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## Release of HMGB1 in response to pro-apoptotic glioma killing strategies: efficacy and neurotoxicity

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### Abstract

#### Purpose

In preparation for a Phase I clinical trial utilizing a combined cytotoxic/immunotherapeutic strategy using adenoviruses expressing Flt3L (Ad-Flt3L) and thymidine kinase (Ad-TK) to treat glioblastoma (GBM), we tested the hypothesis that Ad-TK+GCV would be the optimal tumor killing agent in relation to efficacy and safety when compared to other pro-apoptotic approaches.

#### Experimental Design and Results

The efficacy and neurotoxicity of Ad-TK+GCV was compared with Ads encoding the pro-apoptotic cytokines (TNF- $\alpha$ , TRAIL, FasL), alone or in combination with Ad-Flt3L. In rats bearing small GBMs (day 4), only Ad-TK+GCV or Ad-FasL improved survival. In rats bearing large GBMs (day 9), the combination of Ad-Flt3L with Ad-FasL did not improve survival over FasL alone, while Ad-Flt3L combined with Ad-TK+GCV led to 70% long-term survival. Expression of FasL and TRAIL caused severe neuropathology, which was not encountered when we utilized Ad-TK+/-Ad-Flt3L. In vitro, all treatments elicited release HMGB1 from dying tumor cells. In vivo, the highest levels of circulating HMGB1 were observed after treatment with Ad-TK+GCV+Ad-Flt3L; HMGB1 was necessary for the therapeutic efficacy of AdTK+GCV+Ad-Flt3L, since its blockade with glycyrrhizin completely blocked tumor regression. We also demonstrated the killing efficacy of Ad-TK+GCV in human GBM cell lines and GBM primary cultures; which also elicited release of HMGB1.

#### Conclusions

Our results indicate that Ad-TK+GCV+Ad-Flt3L exhibits the highest efficacy and safety profile amongst the several pro-apoptotic approaches tested. The results reported further support the implementation of this combined approach in a Phase I clinical trial for GBM.

Keywords: glioblastoma, apoptosis, immunotherapy, adenoviral vectors, Flt3L

## INTRODUCTION

Glioblastoma multiforme (GBM) is an invasive brain tumor derived from glial cells. Every year in the United States, 18,000 people are diagnosed with GBM, constituting the most common malignant primary brain tumor. The standard of care for treatment of GBM consists of surgical resection, followed by radiation therapy and chemotherapy with temozolomide. Temozolomide extends the median survival by 2 to 12 months, with 8% to 40% of the patients surviving for up to 2 years depending on the trial and the molecular makeup of GBM (1). Due to the diffuse nature of GBM, tumor resection is unlikely to be complete and recurrence occurs usually within 2 to 3 cm of the resection margins (2). Thus, more effective strategies are urgently needed for patients with GBM. Novel therapies aimed at targeting immune cells to eliminate neoplastic cells within the brain parenchyma far from the main tumor mass, including various vaccination approaches (3, 4), could have a high impact in the treatment of this devastating cancer.

Previous results from our laboratory have shown that an immunotherapy approach using adenoviral vectors (Ad) encoding the cytokine Flt3L and herpes simplex virus type 1-thymidine kinase (TK) induces tumor regression, long-term survival, and immunologic memory in rats and mice bearing large intracranial syngeneic glioblastomas or metastatic melanoma (GBM; refs. 5–9). Intracranial administration of Ad-Flt3L recruits dendritic cells into the brain parenchyma (10), improving brain tumor antigen presentation; Ad-TK exerts a cytotoxic effect exclusively in proliferating GBM cells in the presence of ganciclovir (GCV), leading to the release of tumor antigens and proinflammatory molecules from dying tumor cells (7).

Before clinical translation of the conditional cytotoxic/immunotherapeutic approach that combines Ad-Flt3L with Ad-TK+GCV in a phase I clinical trial for GBM, we wished to test the hypothesis that delivery of the conditionally cytotoxic gene, TK, is the optimal tumor-killing agent to be used in combination with Ad-Flt3L. Thus, we compared the efficacy and neurotoxicity of Ad-TK with Ad vectors encoding the proapoptotic cytokines tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), TNF-related apoptosis-inducing factor (TRAIL), or Fas ligand (FasL). Because expression of death receptors and their ligands has been described in human glioblastoma, targeting of these receptors has been proposed as potential approaches for GBM treatment. Importantly, proapoptotic cytokines released from infected cells could also elicit strong bystander effects.

TNF- $\alpha$  receptor 1 (TNFR1) expression has been detected in human GBM cells; hence, delivery of TNF- $\alpha$  has been attempted in preclinical GBM models and phase I clinical trials for GBM using recombinant proteins or gene therapy vectors (11–14). TRAIL was selected in view that this cytokine exhibits a strong cytotoxic effect on GBM cells in vitro and in vivo that can be enhanced with chemotherapeutic agents and radiotherapy (15–17). Expression of TRAIL receptors has been detected consistently in human GBM (18) and their expression is enhanced by radiation and chemotherapy (15–17, 19). Thus, delivery of TRAIL in combination with irradiation or temozolomide has been attempted in preclinical models for GBM (17, 20–23).

It has also been reported that Fas is expressed in ~90% of human GBM (24), constituting a valuable target for therapy development. FasL showed a very strong proapoptotic effect in several human and rodent GBM cells (25). Moreover, we and others found that intratumoral delivery of an adenovirus expressing FasL improved the survival of rats bearing intracranial GBM (26, 27), constituting a promising therapeutic candidate.

In the present work, we found that in rats bearing small tumors (day 4), only Ad-TK+GCV and Ad-FasL improved survival. Thus, we selected them to be used in combination with immune-stimulatory Ad-Flt3L for the treatment of large tumors (day 9), in which all single therapies fail (5). We found that although Ad-Flt3L only marginally improved the survival of Ad-FasL-treated rats, it significantly increased survival when combined with Ad-TK+GCV, leading to more than 70% of long-term survivors. Administration of Ad-TK+GCV alone or combined with Ad-Flt3L did not significantly alter the structure of the normal brain, whereas expression of FasL or TRAIL had severe neuropathologic consequences. These results suggest that Ad-TK+GCV+Ad-Flt3L is the most effective among the several therapeutic approaches tested and also exhibits the best safety profile.

We recently showed that therapeutic efficacy of Ad-TK+GCV+Ad-Flt3L is dependent on the release of the nuclear protein high mobility group box 1 (HMGB1) from dying tumor cells (7). HMGB1 is a ubiquitous chromatin-binding protein present in the nucleus of virtually all eukaryotic cells (28). When HMGB1 is secreted by inflammatory cells or released from dying cells into the extracellular milieu, it acts as an endogenous TLR agonist (7, 28, 29). We showed that treatment of mice bearing syngeneic intracranial brain tumors with Ad-TK+GCV+Ad-Flt3L induces the release of HMGB1 from dying tumor cells, which in turn activates TLR2 signaling in bone marrow-derived tumor-infiltrating dendritic cells, initiating a specific antitumor immune response (7). Other cytotoxic agents that kill proliferating cells and are routinely used in the treatment of GBM patients, such as radiotherapy and temozolomide, also led to HMGB1 release from GBM cells (7).

In the present work, we wished to test the hypothesis that HMGB1 would be released upon tumor cell death induced not only by cytotoxic agents that inhibit replication but also by proapoptotic cytokines that kill cells by activation of membrane death receptors. In addition, we determined that HMGB1 release is involved in the efficacy of the immunotherapeutic approach in a rat syngeneic model of GBM. All proapoptotic Ads induced the release of HMGB1 from CNS-1 tumor cells *in vitro* and *in vivo* and the therapeutic efficacy of Ad-TK+GCV+Ad-Flt3L was indeed dependent on release of HMGB1 because its blockade with glycyrrhizin completely abolished the efficacy of the treatment. Further, HMGB1 was also released from human GBM cell lines and primary GBM cell cultures obtained from surgical biopsies, in response to tumor cell killing elicited by treatment with Ad-TK+GCV. Collectively, our data strongly support the implementation of the combined TK/Flt3L gene therapy in a phase I trial for human GBM.

## **MATERIALS AND METHODS**

### *Adenoviral vectors*

Ad vectors used are based on adenovirus type 5 (Ad5), with deletion in the E1 and E3 regions; the expression cassette containing the appropriate transgene is inserted within the E1 region (30). Six

different vectors were used: Ad-TRAIL [expresses human TRAIL under the control of the CAG promoter, which combines the human cytomegalovirus (CMV) immediate-early enhancer and a modified chicken  $\beta$ -actin promoter; ref. 31], Ad-TNF- $\alpha$  (expresses human TNF- $\alpha$  under the control of the human CMV promoter, hCMV; ref. 32), Ad-FasL (expresses murine FasL under the control of the hCMV promoter; refs. 25, 32), Ad-TK (expresses HSV1-thymidine kinase under the control of the hCMV promoter; ref. 5), Ad-Flt3L (expresses human soluble fms-like tyrosine kinase ligand under the control of the hCMV promoter; refs. 5, 7, 10, 33), and, as a control, we used an Ad without transgene (Ad0). The Ads were grown and purified as previously described (30). All viral preparations were free from replication-competent adenovirus and lipopolysaccharide contamination (30).

