

Antiglioma Immunological Memory in Response to Conditional Cytotoxic/Immune-Stimulatory Gene Therapy: Humoral and Cellular Immunity Lead to Tumor Regression

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Abstract Purpose: Glioblastoma multiforme is a deadly primary brain cancer. Because the tumor kills due to recurrences, we tested the hypothesis that a new treatment would lead to immunological memory in a rat model of recurrent glioblastoma multiforme. **Experimental Design:** We developed a combined treatment using an adenovirus (Ad) expressing *fms*-like tyrosine kinase-3 ligand (Flt3L), which induces the infiltration of immune cells into the tumor microenvironment, and an Ad expressing herpes simplex virus-1-thymidine kinase (TK), which kills proliferating tumor cells in the presence of ganciclovir. **Results:** This treatment induced immunological memory that led to rejection of a second glioblastoma multiforme implanted in the contralateral hemisphere and of an extracranial glioblastoma multiforme implanted intradermally. Rechallenged long-term survivors exhibited anti-glioblastoma multiforme-specific T cells and displayed specific delayed-type hypersensitivity. Using depleting antibodies, we showed that rejection of the second tumor was dependent on CD8⁺ T cells. Circulating anti-glioma antibodies were observed when glioblastoma multiforme cells were implanted intradermally in naïve rats or in long-term survivors. However, rats bearing intracranial glioblastoma multiforme only exhibited circulating antitumoral antibodies upon treatment with Ad-Flt3L + Ad-TK. This combined treatment induced tumor regression and release of the chromatin-binding protein high mobility group box 1 in two further intracranial glioblastoma multiforme models, that is, Fisher rats bearing intracranial 9L and F98 glioblastoma multiforme cells. **Conclusions:** Treatment with Ad-Flt3L + Ad-TK triggered systemic anti-glioblastoma multiforme cellular and humoral immune responses, and anti-glioblastoma multiforme immunological memory. Release of the chromatin-binding protein high mobility group box 1 could be used as a noninvasive biomarker of therapeutic efficacy for glioblastoma multiforme. The robust treatment efficacy lends further support to its implementation in a phase I clinical trial. (Clin Cancer Res 2009;15(19):6113–27)

Gliomas represent the most common primary brain tumors, and ~50% of these are the most aggressive type, that is, glioblastoma multiforme. The available multimodality clinical treatment for glioblastoma multiforme, that is, surgery,

radiotherapy, and chemotherapy, results in an improved median survival from 6 to 18 months (1, 2). This has led to the development of various novel adjuvant treatments such as gene therapy and immunotherapy. Although numerous clinical

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Translational Relevance

Glioblastoma multiforme, the most common primary brain tumor, carries a dismal prognosis. Glioblastoma multiforme mostly kills due to recurrences; we therefore tested the hypothesis that a combined conditional cytotoxic/immune stimulatory strategy would lead to cellular and humoral immunological memory against recurrent glioblastoma multiforme lesions. Our results indicate that adenovirus (Ad) expressing *fms*-like tyrosine kinase-3 ligand + Ad expressing herpes simplex virus-1-thymidine kinase elicits CD8⁺ T cell-dependent immunological memory that leads to the rejection of a second glioblastoma multiforme implanted in the brain or in the flank. Circulating anti-glioblastoma multiforme antibodies were observed in the long-term survivors. Treatment with Ad expressing *fms*-like tyrosine kinase-3 ligand + Ad expressing herpes simplex virus-1-thymidine kinase induced tumor regression and release of the chromatin-binding protein high mobility group box 1 in two additional intracranial glioblastoma multiforme models, that is, Fisher rats bearing intracranial 9L and F98 glioblastoma multiforme cells, thus circulating levels of high mobility group box 1 could be used as a noninvasive biomarker of therapeutic efficacy for glioblastoma multiforme. Our results show the efficacy of this combined treatment in alternative glioblastoma multiforme models as a prelude for its clinical implementation.

trials have been conducted in patients with glioblastoma multiforme, the observed outcomes have not yet provided a major therapeutic breakthrough (3–7). Hence, continued research efforts are needed to develop and implement more efficacious and safer therapeutic strategies (8).

We previously reported the efficacy of a combined adenovirus (Ad)-mediated conditional cytotoxic/immune stimulatory gene therapy in preclinical rodent glioblastoma multiforme models (9–13). This gene therapy is composed of two transgenes: the conditional cytotoxic gene herpes simplex virus type 1-thymidine kinase (TK) and the immune stimulatory transgene *fms*-like tyrosine kinase-3 ligand (Flt3L); both are delivered simultaneously into the growing glioblastoma multiforme tumor mass using adenoviral vectors (Ad-Flt3L + Ad-TK). This combination therapy results in a powerful therapeutic efficacy that rescues ~70% of the treated rats bearing large intracranial glioblastoma multiforme (10, 14). The therapeutic efficacy of this treatment relies on an immune response mediated by antigen-presenting cells, that is, dendritic cells and macrophages recruited by Flt3L into the glioblastoma multiforme microenvironment and CD4⁺ and CD8⁺ T cells (10, 12). Moreover, this combined therapy inhibits the progression of a second glioblastoma multiforme implanted in the contralateral hemisphere at the same time as the primary treated tumor in a rat model of multifocal glioblastoma multiforme (13). In this report, we show that CD8⁺ T cells mediate the immunological memory induced by Ad-Flt3L + Ad-TK (+ganciclovir) in an intracranial rat model of recurrent glioblastoma multiforme.

It is thought that due to the short survival of patients with glioblastoma multiforme, distant metastases from this tumor outside the cranium are rare. Although rare, systemic metastases of glioblastoma multiforme have been reported in bone marrow, lung, lymph nodes, spinal cord, peritoneum, and bone (15–20). Metastatic glioblastoma multiforme occurs mostly in young patients (<40 years) and is not correlated with overall median survival (17). Molecular genetic analysis of metastatic and primary glioblastoma multiforme suggested that systemic metastases of glioblastoma multiforme emerge from subclones within the primary tumor mass (17). In this study, we determined that the successful treatment of the primary intracranial glioblastoma multiforme mass with Ad-Flt3L + Ad-TK stimulates an immune response strong enough that can inhibit the growth of an extracranial glioblastoma multiforme mass growing outside of the cranial cavity.

We recently reported that the efficacy of the combined conditional cytotoxic/immune stimulatory gene therapy depends on the activation of toll-like receptor-2 (TLR2) on dendritic cells by high mobility group box 1 protein (HMGB1) released by dying tumor cells (12). TLR2 activation by HMGB1 is necessary to induce a therapeutically effective immune response against glioblastoma multiforme. In the present work, we aimed to test the hypothesis that serum levels of HMGB1 could be used as a noninvasive biomarker of therapeutic efficacy in glioblastoma multiforme. Thus, we measured the systemic levels of HMGB1 in several syngeneic glioblastoma multiforme models undergoing gene therapy. We determined the concentration of HMGB1 in serum from F98, 9L, and CNS-1 brain tumor-bearing rats 5 days after the treatment, as well as in naive rats and long-term survivor implanted with CNS-1 tumors in the flank. We found that the systemic levels of HMGB1 were elevated by treatment with Ad-Flt3L + Ad-TK in all glioblastoma multiforme models studied, suggesting that serum HMGB1 levels are a useful biomarker to monitor tumor regression in response to therapeutic intervention.

In summary, our results show that the combined conditional cytotoxic/immune-stimulatory gene therapy induces long-term cellular and humoral immunity against intracranial or extracranial glioblastoma multiforme. These findings bear clinical relevance for primary and recurrent glioblastoma multiforme. Importantly, in this report, we also show that the combined conditional cytotoxic/immune stimulatory gene therapy induces tumor regression and release of the chromatin-binding protein, that is, HMGB1 in several syngeneic glioblastoma multiforme models, which supports its use as a noninvasive biomarker of glioblastoma multiforme progression.

Materials and Methods

Adenoviral vectors

The first-generation, replication-defective recombinant Ad type 5 vectors used in the study expressed soluble human Flt3L (9, 10, 21) or TK (9, 10) under the transcriptional control of human cytomegalovirus intermediate early promoter embedded within the E1 region (22). The construction of these vectors has been described in detail previously (9, 10, 13). The vectors were scaled up by infecting human embryonic kidney HEK 293 cells with a multiplicity of infection of 3 infectious units per cell of the vector seed stock. The cells were harvested 48 h later and lysed with 5% deoxycholate and DNase I, and the Ad vectors were purified by ultracentrifugation over two cesium chloride step gradients (22). The titers of vectors were estimated in triplicate by end point-dilution

