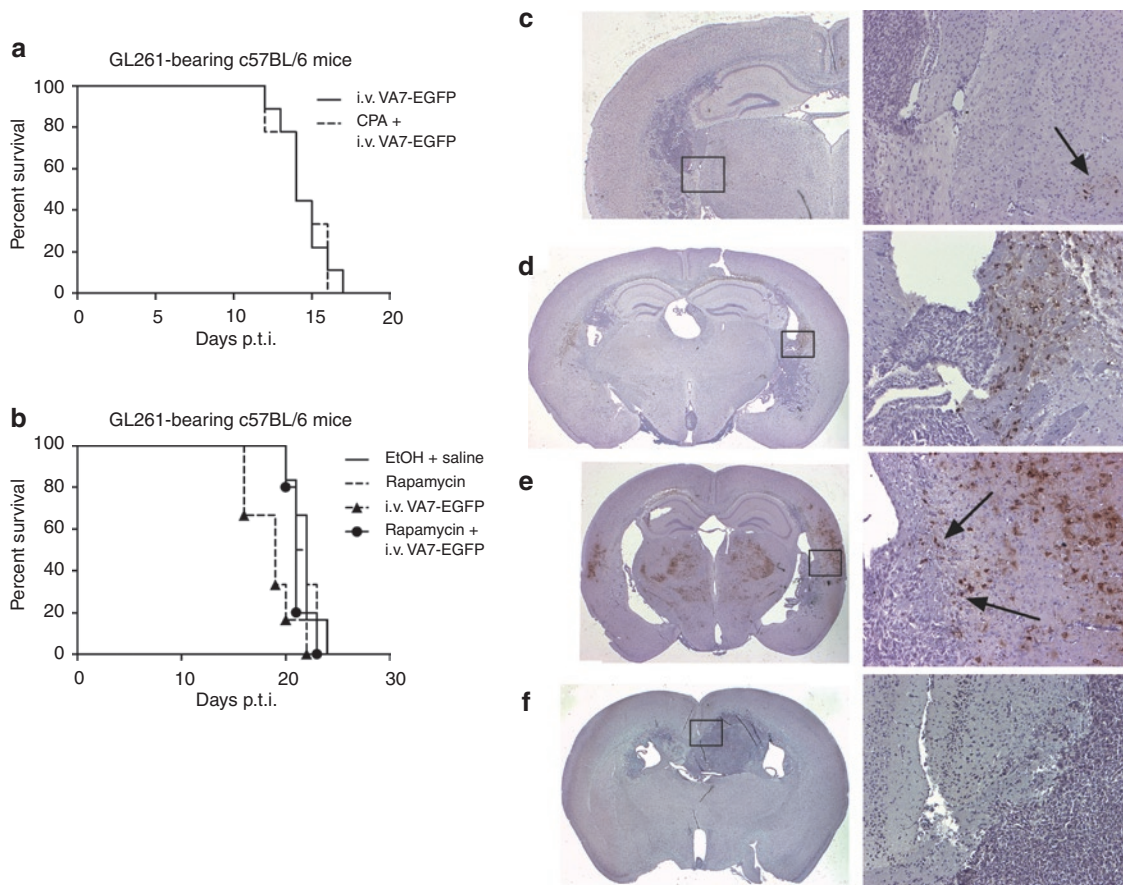
### Immunosuppressive treatment with CPA and rapamycin

To reduce the amount of virus-neutralizing antibodies (NAbs) and the effects of innate immunity, we tested immunosuppressive drugs CPA and rapamycin in combination with the virus. Groups of GL261 tumor-bearing mice were treated with VA7-EGFP with or without preceding i.p. CPA or rapamycin treatment, but no survival benefit was gained (Figure 2a,b). A fivefold decrease of VA7-neutralizing serum antibodies upon CPA and rapamycin treatments was achieved (Table 1). Nevertheless, no virus antigen could be detected in the GL261 tumors by immunohistochemistry

(Figure 2c–f), while infected glial cells could be identified in the *corpus callosum* following i.c. vector injection (Figure 2d) and a few virus foci were seen in healthy brain parenchyma upon i.v. injection (Figure 2c). The CPA treatment strongly enhanced VA7-EGFP replication in healthy brain tissue including neurons but not in tumor (Figure 2e). No survival advantage was gained with either combination therapy (Figure 2a,b). In contrast to CPA, rapamycin did not enhance viral central nervous system replication (Figure 2f).

### Orthotopic GL261 tumors in athymic C57BL/6 mice are refractory to virus

Immunohistochemical analysis of C57BL/6 nude mice killed at 2 (Figure 3a) and 4 days (Figure 3b) after VA7 i.c. injection revealed



**Figure 2** Immunohistochemistry and survival data for GL261-bearing mice. (a) Survival of mice ( $n = 9$ ) treated with CPA and VA7-EGFP combination therapy. CPA injections were given on days 5 (3 mg) and 11 (2 mg) p.t.i. followed by intravenous injection of VA7-EGFP on the next day (days 6 and 12,  $1 \times 10^6$  PFU/dose). Mice ( $n = 9$ ) treated with only VA7-EGFP were used as a control group. (b) Survival of mice ( $n = 5$ ) treated with rapamycin and VA7-EGFP combination therapy. Intraperitoneal (i.p.) rapamycin injection was (5 mg/kg) given on days 5–9, 12 and 13 p.t.i. Intravenous (i.v.) VA7-EGFP ( $1 \times 10^6$  PFU/dose) injections were administered 6–9 and 13 days p.t.i. Survival was compared with groups of mice ( $n = 6$ ) given only rapamycin or VA7-EGFP. The control group ( $n = 6$ ) was mock treated with 30% EtOH and saline instead of rapamycin and VA7-EGFP, respectively. (c–f) Immunohistochemistry of C57BL/6 mouse brain sections using anti-SFV antibody and hematoxylin background stain. VA7-EGFP antigen is seen as brown color. Left column: pictures taken at a magnification of  $\times 1.25$ ; right column: pictures taken from the same section at a magnification of  $\times 10$  (black boxes in the left column indicate the magnified areas). (c) Brain tissue section from glioma-bearing mouse given i.v. VA7-EGFP 10 and 13 days p.t.i. Mouse was killed on day 16 p.t.i. Focal infection highlighted with arrow. (d) Brain tissue section from glioma-bearing mouse administered i.c. VA7-EGFP on day 11 p.t.i. Mouse was killed on day 13 p.t.i. Infected cells of the *corpus callosum* are detected. (e) Brain tissue section from glioma-bearing mouse administered CPA on days 5 and 11 p.t.i. and i.v. VA7-EGFP on days 6 and 12 p.t.i. Mouse was killed on day 12 p.t.i. Enhanced infectivity of healthy brain tissue can be observed. Arrows highlight the border between infected brain tissue and uninfected GL261 glioma tissue. (f) Brain tissue section from mouse administered rapamycin at 5–9, 12, and 13 days p.t.i. and i.v. VA7-EGFP 6–9 and 13 days p.t.i. Mouse was killed on day 20 p.t.i. and infected tissue was not detected. CPA, cyclophosphamide; EtOH, ethanol; i.c., intracranial; PFU, plaque-forming unit; p.t.i., post-tumor induction.