Ads were administered within the intracranial tumors or in naïve striatum as described below using the following doses: Ad-TNF- $\alpha$ , Ad-FasL, Ad-TRAIL, and Ad-TK:  $5 \times 10^7$  plaque-forming units (pfu)/3  $\mu$ L; Ad-Flt3L: 108 pfu/3  $\mu$ L; Ad0:  $5 \times 10^7$  pfu/3  $\mu$ L (to mimic dose of single proapoptotic Ad treatment) or  $1.5 \times 10^8$  pfu/3  $\mu$ L (to deliver equivalent total pfu in all experimental treatment groups).

#### *Brain tumor rodent models*

**Intracranial CNS-1 syngeneic model.** Rat GBM CNS-1 cells (4,500; 3  $\mu$ L) were implanted intracranially in the right striatum of syngeneic Lewis rats (220–250 g, Harlan) as previously described (34). Rats were treated 4 d (small tumor) or 9 d (large tumor) after tumor implantation.

**Recurrent intracranial CNS-1 syngeneic model.** Rat GBM CNS-1 cells (4,500; 3  $\mu$ L) were implanted intracranially in the left striatum of Ad-TK+GCV+Ad-Flt3L-treated rats that survived the primary brain tumor (implanted on the right striatum) for over 90 d. Rechallenged rats were not treated further. Naïve rats were used as controls for CNS-1 cell tumor growth in this experimental paradigm.

CNS-1 cells were grown in DMEM (CellGro), supplemented with 10% FCS, 1% L-glutamine, 1% Pen-Strep, 1% nonessential amino acids, and passaged routinely. The day of surgery, cells were trypsinized, resuspended in DMEM without supplements, and kept on ice for up to 2 h.

Rats were housed in a pathogen-free environment, humidity- and temperature-controlled vivarium on a 12:12 hour light/dark cycle (lights on 07:00) with free access to food and water. All animal experiments were done after prior approval by the Institutional Animal Care and Use Committee at Cedars Sinai Medical Center and conformed to the policies and procedures of the Comparative Medicine Department. After anesthesia, animals were placed in a stereotactic apparatus and injected unilaterally into the right striatum. Rats were injected using a 10- $\mu$ L Hamilton syringe (coordinates: 1 mm forward from bregma, 3.1 mm lateral and 5 mm ventral from the dura). Animals were allowed to recover and their health status was closely monitored. Treatment was done at the times indicated in each figure, using the same drill hole to inject saline or Ad in a volume of 3  $\mu$ L (delivered in three locations ventral of the dura: 5.5, 5.0, and 4.5 mm) into the tumor mass. Twenty-four hours after delivery of viral vectors, animals that received Ad-TK began treatment with GCV (7 mg/100  $\mu$ L i.p.), twice daily for 7 d. To block HMGB1, groups of rats received 100 mg glycyrrhizin (7, 35) i.p. twice a day for 10 to 15 d, starting on the day of the injection of saline or Ad-TK+Ad-Flt3L or the day of GBM rechallenge. Glycyrrhizin (Calbiochem) was diluted to a concentration of 100 mg/mL in 50 mmol/L NaOH at 37°C and pH was adjusted to pH 7.4 using 1 mol/L Tris-HCl. The solution was then filtered through a 0.22- $\mu$ m syringe pump filter and 1 mL was administered per rat per dose.

Animals were monitored daily and euthanized at the first signs of moribund behavior or at predetermined time points for DNA purification, serum HMGB1 ELISA, or immunostaining. Animals were euthanized according to the guidelines of the Institutional Animal Care and Use Committee at Cedars-Sinai Medical Center, by terminal perfusion with Tyrodes solution (132 mmol/L NaCl, 1.8 mmol/L CaCl<sub>2</sub>, 0.32 mmol/L NaH<sub>2</sub>PO<sub>4</sub>, 5.56 mmol/L glucose, 11.6 mmol/L NaHCO<sub>3</sub>, and 2.68 mmol/L KCl) followed by perfusion with 4% paraformaldehyde (PFA) under deep anesthesia. Brains were removed and further fixed in 4% PFA for 4 to 5 d.

### *Immunofluorescence*

Transgene expression of the proapoptotic Ads was evaluated in CNS-1 cells in vitro fixed with 4% PFA (20 min at 4°C) 24 h after infection with 100 pfu/cell (50,000 cells in 24-well plates).

Immunofluorescence was done as described in Supplementary Data.

Expression of therapeutic targets was done in vitro in PFA-fixed CNS-1 cells and in vivo in PFA-fixed free-floating 60- $\mu$ m coronal sections from rat brain 9 d after tumor implantation.

Immunofluorescence was done as described elsewhere (6, 9, 32) using specific antibodies indicated in Supplementary Data.

Nuclei were stained with 4',6-diamidino-2-phenylindole (5  $\mu$ g/mL, Invitrogen Molecular Probes); cells and tissues were mounted with ProLong Antifade (Invitrogen Molecular Probes). Confocal micrographs were obtained using a Leica confocal microscope TCS SP2 with AOBS equipped with 405-nm violet-diode UV laser, 488-nm argon laser, and 594- and 633-nm helium-neon lasers; and using a HCX PL APO 63 $\times$  1.4 numerical aperture oil objective (Leica Microsystems Heidelberg).

### *Neuropathologic analysis*

Neuropathologic analysis was done in naïve rat brain 7 and 60 d after injecting proapoptotic Ads alone or in combination with Ad-Flt3L. Following perfusion with Tyrode's solution and 4% PFA, brains were fixed in 4% paraformaldehyde for 3 additional days. Sixty-micrometer serial coronal sections were cut through the striatum and free-floating immunocytochemistry was done as previously described (9, 34). Nissl staining was used to determine the histopathologic features of the brains. For a brief description of these methods and the antibodies used, see Supplementary Data. Tissues were photographed with Carl Zeiss Optical Axioplan microscope using Axiovision Rel 4.6 and MOSAIX software (Carl Zeiss).

### *ELISA assays*

HMGB1 release was determined in rat serum and cell culture supernatant using a specific anti-HMGB1 ELISA (IBL International) following the manufacturer's protocol (1). Release of TNF- $\alpha$  and TRAIL was evaluated in cell culture supernatant of CNS-1 cells infected with the corresponding proapoptotic Ads (200 pfu/cell for 48 h) by ELISA following the manufacturer's protocol (eBioscience 88-7346-22 and R&D Systems DTRL00, respectively). Wells were read on a 96-well plate reader (Spectramax Plus, Molecular Devices) at 450 nm and at 570 nm to subtract background absorbance.

### *Flow cytometry*

CNS-1 cells were seeded (25,000 per well) and infected, 24 h later, with the proapoptotic Ads (100 pfu/cell). After 24 h, GCV (25  $\mu$ mol/L) was added to Ad-TK-infected cells. Seventy-two hours after infection or addition of GCV, cell death was determined by propidium iodide (PI)-Annexin V staining (see Supplementary Data). Human GBM cell lines (U251 and U87) and human GBM short-term cell cultures (IN2045 and IN859) were seeded (25,000 in 24-well plates) and infected 24 h with Ad-TK or Ad0 (200 pfu/cell). GCV was added to Ad-TK-infected cells 24 h later. Cell death was determined 72 h after addition of GCV.

To detect FasL release from CNS-1 cells, we collected conditioned medium from Ad-FasL-infected (100 pfu/cell) CNS-1 cells and used it to incubate LN18 cells (50,000 per well in 24-well plates), which are highly sensitive to FasL-induced cytotoxicity (26). Twenty-four hours later, cell death was detected by Annexin-PI staining.

#### *DNA ladder*

The cytotoxic effect of the proapoptotic Ads was analyzed in vitro and in vivo by the pattern of DNA fragmentation. DNA was obtained from CNS-1 cells (106 cells in T25 flask) infected with the proapoptotic Ads (100 pfu/cell for 72 h) and from CNS-1 intracranial tumors dissected from the rat brain 5 d after injection of  $5 \times 10^7$  pfu of each Ad. DNA fragmentation was done as described in Supplementary Data.

#### *Statistical analysis*

Sample sizes were calculated 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). Kaplan-Meier survival curves were analyzed using the Mantel log-rank test (GraphPad Prism version 3.00, GraphPad Software). Levels of HMGB1 and cell death percentages were analyzed by one-way ANOVA followed by Tukey's test (NCSS). When data failed normality or Levene's test for variance homogeneity (NCSS), they were log-transformed before analysis. Pearson's test was used to determine correlation coefficient ( $R^2$ ) between HMGB1 release and percentage of cell death (GraphPad Prism). Randomization test was used to analyze body weight curves (NCSS). P values of  $<0.05$  were used to determine the null hypothesis to be invalid. The statistical tests used are indicated in the figure legends.