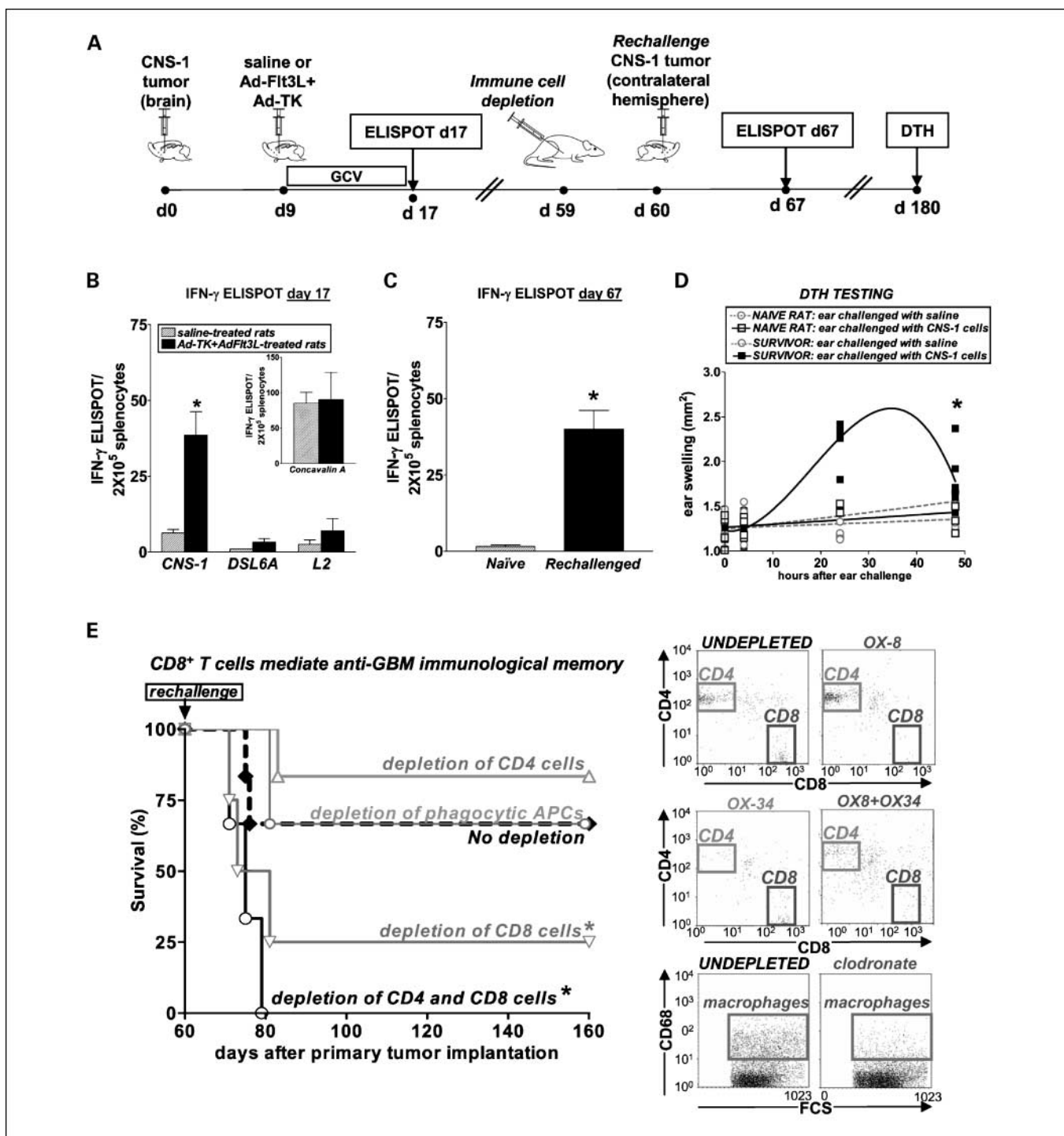


Fig. 1. Ad-Fit3L + Ad-TK elicit CD8⁺ T cell-dependent immunological memory against a second glioblastoma multiforme. Lewis rats received CNS-1 cell injection into the right striatum followed 9 d later by intratumoral injection of either saline or Ad-Fit3L + Ad-TK. After 24 h, they received ganciclovir (25 mg/kg i.p.) twice daily for 10 d. A, the presence of tumor antigen-specific-producing T-cell precursors in the spleen was quantified using an IFN- γ ELISPOT 7 d after the treatment. Splenocytes were challenged using extracts of glioblastoma multiforme CNS-1 cells, pancreatic carcinoma DSL6A cells, and L2 yolk sac carcinoma cells. Concanavalin A (ConA) stimulated splenocytes (+) are shown as a positive control. *, $P < 0.05$ versus saline-treated rats (Student's t test). B, 60 d after primary tumor implantation, Ad-Fit3L + Ad-TK-treated survivors were rechallenged in the contralateral hemisphere with CNS-1 cells. As controls, naïve rats were also implanted with intracranial CNS-1 tumors. Seven days after rechallenge, spleens were collected to detect tumor antigen-specific T-cell precursors. These were quantified using an IFN- γ ELISPOT. *, $P < 0.05$ versus naïve (Student's t test). C, DTH was done in long-term survivors 120 d after rechallenge. Naïve rats that did not receive tumors were used as controls. Irradiated CNS-1 cells were injected intradermally into the pinna of the right ear, and the left pinna received saline. The thickness of the pinna was recorded with slide calipers after 4, 24, and 48 h. *, $P < 0.05$ versus saline ear (randomization test). D, Kaplan-Meier survival curves showing the survival of brain tumor rechallenged rats that were depleted of specific immune cell populations 24 h before the second tumor implantation. Antigen-presenting cells were depleted using clodronate-filled liposomes ($n = 4$). CD4⁺ and CD8⁺ cells were depleted using an anti-CD2 antibody (OX-34; $n = 6$) and an anti-CD8 antibody (OX-8; $n = 4$), respectively. *, $P < 0.05$ versus no depletion (Mantel log rank test). Representative dot plots show the depletion of immune cell populations in the spleen, as assayed by flow cytometry using anti-CD4-PE-Cy5 and anti-CD8-PE or anti-CD68-FITC (macrophages).

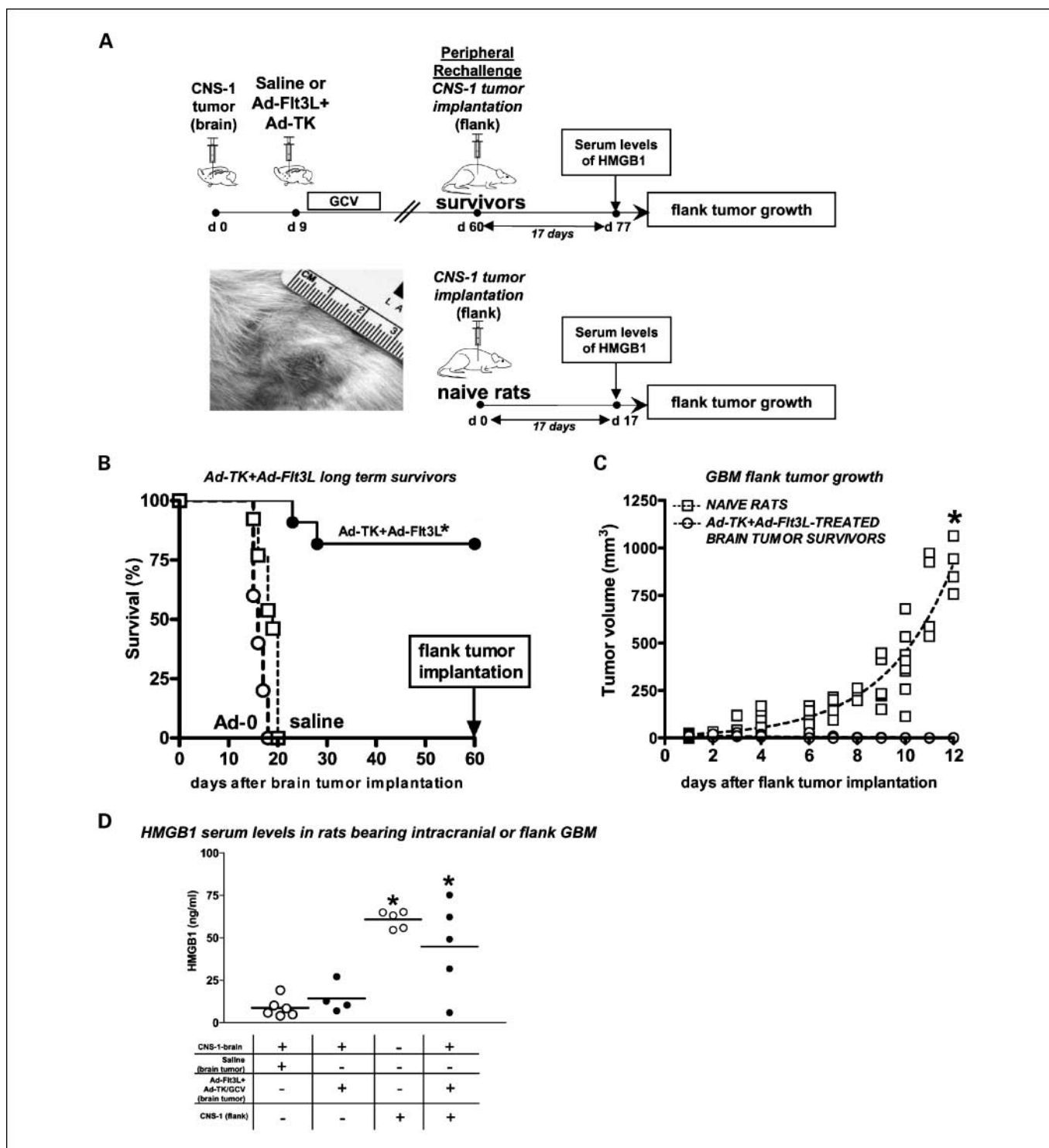


Fig. 2. Extracranial glioblastoma multiforme implantation in Ad-Fit3L + Ad-TK (+ganciclovir)-treated brain tumor survivors. **A**, Lewis rats received 4,500 CNS-1 cell injection into the right striatum, followed 9 d later by intratumoral injection of Ad-Fit3L + Ad-TK or, as controls, saline or an empty Ad (Ad-0). After 24 h, they received ganciclovir (25 mg/kg i.p.) twice daily for 10 d. Ad-Fit3L + Ad-TK-treated rats surviving to 60 d were rechallenged with 3 million CNS-1 cells injected intradermally into the flank. Tumor growth was monitored daily. Rats were euthanized for histopathologic analysis of brain and flank tumors 17 d after glioblastoma multiforme implantation in the flank. **B**, Kaplan-Meier survival curves for intracranial glioblastoma multiforme bearing rats treated 9 d after tumor implantation in the brain with saline ($n = 13$, open squares), empty Ad ($n = 5$, open circles), or Ad-Fit3L + Ad-TK (+ganciclovir; $n = 11$; solid circles). *, $P < 0.05$ versus saline (Mantel log rank test). **C**, flank tumor volume was determined in naïve rats or Ad-Fit3L + Ad-TK (+ganciclovir)-treated brain tumor survivors following injection of 3 million CNS-1 cells into the flank. *, $P < 0.05$ versus Ad-Fit3L + Ad-TK-treated brain tumor survivors (randomization test). **D**, levels of HMGB1 were assessed by ELISA in the serum from saline-treated rats or Ad-Fit3L + Ad-TK (+ganciclovir)-treated rats bearing intracranial CNS-1 tumor 8 d after the treatment (black dots). Ad-Fit3L + Ad-TK (+ganciclovir)-treated brain tumor survivors (~80%) were rechallenged with CNS-1 cells in the flank 60 d after the primary brain tumor implantation. As controls, naïve rats were also implanted in the flank with CNS-1 tumors. Serum was collected 17 d after glioblastoma multiforme implantation in the flank to determine the levels of HMGB1 (gray dots). *, $P < 0.05$ versus saline-treated rats implanted with brain tumors (empty dots), as determined by Kruskal-Wallis multiple comparison followed by Dunn's test.

cytopathic effect assay (22), and the values were 3.28×10^{11} infectious units per milliliter for Ad-TK and 4.10×10^{10} infectious units per milliliter for Ad-Flt3L. All viral stocks used in this study were replication competent Ad and lipopolysaccharide free (22). The two vectors used to treat the glioblastoma multiforme rat models in the present study possessed a viral particles-versus-infectious units ratio of 18:1.

Rat tumor models

Brain tumor models

Intracranial CNS-1 model. Glioblastoma multiforme CNS-1 cells (4,500; 3 μ L) were implanted in the striatum of Lewis rats (220-250 g; Harlan), as described below (coordinates, 5 mm from the dura and 1 mm anterior and 3.2 mm lateral to the bregma), and treated with Ads 9 d later. Ad-Flt3L + Ad-TK (10^8 infectious units each in 3 μ L) were delivered in three locations (1 μ L each) within the tumor (-5.5, 5.0, and -4.5 mm from dura).

Recurrent intracranial CNS-1 model. Ad-Flt3L + Ad-TK-treated brain tumor survivors were implanted in the contralateral striatum with 4,500 glioblastoma multiforme CNS-1 cells 60 d after primary brain tumor implantation. The day before the second tumor implantation, rats were depleted of specific immune cell populations. Phagocytic antigen-presenting cells and macrophages were depleted by i.p. injection of liposome encapsulated clodronate (Cl₂MBP; 12 mg in 2 mL/rat; ref. 23). Clodronate (a gift from Dr. Nico Van Rooijen) was encapsulated in liposomes, as previously described (10, 23). CD4⁺ and CD8⁺ T cells were depleted by i.p. injection of 1 mg anti-CD2 antibody

(OX-34) and 1 mg anti-CD8 antibody (OX-8), respectively. Hybridoma cell lines for CD8⁺ cell depletion (OX-8; European Collection of Animal Cell Cultures) and CD4⁺ cell depletion (OX-34; European Collection of Animal Cell Cultures) were produced by Bioexpress Cell Culture Services.

Intracranial F98 model. Glioblastoma multiforme F98 cells (50,000; 3 μ L) were implanted in the striatum (coordinates, 6 mm from the dura and 1 mm anterior and 3.2 mm lateral to the bregma) of Fisher rats (Harlan) and treated with Ads 7 d later. Ad-Flt3L + Ad-TK (10^8 infectious units each in 3 μ L) were delivered in three locations (1 μ L each) within the tumor (-6.5, -5.5, and -4.5 mm from the dura).

Intracranial 9L model. Glioblastoma multiforme 9L cells (500,000; 3 μ L) were implanted intracranially in the striatum (coordinates, 5 mm from the dura and 1 mm anterior and 3.2 mm lateral to the bregma) of Fisher rats (Harlan) and treated with gene therapy Ads 9 d later. Ad-Flt3L + Ad-TK (10^8 infectious units each in 3 μ L) were delivered in three locations (1 μ L each) within the tumor (-5.5, 5.0, and -4.5 mm from dura).

Rats were kept in controlled conditions of light (12 h light-dark cycles) and temperature (20-25°C) and fed standard laboratory chow and water *ad libitum*. All animal procedures were carried out in accordance with the NIH guide for the care and use of laboratory animals and approved by the Cedars-Sinai Institutional Animal Care and Use Committee.