**Table 1** VA7-neutralizing antibody amounts detected from serum samples

Treatment	Serum sampling (days after first virus injection)	VA7-NAb titer
i.v. VA7-EGFP, 6 and 12 days p.t.i.	8	20
	8	100
	9	100
	9	20
	10	20
i.p. CPA, 5 and 11 days p.t.i. + i.v. VA7-EGFP 6 and 12 days p.t.i.	8	<20
	8	<20
	9	<20
	9	<20
	10	<20
i.v. VA7-EGFP, 6–9 and 13 days p.t.i.	13	20
	13	100
	14	100
	16	100
	16	100
i.p. rapamycin, 5–9, 12, and 13 days p.t.i. + i.v. VA7-EGFP, 6–9 and 13 days p.t.i.	14	<20
	15	<20
	15	<20
	15	<20
	15	<20

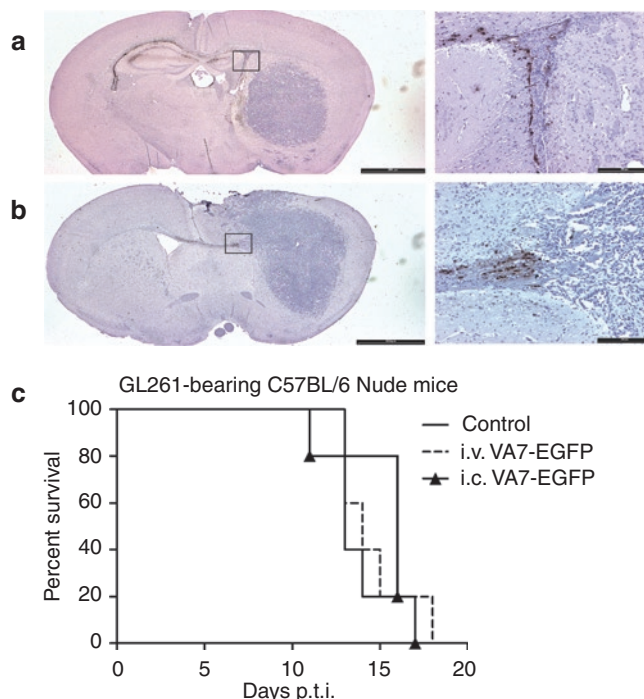
Abbreviations: CPA, cyclophosphamide; i.p., intraperitoneal; i.v., intravenous; p.t.i., post-tumor induction; NAb, neutralizing antibody.

The amount of antibodies is presented as reciprocal of the last serum dilution that inhibited VA7-EGFP infection in Vero(B) or MBA-13 cells *in vitro*.

infected glial cells of *corpus callosum* lining the glioma tumor (similar to **Figure 2d**). However, no virus was detected in tumor itself (**Figure 3a,b**) and no survival benefit was seen (**Figure 3c**).

### Mouse IFN $\beta$ blocks infection of GL261 cells *in vitro*

Pretreatment of GL261 cells with 100 U/ml mouse (m)IFN $\beta$  significantly ( $P < 0.01$ ) inhibited VA7-EGFP infection *in vitro* (**Figure 4a**), and even when added 30 or 90 minutes p.i., rescued the cells from infection. Although majority (88–98%) of the mIFN $\beta$ -treated cells were still alive after 72 hours p.i., the infected cultures without mIFN $\beta$  treatment showed only 28% viability. Similarly, the infection was inhibited by IFN $\beta$  pretreatment also in CT-2A cells (**Figure 4a**). Less than 1% of the infected cells remained viable beyond 48 hours p.i., while the corresponding viability of mIFN $\beta$ -pretreated cells was 65%. No protection of GL261 cells was seen if mIFN $\beta$  was added 4 hours p.i. (**Figure 4b**). To test whether cells from i.c. virus-treated GL261 tumors had become irreversibly refractory, we prepared explant cultures and infected them with VA7-EGFP. The vector efficiently infected the cultures leading to complete cell lysis within 48 hours p.i. (**Figure 4c**). Pretreatment with 100 U/ml of mIFN $\beta$  blocked the infection similar to the inhibition of fresh GL261 cells (**Figure 4c**). The U87-Fluc human glioblastoma cells were readily infected with VA7-EGFP (MOI = 0.1) regardless of pretreatment with 500 U/ml of mIFN $\beta$  or hIFN $\beta$  (**Figure 4d**). The latter efficiently restricted VA7 infection in primary cells derived from human glioma biopsy (data not shown).

