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Study of the Efficacy, Biodistribution, and Safety Profile of Therapeutic Gutless Adenovirus Vectors as a Prelude to a Phase I Clinical Trial for Glioblastoma

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Abstract

Glioblastoma multiforme (GBM) is the most common and most aggressive primary brain tumor in humans. Systemic immunity against gene therapy vectors has been shown to hamper therapeutic efficacy; however, helper-dependent high-capacity adenovirus (HC-Ad) vectors elicit sustained transgene expression, even in the presence of systemic anti-adenoviral immunity. We engineered HC-Ads encoding the conditional cytotoxic herpes simplex type 1 thymidine kinase (TK) and the immunostimulatory cytokine fms-like tyrosine kinase ligand 3 (Flt3L). Flt3L expression is under the control of the regulatable Tet-ON system. In anticipation of a phase I clinical trial for GBM, we assessed the therapeutic efficacy, biodistribution, and clinical and neurotoxicity with escalating doses of HC-Ad-TetOn-Flt3L + HC-Ad-TK in rats. Intratumoral administration of these therapeutic HC-Ads in rats bearing large intracranial GBMs led to long-term survival in ~70% of the animals and development of antiglioma immunological memory without signs of neuropathology or systemic toxicity. Systemic anti-adenoviral immunity did not affect therapeutic efficacy. These data support the idea that it would be useful to develop HC-Ad vectors further as a therapeutic gene-delivery platform to implement GBM phase I clinical trials.

Glioblastoma multiforme (GBM), the most common primary brain tumor in adults, carries the dismal prognosis of 15–21 months median survival.[1–3] Its current clinical management comprises surgical resection followed by chemotherapy and radiotherapy.[4–6] However, given GBM's highly infiltrative nature, total resection is not possible and the tumor inevitably recurs. Recurrent GBM is resistant to chemotherapy and radiotherapy and eventually leads to the patient's death.[2] Immunotherapeutic approaches, currently under preclinical and clinical testing, constitute a promising adjuvant treatment for GBM.[7–13]

We previously engineered first-generation adenoviral vectors (Ads) that deliver a combination of therapeutic transgenes for the treatment of GBM. The conditionally cytotoxic herpes simplex type 1 thymidine kinase (TK)[8,12] kills proliferating tumor cells in the presence of the prodrug ganciclovir (GCV), and human soluble fms-like tyrosine kinase ligand 3 (Flt3L) recruits bone marrow–derived dendritic cells into the brain tumor milieu, triggering an anti-GBM-specific immune response.[8–9,13] It has been shown that the administration of Ad-TK + Ad-Flt3L into the tumor mass leads to long-term survival in rats bearing intracranial CNS-1, 9L, F98, and RG2 tumors[12,13] and in mice bearing intracranial GL26, GL261, and B16-F10 tumors.[8] In addition, Ad-Flt3L + Ad-TK induces GBM-specific immunological memory that improves survival in intracranial multifocal and recurrent models of GBM.[8,9,14,15]

The outcome of delivering TK alone has been studied in clinical trials using first-generation Ads.[16] Injection of Ad-TK into the margins of the tumor cavity after surgical resection of the GBM was well tolerated in more than 70 patients in six early clinical trials.[17,18] Final results from a large, multicenter phase III trial are eagerly awaited; however, the interim data, although they are promising, reveal that there is room for improvement with respect to the efficacy of the treatment.[19] Systemic anti-adenoviral immunity, which is present in most human patients[20] and which curtails transgene expression,[21–23] could limit the therapeutic efficacy of Ad-TK.

In an effort to evade the immune system, "gutless" high-capacity adenovirus (HC-Ad) vectors have been developed that are devoid of all viral genes.[24,25] In the presence of anti-adenoviral immunity, transgene expression from first-generation Ads is eliminated within 4 weeks, whereas expression from HC-Ad vectors remains stable in the brain for at least 1 year.[26–28] We recently showed the lack of efficacy of first-generation Ad-TK vectors in tumor-bearing rats preimmunized against Ads.[23] Conversely, HC-Ad-mediated expression of TK led to long-term survival in >50% of the preimmunized rats.[23] In the light of our earlier finding that single-cytotoxic therapies do not elicit antiglioma immunity,[8–10,12] we combined the HC-Ad-TK vector with a HC-Ad vector encoding Flt3L under the control of a tightly regulatable mCMV-TetOn expression system (HC-Ad-TetOn-Flt3L[29–31]) for future implementation in GBM clinical trials.

This study involved dose escalation of intratumoral injections of HC-Ad-TetOn-Flt3L + HC-Ad-TK in rats bearing intracranial GBM. We assessed the biodistribution and transgene expression as well as putative systemic and neurological toxicity. Therapeutic efficacy was evaluated in naive and preimmunized rats. This is the first study involving the combination of two HC-Ad vectors encoding a conditional cytotoxic gene and a tightly regulatable immunostimulatory gene in a preclinical animal model of brain cancer. Taken together, these data demonstrate the high safety profile and therapeutic efficacy of the HC-Ad vector platform for the delivery of therapeutic genes to the brain and suggest that there are potential advantages in pursuing further downstream process development for their eventual use in phase I clinical trials in human patients.

RESULTS

In vitro characterization of HC-Ad-TetOn-Flt3L + HC-Ad-TK

Characterization of HC-Ads was performed in vitro in rat CNS-1 GBM cells and in primary astrocyte cultures. Infection with HC-Ad-TetON-Flt3L (Figure 1a) led to doxycycline-dependent Flt3L release in both GBM cells and astrocytes, with negligible release in the OFF state (Figure 1b). Flt3L expression

was also confirmed by immunofluorescence. Expression of TK in cells infected with HC-Ad-TK (Figure 1c) was readily observed by immunofluorescence in GBM cells and astrocytes. However, the presence of the prodrug GCV exerted a cytotoxic effect only in CNS-1, leaving primary astrocytes unaffected (Figure 1d).