Brain tumor implantation was done as previously described (24). Briefly, using a 10 μ L Hamilton syringe fitted with a 26-gauge needle,

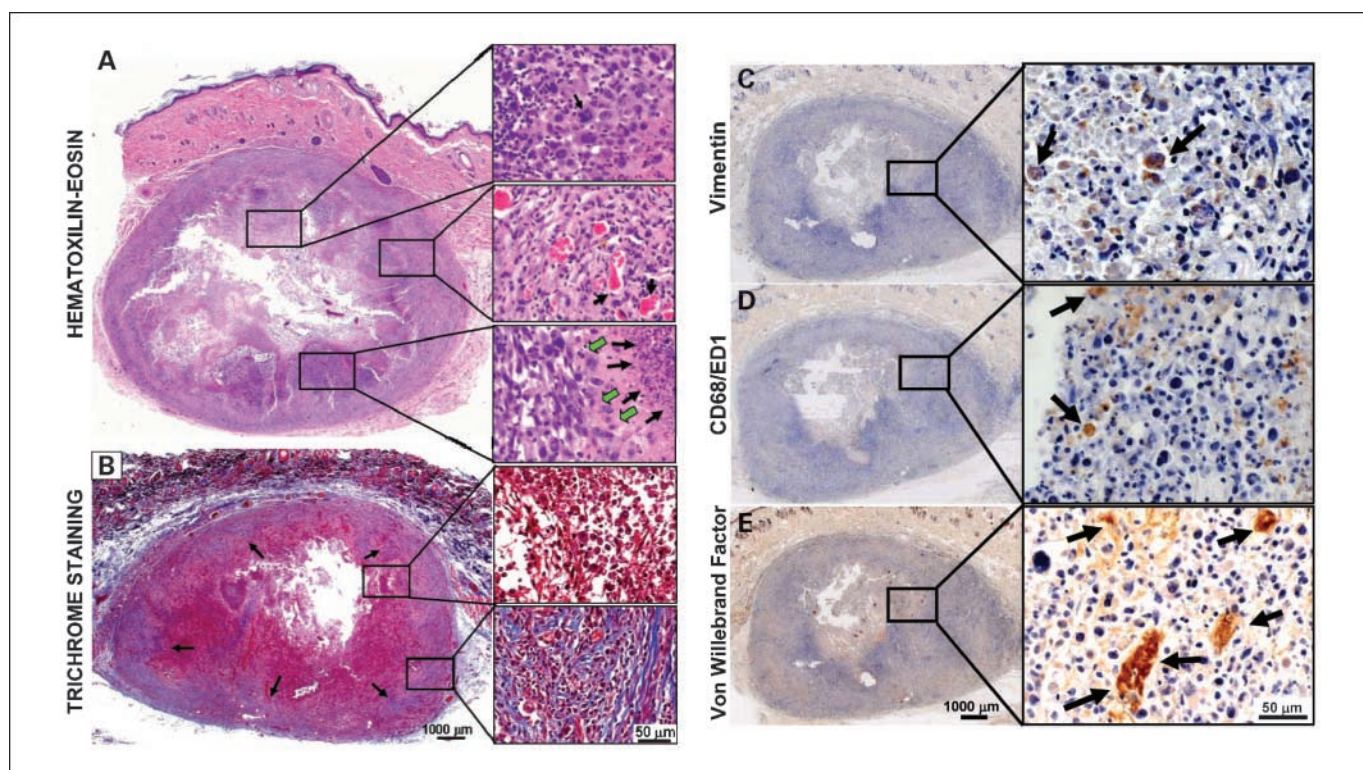


Fig. 3. Histologic features of flank tumors in naïve rats. Flank tumor pathologic features were studied in paraffin-embedded sections of flank glioblastoma multiforme specimens harvested at day 17 post-tumor implantation in naïve rats. **A**, low-magnification image shows the macroscopic appearance and size of a representative flank tumor in naïve Lewis rat, as assessed by hematoxylin-eosin staining. High-magnification microphotographs show nuclear atypia (black arrow, top), profuse neovascularization (black arrows, center), and areas of coagulative necrosis (black arrows, bottom) and pseudopalisading (green arrows, bottom). **B**, trichrome staining was used to detect the presence of collagen fibers in the tissue surrounding the tumor mass (black arrows). High-magnification microphotographs show the absence of collagen tissue in the central tumor mass (top) and the abundance of collagen fibers in periphery of the tumor growing in the flank (bottom). **C**, representative images of flank tumor sections stained with anti-vimentin antibody. High-magnification images show positive vimentin immunoreactivity in atypical cells with large pleomorphic nuclei within the tumor mass (arrows). **D**, representative images of flank tumor sections stained with anti-CD68 antibody. High-magnification microphotograph show infiltration of CD68⁺ cells (macrophages; arrows) within the tumor mass. **E**, representative images of flank tumor sections stained with anti-von Willebrand Factor antibody. High-magnification microphotograph shows immunoreactivity in vascular endothelial cells (arrows).

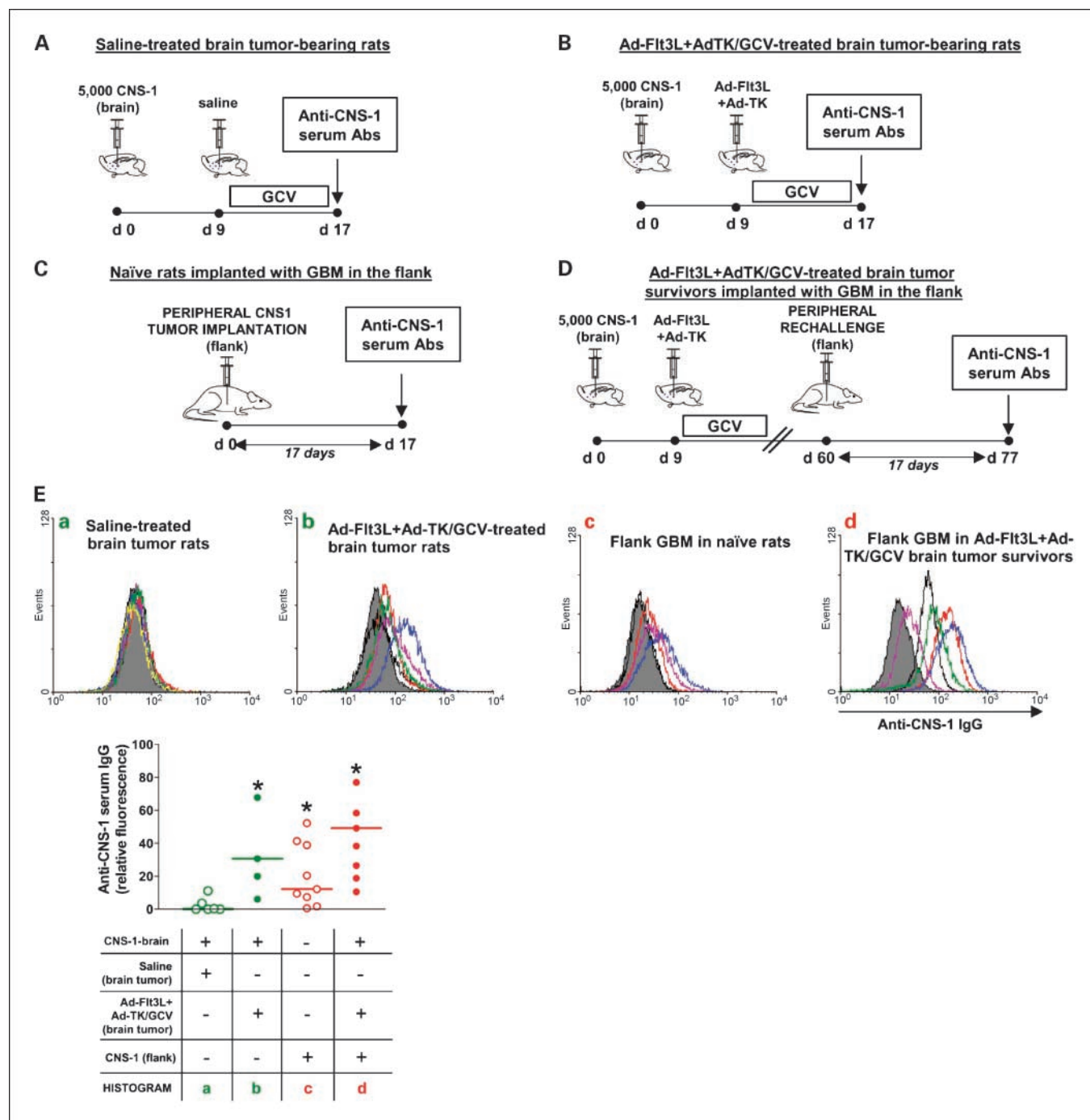


Fig. 4. Humoral immune response against intracranial (A and B) or extracranial (C and D) glioblastoma multiforme. Lewis rats were implanted intracranially with CNS-1 cells (4,500) and 9 d later received intratumoral injection of either saline (A) or Ad-Fit3L + Ad-TK (+ganciclovir; B and D). Serum was collected from saline-treated rats (A) or Ad-Fit3L + Ad-TK (+ganciclovir)-treated rats (B) 8 d after the treatment for detection of anti-CNS-1 antibodies. Ad-Fit3L + Ad-TK (+ganciclovir)-treated brain tumor survivors (~80%) were rechallenged with CNS-1 cells (3×10^6) in the flank (D) 60 d after brain tumor implantation in the flank to determine the presence of anti-CNS-1 antibodies by flow cytometry. Serum from naïve rats that did not receive tumor was used as isotype control (gray histograms). The scatter plot shows the fluorescence of CNS-1 cells bound to putative opsonizing antibodies was determined by flow cytometry. The fluorescence of CNS-1 cells incubated with serum from naïve non-tumor bearing rats was subtracted from test samples. *, $P < 0.05$ versus saline-treated intracranial glioblastoma multiforme-bearing rats (a) as determined by Kruskal-Wallis multiple comparison, followed by Dunn's test. Histograms show the fluorescence intensity of CNS-1 cells labeled with serum from non-tumor bearing rats (isotype control; gray histogram) or with serum from rats from each of the experimental groups (colored lines).

glioblastoma multiforme cell suspensions were injected stereotactically at the coordinates indicated above. A small pocket was created before depositing the cells by holding the needle 0.5 mm below the stated coordinates for 1 min before moving up to the stated coordinates and

injecting the cells slowly over a period of 3 min. The needle was left in place for an additional 5 min before being slowly withdrawn. At the times indicated above, saline or Ad-Fit3L + Ad-TK (10^8 infectious units each) was injected into the growing tumor mass using the