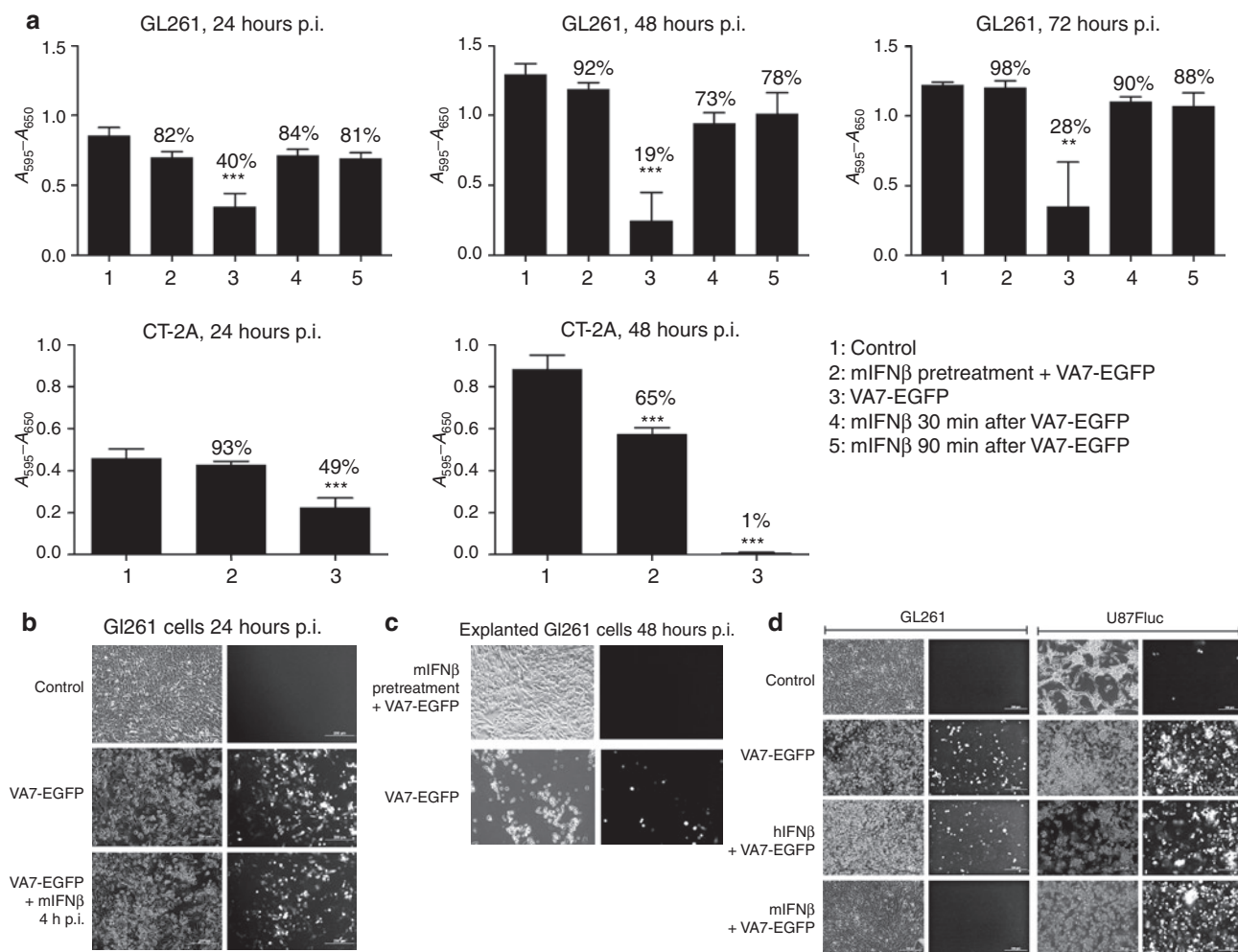


**Figure 3** VA7-EGFP is not effective in therapy of GL261 gliomas in athymic C57BL/6 mice. Immunohistochemistry of athymic C57BL/6 mouse brain sections using anti-SFV antibody and hematoxylin background stain. VA7-EGFP antigen is seen as brown color. Left column: Pictures were taken at a magnification of  $\times 1,25$  (Bar = 2,000  $\mu\text{m}$ ), right column: pictures were taken from the same section at a magnification of  $\times 10$  (Bar = 200  $\mu\text{m}$ , black box in the left column indicates magnified area). (a) Brain section from GL261 glioma-bearing mice killed 2 days after i.c. VA7-EGFP injection. (b) Brain section from GL261 glioma-bearing mice killed 4 days after i.c. VA7-EGFP injection. (c) Survival of glioma-bearing athymic C57BL/6 mice following i.v. ( $n = 5$ ) and i.c. ( $n = 6$ ) VA7-EGFP therapy and untreated control mice ( $n = 5$ ). i.c., intracranial; i.v., intravenous; PFU, plaque-forming unit; p.t.i., post-tumor induction; SFV, Semliki Forest virus.

### VA7-EGFP induces activation of antiviral pathways and production of IFN $\beta$

We then used western blotting to analyze whether VA7 infection caused induction of antiviral proteins in GL261 cells and measured oligoadenylate synthetase (OAS) expression. In concordance with fluorescence development data (**Figure 1a**), the levels of SFV capsid and E1/E2 envelope proteins peaked at 24 hours p.i. (**Figure 5a**), whereas 2',5'-OAS isoforms 1 (OAS1) and 3 (OAS3) were increasing with time and peaked at 48 hours p.i., while the changes in isoform 2 (OAS2) were less prominent (**Figure 5a**). Mobilization of these cellular antiviral proteins did not, however, lead to inhibition of GL261 infection or cell lysis *in vitro*.

We next measured IFN $\beta$  production in culture supernatants of GL261 and DBT (Balb/c) glioma cells after infection with VA7-EGFP or  $\Delta 51$  mutant of Vesicular stomatitis virus (VSV) carrying enhanced green fluorescent protein. Although relatively high levels of mIFN $\beta$  were induced by VSV  $\Delta 51$  in both cell lines, markedly lower amounts were induced by VA7-EGFP in GL261 cells (**Figure 5b**), and no IFN $\beta$  was detected in DBT glioma cells (**Figure 5b**).



**Figure 4** Treatment with mouse IFN $\beta$  hampers VA7-EGFP infectivity and replication in GL261 and CT-2A cells *in vitro*. **(a)** Cell viability measurements performed using MTT-assay. 10,000 GL261 or CT-2A cells cultured on 96-well plate and subjected to mouse IFN $\beta$ . Pretreatment: cells were incubated in 100 U/ml mouse IFN $\beta$  5 hours before infecting with VA7-EGFP, MOI = 1. In addition, mouse IFN $\beta$  was added to other plated cells 30 or 90 minutes p.i. with VA7-EGFP, MOI = 1. Absorbance ( $A_{595} - A_{650}$ ) was measured from parallel prepared plates 24, 48, and 72 hours p.i. Data are presented as mean  $\pm$  SD. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  as analyzed by unpaired, two-tailed *t*-test. **(b)** A volume of  $1 \times 10^5$  GL261 cells cultured on 24-well plate. Top row: uninfected GL261 cells. Middle row: cells infected with VA7-EGFP, MOI = 1. Bottom row: mouse 100 U/ml IFN $\beta$  added to the cells 4 hours p.i. **(c)** GL261 explanted from glioma-bearing mouse given i.c. VA7-EGFP ( $1 \times 10^6$  PFU/dose) on days 6 and 12 p.t.i. and killed on day 17 p.t.i. Cells were seeded on 24-well plate ( $1 \times 10^5$  cells/well) and infected with VA7-EGFP the following day. Top row: cells pretreated 5 hours with 100 U/ml mouse IFN $\beta$  before infecting with VA7-EGFP, MOI = 1. Bottom row: cells infected with VA7-EGFP, MOI = 1. **(d)** Minimal cross-reactivity between human and mouse IFN $\beta$ . Left column:  $1 \times 10^5$  GL261 cells cultured on 24-well plate and pretreated by incubating in culture medium containing 500 U/ml human IFN $\beta$  or 100 U/ml mouse IFN $\beta$  for 5 hours before infection with VA7-EGFP, MOI = 1. Right column:  $1 \times 10^5$  U87-Fluc cells cultured on 24-well plate and pretreated by incubating in culture medium containing 500 U/ml human or mouse IFN $\beta$  for 5 hours before infecting with VA7-EGFP, MOI = 0.1. Top row: uninfected control cells. Second row: cells infected with MOI = 1. Third row: cells pretreated with human IFN $\beta$  before infection. Bottom row: cells pretreated with mouse IFN $\beta$  before infection. IFN $\beta$ , interferon- $\beta$ ; MOI, multiplicity of infection; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; p.i. post infection.