Intratumoral delivery of HC-Ad-TetOn-Flt3L + HC-Ad-TK mediates long-term survival up to 1 year after treatment

As a prelude to a phase I clinical trial in GBM patients, a dose-escalation study was performed in rats to assess the efficacy and neuropathology of intratumoral delivery of HC-Ad-TetOn-Flt3L and HC-Ad-TK (Figure 2a). We also assessed the biodistribution of vector genomes and transgene expression at 5 days, 30 days, 6 months, and 1 year after treatment. Each rat bearing an intracranial CNS-1 tumor received an intratumoral injection of either 5×10^8 vp, 1×10^9 vp, or 5×10^9 vp of each HC-Ad vector or saline. GCV was administered twice daily for 10 days. The rats received doxycycline-containing chow for 4 weeks because this schedule was found to be optimal with respect to efficacy of the therapy (see Supplementary Figure S1 online). All three doses led to tumor regression and survival for at least 1 year in ~70% of rats (Figure 2b).

Flt3L protein expression and copies of Flt3L transgene decrease within 30 days of HC-Ad delivery

The levels of Flt3L protein and transgene copies were determined in the brain 5 and 30 days after intratumoral administration of HC-Ads. Using an enzyme-linked immunosorbent assay, Flt3L protein was detected only in the injected hemisphere of the brain at day 5 after the treatment and became undetectable at day 30 (Figure 2c). Flt3L protein was not found in the contralateral hemisphere of the brain. We also quantified the copies of the Flt3L transgene, using real-time quantitative PCR. Flt3L transgene was detected only in the injected hemisphere of the brain at both day 5 and day 30 after the HC-Ad injection (Figure 2d).

Biodistribution of HC-Ad vector genomes is restricted to the brain hemisphere ipsilateral to the injection site at all vector doses and time points tested

We assessed the biodistribution of HC-Ad vector genomes in the CNS and in peripheral organs at 5 days, 30 days, 6 months, and 1 year after treatment, using quantitative PCR analysis. At all doses and time points, HC-Ad genomes were restricted to the injected hemisphere of the brain (Figure 3 and see Supplementary Figure S3 online), where a substantial decrease in HC-Ad vector genomes was detected between days 5 and 30 after the administration of HC-Ad. These findings are in agreement with the decline in Flt3L transgene copy number observed during the same time period (Figure 2d). Importantly, HC-Ad vector genomes were below detectable limits in all peripheral organs, including other regions of the CNS and the liver, even at the highest dose tested at all time points (Figure 3 and see Supplementary Figure S3 online), thereby indicating the high safety profile of this combined approach.

Analysis of neuropathology and clinical laboratory parameters

To examine the effects of HC-Ad-TetOn-Flt3L and HC-Ad-TK delivery and subsequent brain tumor regression on brain architecture and inflammation, we performed an extensive neuropathological analysis of brain sections at 5 days, 1 month, 6 months, and 1 year after intratumoral HC-Ad delivery. The three doses led to similar findings with respect to neuropathology. Nissl staining revealed a

dramatic reduction in tumor burden within 5 days of HC-Ad delivery as compared to saline-treated animals. Myelin basic protein and tyrosine hydroxylase immunoreactivity indicated rapid restoration of brain architecture, even at this early time point. Profuse infiltration of CD8+ T cells, macrophages, and major histocompatibility complex II+ cells was localized only within the tumor-bearing hemisphere of the brain (Figure 4).

One month after the treatment, although the infiltration of CD8+ T cells in the brain declined, sustained infiltration of macrophages and major histocompatibility complex II+ immune cells was observed (see Supplementary Figure S4 online). At this time point, all the saline-treated rats had succumbed to their tumor burden, whereas no remnants of the tumors were visible in ~70% of the rats that had received HC-Ad treatment, irrespective of the dose (see Supplementary Figure S4 online). At 6 months and at 1 year after HC-Ad delivery, we found complete restoration of the normal brain architecture—with the exception of minor ventriculomegaly, which was more accentuated in the rats that received the highest dose—in the brain hemisphere that harbored the tumor (Figure 5 and see Supplementary Figure S5 online). Residual macrophages were localized in the scar left after tumor regression.

Immunocytochemical characterization of transgene expression revealed abundant cells expressing TK and Flt3L within the tumor mass at day 5 after the treatment (Figure 4). Along with tumor regression, the presence of transgene-expressing cells declined with time; at 6 months and at 1 year after the treatment, only a few positive cells, probably non-neoplastic ones, remained in the scar area (Figure 5 and see Supplementary Figure S5 online). These data further highlight the fact that the conditional cytotoxicity of TK in combination with GCV is such that it does not kill normal brain cells and is a safe cytotoxic approach for brain cancer.

Serum chemistry tests were carried out and blood cell counts were assessed in the blood of tumor-bearing rats at 5 days, 30 days, 6 months, and 1 year after treatment. Analysis of biochemical laboratory parameters indicated normal liver and renal function, with concentrations of aspartate aminotransferase, alanine aminotransferase, bilirubin, urea, and creatinine within the normal range as seen in age-matched naive animals, at all time points and vector doses tested (see Supplementary Tables S1–S4 online). Red and white blood cell counts in the treated animals were also within normal ranges, indicating that Flt3L expression in the brain tumor does not substantially alter the levels of circulating immune cells.

HC-Ad-tetOn-Flt3L- and HC-Ad-TK (+GCV)-treated long-term survivors do not exhibit behavioral deficits

In order to rule out the occurrence of chronic neurological deficits, tumor-bearing animals treated with escalating doses of HC-Ad-TetOn-Flt3L and HC-Ad-TK long-term survivor animals were subjected to a panel of neurobehavioral tests 1 year after treatment. Analysis of amphetamine-induced rotational behavior and total locomotor activity failed to identify any behavioral abnormalities as compared with age-matched naive controls (Figure 6a,c). Abnormalities in limb-use asymmetry test and rearing activity test were detected in HC-Ad-treated long-term survivors after 1 year. However, these differences did not appear to be HC-Ad dose-dependent and are therefore probably a consequence of the regression of a very large brain tumor mass (Figure 6b,d).

HC-Ad-TetOn-Flt3L and HC-Ad-TK therapy improves survival of tumor-bearing animals having a preexisting anti-Ad immune response

Because most patients who undergo gene therapy clinical trials have previously been exposed to Ad in their lifetime,[20] we assessed the efficacy of HC-Ad-TetOn-Flt3L and HC-Ad-TK (5×10^9 vp each) in a preclinical setting that models this likely clinical scenario (Figure 7a). Lewis rats were immunized with an intradermal injection of an Ad vector without a transgene (Ad0). Animals preimmunized against Ad displayed high levels of circulating neutralizing antibodies against Ad 2 weeks later, at the time of tumor implantation (Figure 7b). HC-Ad treatment led to the survival of >70% of the animals in both immunized and nonimmunized groups for at least 1 year (Figure 7b).