## **RESULTS**

In vitro characterization of proapoptotic Ads and their targets in rat glioblastoma cells. In anticipation of phase I clinical trials in patients with GBM, we aimed to test the hypothesis that Ad-TK, which kills dividing cells in the presence of ganciclovir (GCV) in combination with the immunostimulatory Ad-Flt3L, is both the safest and most effective tumor cell-killing approach when compared with Ads expressing proapoptotic TNF- $\alpha$  (Ad-TNF- $\alpha$ ), FasL (Ad-FasL), and TRAIL (Ad-TRAIL). We used immunocytochemistry to determine the presence of the target receptor for each proapoptotic cytokine in CNS-1 cells in culture (Supplementary Fig. S1A). CNS-1 tumor cells in culture expressed all necessary death receptors (i.e., TNFR1, TRAILR2, Fas). Proliferating CNS-1 cells, the target cells for the cytotoxic effects of Ad-TK+GCV, were abundant in culture as determined by staining of the nuclear protein Ki67, a cellular marker of proliferation (Supplementary Fig. S1A).



Transgene expression of the therapeutic Ads was confirmed by immunocytochemistry using specific antibodies against the transgenes (Supplementary Fig. S1B). Release of the proapoptotic cytokines TNF- $\alpha$  and TRAIL was detected by ELISA in cell culture supernatants (Supplementary Fig. S1C). The levels of TNF- $\alpha$  and TRAIL in the cell supernatants were  $\sim$ 0.3 and 1.5 ng/mL, respectively. Release of FasL was evaluated by a biological assay using conditioned medium from Ad-FasL-infected CNS-1 cells to induce cell death in LN18 cells, which are sensitive to FasL-induced cytotoxicity (26). Ad-FasL conditioned medium had strong cytotoxic effects, reducing LN18 cell viability to  $<$ 10% in 24 hours (Supplementary Fig. S1C).

We then tested the proapoptotic effects of Ads in vitro using cultures of rat CNS-1 GBM cells (Fig. 1A). We infected the cells with the proapoptotic Ads, and 24 hours later the cells infected with Ad-TK received GCV (25  $\mu$ mol/L). Seventy-two hours after infection, cells were collected and stained with Annexin-PI. Flow cytometric analysis revealed that Ad-TRAIL induced apoptosis in nearly 100% of the cells, Ad-TK+GCV and Ad-FasL in  $>$ 80%, and Ad-TNF- $\alpha$  in  $\sim$ 40% of the cells (Fig. 1A). Electrophoretic analysis of DNA purified from these cells confirmed that cells underwent cell death by apoptosis (Supplementary Fig. S2A).

Release of HMGB1 from dying GBM cells in vitro upon infection with proapoptotic Ads. We recently determined that mouse GBM cells release HMGB1 upon cell death induced by a variety of genotoxic agents, including Ad-TK, radiotherapy, and temozolomide. HMGB1 is an abundant chromatin protein that acts as an endogenous TLR2 agonist when released by either dying cells and inflammatory cells (7, 28, 29). In these experiments, we aimed to test the hypothesis that HMGB1 would be released upon tumor cell death induced not only by cytotoxic agents that inhibit replication but also following tumor cell death induced by proapoptotic cytokines that kill cells by activation of membrane death receptors. We found that infection of CNS-1 cells with proapoptotic Ads led to release of HMGB1, as detected by ELISA in the cell culture supernatant (Fig. 1B). Pearson correlation analysis was used to determine the correlation coefficient ( $R^2$ ) between the concentration of HMGB1 in the cell supernatants and the percentage of cell death in vitro in CNS-1 cells infected with the proapoptotic Ads. The levels of HMGB1 (Fig. 1B) exhibited strong correlation ( $R^2 = 0.96$ ,  $P < 0.05$ ; Fig. 1B, inset) with the levels of cell death (Fig. 1A). Our results indicate that HMGB1 release following tumor cell death is a widespread phenomenon that is independent of the tumor cell-killing mechanism.

In vivo efficacy of proapoptotic Ads in a rat orthotopic syngeneic glioblastoma model: release of HMGB1 from dying tumor cells into the general circulation, tumor regression, and long-term survival. We tested the efficacy of the proapoptotic Ads in a syngeneic intracranial GBM rat model. We implanted CNS-1 GBM cells in the striatum of syngeneic Lewis rats and treated them 4 days later (small tumor, volume:  $1.6 \pm 0.2$  mm<sup>3</sup>) by intratumoral administration of the proapoptotic Ads. Rats that received Ad-TK were injected with GCV i.p. for 7 days (7 mg/twice a day). Tumor regression and long-term survival was achieved only after the administration of Ad-TK (6 of 7 rats) and Ad-FasL (3 of 9 rats), whereas Ad-TRAIL (0 of 8 rats) or Ad-TNF- $\alpha$  (0 of 5 rats) did not improve the survival of rats when compared with saline-treated animals (Fig. 1C). However, all the Ads induced apoptosis in vivo, as determined by analysis of DNA purified from the intracranial tumor 5 days after treatment. Agarose gel electrophoresis showed the typical laddering of apoptotic DNA fragmentation in the tumors treated with all proapoptotic Ads tested (Supplementary Fig. S2B).

We then tested the hypothesis that the Ads expressing proapoptotic cytokines would induce HMGB1 release from rat GBM *in vivo*. Circulating levels of HMGB1 were determined by ELISA in the serum of tumor-bearing rats 5 days after treatment with Ads (Fig. 1D). We found increased HMGB1 levels in the serum of tumor-bearing rats after administration of the proapoptotic Ads but not with an empty Ad (Ad0) or saline (Fig. 1D), which indicates that HMGB1 release from dying tumor cells occurs *in vivo* upon tumor cell killing induced by a wide spectrum of proapoptotic agents and that this phenomenon can be monitored in the peripheral circulation *in vivo*.

Distribution of therapeutic targets of proapoptotic molecules within intracranial CNS-1 tumors and peritumoral brain tissue. Because in GBM patients Ads may be injected directly into the margins of the tumor after surgical resection (36) or Ad-expressed transgenes can diffuse from the tumor, it is critical to evaluate whether the cytotoxic effect is limited to tumor cells and will not affect normal peritumoral tissues. We performed the qualitative evaluation for the distribution of the targets of the therapeutic genes to allow the assessment of potential effectiveness and side effects. We evaluated the expression of the receptors for the proapoptotic gene therapy approaches, in the tumor and surrounding normal brain, 9 days after GBM cell implantation. To this end, we used immunofluorescence techniques using antibodies specific for death receptors TNFR1, TRAILR2, and Fas, and also determined the distribution of proliferating cells, the target cells for TK+GCV (stained with an anti-Ki67 antibody). We stained tumor cells using anti-vimentin antibodies, and neurons and astrocytes were identified with anti-NeuN and anti-glial fibrillary acidic protein-(GFAP) antibodies, respectively (Fig. 2 and Supplementary Figs. S3 and S4). Tissues were analyzed by confocal microscopy, which revealed that all receptors were expressed in the tumor. TNFR1, Fas, and Ki67 were expressed throughout the tumor mass, whereas TRAILR2 was more concentrated surrounding areas of necrosis. Ki67 was more abundant than the expression of the death receptors within the tumor mass. Nuclear protein Ki67 was rarely detected in the brain tissue adjacent to the tumor mass; expression was only detected within a small number of peritumor reactive GFAP-positive astrocytes. Expression of TNFR1 was also confined to GFAP-positive cells in the brain adjacent to the tumor. Expression of TRAIL was readily detected in neurons surrounding the tumor mass, and Fas was profusely expressed in structures resembling neuronal axons.

For a therapy to be implemented in human clinical trials, it is not only important to determine its efficacy but it is also critical to assess its safety. Considering that Ads can express very powerful proapoptotic molecules and that the targets for some of them, such as TRAILR2 and Fas, were detected in neuronal cell bodies and axons surrounding the tumors (Fig. 2), we tested the neurotoxicity profile of the proapoptotic Ads in the normal brain parenchyma 7 days (Fig. 3A) and 60 days (Supplementary Fig. S5) post vector delivery into the striatum; rats that were injected with Ad-TK also received GCV for 7 days. The brain structure was evaluated by Nissl staining and immunocytochemistry of tyrosine hydroxylase (TH, as an index of striatal tissue integrity) and myelin basic protein (MBP, as an index of oligodendrocyte integrity), whereas infiltration of inflammatory cells was studied by immunostaining of MHCII, CD68 (macrophages), and CD8 (cytotoxic T cells). Injection of Ad-TNF- $\alpha$  and Ad-TK+GCV did not affect the normal structure of the brain or the expression of TH and MBP and only induced a mild infiltration of inflammatory cells. However, Ad-FasL and Ad-TRAIL exerted severe neuropathology (*i.e.*, hemorrhages and loss of brain tissue). Reduction of TH expression and demyelination were detected in large areas of the striatum surrounding the site of injection of Ad-FasL and Ad-TRAIL. Concomitantly, profuse infiltration of macrophages, MHCII-positive cells, and T cells were found in the brains of these rats. Sixty days later,

reduction of TH expression was still evident in Ad-FasL- and Ad-TRAIL-injected animals, as well as large unilateral and sometimes bilateral ventriculomegaly (Supplementary Fig. S5), a result of significant brain tissue loss.