### VA7-EGFP replication is restricted in confluent GL261 cultures *in vitro*

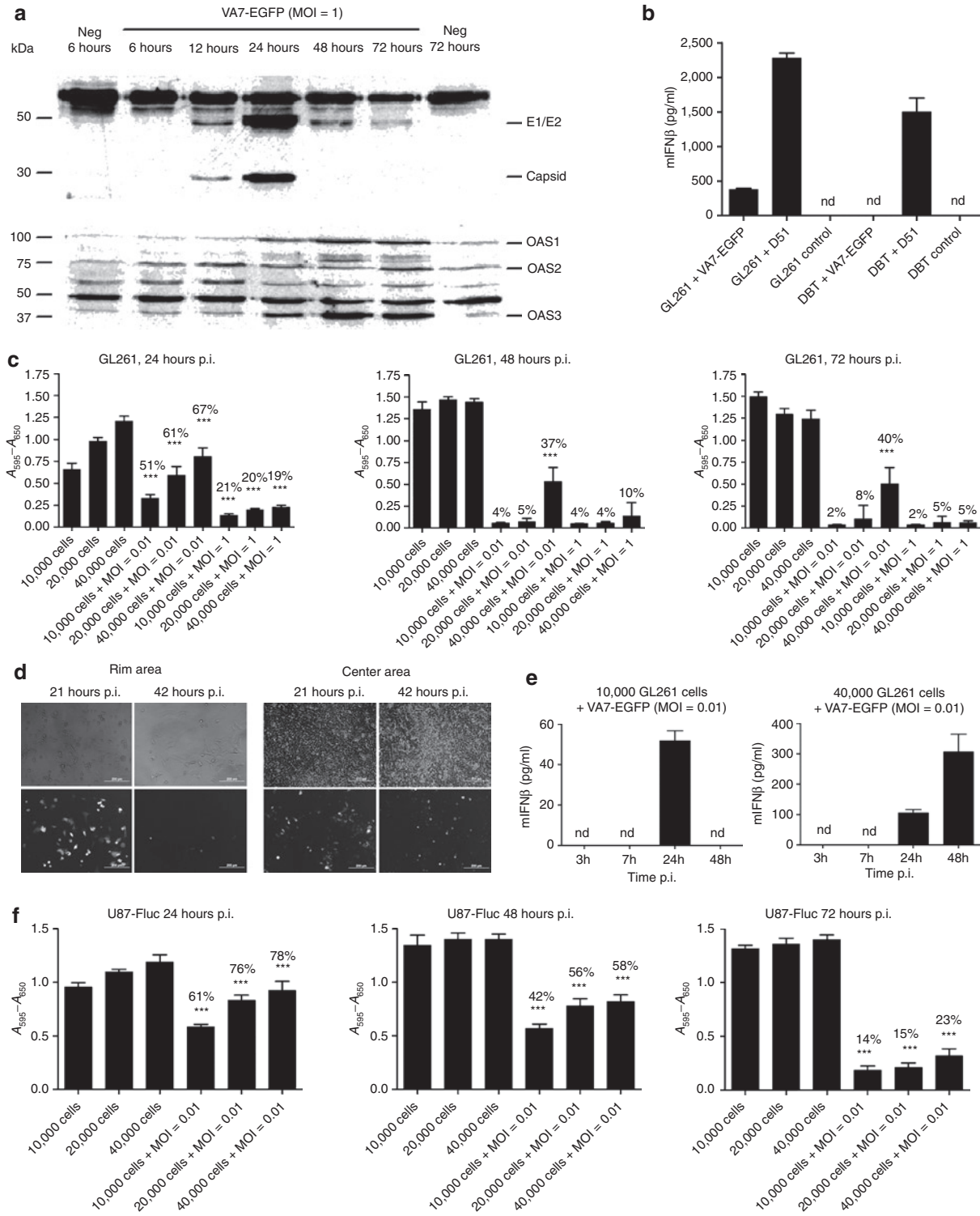
We then tested whether high cell density typical for compact GL261 tumors (Figures 2 and 3) could affect virus permissiveness by infecting confluent and subconfluent GL261 cultures *in vitro*. Although viability of subconfluent cultures declined to 2–5% during infection with significant ( $P < 0.001$ ) decrease as compared with uninfected controls (Figure 5c), VA7 replication and spread were highly restricted in confluent GL261 cultures, and cell viability was significantly (40%,  $P < 0.001$ ) higher. Similar effect was observed also in a separate study, where in GL261 cultures the

subconfluent rim areas were readily infected and lysed, while the inoculated dense centers inside the same wells showed low virus permissiveness (Figure 5d). In contrast, in Vero(B) and CT-2A cultures VA7 spread was efficient regardless of cell density (Supplementary Figure S1). In order to mimic the sparse cultures *in vivo*, we gave i.c. VA7-EGFP injections [ $1 \times 10^6$  plaque-forming units (PFUs)] to C57BL/6 mice already 1 day after tumor induction. Despite the early treatment, no benefit in survival was observed (data not shown).

Next, we tested whether the refractory dense cultures produce more IFN $\beta$  as compared with sensitive sparse monolayers.

The results showed that subconfluent cultures were infected and killed by VA7-EGFP despite the production of IFN $\beta$  (Figure 5e). A volume of  $51.78 \pm 4.96$  pg/ml of IFN $\beta$  could be measured from infected subconfluent cultures 24 hours p.i. ( $n = 2$ ), but declined under detection limit by 48 hours p.i. ( $n = 3$ ) due to cell lysis, whereas confluent cultures showed continuously increasing IFN $\beta$  production up to 48 hours ( $105.42 \pm 11.00$  and  $306.46 \pm 58.02$  pg/ml). IFN $\beta$  levels in samples ( $n = 3$ ) collected at 3 and 7 hours

from infection were below the detection limit ( $<31.2$  pg/ml). We then studied whether VA7-EGFP replication in confluent GL261 cultures could be enhanced by adding anti-mIFN $\beta$  antibody to the culture before infection. As shown in the Supplementary Figure S2, no enhancement was gained but in contrast, slight enhancement of virus spread in confluent cultures was seen when paracellular junctions were disrupted by ethylene glycol tetracetic acid-mediated calcium deprivation (Supplementary Figure S3).





Given that high tumor cell density may compromise virus therapy especially when moderately permissive cells are used, we tested whether spherules obtained in U87 human glioblastoma cultures representing more compact cellular formation were less permissive than monolayers. As compared with GL261 cells, the U87 cells were readily infected with VA7-EGFP with significant ( $P < 0.001$ ) loss of cells at 24, 48, and 72 hours p.i. (MOI = 0.01) with respective viabilities of 61–78%, 42–58%, and 14–23% (Figure 5f). We found that also the neurospheres were infected by VA7-EGFP, but in contrast to the surrounding monolayer cells that were rapidly lysed, the spheres retained their metabolic activity up to 120 hours p.i. as observed by the formation of blue formazan metabolite in 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT)-assay (MOI = 0.01; Figure 6a,b).

### ***In vivo* antiviral assay demonstrates robust antiviral response by the host**

Finally, we designed “*in vivo* antiviral assay” and infected fresh GL261 cells for 1 hour with VA7-EGFP, washed them, and implanted into brains of immunocompetent C57BL/6/J mice. Despite successful infection as determined by fluorescence microscopy from a parallel culture next day, the cells produced aggressive i.c. tumors with only slight delay on median mortality as compared with uninfected controls (23 days and 18 days, respectively, not significant, log-rank test; Figure 7).