Because recurrence of the tumor is one of the hallmarks of GBM, we sought to assess whether, despite anti-Ad immunity, HC-Ad treatment would induce anti-GBM immunological memory. For this purpose, we rechallenged preimmunized long-term survivor animals with a second tumor in the contralateral striatum (Figure 7c). Although no further treatment was administered, ~70% of the animals survived the rechallenge. These findings indicate that, despite the pre-existing anti-Ad immunity, treatment with HC-Ad-TetOn-Flt3L + HC-Ad-TK elicits immunological memory against GBM, and this is capable of preventing the progression of a recurrent brain tumor.

DISCUSSION

The hallmarks of GBM, such as multifocal and recurrent tumors infiltrating the normal brain tissue, make the treatment of this disease particularly challenging.[2] We developed a gene therapy approach that combines the conditionally cytotoxic TK with the immunostimulatory Flt3L.[12] Although the normal brain parenchyma exhibit a paucity of antigen-presenting cells, Ad-Flt3L delivered directly into the brain tumor elicits the recruitment of these cells into the GBM microenvironment. Ad-TK (+GCV) induces the killing of tumor cells, and the brain tumor antigen released in response to the killing is phago cytosed by dendritic cells.[8] Dendritic cells loaded with brain tumor antigen migrate to the draining lymph node, where they present tumor antigen to naive T cells, thereby inducing a brain tumor–specific immune response[8,9] and generating immunological memory that protects against recurrent brain tumors.[8,14] We previously showed the therapeutic efficacy of this approach in syngeneic intracranial rat and mouse models of GBM;[8,13] however, pre-existing systemic immunity against Ad is highly prevalent in humans[20] and could hamper the efficacy of Ad-mediated gene therapy in this setting.[21–23] In this study, therefore, we tested the hypothesis that HC-Ad vectors encoding TK and Flt3L would be efficacious and safe, even in the presence of anti-Ad immunity, as may occur in humans undergoing clinical trials.[20]

In anticipation of a phase I clinical trial for GBM using HC-Ad-TetOn-Flt3L + HC-Ad-TK, we performed a dose-escalation study and performed a comprehensive analysis of the efficacy and toxicity of this therapeutic approach in a syngeneic intracranial GBM model. To our knowledge, these data represent the first from an efficacy and toxicity study utilizing gutless, helper-dependent HC-Ad vectors in an animal model of brain cancer. In fact, the number of preclinical efficacy studies in animal models of disease remains surprisingly limited for such a promising gene delivery technology. The efficacy of HC-Ad vectors has been assessed in animal models of sensory neuronopathies, hemophilia, diabetic retinopathy, glycogen storage disease, monogenic hypoalphalipoproteinemia, and hypertension.[32–38] Two of these studies involved the use of regulatable adenoviral vectors,[35,38]

which, given the large size of regulatable expression cassettes, can be engineered only on HC-Ad vector backbones.

Using escalating doses of combination HC-Ad-TetOn-Flt3L + HC-Ad-TK, we demonstrated high-therapeutic efficacy and concomitant reduction of the tumor mass within 30 days of treatment in ~70% of rats. Tumor regression occurs concomitantly with a reduction in the copies of vector genomes and expression of Flt3L protein. These data suggest that tumor cells transduced with HC-Ad vectors are rapidly eliminated upon treatment.

Our previous studies in normal brains of preimmunized animals demonstrated sustained levels of HC-Ad vector genomes, even in the presence of anti-Ad immunity.[28,39] Although considerably reduced from their levels at day 5 after treatment, sustained levels of HC-Ad vector genomes persist in the brain for up to 1 year after treatment, suggesting that the cytotoxic effects of TK or the immunostimulatory effects of Flt3L can be "reactivated" with the readministration of GCV or DOX, respectively, if necessary.

The restoration of the normal brain architecture, the absence of severe long-term behavioral deficits 1 year after treatment, and the absence of significant chronic inflammation in the brain provide strong evidence of the safety of this combined approach. The decrease in the number of immune cells in the brain at 6 months after treatment may be attributed, at least partly, to the regulatable features engineered into HC-Ad-TetOn-Flt3L, which expresses Flt3L only in the presence of DOX. The absence of demyelination at all time points tested attests to the overall safety of HC-Ad-mediated delivery of TK and Flt3L. Importantly, the delivery of HC-Ads into the brains of tumor-bearing animals did not result in an alteration of the biochemical or hematological parameters. This was in contrast to the findings of a recent study that used a retargeted adenoviral vector encoding TK in an animal model of recurrent ovarian cancer.[40]

Importantly, our data, showing that the HC-Ad vector genomes are restricted to the injected brain hemisphere, demonstrate that HC-Ad vectors do not diffuse to other regions of the CNS or peripheral organs. This finding is in agreement with those of several preclinical studies that assessed the safety and biodistribution of first-generation and oncolytic adenoviral vectors after intracranial injection in naive rodents.[41,42]

The demonstration of therapeutic efficacy of HC-Ad-TetOn-Flt3L + HC-Ad-TK in a large brain tumor model in animals preimmunized against Ad highlights the high efficacy of HC-Ad vectors in delivering therapeutic genes at biologically appropriate levels in an animal model of GBM exhibiting anti-Ad systemic immunity. This is important because it mirrors the scenario likely to be encountered in a clinic setting.[20] Most importantly, long-term immunological memory protects against tumor rechallenge, even at 1 year after treatment, which demonstrates the therapeutic efficacy of this approach in countering recurrences of GBM. The data reported represent the first experimental evidence to indicate the persistence of immunological memory that is sustained for up to 1 year after HC-Ad-mediated combined delivery of Flt3L and TK, even in the presence of a systemic anti-Ad immune response. We previously demonstrated that HC-Ad-TK alone is effective in eradicating intracranial brain tumors in preimmunized animals.[23] However, when administered alone, it failed to induce anti-GBM immunological memory. This shows that TK alone cannot provide protection in cases of tumor recurrence as would be encountered in the clinic (data not shown). In summary, the efficacy and safety of this approach, as demonstrated in clinically relevant animal models, strongly

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support the further development of HC-Ad-TetOn-Flt3L + HC-Ad-TK as a second-generation delivery platform for implementation in phase I clinical trials for GBM.