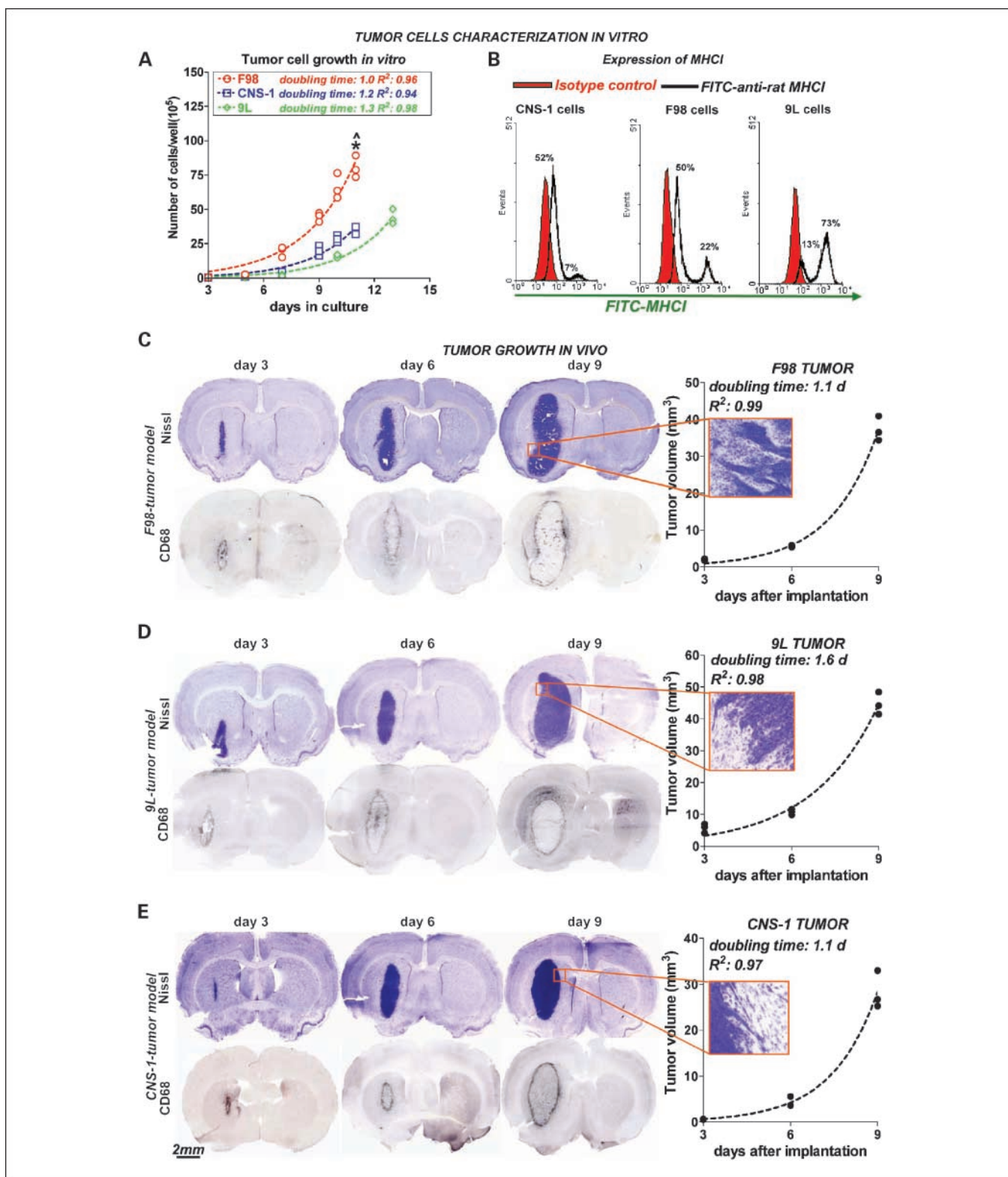


Fig. 5. Characterization of rat orthotopic glioblastoma multiforme models. *A*, *in vitro* cell growth rates of F98, 9L, and CNS-1 rat glioblastoma multiforme cells ($n = 3$ wells per group). Cells (5,000) were seeded on day 0, and cells were harvested and counted every day for up to 15 d. *, $P < 0.05$ versus CNS-1, ^, $P < 0.05$ versus 9L (randomization test). *B*, expression of MHCII was assessed in CNS-1, 9L, and F98 cells by flow cytometry. Representative histograms show the intensity of FITC-mouse anti-rat MHCII or isotype control (FITC-mouse IgG1k1; red area). Numbers indicate the percentage of low- and high-expressing cells. *C-E*, *in vivo* tumor growth rates of intracranial F98, 9L, and CNS-1 glioblastoma multiforme in rats. Fisher rats were implanted in the striatum with F98 cells (*C*) or 9L cells (*D*), and Lewis rats were injected with CNS-1 cells (*E*). Brains were processed for stereology to determine tumor volume 3, 6, and 9 d after tumor implantation. Graphs show tumor growth rates for each tumor model ($n = 3$ per group). Doubling time and regression coefficient (R^2) are indicated in the graphs. Microphotographs show the appearance of representative brain tumor sections stained with Nissl. Infiltration of inflammatory cells was detected using antibodies against CD68 (macrophages/activated microglia).

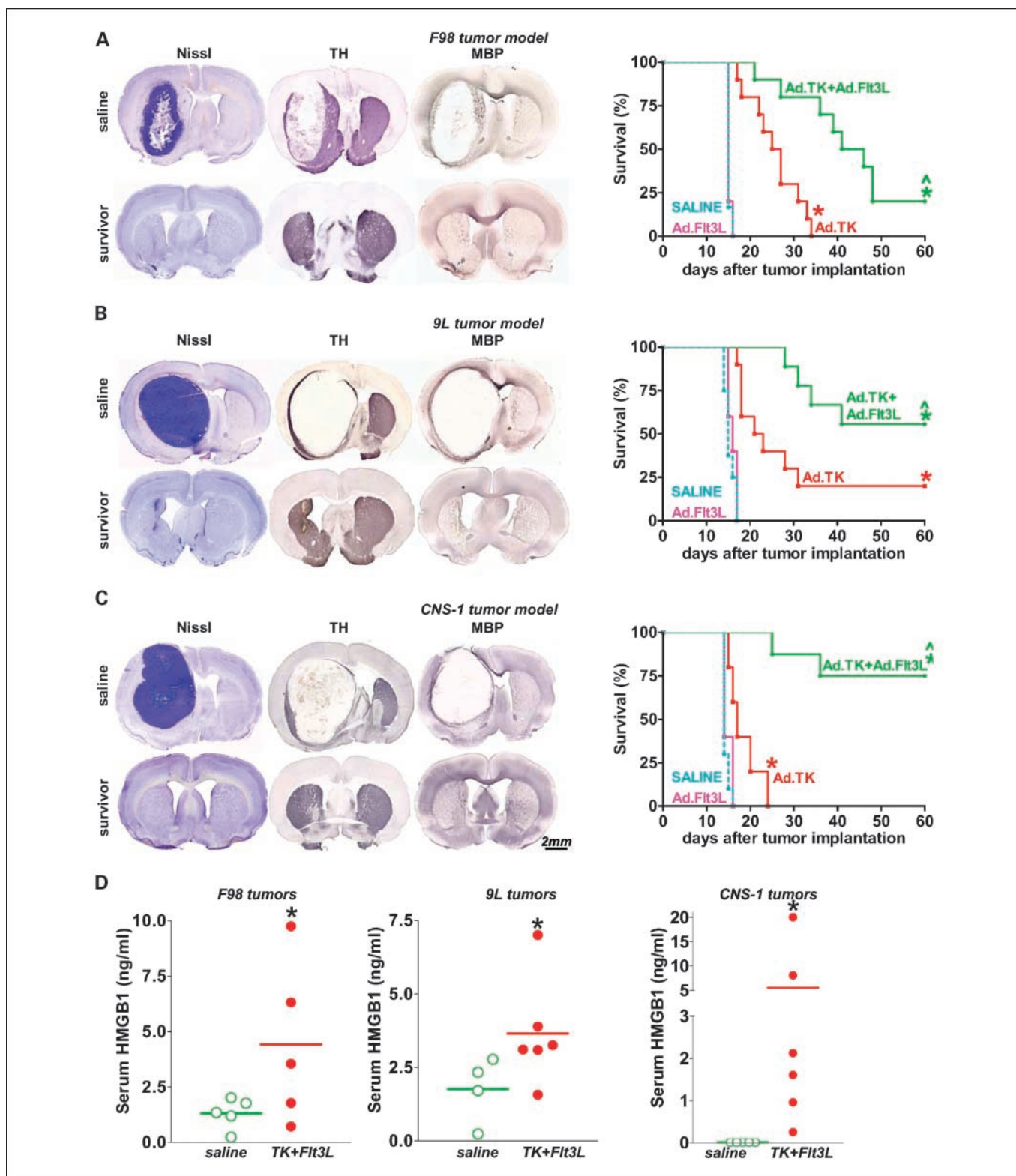


Fig. 6. Efficacy of the combined Ad-TK and Ad-Fit3L gene therapy in several syngeneic intracranial rat glioblastoma multiforme models. *A*, Fisher rats were implanted with F98 cells and treated 7 d later with saline ($n = 6$), Ad-TK ($n = 10$), Ad-Fit3L ($n = 5$), or Ad-Fit3L + Ad-TK ($n = 10$). *B*, Fisher rats were implanted in the striatum with 9L cells and treated 9 d later with saline ($n = 8$), Ad-TK ($n = 10$), Ad-Fit3L ($n = 5$), or Ad-Fit3L + Ad-TK ($n = 9$). *C*, Lewis rats were implanted in the striatum with CNS-1 cells and treated 9 d later with saline ($n = 10$), Ad-TK ($n = 5$), Ad-Fit3L ($n = 5$), or Ad-Fit3L + Ad-TK ($n = 8$). Rats received ganciclovir (25 mg/kg i.p.) twice daily for 10 d. Graphs show Kaplan-Meier survival curves of tumor-bearing rats. *, $P < 0.05$ versus saline; ^, $P < 0.05$ versus Ad-TK (log rank test). Microphotographs show the neuropathology of moribund saline-treated rats and of Ad-Fit3L + Ad-TK-treated long-term survivors (60 d after tumor implantation). Brains were stained using Nissl, and immunocytochemistry was done using antibodies against TH and MBP. *D*, 5 d after the treatment, tumor-bearing rats treated with saline or Ad-Fit3L + Ad-TK were euthanized, and the circulating levels of HMGB1 were measured in serum by ELISA. *, $P < 0.05$ versus saline (Student's t test).

previously drilled burr hole. As controls, a group of rats bearing intracranial CNS-1 tumors were injected intratumorally with an empty Ad (2×10^8 infectious units), as described above. Twenty-four hours after treatment, rats were injected with ganciclovir (25 mg/kg i.p.; Roche Laboratories) twice daily for 10 consecutive days.

Flank CNS-1 model. CNS-1 cells (3×10^6) in 50 μ L DMEM (CellGro) were injected intradermally in the flank of naïve Lewis rats or Ad-F13L + Ad-TK-treated survivors after 60 d of CNS-1 tumor implantation in the brain. The length (*L*) and the width (*W*) of the skin lesion were measured daily with slide calipers. Tumor volume (*V*) was calculated using the longest measurement as length and using: V (cubic millimeters) = $(L \times W^2) / 2$ (25). Optimization of this model was done by injecting 5×10^4 , 1×10^6 , or 3×10^6 CNS-1 cells in 50 μ L DMEM intradermally in the flanks of naïve rats (data not shown).

Animals were monitored daily and euthanized at the first signs of moribund behavior or at predetermined time points for collection of splenocytes for enzyme-linked immunosorbent spot, for detection of HMGB1 or anti-CNS-1 antibodies in serum, or for analysis of brain and flank tumor pathology. Animals were euthanized according to the guidelines of the Institutional Animal Care and Use Committee at Cedars-Sinai Medical Center, by terminal perfusion with Tyrode's solution (132 mmol/L NaCl, 1.8 mmol/L CaCl_2 , 0.32 mmol/L NaH_2PO_4 , 5.56 mmol/L glucose, 11.6 mmol/L NaHCO_3 , and 2.68 mmol/L KCl) followed by perfusion with 4% paraformaldehyde under deep anesthesia. Brains were removed and further fixed in 4% paraformaldehyde for 3 d.

Delayed-type hypersensitivity (DTH) tests

DTH was done in long-term survivors 120 d after rechallenge. CNS-1 cell suspensions were prepared in PBS and then irradiated (30 Gy). Irradiated CNS-1 cells (1×10^6 ; 50 μ L) were injected intradermally into the pinna of right ear of each rat, and the left pinna received 50 μ L of saline. Baseline measurements of the thickness of pinna were recorded with slide calipers, and the measurement was repeated after 4, 24, and 48 h.

Immunohistochemistry

Following perfusion with Tyrode's solution and 4% paraformaldehyde, brains were fixed in 4% paraformaldehyde for 3 additional days. Free-floating immunohistochemistry was done in serial coronal sections (50 μ m). Sections were treated with 0.3% hydrogen peroxide to inactivate endogenous peroxidase and blocked with 10% horse serum, followed by incubation with the primary antibodies diluted in TBS (pH 7.4) containing 1% horse serum and 0.5% Triton X-100. Sections were incubated for 48 h with the following antibodies: anti-tyrosine hydroxylase (TH) in rabbit (1:5,000; Calbiochem 657012), anti-myelin basic protein (MBP) in mouse (1:1,000; Chemicon MAB1580), and anti-rat CD68 in mouse (clone ED1 to identify macrophages/activated microglia; 1:1,000; Serotec MCA341R). Then, the sections were incubated for 4 h with biotin-conjugated anti-rabbit goat IgG or anti-mouse rabbit IgG (1:800; DAKO E0432 and E0464, respectively), followed by 4 h additional incubation with avidin-biotin complex (Vectastain Elite ABC Kit, Vector Laboratories, Inc.). Nickel-enhanced 0.02% 3,3'-diaminobenzidine in sodium acetate was used as the chromogen. Finally, the sections were mounted onto gelatin-coated slides, dehydrated, and cover slipped using DPX mountant for histology (Sigma-Aldrich).