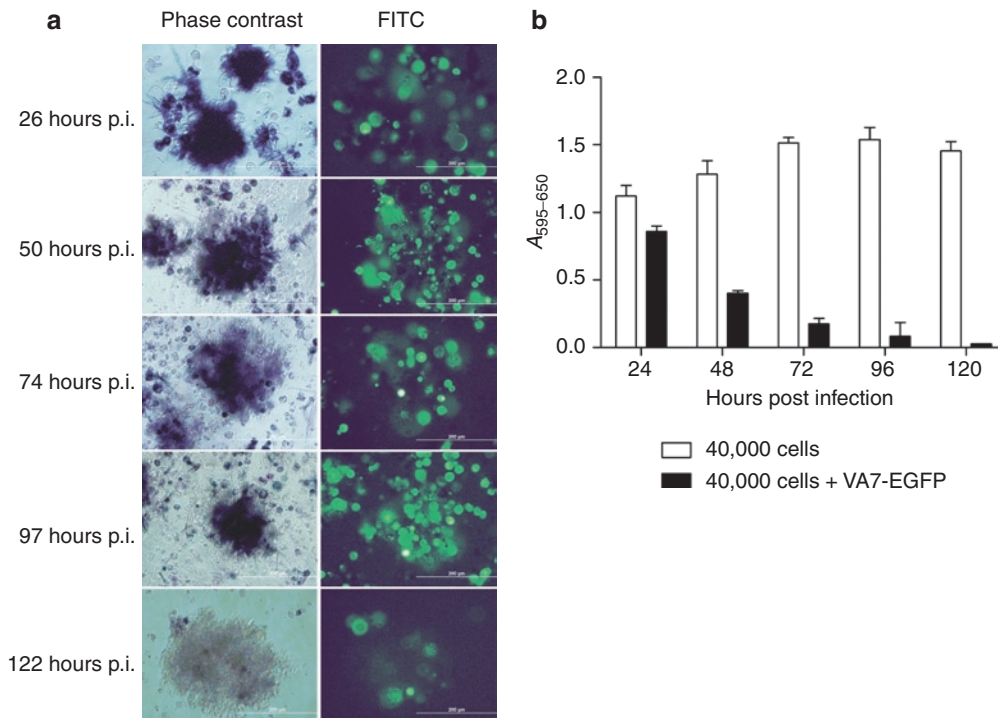
## **DISCUSSION**

The observed discrepancy that VA7-EGFP readily lysed GL261 and CT-2A cells *in vitro* but neither infected tumor tissue (brain or s.c.) nor survival benefit was seen after *in vivo* treatments (Figures 1c–e and 2c,d) is multifaceted. The attempts to enhance the efficacy of the therapy by combining the virus with immunosuppressive drugs, CPA and rapamycin, both known to affect humoral and innate immunity, were unsuccessful. The effects of CPA are known to be pleiotropic; it can on one hand stimulate virus replication by reducing NAb<sup>18,20</sup> or suppressing innate immunity,<sup>17–20</sup> but it can also stimulate antiviral IFN $\alpha/\beta$  production.<sup>24,25</sup> Oncolytic viruses themselves are immunomodulatory and sometimes CPA/virus combinations do not produce additive or synergistic, but rather antagonistic effects.<sup>26</sup> Rapamycin can inhibit IFN $\alpha/\beta$  production

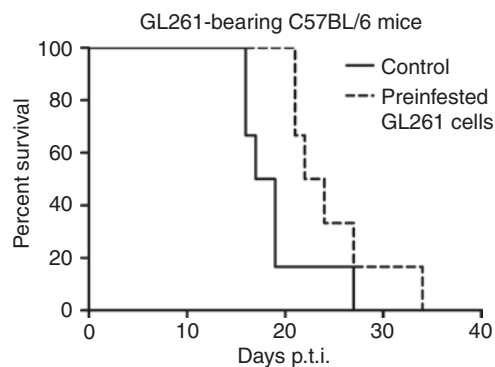
by the tumor or systemically by plasmacytoid dendritic cells thus enabling specific tumor infection when the neoplastic cells have lowered ability to respond to type I IFNs.<sup>23</sup>

Strikingly, CPA enhanced viral replication throughout the healthy brain including cortex, thalamic, hypothalamic, and periventricular regions with an infection pattern resembling the virulent SFV strains,<sup>11</sup> whereas infected tumor cells could not be found and survival was not improved as compared with controls (Figure 2a,b,e,f). The infected brain parenchyma rather seemed to form an absolute border to the tumor lining indicating a strict cell type–specific inhibition (Figure 2e). This was probably not due to physical barriers (e.g., encapsidation) as the infection borders were evident also upon i.c. virus injections (Figure 2d). CPA counteracts Th1 responses and it has been used in treating autoimmune diseases.<sup>27,28</sup> Antibodies and IFN $\gamma$  secreted by T cells are known mediators of alphavirus clearance from central nervous system neurons,<sup>29</sup> and thus it is not surprising that several Th1/IFN $\gamma$  inhibitors in addition to CPA stimulate central nervous system replication of avirulent SFV strains.<sup>30,31</sup> IFN $\gamma$  alone inhibits only minimally VA7-EGFP replication in GL261 glioblastoma cells *in vitro* (data not shown) but as we have shown these cancer cells are sensitive to IFN $\beta$ , produced readily by most somatic cell types.<sup>16</sup> As type I IFNs do not play a major role in the control of SFV neuronal replication *in vivo*,<sup>14,15</sup> we speculate that the different target cells of type I and II IFNs (glial and neuronal, respectively) could explain the infection pattern induced by CPA pretreatment. This hypothesis awaits yet further corroboration. The GL261 model was recently proven resistant also to vaccinia virus even in combination with rapamycin, whereas similar combination showed partial effect in racine RG2 glioma model.<sup>22</sup> To exclude the possibility that the therapy failure was due to residual serum NAb despite CPA or rapamycin treatments or was caused by cellular adaptive responses, we treated orthotopic gliomas in athymic C57BL/6 mice. Virus could not be detected in tumor tissue by immunohistochemistry (Figure 3) and similar borders were seen between the infected brain parenchyma and the tumor as in the immunocompetent model. Taken together, the data strongly suggested that the therapy failure was not due to the adaptive immunity but rather mediated by components of the antiviral innate immunity, which we then proceeded to study. Intriguingly,

**Figure 5** VA7-EGFP induces antiviral responses in GL261 cells *in vitro*. **(a)** Viral and virus-induced antiviral proteins detected from VA7-EGFP-infected GL261 cells with western blotting. A volume of  $1 \times 10^5$  GL261 cells were seeded on 12-well plate and infected with VA7-EGFP, MOI = 1. Cells were collected 6, 12, 48, and 72 hours p.i., lysed, and proteins analyzed with western blotting (15  $\mu$ g protein/sample). First lane: uninfected cells collected 6 hours p.i.; second to sixth lanes: infected cells collected 6–72 hours p.i.; 7th lane: uninfected cells collected 72 hours p.i. Top picture: SFV-protein expression. E1/E2 (50 kDa) and capsid protein (30 kDa) detected with SFV-antibody. Additionally a high molecular weight band is detected in infected and uninfected samples. Bottom picture: OAS1-3 isoforms detected with OAS antibody. **(b)** IFN $\beta$  ELISA on GL261 and DBT cells. A total of 300,000 cells in 6-well plate were either infected with VSV $\Delta$ 51-EGFP or VA7-EGFP at MOI = 0.01 or left uninfected with 1 ml medium. After 24 hours supernatants were collected and mouse IFN $\beta$  was quantified according to manufacturer’s instructions (Verikine IFN $\beta$  ELISA; PBLBiosource). **(c)**  $A_{595} - A_{650}$  measured with MTT-assay from GL261 cells.  $1 \times 10^4$ ,  $2 \times 10^4$ , or  $4 \times 10^4$  cells plated on 96-well plate and infected the next day with MOI = 0.01 or 1. Cell viability measured from different parallel prepared plates 24, 48, and 72 hours p.i. Data are presented as mean  $\pm$  SD. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  as analyzed by unpaired, two-tailed *t*-test. **(d)** Confluent centers of the wells were supplemented with subconfluent rims on a 24-well plate (50,000 GL261 cells/well in total) and infected with VA7-EGFP (MOI = 1). Pictures were taken 21 and 42 hours p.i. from the rim and center areas of the same well. **(e)** mIFN $\beta$  measured from 10,000 or 40,000 GL261 cells seeded on 96-well plate and infected with VA7-EGFP (MOI = 0.01) in 200  $\mu$ l of culture medium. Medium was collected from wells at 3, 7, 24, and 48 hours p.i. and mIFN $\beta$  measured using ELISA kit (Verikine IFN $\beta$  ELISA; PBLBiosource). Measurements were done from 2 to 3 parallel wells/time point. **(f)**  $A_{595} - A_{650}$  measured with MTT-assay from U87-Fluc cells infected with VA7-EGFP. A volume of  $1 \times 10^4$ ,  $2 \times 10^4$ , or  $4 \times 10^4$  cells were plated on 96-well plate and infected the next day with MOI = 0.01. Cell viability was measured from different parallel prepared plates at time points 24, 48, and 72 hours p.i. Data are presented as mean  $\pm$  SD. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  as analyzed by unpaired, two-tailed *t*-test. ELISA, enzyme-linked immunosorbent assay; mIFN $\beta$ , mouse interferon- $\beta$ ; MOI, multiplicity of infection; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; nd, not detected; OAS, 2'-5'-oligoadenylate synthetase; SFV, Semliki Forest virus.