METHODS

Please see Supplementary Materials and Methods S1 below for a more detailed description of materials and methods used.

Adenoviral vectors

We have previously described the molecular characterization, rescue, and amplification of HC-Ad-TK, HC-Ad-TetOn-Flt3L, and HC-Ad-TetOn-βgal and Ad0.[29,31]

In vitro characterization of HC-Ad vectors

CNS-1 cells and astrocytes were seeded (25,000/well) and infected 24 h later with either HC-Ad vector (2,000 vp/cell). Transgene expression was assessed using enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN) and immunocytochemistry.[8,43] TK cytotoxicity was assessed by flow cytometric analysis of propidium iodide/Annexin V–stained cells.[8,9,44]

Brain tumor rodent models

Rat GBM CNS-1 cells (4,500 cells, 3 µl) were stereotactically implanted in the right striatum of the brain in syngeneic Lewis rats (220–250 g; Harlan, Indianapolis, IN) as previously described.[9,45] Four days after cell implantation (2 days before HC-Ad administration), the rats were started on doxycycline-containing chow ad libitum (see Supplementary Materials and Methods S1 online). Six days after cell implantation (after 2 days on the doxycycline chow), the rats received an intratumoral injection of HC-Ad-TetOn-Flt3L + HC-Ad-TK, the control vector HC-Ad-TetOn-βgal, or saline. Starting at 24 h after treatment, the rats received GCV (25 mg/kg, intraperitoneal) twice daily for 10 days. All experimental procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Biodistribution of vector genomes and quantification of Flt3L transgene copies in the brain

Analysis of the biodistribution of vector genomes was performed at 5 days, 30 days, 6 months, or 1 year after treatment. Striatal tissue (25 mg) samples were dissected from the brain tumor and the contralateral hemisphere, the cerebellum, and the brain stem. Tissue samples (25 mg) were also obtained from the spleen, liver, testes, gut, lung, heart, cervical draining lymph nodes, kidney, and lumbar spinal cord (Supplementary Figure S2 online). Total DNA was purified and used for the quantitation of vector genomes and Flt3L transgene copies by real-time quantitative PCR[46] as described in Supplementary Materials and Methods S1 online. The sequence of HC-Ad primers and probe for detection of HC-Ad vector genomes are shown in Supplementary Figure S2 online. Vector genomes and Flt3L transgene copies are shown as ratios relative to 25 mg of tissue. Graphs show the values and means of n = 5 per group.

Neuropathological analysis

Neuropathological analysis of the brain was performed at 5 days, 30 days, 6 months, or 1 year after treatment. The analysis is described in Supplementary Materials and Methods S1 online.

Assessment of Flt3L expression in brain tissue

Analysis of Flt3L expression in the brain of treated animals was performed 5 days and 30 days after treatment (as described in Supplementary Materials and Methods S1 online), using an enzyme-linked immunosorbent assay kit (DFK00; R&D Systems) specific for human soluble Flt3L.

Analysis of blood biochemistry

At 5 days, 30 days, 6 months, and 1 year after treatment, blood was collected and a comprehensive panel of tests involving serum chemistry and hematologic parameters was performed by Antech Diagnostics (Irvine, CA). Normative reference values were established using blood from naive, age-matched animals. The median, minimum, and maximum values for each parameter are shown in Supplementary Tables S1–S4 online.

Behavioral analysis

The long-term behavioral impact of intratumoral delivery of HC-Ad was evaluated at 1 year after treatment by assessing amphetamine-induced rotational behavior, abnormalities in limb-use asymmetry test and spontaneous motor and rearing behavior as described elsewhere (see ref. 14 and Supplementary Materials and Methods S1 online). Naive, age-matched Lewis rats were used as controls.

Preimmunization and rechallenge studies

Anti-Ad immunization was performed by administering an intradermal injection in the back of the neck of the animal, with 1×10^9 infectious units of first-generation adenoviral vector Ad0, or saline as a control, 2 weeks before implantation of CNS-1 tumor cells. In order to confirm the presence of an anti-Ad immune response, blood was collected by retro-orbital bleeding during implantation of the CNS-1 tumors, and the circulating levels of anti-Ad neutralizing antibodies were assessed as described earlier (see ref. 23 and Supplementary Materials and Methods S1 online). Six days after tumor implantation, each rat received an intratumoral injection of 5×10^9 vp each of HC-Ad-TetOn-Flt3L + HC-Ad-TK or saline. At 1 year after treatment, long-term survivors were rechallenged with a second tumor in the contralateral striatum. No further treatment was given after the rechallenge.

Statistical analysis

Sample sizes were calculated in order to detect differences between groups with a power of 80% at a 0.05 significance level, using PASS 2008 (Power and Sample Size software; NCSS, Kaysville, UT). Data were analyzed using one-way analysis of variance followed by Tukey's post-test or two-tailed Student's t-test (NCSS). Where data were not normally distributed, they were either log-transformed or analyzed using a Mann–Whitney U nonparametric post-test. Kaplan–Meier survival curves were analyzed using the Mantel-log rank test (GraphPad Prism version 3.00; GraphPad Software, San Diego, CA). P values <0.05 were used to determine the null hypothesis to be invalid. The statistical tests used are indicated in the figure legends.

Supplementary Material

Please see the accompanying supplementary material file online for additional data, materials and methods.