Putative systemic toxicity was assessed by weighing these rats daily after intracranial injection of the proapoptotic Ads. We detected a rapid drop of 15% to 25% in the weight of rats injected in the striatum with Ad-FasL (Fig. 3B). The reduction in body weight was sustained for 9 to 10 days, after which rats started slowly gaining weight, without, however, reaching the weight of control rats throughout the 4-week duration of the study. In rats injected in the brain with Ad-TRAIL, their weight remained stable ~300 g until day 10; at this time point, their weight dropped ~15% to 25%, and remained low for 3 days, after which they started to gain weight and recovered. On the other hand, the body weight of rats that were injected in the brain with Ad-TNF- $\alpha$  or Ad-TK showed a similar pattern to the rats injected with saline and Ad0, maintaining their weight at ~300 g for 10 days; from that time point onward, the rats started gaining weight at a rate of ~2.5 g/d.

Efficacy of proapoptotic viruses in combination with Ad-Flt3L in large CNS-1 tumors; release of HMGB1. Considering that the proapoptotic Ads with the highest efficacy in vivo in small tumors were Ad-TK+GCV and Ad-FasL (Fig. 1C), we selected these Ads to use in combination with the immunostimulant Ad-Flt3L for the treatment of rats bearing large tumors (9 days postimplantation, volume:  $35 \pm 5.5$  mm<sup>3</sup>). We implanted CNS-1 GBM cells in the striatum of syngeneic Lewis rats and treated them 9 days later with either Ad-TK or Ad-FasL alone or in combination with Ad-Flt3L (Fig. 4A). These tumors are ostensibly larger at the time of treatment than those treated 4 days after the implantation (Fig. 4A). In this model, Ad-TK+GCV and Ad-FasL alone failed to induce tumor regression and long-term survival. Although Ad-TK+GCV exerted a slight increase in median survival (MSR, 1.17), all of the rats succumbed due to tumor growth. When Ad-Flt3L was combined with Ad-FasL, the survival did not improve compared with the saline-treated rats, and only 1 of 10 rats had long-term survival. However, when Ad-Flt3L was used together with Ad-TK+GCV, the treatment led to long-term survival of 7 of 10 rats (Fig. 4A). Supplementary Table S1 shows the median survival or percentage of long-term survival upon treatment with all the therapeutic approaches described. Note that the combination of Ad-TK+GCV+Ad-Flt3L led to ~70% survival, and thus the median survival was not reached.

Circulating serum levels of HMGB1 were determined by ELISA in tumor-bearing rats 5 days after the treatment with Ad-TK+GCV or Ad-FasL alone or in combination with Ad-Flt3L; as controls, rats were injected with saline or Ad0 (Fig. 4B). Whereas control rats exhibited basal levels of HMGB1, we detected HMGB1 in the serum of most rats treated with Ad-TK+GCV or Ad-FasL alone or in combination with Ad-Flt3L. However, Ad-TK+GCV+Ad-Flt3L led to the highest levels of serum HMGB1 (Fig. 4B).

Efficacy of combined conditional cytotoxicity and immunotherapy depends on circulating HMGB1. To assess the hypothesis that the endogenous TLR ligand HMGB1 released from dying tumor cells is necessary for efficacy of the immunotherapy in the CNS-1 GBM rat model, we blocked its activity using glycyrrhizin, which binds to both of the box domains on HMGB1 and prevents subsequent HMGB1 signaling (7, 35). Tumor-bearing rats received intratumoral injection of saline or Ad-TK+Ad-Flt3L, followed by the administration of GCV and glycyrrhizin, starting on the day of the vector administration (Fig. 4C). Rats treated with Ad-TK+GCV+Ad-Flt3L (four of six) survived long term,

whereas simultaneous administration of glycyrrhizin completely blocked the therapeutic effect of the immunotherapy and all the rats succumbed due to tumor burden (Fig. 4C). Taken together, these results suggest that the efficacy of the combined treatment is strongly dependent on the release of HMGB1 from dying tumor cells. Further, HMGB1 could be used as a biomarker to assess therapeutic efficacy *in vivo*.

Role of HMGB1 in the induction of anti-GBM immunologic memory induced by the combined Ad-TK+GCV+Ad-Flt3L treatment. Treatment with Ad-TK+GCV+Ad-Flt3L induces brain tumor regression and immunologic memory in both murine and rat syngeneic GBM models (7, 8). Now we aimed to uncover the role of HMGB1 in the rejection of a second brain tumor in a model of recurrent GBM. Ad-TK+GCV+Ad-Flt3L-treated rats that survived a primary CNS-1 tumor were rechallenged with a second CNS-1 tumor in the contralateral striatum 90 days after primary tumor implantation (Fig. 4D). To block HMGB1, rats received glycyrrhizin for 15 days, starting the day of the second tumor implantation. Naïve rats were used as controls of tumor growth. We found that 50% of Ad-TK+GCV+Ad-Flt3L-treated long-term survivors that were implanted in the contralateral hemisphere survived the rechallenge without further treatment. Blocking HMGB1 by administration of glycyrrhizin did not block anti-GBM immunologic memory induced by the combined treatment (Fig. 4D), thus suggesting that HMGB1 is not essential for memory T-cell elimination of CNS-1 cells.

Acute and chronic neurotoxicity of the combined therapy in the normal brain. We next assessed whether Ad-TK+GCV or Ad-FasL combined with Ad-Flt3L are neurotoxic when injected into the normal brain parenchyma. We injected the Ads in the striatum of naïve Lewis rats and performed neuropathologic analysis 7 days (Fig. 5) and 60 days later (Supplementary Fig. S6). As controls, we administered saline or Ad0. We found that Ad-TK+GCV+Ad-Flt3L did not affect the brain structure or the expression of TH and MBP and induced a mild infiltration of inflammatory cells similar to that observed using Ad0. However, Ad-FasL+Ad-Flt3L injection led to severe neuropathology, with hemorrhages and large areas of tissue loss (Fig. 5). Reduction of TH expression and patches of demyelination were seen in the striatum surrounding the injection site of Ad-FasL+Ad-Flt3L, as well as large infiltration of macrophages, MHCII-positive cells, and CD8+ cells. Sixty days later, inflammation declined, but ventriculomegaly secondary to brain tissue loss was evident in Ad-FasL+Ad-Flt3L-injected rats (Supplementary Fig. S6).

Induction of cell death and HMGB1 release *in vitro* from human GBM cell lines and primary GBM cell cultures. Human GBM cell lines (U251 and U87) and short-term cultures of human GBM (IN859 and IN2045) were infected with Ad0 or Ad-TK followed by addition of GCV. HMGB1 release was evaluated in the cell culture supernatants 72 hours after addition of GCV and cell death was determined by Annexin-PI staining and flow cytometry (Fig. 6). We found that human GBM cells were very sensitive to Ad-TK+GCV-induced cell death, exhibiting 60% to 80% cell death (Fig. 6). Accordingly, HMGB1 release was greatly increased when cells were treated with Ad-TK+GCV. These results support the notion that HMGB1 is released upon killing of human GBM cells, suggesting that this is a universal mechanism independent of the tumor cell origin.

## DISCUSSION

In anticipation of a phase I clinical trial in GBM patients using an immunotherapeutic approach that combines Ad-Flt3L with Ad-TK+GCV, it was critical to determine the optimal cytotoxic agent to use in this approach. Therefore, we compared the efficacy and neurotoxicity of Ad-TK+GCV with Ad vectors encoding the proapoptotic cytokines TNF- $\alpha$ , TRAIL, and FasL. Our hypothesis was that Ad-TK+GCV would exhibit superior efficacy and safety when compared with Ads expressing proapoptotic cytokines. Because Ad-TK kills proliferating cells in the presence of GCV (37), we expected this agent to have a powerful antitumor effect due to the presence of mitotic tumor cells within GBM. Also, the bystander effect of phosphorylated GCV would amplify the cytotoxic effect of this approach (37). The highest therapeutic efficacy was indeed achieved when using Ad-TK+GCV by itself for small tumors, and in combination with Ad-Flt3L for large tumors. Although all the proapoptotic Ads certainly induced apoptosis *in vitro* and *in vivo* in tumor cells, delivery of proapoptotic cytokines was insufficient to induce therapeutically effective tumor regression *in vivo*. This could be related to the relative low levels of death receptor expression. In fact, weak expression of TRAILR2 in GBM cells has been suggested to limit the therapeutic efficacy of TRAIL delivery in GBM patients (38). Preclinical research showed that radiation and chemotherapeutic agents increase TRAILR expression and GBM sensitivity to TRAIL-induced apoptosis (15, 17, 39), although the therapeutic implications of this increase remain to be determined.