Nissl staining was done on brain sections to delineate the glioblastoma multiforme tumor mass. The sections were mounted and incubated in cresyl violet (0.1%; Sigma) for 15 min. They were washed in destain solution (70% ethanol, 10% acetic acid) for 1 min and then dehydrated (100% ethanol and xylene) and mounted.

The skin lesions were fixed in 4% paraformaldehyde and embedded in paraffin, and 4- μ m serial sections were processed. Following deparaffinization, hematoxylin-eosin staining was done to evaluate tissue histology. Trichrome staining was done to look for the presence of collagen tissue within the lesion and to furnish supplementary evidence that the skin lesions were actually tumor masses and not merely

inflammatory lesions. In addition, after antigen retrieval by heated citrate buffer (10 mmol/L citric acid; pH 6.0) followed by trypsin or proteinase K treatment, immunocytochemistry was done using the following antibodies: mouse anti-vimentin (1:1,000; Sigma V6630), mouse anti-human von Willebrand Factor (1:50; DAKO A0082), and anti-rat CD68 in mouse, as described above. Sections were evaluated and photographed with a Zeiss Axioplan microscope.

In vitro growth profile of CNS-1, 9L, and F98 glioblastoma multiforme cells

The glioblastoma multiforme cell lines (5,000 cells per well) were seeded in 12-well plates in triplicate and maintained in culture in DMEM supplemented with 10% fetal bovine serum (FBS; Omega Scientific) and 1% penicillin-streptomycin (CellGro). The growing cells were checked under the microscope daily, and the culture media were changed every 3 d. For counting the cells, wells in triplicate were trypsinized and the cells were harvested. The number of cells per well was then counted by mixing the cell suspension with trypan blue stain 0.4% (Gibco; Invitrogen Co.) to exclude dead cells.

In vivo growth profile of CNS-1, 9L, and F98 glioblastoma multiforme cells

The tumor volumes at 3, 6, or 9 d following implantation of the corresponding glioblastoma multiforme cells in the striatum ($n = 3$ per cell line) were estimated using unbiased stereological techniques. The postfixed brain was cut into consecutive 30- μ m-thick coronal brain sections. A random selection of one twelfth of the brain sections was made, and the sections were processed for Nissl staining. With a Zeiss Axioplan 2 microscope controlled by Ludl electronic MAC 5000 XY stage control (Ludl Electronics Products Ltd.) and Axioplan Z-axis control (Carl Zeiss, Inc.), the tumor mass was visualized under low magnification (original magnification, $\times 1.25$). All of the sections that had tumor mass as revealed by Nissl staining were sampled for tumor volume estimation (6-10 sections per animal). Stereo Investigator software version 8.00.0 (Microbrightfield, Inc.) was used to estimate the tumor volume using the Cavalieri estimator, with grid spacing set at 250 μ m; the tumor mass was measured on all sections using point counting (26).

Flow cytometry

Evaluation of immune cell depletion. Confirmation of immune cell depletion was done in naïve Lewis rats injected i.p. with clodronate, OX-34, or OX-8 antibodies, as described above. Seven days after depletions, spleens were analyzed by flow cytometry. Splenocytes were harvested, and RBCs were removed by incubating in 3 mL ammonium chloride-potassium carbonate solution (0.15 mmol/L NH_4Cl , 10 mmol/L KHCO_3 , and 0.1 mmol/L sodium EDTA at pH 7.2) for 3 min. Splenocytes were washed in RPMI media (containing 10% FBS, 1% penicillin-streptomycin, 1% L-glutamine); 1×10^6 splenocytes were resuspended in FACS buffer (PBS with 1% FBS and 0.1% sodium azide) containing anti-CD3-fluorescein isothiocyanate, anti-CD4-PE-Cy5, and anti-CD8-PE antibodies (BD Biosciences 554857, 554839, 557354). Macrophages were detected by CD68-FITC (MCA341F; Serotec) after fixation with 2% paraformaldehyde. Cells were analyzed on a FACScan flow cytometer (Beckman Coulter).

Detection of anti-CNS-1 antibodies. Serum samples were collected 7 d after CNS-1 tumor implantation in the brain or the flank. Fixed CNS-1 cells (50,000; 4% paraformaldehyde for 15 min on ice) were incubated in 50 μ L of sample serum, naïve rat serum (isotype control), or FACS buffer (PBS with 1% FBS and 0.1% sodium azide) for 30 min on ice. After washing, cells were incubated with 50 μ L of FITC-goat anti-rat immunoglobulin (1:200; Jackson Labs 112-096-003) for 30 min on ice. After washing, cells were analyzed on a FACScan flow cytometer (Beckman Coulter).

Expression of MHC I and MHC II. Trypsinized glioblastoma multiforme cells were incubated with FITC-conjugated mouse anti-rat major histocompatibility complex class II (BD Pharmingen 554919) or

FITC-conjugated mouse IgG1 κ isotype control, peridin chlorophyll protein-conjugated mouse anti-rat MHCII (BD Pharmingen 557016), or Per.CP-conjugated Ig1 κ isotype control antibodies (4°C for 30 min). Cells were analyzed on a FACScan flow cytometer (Beckman Coulter). WinMDI 2.9 software (J. Trotter, Scripps Research Institute, La Jolla, CA) was used to determine fluorescence intensity.

Analysis of antigen-specific T cells by IFN- γ -ELISPOT

ELISPOT assay was carried out using the rat IFN- γ development module (R&D Systems), according to the manufacturer's instructions. Splenocytes were collected as described above, resuspended in 90% FBS + 10% dimethyl sulfoxide and frozen in liquid nitrogen. Frozen splenocytes were thawed rapidly and resuspended in RPMI-10 (RPMI medium supplemented with 10% FBS, 50 units/mL penicillin, 50 μ g/mL streptomycin) and incubated at 37°C in 5% CO₂ for 48 h in the presence of 10 U/mL recombinant human IL-2. One million cells per well were then plated in Imobilon-P membrane-lined 96-well plates (Millipore), precoated with mouse anti-rat IFN antibody. Stimulants were added as follows: (a) Concanavalin A at 1.6 μ g/mL and (b) cell lysates, 100 μ g/mL protein equivalent of cell lysate prepared from CNS-1, DSL6A, or L2 cells. Each well was supplemented with a further 10 U/mL of IL-2, and the plate was incubated as above for a further 48 h before discarding the cells and detecting IFN spots with a biotinylated secondary antibody, alkaline phosphatase-conjugated streptavidin, and a chromagenic substrate mixture of 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (R&D Systems). Spots were counted manually under a dissection microscope.

HMGB1 ELISA

HMGB1 expression was determined in rat serum using a specific anti-HMGB1 ELISA (IBL International), following the manufacturer's protocol. Briefly, 100 μ L sample diluent was added to each well. Next, 10 μ L of rat serum was added to each well and incubated at 37°C for 24 h. Wells were washed five times with wash buffer and incubated for 2 h at 25°C with 100 μ L peroxidase-conjugate solution. Wells were washed a further five times in wash buffer and incubated for 30 min at room temperature with substrate solution. The reaction was stopped by adding 100 μ L stop solution to each well, and the absorbance was read at 450 nm (the background was subtracted by measuring absorbance at 570 nm).

Trp₂₁₈₀₋₁₈₈ RT-PCR

RNA was extracted from rat glioma cell lines or mouse melanoma cell line B16 using the RNeasy Plus Mini kit (Qiagen). RNA quality and concentration was measured using DU640 Spectrophotometer (Beckman Coulter, Inc.). A total of 1 μ g of RNA, 25 ng of Random primer, 20 units of Recombinant RNasin RNase Inhibitor, and 200 units of M-MLV Reverse transcriptase (Promega) were used in one reverse transcription reaction. The RT reaction was run at 37°C for 1 h. cDNA (1 μ L), 800 nmol of both reverse and forward primers, and Go-Taq Flexi DNA polymerase (Promega) were used for PCR. PCR was done using Gene Amp PCR system 9700 (AB Applied Biosystem). The thermal cycles used for Trp-2 amplification were 10 min at 95°C, followed by 35 cycles of 95°C for 50 s, 56°C for 50 s, and 72°C for 50 s, ending with 10 min at 72°C. The thermal cycles used for GAPDH amplification were 10 min at 95°C, followed by 30 cycles of 95°C for 50 s, 58°C for 50 s, and 72°C for 50 s, ending with 10 min at 72°C. The reaction mixture was run on 0.8% w/v agarose gel with ethidium bromide. Primers, corresponding annealing temperature, and fragment length of PCR product size are listed in a table in Supplementary Fig. S1.

Statistical analysis

The survival curves were analyzed using the log-rank test using Prism GraphPad software (version 3.03). *In vitro* and *in vivo* tumor growth rates were determined by nonlinear regression analysis using Prism GraphPad software. NCSS statistical and power analysis software

was used for the statistical analysis for flank tumor size, *in vitro* cell growth, and DTH data using randomization test. The flow cytometry, ELISPOT, and ELISA data were analyzed by Student's *t* test or ANOVA. When data failed normality or Levene's test for variance homogeneity, they were analyzed by Kruskal-Wallis multiple comparison followed by Dunn's test. Differences between groups were considered significant at *P* < 0.05.

Results

Anti-glioblastoma multiforme immunological memory elicited by Ad-Flt3L + Ad-TK gene therapy is mediated by CD8⁺ T cells. We have previously showed that gene therapy using Ad-Flt3L + Ad-TK induces brain tumor regression and immunological memory in rodent models of glioblastoma multiforme (12–14). Here, we characterize the phenotype of the immune cells that mediate the effector phase of the combined gene therapy approach using the recurrent rat glioblastoma multiforme model in Lewis rats. We first determined whether Ad-Flt3L + Ad-TK induces clonal expansion of tumor antigen-specific T lymphocytes and also the specificity of this response. Thus, we collected splenocytes from rats bearing intracranial CNS-1 tumors 7 days after the treatment with saline or Ad-Flt3L + Ad-TK and exposed them to cell extracts from CNS-1 cells or, as controls, DSL6A cells (syngeneic pancreatic carcinoma) or L2 cells (syngeneic rat yolk sac carcinoma cells). An increase in the frequency of T-cell precursors that released IFN- γ in response to exposure to specific tumor antigen was only detected in splenocytes from Ad-Flt3L + Ad-TK-treated rats that were challenged with CNS-1 cell extracts (Fig. 1A). No response was observed when splenocytes were exposed to DSL6A or L2 cell extracts or when the splenocytes originated from rats treated with saline. In addition, when Ad-Flt3L + Ad-TK (+ganciclovir)-treated long-term survivors were rechallenged in the contralateral brain hemisphere with a second CNS-1 tumor (60 days after primary tumor implantation), we observed an increase in the number of tumor antigen-specific T-cell precursors that release IFN- γ (Fig. 1B) when compared with tumor-bearing naïve rats, used as controls. Cellular immunity against CNS-1 cells in rechallenged long-term survivors was also assessed using the DTH reaction (Fig. 1C). DTH test was done 120 days after intracranial rechallenge of Ad-Flt3L + Ad-TK-treated long-term survivors. Naïve rats, used as controls, did not exhibit a positive DTH reaction over the 48-hour period of observation (Fig. 1C). However, rechallenged Ad-Flt3L + Ad-TK (+ganciclovir)-treated long-term survivors developed a strong DTH reaction, detected at 24 and 48 h after CNS-1 injection in the ear (Fig. 1C).