Footnotes

The first two authors contributed equally to this work.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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FIGURE LEGENDS

Figure 1: Structure and *in vitro* characterization of therapeutic HC-Ads. (**a**) Structure and transcriptional regulation of HC-Ad-TetOn-Flt3L. (**b**) CNS-1 GBM cells and astrocytes in primary culture were infected with HC-Ad-TetOn-Flt3L with or without the inducer, DOX. Flt3L expression was assessed by immunofluorescence and ELISA. *$P < 0.05$ vs. mock infection. One-way ANOVA followed by Tukey's test. (**c**) Illustration depicting structure and function of HC-Ad-TK. (**d**) CNS-1 GBM cells and astrocytes in primary culture were infected with HC-Ad-TK and incubated with or without the prodrug GCV. TK expression was assessed by immunofluorescence, and cell death was determined by flow cytometric analysis of Annexin V/PI-stained cells. *$P < 0.05$ vs. mock infection. One-way ANOVA followed by Tukey's test. ANOVA, analysis of variance; DOX, doxycycline; ELISA, enzyme-linked immunosorbent assay; Flt3L, fms-like tyrosine kinase ligand 3; GBM, glioblastoma multiforme; GCV, ganciclovir; HC-Ad, high-capacity adenovirus; PI, propidium iodide; TK, thymidine kinase.

Figure 2: Efficacy and Flt3L expression after intratumoral injection of escalating doses of HC-Ads. (**a**) Three escalating doses of HC-Ad-TK and HC-Ad-TetOn-Flt3L (5×10^8 vp, 1×10^9 vp, and 5×10^9 vp, *n* = 18–19/group) or saline (*n* = 10/group) were delivered intratumorally into 6-day intracranial CNS-1 tumors in rats. (**b**) Kaplan–Meier survival curves show efficacy of HC-Ad treatment. *$P < 0.05$ vs. saline (log-rank test). (**c**) Levels of Flt3L protein in brain hemispheres ipsilateral and contralateral to HC-Ad injection site were assessed by ELISA at 5 and 30 days after treatment (*n* = 5/group). *$P < 0.05$ vs. contralateral (Student's *t*-test). (**d**) DNA was isolated from both brain hemispheres (*n* = 5/group), and Flt3L transgene copies were quantified using quantitative PCR at 5 and 30 days after HC-Ad delivery. *$P < 0.05$ vs. contralateral (Student's *t*-test). cont, contralateral; ELISA, enzyme-linked immunosorbent assay; Flt3L, fms-like tyrosine kinase ligand 3; GCV, ganciclovir; HC-Ad, high-capacity adenovirus; ips, ipsilateral; TK, thymidine kinase.

Figure 3: Biodistribution of vector genomes in tumor-bearing animals. HC-Ad vector genomes were quantified in the tissues indicated at 5 days after intratumoral injection of escalating doses of HC-Ad-TetOn-Flt3L and HC-Ad-TK (**a**, 5×10^8 vp; **b**, 1×10^9 vp; and **c**, 5×10^9 vp). The dotted line indicates the detection limit. Flt3L, fms-like tyrosine kinase ligand 3; HC-Ad, high-capacity adenovirus; TK, thymidine kinase.

Figure 4: Neuropathological analysis of samples from tumor-bearing Lewis rats treated with HC-Ads. Neuropathological analysis of the brains of rats at 5 days after intratumoral administration of escalating doses of HC-Ad-TetOn-Flt3L and HC-Ad-TK. Flt3L, fms-like tyrosine kinase ligand 3; MBP, myelin basic protein; MHC II, major histocompatibility complex II; TH, tyrosine hydroxilase; TK, thymidine kinase.

Figure 5: Long-term survivors do not show evidence of neuropathology at 1 year after treatment. Neuropathological analysis was performed of the brains of the rats that were long-term survivors, at 1 year after intratumoral administration of escalating doses of HC-Ad-TetOn-Flt3L and HC-Ad-TK. Flt3L, fms-like tyrosine kinase ligand 3; HC-Ad, high-capacity adenovirus; MBP, myelin basic protein; MHC II, major histocompatibility complex II, TH, tyrosine hydroxilase; TK, thymidine kinase.
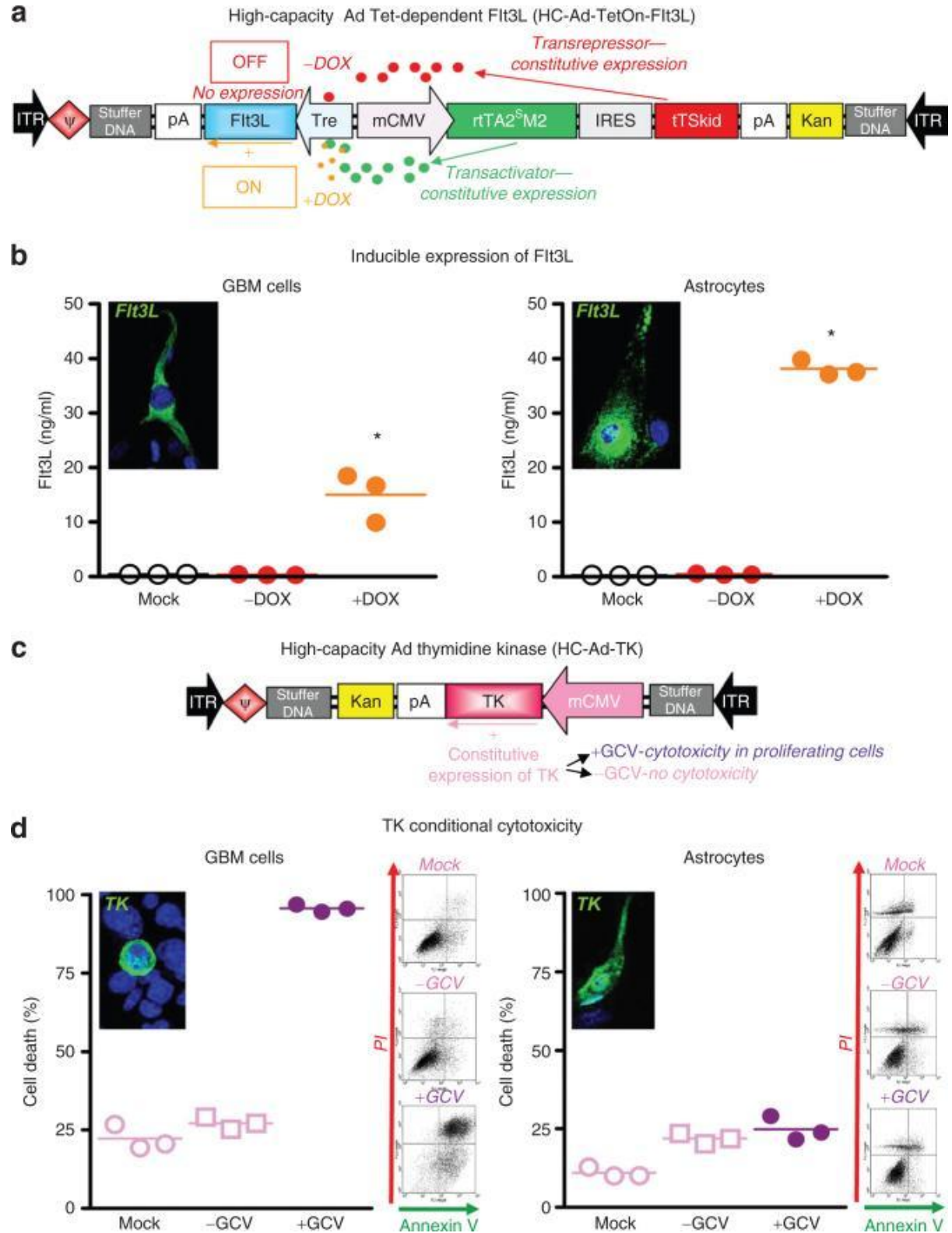
Figure 6: Behavioral assessment of long-term survivors 1 year after treatment with HC-Ad-TetOn-Flt3L and HC-Ad-TK. Behavioral assessment was performed before and after amphetamine