Another possible cause for the lack of efficacy of proapoptotic cytokines could be related to their effect on the immune cells that infiltrate the tumors. The Fas/FasL system has been implicated in the immune privilege of GBM (40). FasL expression has been detected in GBM patients' tumor cells as well as in endothelial cells of the tumor blood vessel, which has been postulated as a mechanism of depletion of Fas+ T cells in these tumors (41). In fact, expression of FasL in human GBM was found to negatively correlate with the degree of intratumoral CD4+ and CD8+ T-cell infiltration (41). Also, expression of Fas was found to positively correlate with the malignancy grade of astrocytomas in brain tumor patients (24).

Soluble receptors have also been involved in the mechanism by which GBM cells down-regulate the effects of proapoptotic cytokines (42). Expression of soluble receptors for FasL by tumor cells has been suggested to mediate GBM escape from FasL-induced apoptosis (42). Expression of soluble TNFR1 has also been detected in GBM specimens from patients. These receptors were found to reduce the function of TNF- $\alpha$  in GBM cells (43) and may be playing a role in the lack of efficacy of Ad-TNF- $\alpha$  observed in our study. Also, delivery of proapoptotic cytokines that only target tumor cells expressing a specific death receptor may lead to the selection of nonexpressing cells that are resistant to the targeted therapy.

Because proliferating cells are encountered within the tumor in all the stages and tumor cell replication is a requirement for tumor progression, targeting these cells with intratumoral delivery of TK is a very attractive candidate to induce apoptosis in GBM (36). Importantly, synergy between TK+GCV and temozolamide, an alkylating agent routinely used in the treatment of GBM patients (1), has been reported in preclinical mouse models of GBM (44). Because phosphorylated GCV was found to inhibit DNA polymerase  $\delta$ , an enzyme involved in repair of DNA cross-links, this synergy has been explained by the TK+GCV-mediated inhibition of the repair of temozolamide-induced cross-links in tumor cell DNA (44).

Considering that in some clinical trials Ads are injected in the tumor cavity margins following surgical resection (36), it is critical to use proapoptotic agents whose cytotoxic effect is restricted to tumor cells. To determine the specificity of each cytotoxic agent, we studied the presence of their therapeutic targets (i.e., TNFR1, TRAILR2, Fas); to detect proliferating cells, we immunolabeled for nuclear protein Ki67 in rat intracranial CNS-1 GBM and in nonneoplastic brain surrounding the tumor. A small number of astrocytes expressing TNFR1 or Ki67 were detected in the nonneoplastic brain surrounding the tumor. However, we found that TRAILR2 and Fas are expressed in neuronal cell bodies and fibers in the normal brain adjacent to CNS-1 tumors. Expression of these death receptors has also been detected in the normal human brain. Fas has been detected in neurons in the cerebral cortex of normal human brains, as well as in neurophil and white matter fibers (45), whereas TRAILR2 is expressed in neurons and oligodendrocytes, as well as in endothelial cells in the meninges and capillaries of the normal human brain (46, 47). In fact, TRAIL-induced apoptosis was reported in human normal brain cells, including neurons and glial cells, in temporal lobe sections *ex vivo* (48), and FasL has been postulated to be involved in neuronal damage following brain injury (49). Considering the bystander effect exerted by the release of the proapoptotic cytokines from tumor cells and the expression of TRAILR2 and Fas in normal brain cells surrounding the tumor, administration of Ads encoding FasL or TRAIL into the normal brain bears a high risk of neurotoxicity.

Tallying with the presence of death receptors in neuronal cell bodies and fibers, delivery of FasL and TRAIL caused severe neuropathologic side effects. Importantly, we also detected systemic toxicity as assessed by a reduction in the body weight (50-75 g) of Ad-TRAIL- and Ad-FasL-injected rats. On the other hand, administration of Ad-TK+GCV alone or combined with Ad-Flt3L did not significantly alter the structure of the normal brain and induced only a mild, transient local inflammation. Intracranial delivery of TK using Ads has been tested in several clinical trials in GBM patients with a very good safety profile (36). Importantly, an extra safety feature of this approach is that the withdrawal of the GCV can limit any potential therapy-associated toxic events (50). Also, we have assessed the toxicity of vectors by performing dose-response curves with Ads expressing several transgenes (51–54). The summary of the data (55) indicates that vector toxicity is dependent on vector dose, but requires the structural integrity of vector capsids (independently of transgenes expressed); thus, doses of vector need to be kept below  $1 \times 10^9$  pfu to avoid serious, deleterious, long-term brain toxicity. Concerning transgene expression, we have determined that even low doses of vectors can provide expression detectable by immunocytochemistry (53). However, to elicit therapeutic efficacy, doses of at least  $5 \times 10^7$  pfu need to be delivered into brain tumors. This explains the doses used in this article (i.e., the safest and most effective doses). In preparation for the clinical trial (i.e., for the submission of the investigational new drug application (IND) to the Food and Drug Administration), the clinical-grade vectors will be tested for toxicity and efficacy using at least three doses of each vector. Clinical dose in human brain tumor patients is otherwise limited by the MTD for adenoviral vectors in the human brain, which has been determined to be  $1 \times 10^{12}$  vp (56). Thus, all doses to be used in humans will be kept below the adenoviral vector MTD.

We have recently reported that mouse GBM cells release HMGB1 upon cell death induced by cytotoxic agents that inhibit DNA replication and thus kill proliferating cells, such as temozolomide, irradiation, and Ad-TK+GCV (7). In the present work, we tested the hypothesis that HMGB1 would not only be released upon tumor cell death induced by cytotoxic agents that inhibit replication but also due to tumor cell death induced by proapoptotic cytokines that kill cells by activation of membrane death receptors. Here, we show that HMGB1 is also released from rat GBM cells when

they are killed by proapoptotic cytokines upon death receptor activation. Importantly, our data indicate that circulating levels of HMGB1 could have potential application as biomarker of therapeutic efficacy *in vivo*. The fact that human GBM cells also responded to the cell-killing effect of Ad-TK+GCV by releasing HMGB1 supports the notion that this molecule could be used as a pharmacodynamic predictor of tumor regression in GBM patients.

We previously showed in a mouse GBM model that HMGB1 released from dying tumor cells activates TLR2 signaling in bone marrow–derived dendritic cells that infiltrate the tumor in response to the immunotherapy with Ad-TK+GCV+Ad-Flt3L (7). In this article, we show that circulating levels of HMGB1 increase in parallel with the efficacy of the treatment in the rat GBM model. We found that of all the treatments tested, the highest circulating levels of HMGB1 are reached when tumor-bearing rats are treated with Ad-TK+GCV+Ad-Flt3L. These levels were indeed higher than those observed in the rats treated with Ad-TK+GCV alone. This could be due to the release of HMGB1 by immune cells (28) recruited by Ad-Flt3L (6, 7, 10) or by the induction of additional tumor cell death by cytotoxic T cells, macrophages, or NK cells, which infiltrate the tumor and we have shown to be crucial for the therapeutic efficacy of this immunotherapy (5, 7). Release of HMGB1 from dying tumor cells has been postulated to direct the immunologic response to dying cells, which determines the clinical outcome of anticancer therapies (7, 57, 58). In fact, we show here that HMGB1 release from dying tumor cells is crucial for the efficacy of Ad-TK+GCV+Ad-Flt3L in GBM-bearing rats and its blockade completely abolishes the efficacy of the therapy. These results are in accordance with those obtained in the mouse GBM model (7). The data reported strongly support the use of cytotoxic therapies to enhance the efficacy of immunotherapeutic approaches in GBM patients (59).

Considering that the majority of GBM patients succumb due to recurrence of tumors that have become completely resistant to any form of chemotherapy and radiation therapy (36), it is crucial to develop immunotherapeutic approaches that induce immunologic memory against the tumor. Tallying with our previous results (7, 8), ~50% of Ad-TK+GCV+Ad-Flt3L–treated long-term survivors survived the rechallenge without further treatment. HMGB1 did not seem to play a critical role in the induction of anti-GBM immunologic memory induced by the combined therapy.

Translation of a novel therapeutic approach into clinical trial requires assessing therapeutic efficacy in other tumor models. We recently showed that this approach is also effective in eradicating B16-F10 melanomas implanted in the brain of syngeneic mice (7). These results are very encouraging because metastatic brain tumors are very frequent and its incidence is predicted to increase with the increasing survival of patients with extracranial cancers that metastasize to the central nervous system (60).