**Figure 6** Spheres formed by U87-Fluc show prolonged survival after VA7-EGFP infection *in vitro*. **(a)** U87-Fluc spheres stay metabolically active until 122 hours p.i. upon infection. Left side: phase-contrast image indicating blue formazan metabolite in the spheres. Right side: FITC image indicating VA7-EGFP infection of the spheres. Images were taken 26–122 hours p.i. (MOI = 0.01). Bar = 200  $\mu$ m. **(b)** MTT-assay from U87-Fluc cells infected with VA7-EGFP. A density of  $4 \times 10^4$  cells plated on 96-well plate and infected the next day with MOI = 0.01. Cell viability was measured from different parallel prepared plates at times 24, 48, 72, 96, and 120 hours p.i. Data are presented as mean  $\pm$  SD. FITC, fluorescein isothiocyanate; MOI, multiplicity of infection; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; p.i., post infection.



**Figure 7** VA7-EGFP preinfected GL261 cells form intracranial gliomas when implanted to C57BL/6 mice. Subconfluent GL261 cells were infected with VA7-EGFP for 1h and implanted intracranially into c57BL/6 mice. Survival of preinfected GL261 group ( $n = 6$ ) was not statistically changed (log-rank test) as compared with control group ( $n = 6$ ) implanted with uninfected GL261 cells. The success of the infection was controlled using fluorescence microscopy of a parallel flask left to incubator over night. p.t.i., post-tumor induction.

in a parallel study by M. Vähä-Koskela, F. Le Boeuf, C. Lemay, N. De Silva, J.-S. Diallo, J. Cox *et al.* (manuscript submitted), VA7 demonstrated efficacy in syngeneic CT26-LacZ colon carcinoma model. This tumor model could not respond to type I IFN further proposing that syngeneic tumors *per se* are amenable to VA7 virotherapy as long as the cells have acquired mutations rendering them defective in their response to IFN $\alpha/\beta$ .

We saw that VA7-EGFP-infected GL261 cells are potent producers and responders to IFN $\beta$  alike (Figures 4 and 5). Mouse IFN $\beta$  was able to protect GL261 cells from VA7-EGFP infection and rescue them when administered as late as 90 minutes but not anymore 4 hours p.i. (Figure 4a,b). These data support existence of a VA7-EGFP replication threshold (quantitative or temporal) that needs to be passed before effective cell lysis is reached. Such turning point could be for example the shift from negative sense RNA synthesis to genomic plus strand RNA production occurring 4–6 hours p.i.<sup>32–34</sup> The explanted GL261 cells had retained their infectivity and IFN $\beta$  sensitivity *ex vivo*, indicating that virus resistant tumor cell populations had not been evolved (Figure 4c). U87 human glioblastoma multiforme cells neither produce nor respond to type I IFN $\beta$ <sup>35</sup> (Figure 4d) providing the most apparent explanation for their sensitivity to VA7-EGFP infection *in vivo* we observed earlier.<sup>4</sup>

The VA7-EGFP infection of GL261 cells was highly restricted in confluent cultures (Figure 5c and Supplementary Figure S1) resembling the poor infectivity of the dense GL261 tumors observed *in situ* (Figure 2d). In contrast, U87-Fluc, Vero(B), and CT-2A cell lines were highly susceptible regardless of cell density (Figure 5f and Supplementary Figure S1). Amongst the potential explanations are the known defects in IFN $\beta$  synthesis of U87 and Vero(B) cells,<sup>23,36</sup> and we saw that also CT-2A astrocytoma cells produced markedly lower levels of IFN $\beta$  as compared with GL261 cells. We could also detect induction of intracellular antiviral proteins OAS1 and OAS3 in infected GL261 cells peaking 48 hours p.i. indicating



that these cells were indeed autonomic in their type I IFN synthesis and response (Figure 5a). We observed spontaneously forming spheres in U87-Fluc cultures, which have earlier been reported to be enriched in cancer stem cells by others.<sup>37</sup> Although susceptible to VA7-EGFP infection, their lysis was retarded until 120 hours p.i. further supporting the importance of the tumor density for infectivity. The amount of IFN $\beta$  produced by GL261 cells correlated linearly with the seeded cell number (Figure 5e) but was clearly less than the respective amount induced by VSV  $\Delta$ 51 used as a control. Interestingly, DBT glioma cells did not produce IFN $\beta$  upon VA7-EGFP infection (Figure 5b), but this did not translate into better *in vivo* efficacy (M. Vähä-Koskela, F. Le Boeuf, C. Lemay, N. De Silva, J.-S. Diallo, J. Cox *et al.*, manuscript submitted) underlining the significant role of the tumor cell-independent local and systemic IFN response elicited by the host.

Cells in close contact might also resist infection *via* IFN-independent mechanisms. Recent data suggest that some dense cultures are able to transmit a rapid antiviral response through cell-to-cell gap junctions,<sup>38</sup> and glial cells may communicate through such junctions.<sup>39</sup> Indeed, only subconfluent GL261 rims, but not the densely growing centers inside the same wells were efficiently infected *in vitro* (Figure 5d). Improved infection may be obtained by disruption of paracellular junctions using ethylene glycol tetraacetic acid-mediated calcium depletion<sup>40–43</sup> and we did observe slightly enhanced infection of confluent GL261 cell cultures upon ethylene glycol tetraacetic acid treatment (Supplementary Figure S3). Inhibition of infection upon close cellular contact may, at least in some cases, be related to the basolateral or intercellular location of the viral receptor.<sup>44</sup> We could not observe increase in viral replication in confluent GL261 cultures upon anti-IFN $\beta$  antibody treatment (Supplementary Figure S2). This further suggested parallel existence of IFN-independent virus resistance mechanisms in the dense tumor cell cultures.