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treatment in the animals that were long-term survivors, at 1 year after intratumoral administration of escalating doses of HC-Ad-TetOn-Flt3L and HC-Ad-TK. Naive, age-matched rats were used as controls. (**a**) Rotational behavior; (**b**) asymmetrical right-limb use; (**c**) spontaneous motor behavior; and (**d**) rearing behavior. Flt3L, fms-like tyrosine kinase ligand 3; HC-Ad, high-capacity adenovirus; TK, thymidine kinase.

Figure 7: HC-Ad-induced tumor regression and antiglioma immunological memory, despite active systemic anti-Ad immunity. (**a**) Illustration depicting the experimental design. Rats peripherally immunized against Ads (Ad0) were implanted with CNS-1 tumors in the brain and treated with 5×10^9 of each HC-Ad (*n* = 8–14/group), or saline (*n* = 6–7/group). (**b**) Kaplan–Meier survival curves show the survival of preimmunized and nonpreimmunized rats. *P < 0.05 vs. saline. Log-rank test. Scatter plot shows the titers of circulating anti-Ad NAB in each group. (**c**) Kaplan–Meier survival curves show the survival of preimmunized long-term survivors rechallenged in the contralateral striatum of the brain at 1 year after treatment. Naive animals were implanted with CNS-1 cells as controls. *P < 0.05 vs. naive. Log-rank test. DOX, doxycycline; GCV, ganciclovir; HC-Ad, high-capacity adenovirus; NAB, neutralizing antibodies.
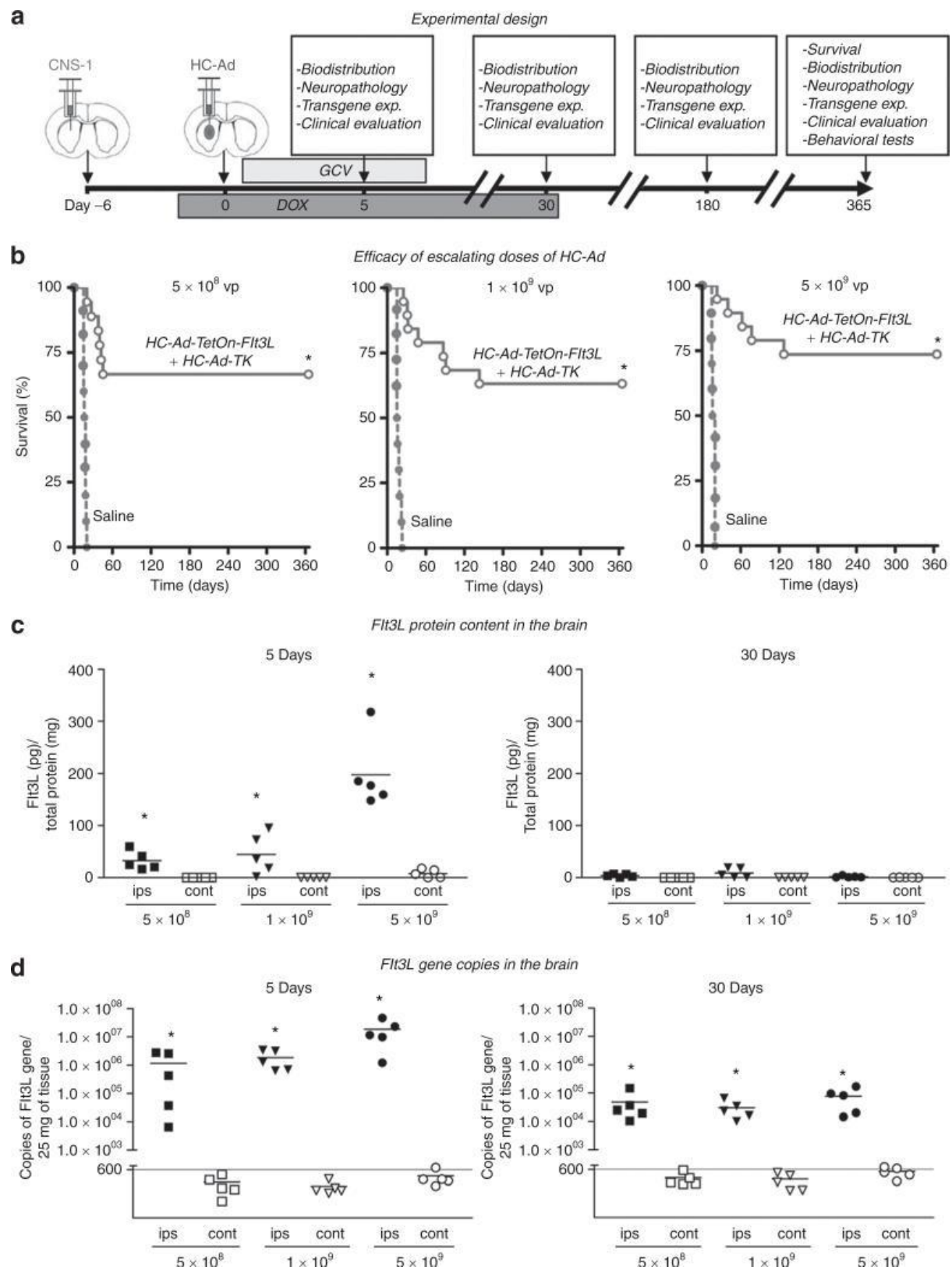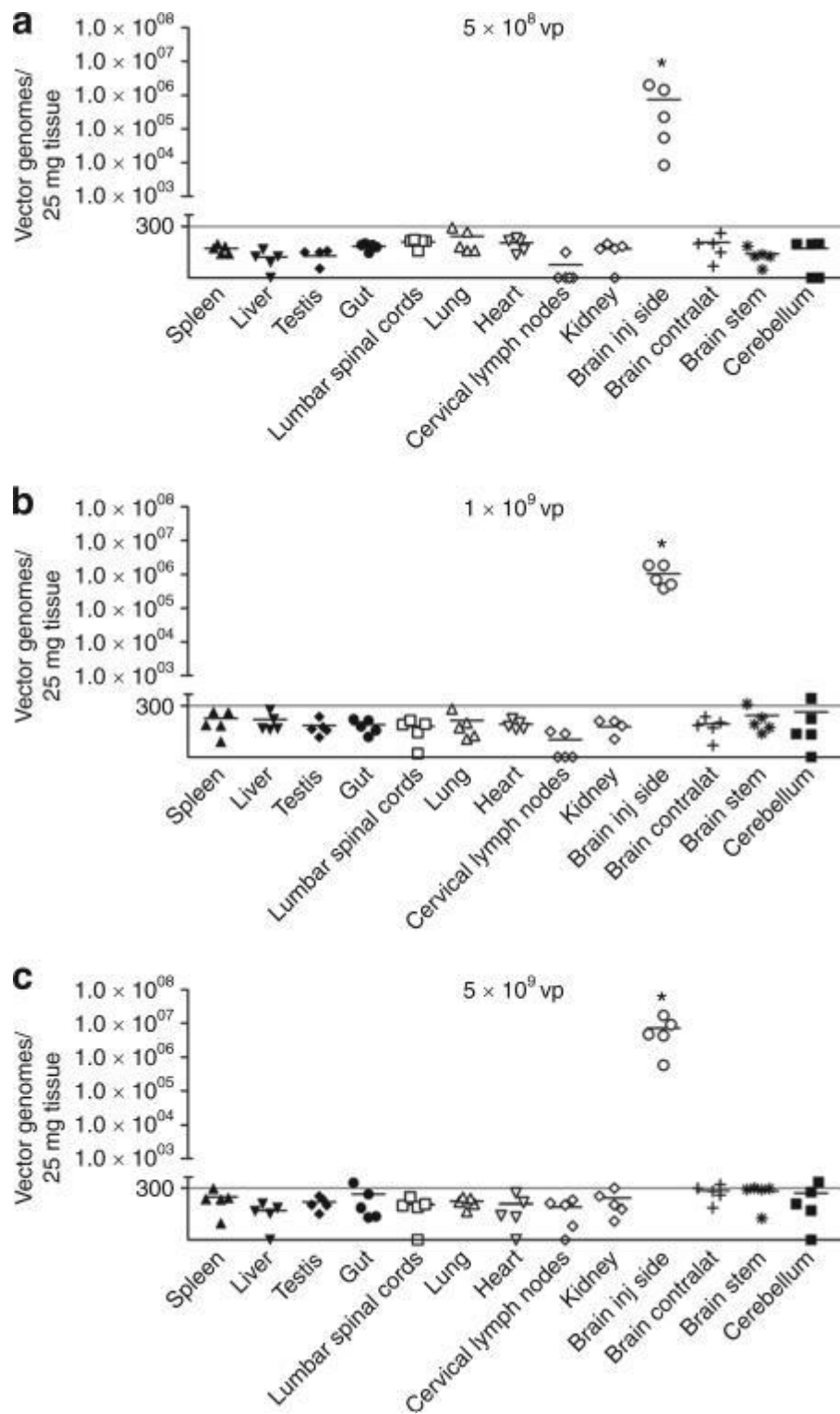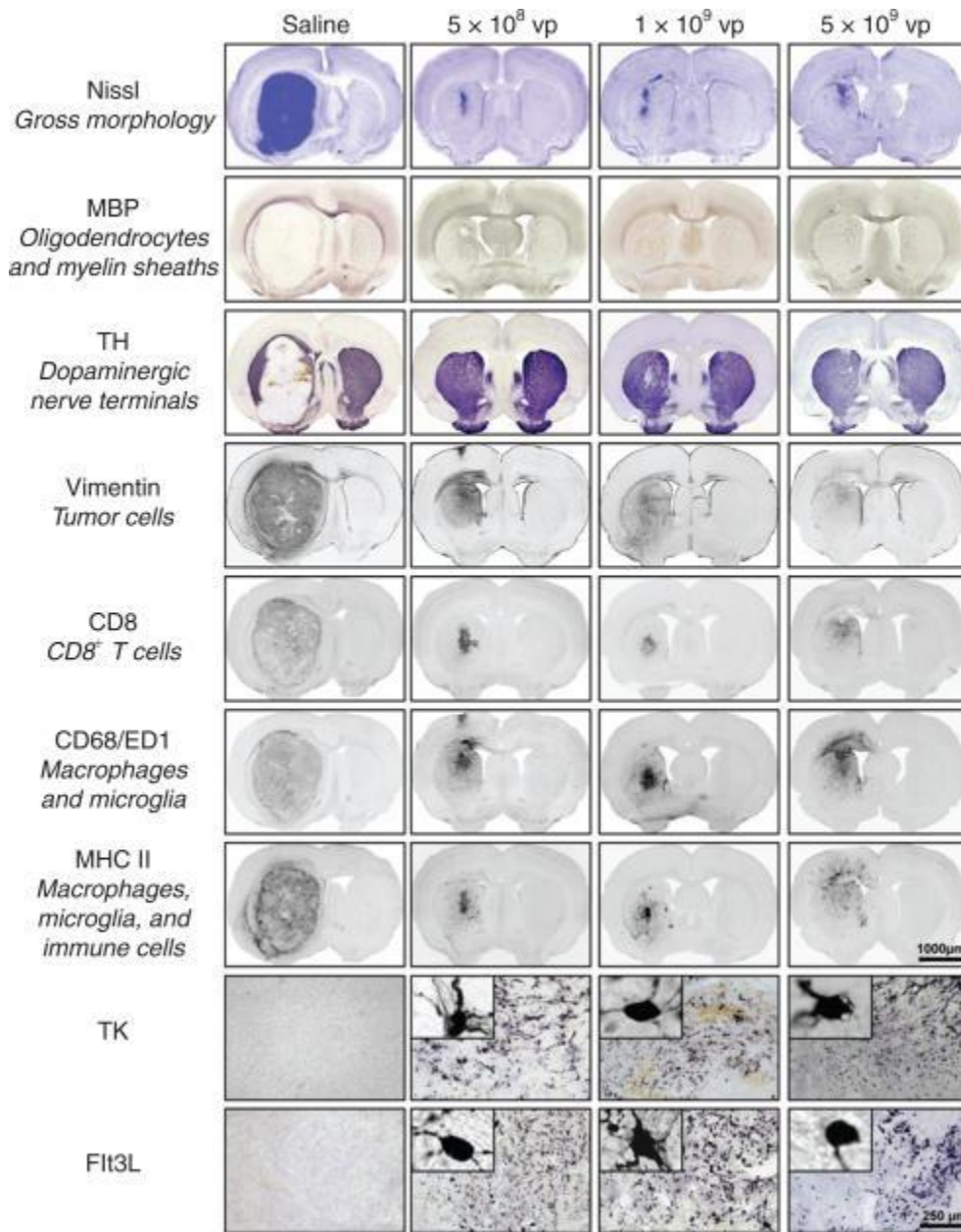
Figure 1