In summary, our study provides the first systematic, comparative assessment of the neurotoxicity and efficacy of several proapoptotic molecules, some of which have already progressed to phase I clinical trials for GBM. Further, we show that HSV1-TK in combination with GCV exerted the most potent antitumor activity and also displayed the most satisfactory safety profile when used as single therapy. Our data also show that the combination of Ad-TK+GCV and Ad-Flt3L exerts a strong antitumoral effect in several intracranial rodent models of GBM and has the safest neurotoxic profile of all the approaches tested. Thus, Ad-TK+GCV+Ad-Flt3L displays the highest therapeutic efficacy of all the therapies tested thus far in preclinical experimental GBM models. Further, the efficacy of the combined treatment is mediated by the release of the endogenous ligand HMGB1, which we have

previously shown signals via TLR2 receptors on tumor-infiltrating dendritic cells (7). These results strongly support the translation of this immunotherapy in a phase I clinical trial for GBM.

#### **DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

No potential conflicts of interest were disclosed.

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## FIGURE LEGENDS

**Fig. 1.** Efficacy of adenoviral vectors expressing proapoptotic transgenes in vitro and in vivo. A, CNS-1 cells were infected with Ads expressing proapoptotic transgenes; that is, HSV1-thymidine kinase (Ad-TK), TNF- $\alpha$  (Ad-TNF- $\alpha$ ), FasL (Ad-FasL), or TRAIL (Ad-TRAIL). Twenty-four hours after infection, cells infected with Ad-TK were incubated with GCV. Untreated cells and cells infected with an Ad containing no transgene (Ad0) were used as controls. Cell death was determined 72 h after infection or addition of GCV by flow cytometric analysis of Annexin V-PI-stained cells. B, release of HMGB1 was assessed in the cell culture supernatant by ELISA. \*,  $P < 0.05$  versus mock (one-way ANOVA followed by Tukey's test). Inset, Pearson correlation analysis was used to determine the correlation coefficient ( $R^2$ ) between the concentration of HMGB1 in the cell supernatant and the percentage of cell death in vitro in CNS-1 cells infected with the proapoptotic Ads. \*,  $P < 0.05$ . C, Kaplan Meier survival curves of rats implanted with CNS-1 cells in the brain and treated 4 d later with intratumoral injection of saline ( $n = 9$ ), Ad-TK ( $n = 7$ ), Ad-TNF- $\alpha$  ( $n = 5$ ), Ad-FasL ( $n = 9$ ), or Ad-TRAIL ( $n = 8$ ). Ad-TK-treated rats received GCV. \*,  $P < 0.05$  versus saline; ^,  $P < 0.05$  versus Ad-FasL (Mantel log-rank test). Representative microphotographs show the appearance of the tumor at the time of treatment (day 4), as assessed by vimentin staining. Tumor volume is indicated; scale bar, 1 mm. D, serum levels of HMGB1 were determined by ELISA 5 d after the treatment. \*,  $P < 0.05$  versus saline (one-way ANOVA followed by Tukey's test).

**Fig. 2.** Distribution of therapeutic targets of proapoptotic molecules within intracranial CNS-1 tumors and peritumoral brain tissue. Rats were implanted in the striatum with CNS-1 tumors and 9 d later brains were processed for immunocytochemistry. Confocal microphotographs show detection of therapeutic targets (green) using specific antibodies against the death receptors TNFR1, TRAILR2, and Fas, whereas proliferating cells, the target for TK+GCV, were stained with an anti-Ki67 antibody. Tumor cells were labeled with anti-vimentin antibodies (red); neurons were stained with anti-NeuN (red); and astrocytes were labeled with anti-GFAP antibodies (red). Nuclei were stained with 4',6-diamidino-2-phenylindole (blue). T, tumor area; N, necrotic patch. Arrows, cells expressing the therapeutic target indicated. Dashed line, tumor border. Scale bars, 10  $\mu$ m.

**Fig. 3.** Acute toxicity of proapoptotic Ads after injection into normal brain. Lewis rats ( $n = 4$ /treatment) were injected in the striatum with saline, Ad-TNF- $\alpha$ , Ad-TRAIL, Ad-FasL, or Ad-TK. Rats treated with Ad-TK received GCV. After 7 d (A) post vector delivery, neuropathologic analysis of the brain was assessed by Nissl staining and immunocytochemistry using antibodies against TH, MBP, MHCII, CD68 (macrophages), and CD8 (cytotoxic T cells). Scale bar, 2 mm. B, the body weight of the rats was assessed daily. \*,  $P < 0.05$  versus saline (randomization test).

**Fig. 4.** Role of HMGB1 in mediating the efficacy of immunotherapy using proapoptotic Ads combined with Ad-Flt3L. A, Kaplan-Meier survival curve of Lewis rats that were implanted in the brain with CNS-1 tumors and treated 9 d later with an intratumoral injection of saline ( $n = 9$ ), Ad-TK ( $n = 11$ ), or Ad-FasL ( $n = 8$ ) alone or in combination with Ad-Flt3L ( $n = 10$ /group). Rats treated with Ad-TK received GCV. \*,  $P < 0.05$  versus saline; ^,  $P < 0.05$  versus Ad-TK; o,  $P < 0.05$  versus Ad-FasL+Ad-Flt3L (Mantel log-rank test). Representative microphotographs show the appearance of the tumor at the time of treatment, as assessed by vimentin staining. Tumor volume is indicated. Scale bar, 1 mm. B, serum levels of HMGB1 were determined by ELISA 5 d after the treatment. \*,  $P < 0.05$  versus saline (one-way ANOVA followed by Tukey's test). C, tumor-bearing rats received intratumoral injection of

saline (n = 10) or Ad-TK+Ad-Flt3L (n = 12), followed by GCV and glycyrrhizin (Gly), an antagonist of HMGB1 (n = 5-6/group). \*, P < 0.05 versus saline; ^, P < 0.05 versus Ad-TK+Ad-Flt3L+Gly (Mantel log-rank test). D, Ad-TK+GCV+Ad-Flt3L-treated rats that survived over 90 d after primary tumor implantation were rechallenged in the contralateral striatum (left) with a second CNS-1 implant. Rechallenged long-term survivors received glycyrrhizin (SURVIVOR+GLY, n = 6) or vehicle (SURVIVOR, n = 6) for 15 d. Naïve rats were implanted with CNS-1 tumor as controls for tumor growth (NAÏVE, n = 6). \*, P < 0.05 versus naïve (Mantel log-rank test).

**Fig. 5.** Acute neurotoxicity of combined proapoptotic/immune-stimulatory gene therapy after injection into normal brain tissue. Lewis rats were injected in the striatum with saline, Ad-FasL+Ad-Flt3L, Ad-TK+Ad-Flt3L, or an Ad without transgene (Ad0). Rats treated with Ad-TK+Ad-Flt3L received GCV. Seven days post vector delivery, neuropathologic analysis of the brain was assessed by Nissl staining and immunocytochemistry using antibodies against TH, MBP, MHCII, CD68 (macrophages), and CD8 (cytotoxic T cells). Scale bar, 2 mm.

**Fig. 6.** Induction of cell death and release of HMGB1 from human GBM cells in vitro. A, human GBM cell lines (U251 and U87) and primary GBM cell cultures (IN2045 and IN859) were infected with Ad-TK. Untreated cells and cells infected with an Ad containing no transgene (Ad0) were used as controls. Twenty-four hours after infection, cells infected with Ad-TK were incubated with 25 µmol/L GCV. Cell death was determined 72 h after addition of GCV by flow cytometric analysis of Annexin V-PI-stained cells. \*, P < 0.05 versus mock (one-way ANOVA followed by Tukey's test). B, release of HMGB1 was assessed in the cell culture supernatant by ELISA. \*, P < 0.05 versus mock (one-way ANOVA followed by Tukey's test).