We treated orthotopic GL261 tumors with VA7 soon (24 hours p.t.i) after tumor induction mimicking sparse *in vitro* conditions, but saw no improvement in survival (data not shown). We then advanced to study the role of systemic and local host IFN response and designed “*in vivo* antiviral assay.” Antiviral assays are the most commonly used biological assays for IFN potency determinations.<sup>45</sup> We preinfected GL261 cells for 1 hour before their i.c. implantation and could see no change in survival suggesting that physical hindrance in tumor microenvironment such as encapsulation or fibrotic barriers<sup>9</sup> was not sufficient explanation and indeed, the *in vivo* therapy failure had to be accounted for host antiviral IFN activity (Figure 7). The local type I IFN production by the tumor cells themselves might have furthered the process, but was not alone enough for saving the tumor as the cells not used for grafting were successfully infected *ex vivo* (data not shown).

In conclusion, we report here that VA7-EGFP virotherapy does not prolong the survival of mice in CT-2A and GL261 glioma models despite obvious lytic efficacy *in vitro*. CPA or rapamycin immunosuppression did not improve tumor infectivity and CPA caused strong increase of viral replication in healthy brain parenchyma. This finding together with earlier reports suggests that combining Th1-type immunosuppressive therapy with neurotropic

alphaviruses may pose serious safety concerns. Our data indicate that type I IFN produced both by the tumor cells themselves and, maybe even more importantly, by the host, is one of the major factors limiting the oncolytic efficacy of VA7-EGFP. This is in agreement with a recent report using Sindbis virus, a close relative of SFV, in virotherapy of different immunocompetent mouse tumor models.<sup>46</sup> Furthermore, considering the observed importance of the tumor cell-independent host IFN response and the reported lack of cross-species reactivity of type I IFNs by us (Figure 4) and others,<sup>47–49</sup> the data suggest that immunocompromised mouse xenograft models where human tumors are implanted into mouse IFN milieu might be of limited translational value in oncolytic virotherapy.

## MATERIALS AND METHODS

**Cell cultures and viruses.** Cell cultures of mouse glioma GL261 (kind gift from G. Safrany), DBT (kindly provided by Daniel Silbergeld, University of Washington, WA), CT-2A (kindly provided by Thomas Seyfried, Boston College, MA), human glioblastoma U87-Fluc (ref. 4) and mouse glial MBA-13 cells were established. GL261 and DBT cells were cultured in Dulbecco's modified Eagle's medium (D6046; Sigma-Aldrich, St Louis, MO) MBA-13 and U87-Fluc cells were cultured in minimum essential medium Eagle (M4655; Sigma-Aldrich). CT-2A cells were cultured in RPMI-1640 medium (R0883; Sigma-Aldrich). Dulbecco's modified Eagle's medium for GL261, DBT, and U87-Fluc was supplemented with 10% fetal calf serum (FCS; Autogen Bioclear, Wiltshire, UK), 1% L-glutamine, and 1% (G57513; Sigma-Aldrich) penicillin-streptomycin (P0781; Sigma-Aldrich). Minimum essential medium for MBA-13 was supplemented with 5% FCS and 1% penicillin-streptomycin. RPMI-1640 medium for CT-2A cells was supplemented with 10% FCS, 1% L-glutamine, and 1% penicillin-streptomycin. A fluorescent derivative VA7-EGFP, based on avirulent SFV A7(74) (ref. 11) was used as reporter virus. Infectivity was analyzed with fluorescence microscopy. Preparation of VA7-EGFP has been described<sup>10</sup> and virus was produced from *in vitro* transcribed RNA by transfection in BHK-21 cell line cultured in Dulbecco's modified Eagle's medium with 10% FCS, 1% L-glutamine, and 1% penicillin-streptomycin. Titering of virus was done by plaque analysis.<sup>50</sup>

**Infection of cell cultures and IFN $\beta$  treatment.** GL261, CT-2A, or U87-Fluc cells were plated on 12- or 24-well plates ( $5 \times 10^4$  to  $3 \times 10^5$  cells/well) and infected with VA7-EGFP (MOI = 0.01–1) the next day. IFN $\beta$  pretreatment was done by incubating the cells in culture medium containing 100 or 500 U/ml of mouse or human recombinant IFN $\beta$  (Sigma-Aldrich) for 5 hours before infection with VA7-EGFP. Addition of IFN $\beta$  was done by replacing the medium used in infection step with medium containing IFN $\beta$ . The infection was monitored with a fluorescence microscope.

**Cell viability measurements.** Cell viability was measured using MTT Cell Proliferation Kit I (Roche) according to manufacturer's instructions. A volume of  $1 \times 10^4$  to  $4 \times 10^4$  GL261, CT-2A, or U87-Fluc cells were plated on 96-well plates and infected with VA7-EGFP (MOI = 0.01 or 1.0) in 100  $\mu$ l of culture medium. IFN $\beta$  treatment was done by incubating GL261 cells 3.5 hours in 100  $\mu$ l of medium containing 100 U/ml mouse IFN $\beta$ . In addition, 100 U/ml mouse IFN $\beta$  was added to VA7-EGFP infected cells 30 or 90 minutes after infection (MOI = 1). MTT reagent was added 24, 48, or 72 hours p.i. followed by solubilization buffer 4 hours later. Absorbance at wavelength 590 nm and 650 nm was measured with VICTOR-plate reader (PerkinElmer, Waltham, MA) the following day. Results are presented by mean absorbance ( $A_{595}$  subtracted by  $A_{650}$ )  $\pm$  SD. Statistical analysis was done using unpaired, two-tailed *t*-test (GraphPad Prism; GraphPad Software, La Jolla, CA). Cell viability was determined by calculating the mean absorbance ( $A_{595} - A_{650}$ ) of treated sample divided by mean absorbance of respective untreated control and indicated as percentage (marked above the columns).

**In vivo animal experiments.** Gliomas were implanted to groups of normal C57BL/6 mice (Harlan Laboratories, Indianapolis, IN) by injecting  $1 \times 10^5$  GL261 or  $5 \times 10^4$  firefly luciferase expressing CT-2A-Fluc cells suspended in 5  $\mu$ l of OPTI-MEM (Sigma-Aldrich) i.c. 2 mm right from bregma in 3 mm depth. The cells were stored on ice before injection. For tumor induction, the animals were sedated with ketamine and medetomidine (75 mg/kg and 1 mg/kg, respectively) and kept under 2% isoflurane gas anesthesia during the operation. Antisiedative drug atipamezole (1 mg/kg, i.p.) was administered after operation and carprofen (5 mg/kg, s.c.) and buprenorphine (0.05–0.1 mg/kg, s.c.) were used for analgesia. Tumor development was monitored with T<sub>2</sub>-weighted magnetic resonance imaging using 9.7 T vertical magnet (Oxford Instruments, Oxford, UK). Mice were weighed daily and their condition and possible distress were monitored carefully. If signs of severe distress (e.g., paralysis) or weight loss of >20% were observed, the animal was euthanized. The animal experiments were performed at biosafety 2 containment level obeying the guidelines of the local committee of animal welfare.