To determine which immune cells are responsible for the immunological memory against glioblastoma multiforme, Ad-Flt3L + Ad-TK-treated rats that survived a primary CNS-1 tumor were rechallenged with a second CNS-1 tumor in the contralateral striatum 60 days after primary tumor implantation and depleted of specific immune cell populations immediately before implantation of the second tumor. Phagocytic antigen-presenting cells were depleted by i.p. injection of liposome encapsulated clodronate, and CD4⁺ and CD8⁺ T cells were depleted by i.p. injection of anti-CD2 antibody (OX-34) and anti-CD8 antibody (OX-8), respectively. Depletion of the specific immune cell populations was confirmed in naïve Lewis rats 7 days following antibody administration (Fig. 1D). We found that depletion of antigen-presenting cells and CD4⁺ cells does not

interfere with the rejection of the second glioblastoma multiforme, whereas the depletion of CD8⁺ T cells abrogates the anti-tumor immunological memory elicited by Ad-Flt3L + Ad-TK. Note that the rechallenged long-term survivors that were not depleted rejected the second tumor and survived long term after rechallenge (Fig. 1D).

Inhibition of glioblastoma multiforme progression in the flank of long-term survivors of intracranial glioblastoma multiforme after treatment with combination Ad-Flt3L + Ad-TK gene therapy. In these experiments, we aimed to assess if the combined Ad-Flt3L + Ad-TK (+ganciclovir) gene therapy delivered into large intracranial glioblastoma multiforme was effective at inhibiting the progression of a glioblastoma multiforme implanted in the flank. The experimental paradigm is depicted in Fig. 2A. Lewis rats were implanted intracranially with 4,500 CNS-1 cells in the right striatum, and 9 days later, they were treated by intratumoral injection of either saline, an empty Ad, or Ad-Flt3L + Ad-TK, followed by administration of ganciclovir. Although all the saline- and empty Ad-treated rats succumbed because of tumor burden (median survival, 19 and 16, respectively), the combined treatment rescued ~80% of the rats bearing large intracranial glioblastoma multiforme (Fig. 2B). Sixty days after primary glioblastoma multiforme implantation in the brain, long-term survivors were implanted with CNS-1 cells (3×10^6) in the flank. Naïve rats implanted in the flank with CNS-1 cells served as controls (Fig. 2A). Tumor size was measured regularly in both groups. In the long-term survivors following successful treatment of intracranial glioblastoma multiforme by Ad-Flt3L + Ad-TK, the skin lesions were very small, attaining a maximum size of $14.8 \pm 4.1 \text{ mm}^3$ (mean \pm SE) on day 3 and became undetectable 10 days of post-CNS-1 cell implantation. The skin lesions in naïve control rats grew rapidly to a maximum size of $903.3 \pm 58.5 \text{ mm}^3$ at 12 days postimplantation (Fig. 2C) and then started to regress spontaneously (not shown). The size and macroscopic appearance of a representative skin lesion 12 days following CNS-1 cell implantation in the flank is shown in Fig. 2A.

We have recently determined that mouse, rat, and human glioblastoma multiforme cells release HMGB1 upon cell death induced by Ad-TK + ganciclovir (12, 14). HMGB1 is an abundant chromatin-binding protein that acts as an endogenous TLR2 and TLR4 agonist when released by dying cells or inflammatory cells (27–29). In our experiments, systemic levels of HMGB1 increased rapidly during tumor cell death *in vivo*, and this was essential for the efficacy of Ad-Flt3L + Ad-TK in eradicating primary intracranial tumors in rodent models of glioblastoma multiforme (12, 14). We therefore aimed to determine whether this protein would also be released during the regression of an extracranial glioblastoma multiforme lesion. We found that 17 days after flank tumor implantation, the systemic levels of HMGB1 were increased in naïve and Ad-Flt3L + Ad-TK-treated long-term survivors (Fig. 2D).

Histopathologic evaluation of skin lesions following intradermal CNS-1 implantation. Seventeen days after intradermal glioblastoma multiforme implantation (77 days after intracranial glioblastoma multiforme implantation), rats were euthanized, and the flank lesions were harvested for histopathologic analysis (Fig. 3). Although Ad-Flt3L + Ad-TK-treated brain tumor survivors did not exhibit tumor remnants in the flank,

hematoxylin-eosin-stained extracranial glioblastoma multiforme lesions from naïve rats revealed tumors exhibiting necrosis with surrounding pseudopalisading cells (pseudopalisading necrosis), coagulative necrosis, microvascular hyperplasia, and also areas of hemorrhages (Fig. 3A), features that are all typical of glioblastoma multiforme. Trichrome Masson staining (Fig. 3B) showed the presence of abundant collagen along the tumor periphery but not in the central tumor mass. In addition, immunocytochemistry was done with antibodies against vimentin (to visualize CNS-1 cells that express vimentin), CD68/ED1 (macrophages), and von Willebrand factor (vascular endothelial cells). Microscopic examination uncovered areas of positive immunoreactivity against vimentin (Fig. 3C) and profuse CD68/ED1⁺ macrophages (Fig. 3D). Positive staining against von Willebrand factor was also evident and revealed characteristic microvascular hyperplasia (Fig. 3E).

Neuropathology of the Ad-Flt3L + Ad-TK (+ganciclovir)-treated long-term survivors that also rejected the extracranial glioblastoma multiforme implanted in the flank was also assessed (Supplementary Fig. S2). Long-term survivors showed no significant structural alterations in the striatum when compared with the untreated contralateral hemisphere. The brains of these rats did not display residual tumor, as determined by Nissl staining and vimentin immunocytochemistry, but exhibited abundant vimentin⁺ reactive astrocytes in the corpus callosum, external capsule, and also along the injection tract, typical of overreactive astrocytes (Supplementary Fig. S2). Immune staining of striatal dopaminergic fibers using anti-TH antibodies revealed similar density of TH⁺ fibers in both hemispheres. The density of myelinated fibers was observed by immune staining of MBP staining. MBP⁺ oligodendrocytes seemed to be intact in both hemispheres; no signs of demyelination were observed. Scarce CD8⁺ T cells and abundant CD68⁺ macrophages/activated microglia were detected at and around the scar at the site of tumor implantation. MHCII immunoreactive cells were seen not only in the injection site but also in the corpus callosum and external capsule of brain parenchyma (Supplementary Fig. S2).

Humoral immunity against glioblastoma multiforme. To evaluate whether humoral immunity was stimulated in response to Ad-Flt3L + Ad-TK (+ganciclovir) treatment during the rejection of the primary glioblastoma multiforme or the second glioblastoma multiforme implanted in the flank, we assessed the presence of circulating anti-CNS-1 antibodies in Lewis rats bearing intracranial and/or flank glioblastoma multiforme cells. Lewis rats were implanted intracranially with CNS-1 cells and 9 days later received intratumoral injection of either saline (Fig. 4A) or Ad-Flt3L + Ad-TK (+ganciclovir; Fig. 4B). All the saline-treated and a set of Ad-Flt3L + Ad-TK (+ganciclovir)-treated rats were euthanized 8 days after the treatment (17 days after intracranial glioblastoma multiforme implantation), and the serum was collected for detection of anti-CNS-1 antibodies. Control naïve rats (Fig. 4C) and a set of Ad-Flt3L + Ad-TK (+ganciclovir)-treated rats that survived to 60 days (Fig. 4D) were implanted with CNS-1 cells in the flank and were euthanized 17 days later (77 days after intracranial glioblastoma multiforme implantation in long-term brain tumor survivors). Serum was collected and used to determine the presence of anti-CNS-1 antibodies. Anti-glioblastoma multiforme cell antibodies were determined by incubating CNS-1

cells with serum from glioblastoma multiforme-bearing rats or from non-tumor bearing naïve rats, used as isotype control. The cells were then incubated with a FITC-tagged anti-rat secondary antibody, and mean fluorescence of CNS-1 cells bound to putative opsonizing antibodies was determined by flow cytometry. We found that, whereas rats with intracranial glioblastoma multiforme that were injected with saline had undetectable levels of anti-CNS-1 antibodies (Fig. 4A), rats treated with Ad-Flt3L + Ad-TK exhibited circulating antibodies against CNS-1 antigens 7 days after the treatment (Fig. 4B). In addition, anti-CNS-1 antibodies were detected in naïve rats (Fig. 4C) and Ad-Flt3L + Ad-TK-treated long-term survivors (Fig. 4D) implanted with glioblastoma multiforme in the flank and euthanized 17 days later.

Efficacy of the combined Ad-TK and Ad-Flt3L gene therapy in syngeneic intracranial rat glioblastoma multiforme models. One important criterion to plan the implementation of novel treatment strategies in phase I clinical trials is to show their efficacy and safety in preclinical animal models. We therefore tested the efficacy of the combined conditional cytotoxic/immune-therapeutic strategy in two further syngeneic brain tumor models. We evaluated therapeutic efficacy in intracranial rat glioblastoma multiforme F98 and 9L orthotopic tumors in Fisher rats, using the CNS-1 model in Lewis rat as reference. *In vitro*, the proliferation rate of F98 cells (doubling time, 1.033 days) was significantly higher than 9L cells (doubling time, 1.328 days) and CNS-1 cells (doubling time, 1.167 days; Fig. 5A). In all the three cell lines, we found expression of MHCII (Fig. 5B) and lack of expression of MHCII (Supplementary Fig. S3). In addition, we did not detect expression of the tumor antigen Trp-2 in any of the three glioblastoma multiforme cell lines (Supplementary Fig. S1A); this antigen is commonly overexpressed in melanoma cells and has been detected in mouse glioblastoma multiforme cells (12, 30) and glioblastoma multiforme specimens from human patients (31). We characterized the tumor growth rate and the histopathologic characteristics of these glioblastoma multiforme models *in vivo* at 3, 6, and 9 days after implantation using Nissl staining (Fig. 5C-E). The tumors were already evident at day 3 in all three models; the doubling time was similar, ranging from 1.1 to 1.6 days. Although the tumor size at day 9 after implantation was similar for the three tumor models, F98 (~40 mm³) and 9L tumors (45 mm³) were slightly larger than CNS-1 tumors (~30 mm³). The three models exhibited infiltration of immune cells, as assessed by staining of CD68⁺ macrophages (Fig. 5C-E) and CD8⁺ T cells (not shown).

To evaluate therapeutic efficacy in these glioblastoma multiforme models, F98 cells were implanted in the striatum of Fisher rats and treated 7 days later with intratumoral injection of saline, Ad-TK, Ad-Flt3L, or the combination Ad-Flt3L + Ad-TK (Fig. 6A). Rats treated with Ad-TK (+ganciclovir) alone exhibited a median survival of 26 days, which was significantly longer than the 15-day survival of saline-treated rats (median survival ratio, 1.73; 95% confidence interval, 1.4-2.1). Although Ad-Flt3L alone did not improve survival, combination with Ad-TK (+ganciclovir) led to a significant extension of the median survival compared with rats that received single treatment or saline-treated rats (median survival ratio, 2.9; 95% confidence interval, 2.5-3.2). Fisher rats harboring 9L tumors were treated with intratumoral injection of saline, Ad-TK, Ad-Flt3L, or Ad-Flt3L + Ad-TK 9 days after tumor implantation

(Fig. 6B). Whereas Ad-TK (+ganciclovir) significantly improved the survival of tumor-bearing rats (median survival ratio, 1.5; 95% confidence interval, 1.16-1.9) and even led to long-term survival in 2 of 10 rats, Ad-Flt3L alone did not improve survival. However, the combined therapy using Ad-Flt3L + Ad-TK induced long-term survival in >50% of the animals treated. Tallying with our previous results, combined therapy using Ad-Flt3L + Ad-TK 9 days after CNS-1 tumor implantation in Lewis rats (used as positive controls) led to >70% long-term survival (Fig. 6C). Images in Fig. 6 show the appearance of the brain in saline-treated moribund rats bearing F98, 9L, or CNS-1 tumors, and the neuropathologic analysis of Ad-Flt3L + Ad-TK (+ganciclovir)-treated long-term survivors 60 days after tumor implantation. Note that the brains of long-term survivors did not exhibit demyelination or loss of TH expression after tumor rejection induced by Ad-Flt3L + Ad-TK (+ganciclovir) treatment (Fig. 6A-C).