FIGURE 1

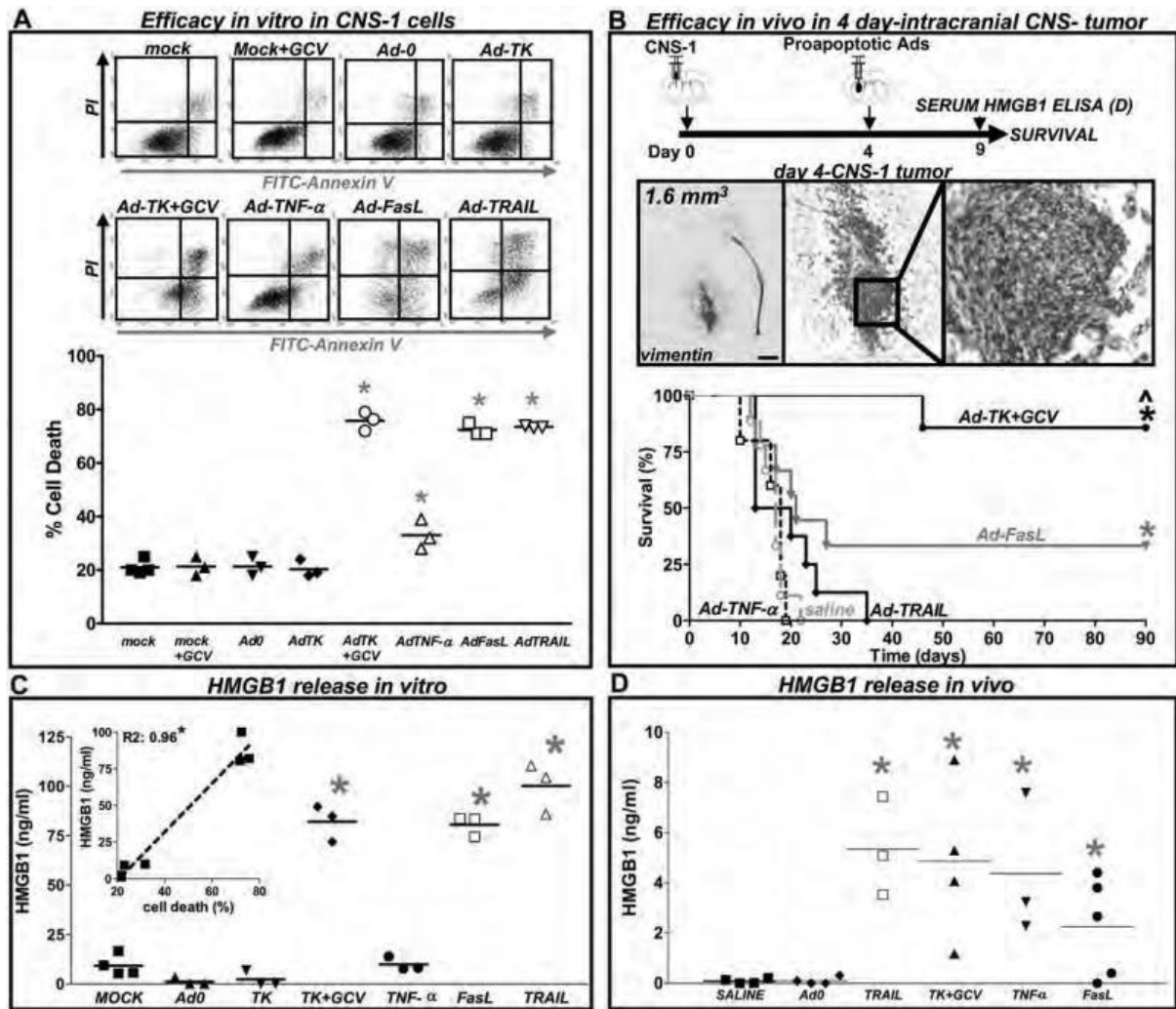




FIGURE 2

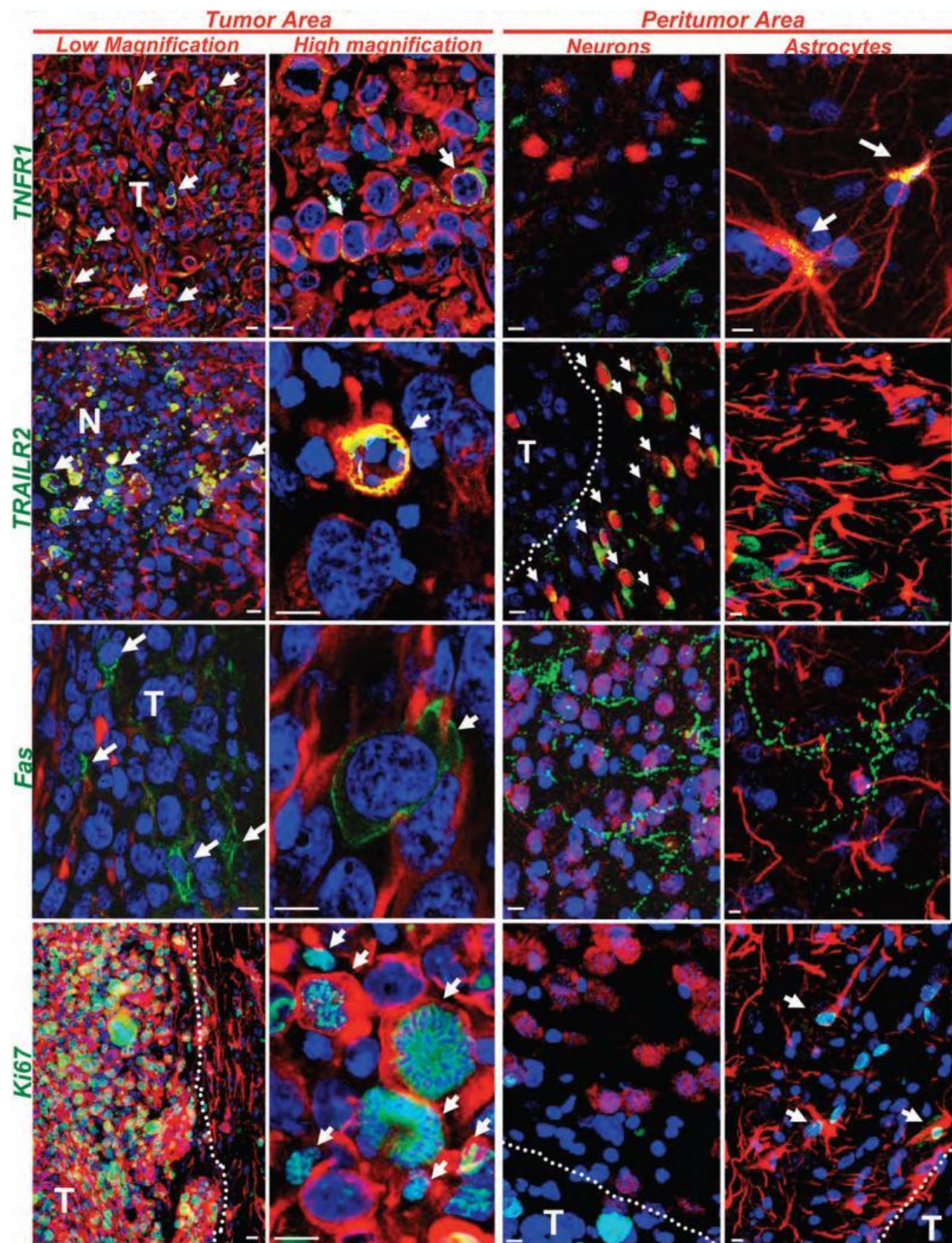
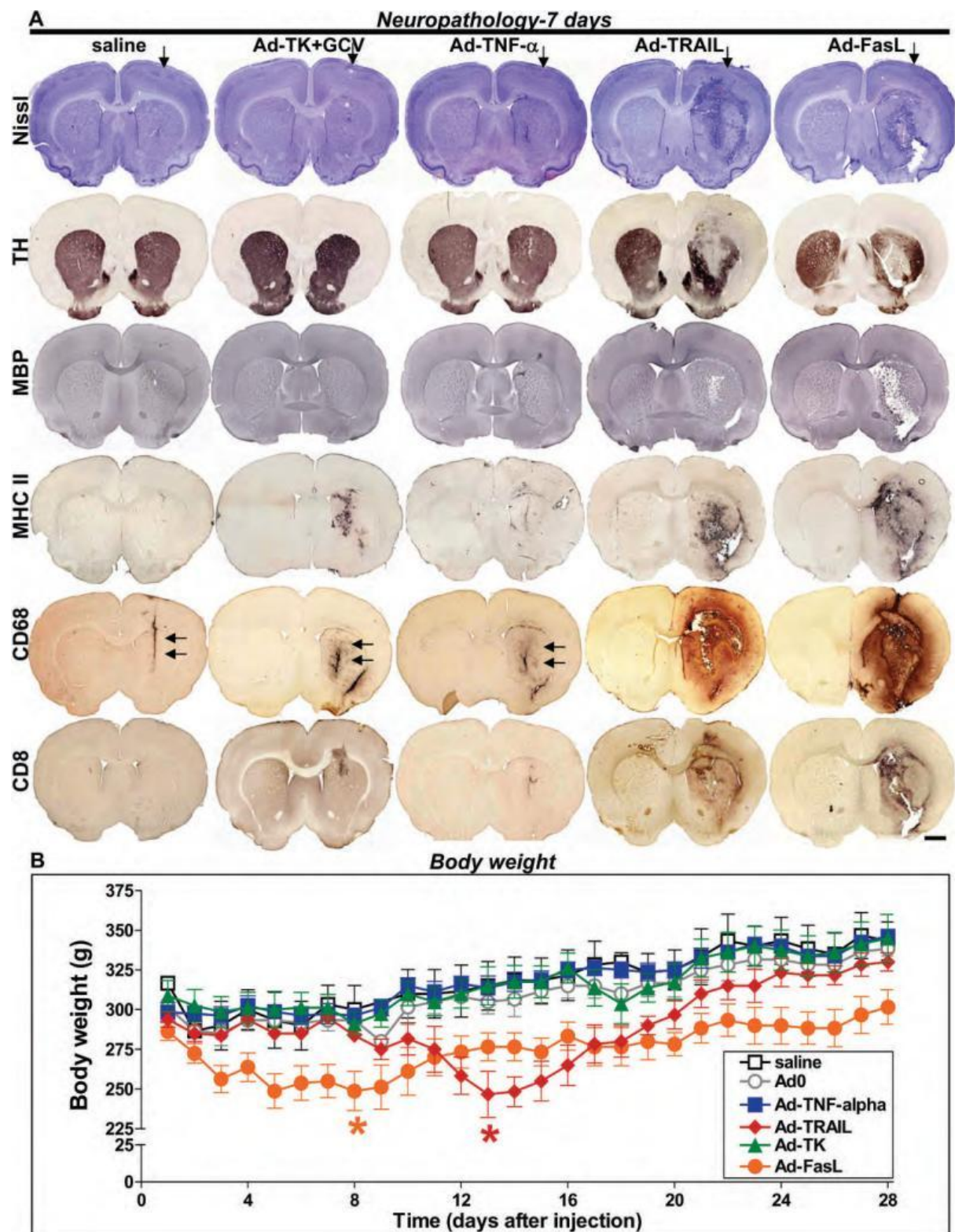