First study group ( $n = 8$ ) was given three i.v. injections of VA7-EGFP ( $1 \times 10^6$  PFU/dose) on days 10, 13, and 16 p.t.i. In this study, an untreated tumor-bearing group ( $n = 8$ ) was used as a control. To enhance the probability of *in vivo* tumor infection, we performed a second study where the mice ( $n = 9$ ) were given three i.c. injections of VA7-EGFP ( $1 \times 10^6$  PFU/dose). Injections were given on days 6, 12, and 18 p.t.i. In this study, the control group ( $n = 9$ ) was given i.c. saline injections instead of virus. CT-2A-Fluc bearing groups of mice were given one i.v. injection of VA7-EGFP ( $1 \times 10^8$  PFU) 12 days p.t.i. ( $n = 9$ ). Control group ( $n = 9$ ) received no treatment.

Immunosuppressive combination therapy employing CPA with VA7-EGFP virus was carried out by giving the mice ( $n = 9$ ) i.p. injections of CPA on day 5 (3 mg) and 11 (2 mg) p.t.i. and i.v. injection of VA7-EGFP ( $1 \times 10^6$  PFU/dose) on day 6 and 12 p.t.i. The control group ( $n = 9$ ) for this study was administered only VA7-EGFP. For rapamycin (diluted in 30% ethanol) combination therapy studies, the gliomas were induced as previously by injecting  $1 \times 10^4$  GL261 cells i.c. to C57BL/6 mice. The combination therapy group ( $n = 5$ ) was given i.p. 5 mg/kg rapamycin on days 5–9, 12, and 13 p.t.i. and VA7-EGFP ( $1 \times 10^6$  PFU/dose i.v.) was given on days 6–9 and 13 p.t.i. Control groups ( $n = 6$ ) were given only rapamycin or VA7-EGFP injections. Third control group ( $n = 6$ ), was given injections of 30% ethanol i.p. and saline i.v. instead of rapamycin and VA7-EGFP.

In experiments utilizing athymic C57BL/6 nude mice (B6.Cg/N-Tac-Foxn1<sup>tm</sup> NE10),  $1 \times 10^5$  GL261 cells were implanted i.c. as described previously. Groups of mice were treated with i.v. ( $n = 5$ ) or i.c. ( $n = 6$ ) VA7-EGFP at days 6 and 12 p.t.i. or left untreated as control ( $n = 5$ ). Mice were monitored as previously.

In the “*in vivo* antiviral assay” subconfluent GL261 cells grown in 175-cm<sup>2</sup> culture flask were infected with VA7-EGFP ( $\sim 1.4 \times 10^8$  PFUs) for 1 hour, washed and implanted i.c. into immunocompetent C57BL/6 mice. Survival of preinfected GL261 group ( $n = 6$ ) was compared with control group ( $n = 6$ ) implanted with uninfected GL261 cells. The success of the infection was controlled by fluorescence microscopy of a parallel flask left to incubator over night.

**Immunohistochemical analysis of brain tissue samples.** After CO<sub>2</sub> killing, the mice were perfused with 4% formalin and the brains were collected. Brain tissue samples were first stored at +4°C in 4% paraformaldehyde (dissolved in phosphate-buffered saline) and transferred to 70% ethanol on the following day. Samples were embedded in paraffin and sliced into 7- $\mu$ m sections with microtome. Immunohistochemistry was performed to detect SFV antigens in paraffin-embedded brain tissue sections using polyclonal rabbit antibody and Vectastain ABC kit (Vector Laboratories, Burlingame, CA).

**Explanted GL261 glioma cells.** Explanted GL261 cell cultures were established from glioma tissue samples taken from orthotopic tumors

of C57BL/6 mice after i.c. VA7-EGFP treatment. Glioma samples were pressed through fine metal mesh onto tissue culture plates and cultured under normal conditions in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% FCS, 1% L-glutamine, and 1% penicillin–streptomycin. Explant GL261 cultures were analyzed for VA7-EGFP infectivity and IFN $\beta$ -sensitivity as described previously.

**Detection of VA7-EGFP NAb in serum samples.** Blood samples from the mice were collected immediately after killing and serum was separated using Microvette 500 Z-gel tubes (SARSTEDT). Serum samples were stored at –20°C. Titering of NAb was carried out on either MBA-13 or Vero (B) cells seeded onto 12-well plates ( $2 \times 10^6$  cells/well). Fivefold serial dilutions (1:20, 1:100, 1:500, and 1:2,500) were made from the serum samples and 40  $\mu$ l of the dilutions were mixed with 100  $\mu$ l of VA7-EGFP sample (containing 30–50 PFU) and incubated for 30 minutes at room temperature. Serum from an untreated mouse was used as a control. After incubation the mixtures were transferred onto 12-well plates containing the cells in 0.5 ml medium. The cells were observed with fluorescence microscopy 24 and 48 hours after infection. Neutralization titer was defined as reciprocal of the last dilution that completely inhibited VA7-EGFP infection visualized as cells expressing EGFP. The choice of cells [MBA or Vero (B)] did not affect the titering result. No neutralization was observed in the untreated samples.

**Western blot analysis.** Cell lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (15  $\mu$ g protein/sample, measured with Bradford method) and transferred to 0.1  $\mu$ m Protran nitrocellulose membrane (Schleicher & Schuell, Keene, NH). Membranes were incubated in anti-SFV or anti-OAS (kind gift from Ilkka Julkunen, KTL, Finland) polyclonal antibody followed by peroxidase-conjugated secondary antibody. Precision Plus Protein Dual Color was used as marker (BioRad, Hercules, CA). Blots were developed using chemiluminescence detection to X-ray film (Kodak, Rochester, NY).

**Measuring IFN $\beta$  with enzyme-linked immunosorbent assay.** A volume of  $3 \times 10^5$  GL261 or DBT cells were seeded in 6-well plate and either infected with VA7-EGFP or VSV carrying EGFP (VSV  $\Delta$ 51-EGFP) at MOI = 0.01 or left uninfected with 1 ml medium. After 24 hours supernatants were collected and kept at –20°C until analyzed. Measurement was done from two parallel wells using Verikine IFN $\beta$  ELISA kit (PBL InterferonSource, Piscataway, NJ) according to manufacturer's instructions. To measure IFN $\beta$  production from subconfluent and confluent GL261 cultures, cells were seeded on 96-well plate ( $1 \times 10^4$  or  $4 \times 10^4$  cells/well, respectively) and infected with VA7-EGFP (MOI = 0.01) in 200  $\mu$ l of culture medium. Supernatants were collected 3, 7, 24, and 48 hours p.i. and kept at –20°C until analyzed as above using half diluted samples (two or three parallels) with Verikine IFN $\beta$  ELISA kit (PBL InterferonSource) according to manufacturer's instructions.

## SUPPLEMENTARY MATERIAL

### Materials and Methods.

**Figure S1.** Inhibition of VA7-EGFP infectivity in confluent GL261 but not in confluent Vero(B) or CT-2A cultures *in vitro*.

**Figure S2.** Anti-mIFN $\beta$  antibody does not enhance VA7-EGFP infectivity.

**Figure S3.** EGTA pretreatment slightly enhances VA7-EGFP infection in GL261 cells *in vitro*.

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