We recently showed that therapeutic efficacy of Ad-Flt3L + Ad-TK (+ganciclovir) depends on the release of HMGB1 from dying tumor cells in a syngeneic mouse model (12) and in the CNS-1 glioblastoma multiforme model in Lewis rats (14). We therefore assessed whether HMGB1 would also be released upon treatment with Ad-Flt3L + Ad-TK in F98 and 9L glioblastoma multiforme model in Fisher rats (Fig. 6D). We found that the systemic levels of HMGB1 were elevated 5 days after the treatment with Ad-Flt3L + Ad-TK in the three rat tumor models studied. This supports the notion that serum HMGB1 levels could be useful to monitor tumor regression in response to the treatment.

Supplementary Table S1 shows the median survival or percentage of long-term survival in the different brain tumor models treated with the combined therapeutic approach described. Note that combination of Ad-Flt3L + Ad-TK lead to at least 60% survival in 9L and CNS-1 tumor models, and thus, the median survival was not reached.

Discussion

Glioblastoma multiforme grows within the brain; because of the lack of classic lymphatic outflow channels, the secretion of immune-inhibitory molecules by glioblastoma multiforme cells and a paucity of antigen-presenting cells within the brain parenchyma, it is challenging to elicit strong and therapeutically effective anti-glioblastoma multiforme immune responses (32). To elicit an anti-glioblastoma multiforme immune response from the brain tumor microenvironment, we developed a combined gene therapy strategy involving Ad-mediated delivery of Flt3L that recruits antigen-presenting cells into the brain tumor mass (9, 10, 13, 21), coupled with local release of tumor antigen and inflammatory molecules upon Ad-TK + ganciclovir-mediated tumor cell death (9, 10, 12-14). This therapeutic approach induces an immune response against the primary intracranial tumor that is dependent on phagocytic immune cells, that is, macrophages and dendritic cells, as well as CD4⁺ and CD8⁺ T cells (10). Considering the high rate of tumor recurrence in glioblastoma multiforme patients, the ability to suppress the growth of recurrent brain tumors growing at the original site or even at locations distant from the primary lesion without further treatment has important clinical implications for brain tumor therapy (19, 20, 33-36). We have

previously shown that Ad-Flt3L + Ad-TK treatment induces immunological memory, and most treated rodents that survive the primary tumor are able to reject a second tumor implanted in the contralateral hemisphere without further treatment (10, 12, 14). In this article, we show that long-term survivors that reject the second tumor exhibit a high frequency of tumor antigen-specific T-cell precursors in the spleen and display DTH in response to tumor antigen, suggesting that cellular immunity is responsible for the anti-glioblastoma multiforme immunological memory. Furthermore, using depleting antibodies for specific immune cell types, we found that rejection of the second tumor is dependent on CD8⁺ memory T cells.

In the present study, we also tested the efficacy of this immune-therapeutic gene therapy strategy to inhibit the progression of glioblastoma multiforme implanted in the flank of long-term glioblastoma multiforme survivors in the syngeneic Lewis rat model. Our results show that the Ad-Flt3L + Ad-TK (+ganciclovir) treatment of intracranial glioblastoma multiforme induces cellular anti-glioblastoma multiforme immunological response that hampers the growth of a second glioblastoma multiforme lesion implanted in the flank. Histopathologic analysis of brain and skin glioblastoma multiforme sections from Ad-Flt3L + Ad-TK (+ganciclovir)-treated long-term survivors revealed the absence of any residual tumor in the brain or the flank. On the other hand, following intradermal implantation of CNS-1 cells in the flank of naïve rats, the tumor mass grew rapidly up to day 12. These flank tumors in naïve rats exhibited histologic features compatible with glioblastoma multiforme, namely, pseudopalisading necrosis, microvascular hyperplasia, and hemorrhages. Although these tumors were still visible to the naked eye 17 days postimplantation, they started regressing spontaneously after day 12. This finding is in line with previous reports showing spontaneous regression of peripherally implanted glioblastoma multiforme without any further treatment (37–39). Glioblastoma multiforme cells may be immunogenic in the periphery, as suggested by the presence of high levels of circulating anti-CNS-1 antibodies. Immunity against extracranial glioblastoma multiforme could be related to the fact that, in response to rapid tumor growth, areas of the tumor become necrotic and/or apoptotic, causing the release of proinflammatory molecules (i.e., HMGB1) and eliciting danger signals, which in turn could trigger the priming of an antitumor immune response (40). In agreement with this, we detected high levels of circulating HMGB1 in the serum of naïve rats implanted with flank glioblastoma multiforme tumors. HMGB1 is a nuclear protein that, when released from dying cells, acts as a cytokine with potent proinflammatory and chemotactic activity (27, 28, 41). HMGB1 levels were also high in the serum of Ad-Flt3L + Ad-TK-treated long-term survivors that rejected the extracranial glioblastoma multiforme tumor. However, because the growth of the flank tumor is abrogated in these animals by the immune response, it is possible that another source of circulating HMGB1 are the immune cells involved in the rejection of the extracranial tumor. In fact, we have recently shown that systemic levels of HMGB1 in Ad-Flt3L + Ad-TK-treated rats are higher than those observed in the rats treated with Ad-TK + ganciclovir alone (14). It has been shown that HMGB1 can also be actively released from immune cells, including macrophages/monocytes (42).

The fact that CNS-1 tumors are rejected from an extracranial site but not from an intracranial implantation site confirms once more that antitumor immune priming does not occur when antigens are injected carefully in the brain parenchyma. Upon implantation into the brain parenchyma, unless dendritic cells are recruited to the brain tumor microenvironment, the brain tumors will continue to grow without being challenged by an immune response. If dendritic cells are recruited to the brain and an immune response is stimulated, then the immune response is able to abrogate intracranial tumor growth. This is not necessary when glioblastoma multiforme cells are implanted in the flank because local infiltrating dendritic cells will be able to take up glioblastoma multiforme antigens that are released because of spontaneous tumor necrosis.

Circulating antibodies against CNS-1 cells were observed when glioblastoma multiforme cells were implanted in the flank of naïve or long-term survivors. However, rats bearing intracranial glioblastoma multiforme only exhibited circulating anti-glioblastoma multiforme antibodies upon treatment with Ad-Flt3L + Ad-TK. The presence of antibodies against intracranial brain tumors has been previously reported, and there is a decline of seroreactivity with increased malignancy, which has been related to loss of antigenicity as part of tumor escape mechanisms (43). Serum antibodies against mutant p53 have been detected only occasionally in glioblastoma multiforme patients, and the humoral immune response is known to be rather weak compared with antibody reactivity reported in other cancers (44). The presence of antibodies against brain tumor cells in Ad-Flt3L + Ad-TK rats suggest that, in addition to the CD4⁺ and CD8⁺ T cell-mediated cellular immunity (10), Ad-Flt3L + Ad-TK also induces a humoral immune response against the intracranial tumor.

To translate novel therapeutic approaches into clinical trials, it is critical to show their efficacy and safety in relevant preclinical animal models. We evaluated the efficacy of the combined conditional cytotoxic-immunotherapeutic approach in two further syngeneic brain tumor models. We found that treatment with Ad-Flt3L + Ad-TK also induces tumor regression in large intracranial 9L and F98 glioblastoma multiforme cells in Fisher rats. These data suggest that intratumoral administration of Ad-Flt3L + Ad-TK could be effective against a wide range of primary brain tumors. In addition, increased levels of HMGB1 in the serum of rats bearing 9L and F98 tumors after treatment with Ad-Flt3L + Ad-TK suggests that HMGB1 release is a wide spread event upon tumor regression. These results support the use of serum HMGB1 levels as a biomarker of antitumor therapeutic efficacy (12, 14).

Glioblastoma multiforme is an invasive primary brain tumor that recurs in most patients (1, 2, 45); therapies that stimulate the immune system to target and eliminate glioblastoma multiforme cells that migrated far from the main tumor mass have been developed with encouraging results (46, 47). Vaccination of a glioblastoma multiforme patient with dendritic cells pulsed with autologous tumor lysate following surgery, radiotherapy, and temozolomide led to robust CD8⁺ T-cell response against the tumor, without adverse side effects (46). Type I cytokine responses were also reported in glioblastoma multiforme patients vaccinated with dendritic cells in a phase II clinical trial (48). The epidermal growth factor receptor variant III, a mutated protein that is expressed in 20% to 30% of primary

glioblastoma multiforme (49, 50), constitutes a tumor antigen that has been targeted using immunotherapy. Anti-epidermal growth factor receptor variant III antibodies have been detected in the serum of glioblastoma multiforme patients after immunization against this protein (47).

Our therapeutic approach aims at modifying the brain tumor microenvironment to recruit antigen-presenting cells into the brain tumor mass and expose these tumor infiltrating immune cells to tumor antigens and endogenous immune stimulatory molecules, that is, HMGB1. This treatment could be administered at the time of glioblastoma multiforme resection, delivering the combined therapy into the tumor mass or into the brain region surrounding the resection cavity. Finally, release of the chromatin-binding protein HMGB1 during tumor regression could be monitored in serum as a noninvasive biomarker of therapeutic efficacy. This combined gene therapy strategy, to-

gether with the potential use of HMGB1 as a biomarker of tumor response to treatment, warrants to be tested in a phase I clinical trial for glioblastoma multiforme.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

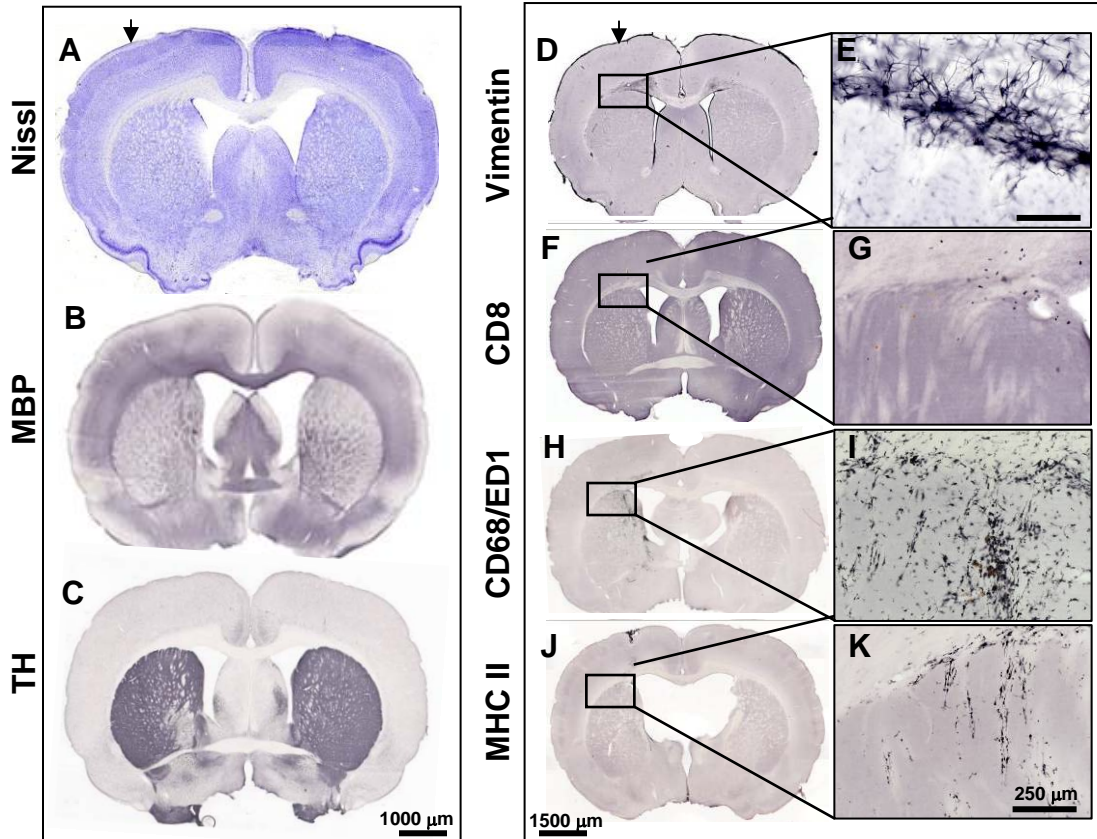
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References