FIGURE 3



**FIGURE 4**

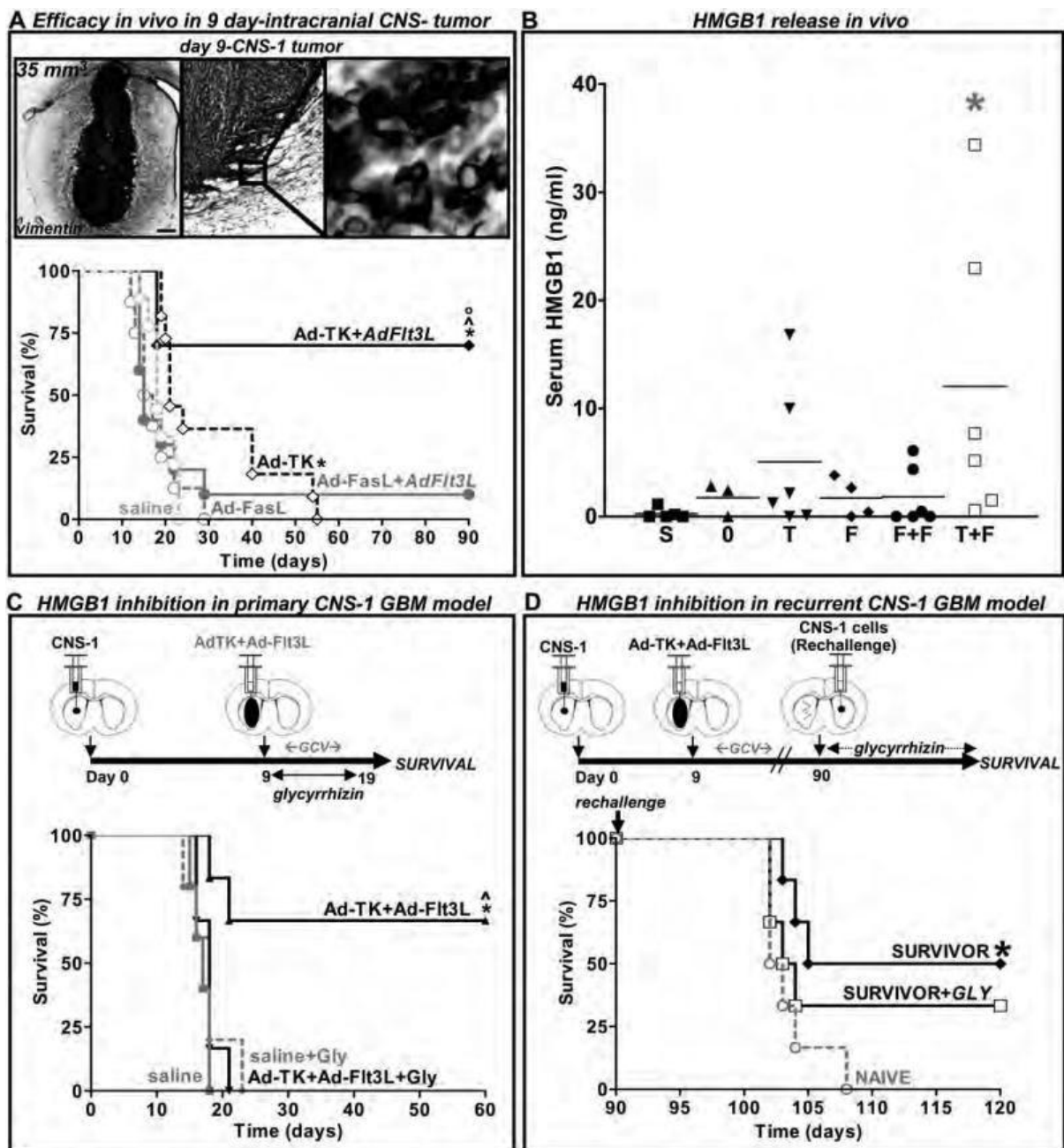


FIGURE 5

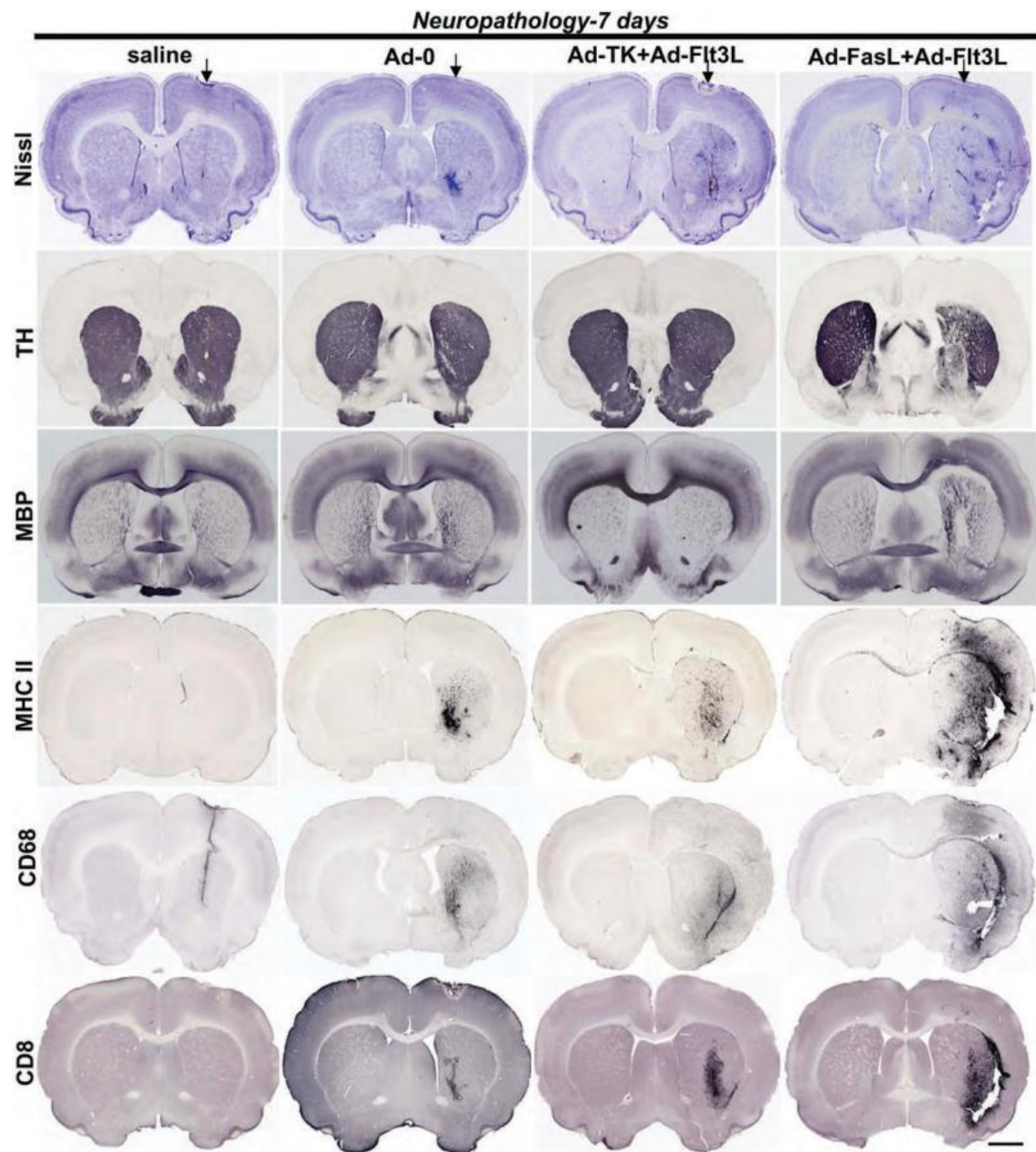


FIGURE 6