a

High-capacity Ad Tet-dependent Flt3L (HC-Ad-TetOn-Flt3L)

b   Inducible expression of Flt3L

GBM cells   Astrocytes

c   High-capacity Ad thymidine kinase (HC-Ad-TK)

d   TK conditional cytotoxicity

GBM cells   Astrocytes

Figure 2

Figure 3

Figure 4

Figure 5

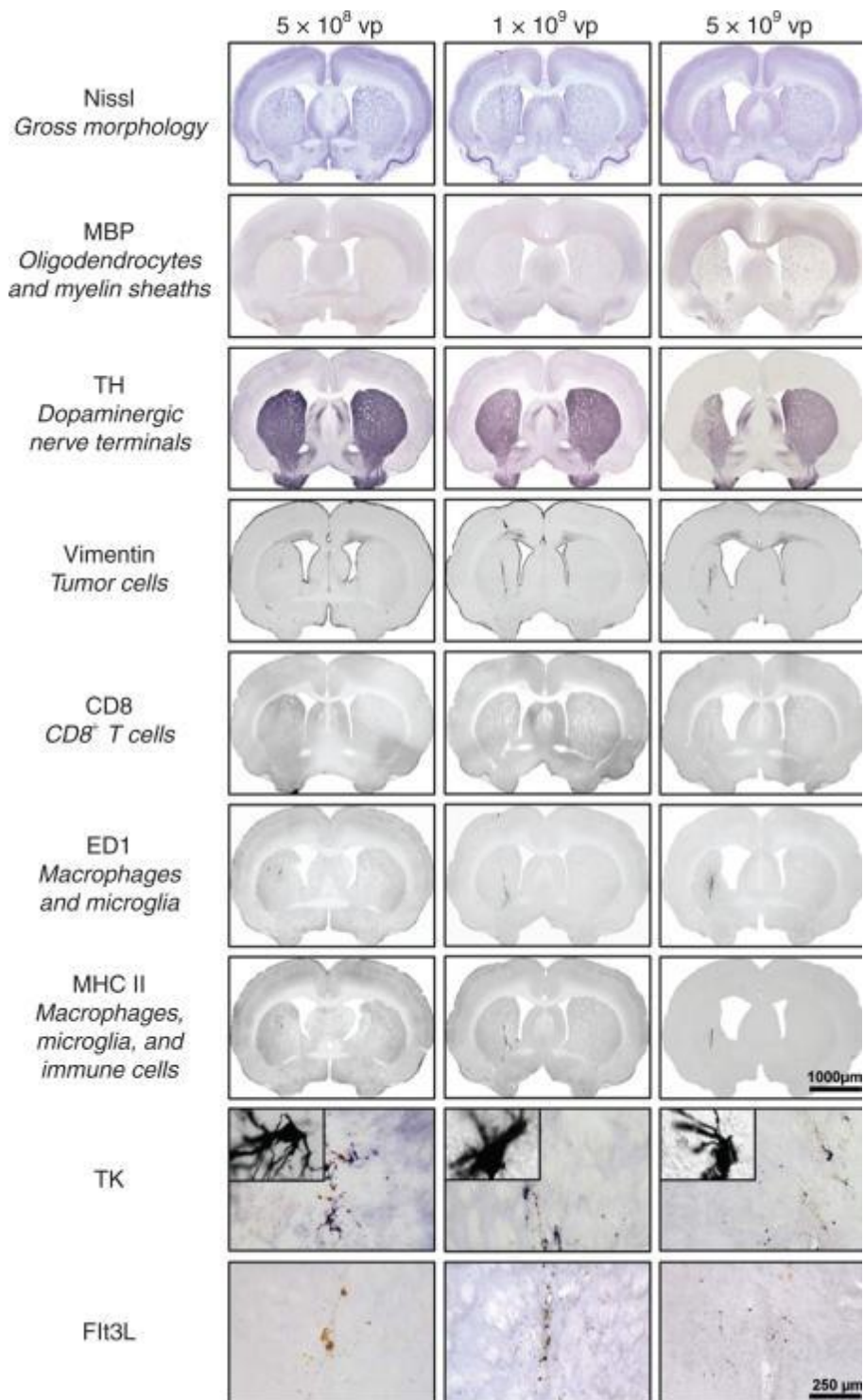**Figure 6**

Figure 7

a Experimental design

b Efficacy of HC-Ad treatment in preimmunized rats

c Antitumor immunological memory in preimmunized rats