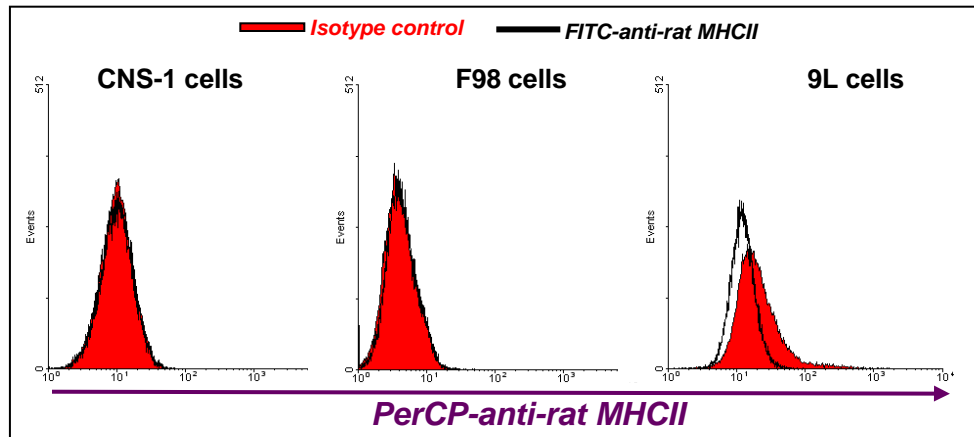
- Chinot OL, Barrie M, Fuentes S, et al. Correlation between O6-methylguanine-DNA methyltransferase and survival in inoperable newly diagnosed glioblastoma patients treated with neoadjuvant temozolomide. *J Clin Oncol* 2007; 25:1470-5.
- Hegi ME, Diserens AC, Gorlia T, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. *N Engl J Med* 2005; 352:997-1003.
- Izquierdo M, Martin V, de Felipe P, et al. Human malignant brain tumor response to herpes simplex thymidine kinase (HSVtk)/ganciclovir gene therapy. *Gene Ther* 1996;3:491-5.
- Klatzmann D, Valery CA, Bensimon G, et al. A phase I/II study of herpes simplex virus type 1 thymidine kinase "suicide" gene therapy for recurrent glioblastoma. Study Group on Gene Therapy for Glioblastoma. *Hum Gene Ther* 1998;9:2595-604.
- Rainov NG. A phase III clinical evaluation of herpes simplex virus type 1 thymidine kinase and ganciclovir gene therapy as an adjuvant to surgical resection and radiation in adults with previously untreated glioblastoma multiforme. *Hum Gene Ther* 2000;11:2389-401.
- Ram Z, Culver KW, Oshiro EM, et al. Therapy of malignant brain tumors by intratumoral implantation of retroviral vector-producing cells. *Nat Med* 1997;3:1354-61.
- Immonen A, Vapalahti M, Tyynela K, et al. *AdvHSV-tk* gene therapy with intravenous ganciclovir improves survival in human malignant glioma: a randomised, controlled study. *Mol Ther* 2004;10:967-72.
- Castro MG, Cowen R, Williamson IK, et al. Current and future strategies for the treatment of malignant brain tumors. *Pharmacol Ther* 2003;98:71-108.
- Ali S, Curtin JF, Zirger JM, et al. Inflammatory and anti-glioma effects of an adenovirus expressing human soluble Fms-like tyrosine kinase 3 ligand (hsFt3L): treatment with hsFt3L inhibits intracranial glioma progression. *Mol Ther* 2004; 10:1071-84.
- Ali S, King GD, Curtin JF, et al. Combined immunostimulation and conditional cytotoxic gene therapy provide long-term survival in a large glioma model. *Cancer Res* 2005;65: 7194-204.
- Curtin JF, Candolfi M, Fakhouri TM, et al. Treg depletion inhibits efficacy of cancer immunotherapy: implications for clinical trials. *PLoS ONE* 2008;3:e1983.
- Curtin JF, Liu N, Candolfi M, et al. HMGB1 mediates endogenous TLR2 activation and brain tumor regression. *PLoS Med* 2009;6:e10.
- King GD, Muhammad AK, Curtin JF, et al. *Ft3L* and *TK* gene therapy eradicate multifocal glioma in a syngeneic glioblastoma model. *Neuro-oncol* 2008;10:19-31.
- Candolfi M, Yagiz K, Foulad D, et al. Release of HMGB1 in response to pro-apoptotic glioma killing strategies: efficacy and neurotoxicity. *Clin Cancer Res* 2009, In press.
- Newton HB, Rosenblum MK, Walker RW. Extraneural metastases of intracranial glioblastoma multiforme to the peritoneal cavity. *Cancer* 1992;69:2149-53.
- Wallace CJ, Forsyth PA, Edwards DR. Lymph node metastases from glioblastoma multiforme. *AJNR Am J Neuroradiol* 1996;17:1929-31.
- Park CC, Hartmann C, Folkherth R, et al. Systemic metastasis in glioblastoma may represent the emergence of neoplastic subclones. *J Neuro-pathol Exp Neurol* 2000;59:1044-50.
- Buhl R, Barth H, Hugo HH, Hutzelmann A, Mehdorn HM. Spinal drop metastases in recurrent glioblastoma multiforme. *Acta Neurochir (Wien)* 1998;140:1001-5.
- Didelot A, Taillandier L, Grignon Y, Vespignani H, Beauchesne P. Concomitant bone marrow metastasis of a glioblastoma multiforme revealed at the diagnosis. *Acta Neurochir (Wien)* 2006;148:997-1000.
- Rajagopalan V, El Kamar FG, Thayaparan R, Grossbard ML. Bone marrow metastases from glioblastoma multiforme—a case report and review of the literature. *J Neurooncol* 2005;72: 157-61.
- Curtin JF, King GD, Barcia C, et al. Fms-like tyrosine kinase 3 ligand recruits plasmacytoid dendritic cells to the brain. *J Immunol* 2006; 176:3566-77.
- Southgate T, Kroeger KM, Liu C, Lowenstein PR, Castro MG. Gene transfer into neural cells *in vitro* using adenoviral vectors. *Curr Protoc Neurosci* 2008;Chapter 4:Unit 4 23.
- Van Rooijen N, Sanders A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J Immunol Methods* 1994;174:83-93.
- Candolfi M, Curtin JF, Nichols WS, et al. Intracranial glioblastoma models in preclinical neuro-oncology: neuropathological characterization and tumor progression. *J Neurooncol* 2007;85: 133-48.
- Murphy S, Davey RA, Gu XQ, et al. Enhancement of cisplatin efficacy by thalidomide in a 9L rat gliosarcoma model. *J Neurooncol* 2007;85: 181-9.
- Gundersen HJ, Jensen EB. The efficiency of systematic sampling in stereology and its prediction. *J Microsc* 1987;147:229-63.
- Palumbo R, Sampaolesi M, De Marchis F, et al. Extracellular HMGB1, a signal of tissue damage, induces mesoangioblast migration and proliferation. *J Cell Biol* 2004;164:441-9.
- Yu M, Wang H, Ding A, et al. HMGB1 signals through toll-like receptor (TLR) 4 and TLR2. *Shock* 2006;26:174-9.
- Apetoh L, Ghiringhelli F, Tesniere A, et al. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. *Nat Med* 2007;13:1050-9.
- O I, Blaszczyk-Thurin M, Shen CT, Ertl HC. A DNA vaccine expressing tyrosinase-related protein-2 induces T-cell-mediated protection against mouse glioblastoma. *Cancer Gene Ther* 2003;10: 678-88.
- Saikali S, Avril T, Collet B, et al. Expression of nine tumour antigens in a series of human glioblastoma multiforme: interest of EGFRvIII, IL-13R α 2, gp100 and TRP-2 for immunotherapy. *J Neurooncol* 2007;81:139-48.
- Mitchell DA, Fecci PE, Sampson JH. Immunotherapy of malignant brain tumors. *Immunol Rev* 2008;222:70-100.
- Gavrilovic IT, Posner JB. Brain metastases: epidemiology and pathophysiology. *J Neurooncol* 2005;75:5-14.
- Rickett CH. Extraneural metastases of paediatric brain tumours. *Acta Neuropathol* 2003;105: 309-27.
- Utsuki S, Tanaka S, Oka H, Iwamoto K, Sagiuchi T, Fujii K. Glioblastoma multiforme metastasis to the axis. Case report. *J Neurosurg* 2005;102:540-2.
- Yokosuka K, Ishii R, Suzuki Y, et al. Extraneural metastasis of high grade glioma without simultaneous central nervous system recurrence: case report. *Neurol Med Chir (Tokyo)* 2007;47:273-7.
- Mourad PD, Farrell L, Stamps LD, Chicoine MR, Silbergeld DL. Why are systemic glioblastoma metastases rare? Systemic and cerebral growth of mouse glioblastoma. *Surg Neurol* 2005;63:511-9; discussion 519.
- Vince GH, Bendszus M, Schweitzer T, et al. Spontaneous regression of experimental gliomas—an immunohistochemical and MRI study of the C6 glioma spheroid implantation model. *Exp Neurol* 2004;190:478-85.
- Parsa AT, Chakrabarti I, Hurley PT, et al. Limitations of the C6/Wistar rat intracerebral glioma model: implications for evaluating immunotherapy. *Neurosurgery* 2000;47:993-9; discussion 999-1000.

40. Marshak-Rothstein A. Toll-like receptors in systemic autoimmune disease. *Nat Rev Immunol* 2006;6:823–35.
41. Bianchi ME, Manfredi AA. High-mobility group box 1 (HMGB1) protein at the crossroads between innate and adaptive immunity. *Immunol Rev* 2007;220:35–46.
42. Chen G, Ward MF, Sama AE, Wang H. Extracellular HMGB1 as a proinflammatory cytokine. *J Interferon Cytokine Res* 2004;24:329–33.
43. Comtesse N, Zippel A, Walle S, et al. Complex humoral immune response against a benign tumor: frequent antibody response against specific antigens as diagnostic targets. *Proc Natl Acad Sci U S A* 2005;102:9601–6.
44. Weller M, Bornemann A, Stander M, Schabet M, Dichgans J, Meyermann R. Humoral immune response to p53 in malignant glioma. *J Neurol* 1998;245:169–72.
45. Wallner KE, Galicich JH, Krol G, Arbit E, Malkin MG. Patterns of failure following treatment for glioblastoma multiforme and anaplastic astrocytoma. *Int J Radiat Oncol Biol Phys* 1989; 16:1405–9.
46. Prins RM, Cloughesy TF, Liau LM. Cytomegalovirus immunity after vaccination with autologous glioblastoma lysate. *N Engl J Med* 2008; 359:539–41.
47. Schmittling RJ, Archer GE, Mitchell DA, et al. Detection of humoral response in patients with glioblastoma receiving EGFRvIII-KLH vaccines. *J Immunol Methods* 2008;339:74–81.
48. Wheeler CJ, Black KL, Liu G, et al. Vaccination elicits correlated immune and clinical responses in glioblastoma multiforme patients. *Cancer Res* 2008;68:5955–64.
49. Pelloski CE, Ballman KV, Furth AF, et al. Epidermal growth factor receptor variant III status defines clinically distinct subtypes of glioblastoma. *J Clin Oncol* 2007;25: 2288–94.
50. Yoshimoto K, Dang J, Zhu S, et al. Development of a real-time RT-PCR assay for detecting EGFRvIII in glioblastoma samples. *Clin Cancer Res* 2008;14:488–93.

Supp. Figure 2. Neuropathology of Ad-Flt3L + Ad-TK/GCV-treated brain tumor survivors that were implanted with GBM in the flank



Supplementary Figure 3. Expression of MHCII



Supplementary Table I. Median survival of rat GBM models (days)

TREATMENT	F98	9L	CNS-1
SALINE	15	15	14
Ad.Flt3L	15	16	14
Ad.TK	23*	23*	17*
Ad.TK+Ad.Flt3L	41*^	~60% long term survival*^	~75% long term survival*^

*note: *p<0.05 vs saline; ^p<0.05 vs Ad.TK (Log rank test)*