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ORIGINAL ARTICLE

Gene Therapy-Mediated Reprogramming Tumor Infiltrating T Cells Using IL-2 and Inhibiting NF-κB Signaling Improves the Efficacy of Immunotherapy in a Brain Cancer Model

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Abstract Immune-mediated gene therapy using adenovirus expressing Flt3 ligand and thymidine kinase followed by ganciclovir administration (Flt3/TK) effectively elicits tumor regression in preclinical glioma models. Herein, we assessed new strategies to optimize Flt3L/TK therapeutic efficacy in a refractory RG2 orthotopic glioblastoma model. Specifically, we aimed to optimize the therapeutic efficacy of Flt3L/TK treatment in the RG2 model by overexpressing the following genes within the brain tumor microenvironment: 1) a TK mutant with enhanced cytotoxicity (SR39 mutant TK), 2) Flt3L-IgG fusion protein that has a longer half-life, 3) CD40L to stimulate DC maturation, 4) T helper cell type 1 polarizing dendritic cell cytokines interleukin-12 or C-X-C motif ligand 10 chemokine (CXCL)-10, 5) C-C motif ligand 2 chemokine (CCL2) or C-C motif ligand 3 chemokine (CCL3) to enhance dendritic cell recruitment into the tumor microenvironment, 6) T helper cell type 1 cytokines interferon-γ or interleukin-2 to enhance effector T-cell functions, and 7) IκBα or p65RHD (nuclear factor kappa-B [NF-κB] inhibitors) to suppress the function of Foxp3 + Tregs and enhanced effector T-cell functions. Anti-tumor

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immunity and tumor specific effector T-cell functions were assessed by cytotoxic T lymphocyte assay and intracellular IFN-γ staining. Our data showed that overexpression of interferon-γ or interleukin-2, or inhibition of the nuclear factor kappa-B within the tumor microenvironment, enhanced cytotoxic T lymphocyte-mediated immune responses and successfully extended the median survival of rats bearing intracranial RG2 when combined with Flt3L/TK. These findings indicate that enhancement of T-cell functions constitutes a critical therapeutic target to overcome immune evasion and enhance therapeutic efficacy for brain cancer. In addition, our study provides novel targets to be used in combination with immune-therapeutic strategies for glioblastoma, which are currently being tested in the clinic.

Key Words Glioblastoma · immunotherapy · adenoviral vectors · gene therapy · HSV1-TK

Introduction

Glioblastoma multiforme (GBM) is a devastating brain cancer, with high mortality and morbidity [1, 2]. Despite advances in the current standard of care, including surgery, radiotherapy, and chemotherapy, the median survival remains at 15 to 18 months postdiagnoses [3]. Immunotherapy has emerged as a promising approach for GBM, which could eradicate residual tumor cells that infiltrate adjacent areas of the brain parenchyma.

Numerous immunotherapy approaches, such as vaccination with tumor associated peptides, autologous tumor cells, or dendritic cells (DCs) have been assessed in human clinical trials for GBM [4, 5]. Vaccination strategies using DCs pulsed with Epidermal growth factor receptor type III deletion mutant gene (EGFRvIII), a tumor antigen which is expressed in ~30 % of human GBM patients [6–8], and vaccination with DC loaded *ex vivo* with autologous tumor lysates [9–15] have shown promise in clinical trials for GBM. By facilitating the presentation of brain tumor antigens to naïve T cells, these approaches induce the proliferation of brain tumor antigen-specific cytotoxic T cells. Although these vaccination strategies enhanced anti-tumor cellular and humoral immune responses against brain tumor antigens, they have not led to significant improvements in median survival yet.

Adoptive T-cell therapy is another immunotherapy strategy that is in clinical development [16–18] to combat malignancies. Adoptive transfer of antigen-specific T cells has been shown to be highly effective for patients with relapsing leukemia after allogeneic bone marrow transplantation [19, 20]. Two main obstacles for the successful clinical translation of adoptive T-cell therapy include the short survival of infused T cells *in vivo* and low-affinity T-cell receptors (TCRs) for tumor associated antigens [17].

Several strategies have been developed to enhance the effectiveness of this approach. To enhance the survival of infused T cells *in vivo*, systemic interleukin (IL)-2 injection or lymphodepleting chemotherapy prior to T-cell infusion have improved clinical responses [21]. To circumvent the low affinity of TCRs, they can be genetically engineered to express high-affinity α/β TCRs specific for a given tumor-associated antigen or chimeric antigen receptors, which are generated by joining the variable regions of a monoclonal antibody with the transmembrane and cytoplasmic signaling domains of CD3ζ chain [16, 17]. A recent study demonstrated the efficacy of genetically engineered T cells from GBM patients expressing HER2-specific chimeric antigen receptor [22]. HER-2 specific T cells secreted interferon (IFN)-γ and IL-2 and killed HER-2-positive glioma cells, including CD133+ glioma stem cells *in vitro*. They also showed therapeutic efficacy *in vivo* in an orthotopic brain tumor model.

We developed an immunotherapeutic approach for GBM, which involves *in situ* priming of anti-tumor immunity, recreating a missing immune circuit from the normal brain. To do so, we delivered 2 therapeutic adenoviral vectors into the tumor mass encoding either the immune-stimulating cytokine fms-like tyrosine kinase-3 ligand (Ad-Flt3L) or the conditionally cytotoxic herpes simplex type 1-thymidine kinase (Ad-TK). Using multiple syngeneic, orthotopic rodent brain tumor models, we have shown that Ad-Flt3L/Ad-TK immunotherapy induces anti-tumor T-cell immune responses leading to high therapeutic efficacy [23–27]. Flt3L induces the proliferation and recruitment of DCs into the brain tumor microenvironment where they capture tumor antigens released by dying tumor cells Ad-TK/GCV-treated tumor cells. Dying tumor cells also release the potent immune adjuvant HMGB1 [24, 27], which further potentiates the efficacy of this approach by stimulating DC maturation via toll-like receptor 2 signaling (TLR-2) [24].

Recent evidence suggests that CD4+ T helper cell type 1 (Th1)-skewed immune responses play a role in anti-tumor effects [28]. IFN-γ and IL-2 are Th1 cytokines released from helper CD4+ T cells and effectively enhance cytotoxic T lymphocyte (CTL)-mediated anti-tumor immunity. Importantly, IL-2 can reverse suppression of CTL responses induced by transforming growth factor-β [29]. Retroviral-mediated IL-2 expression in combination with HSV1-TK enhanced anti-tumor CTL responses in recurrent GBM patients [30]. In addition, Foxp3 positive regulatory T cells are known to act as negative controllers of T-cell function [31] and are known to be present in human GBM [32]. Recent evidence revealed that Foxp3 also suppresses IFN-γ and IL-2 secretion from CD4+ T cells [31]. Nuclear factor kappa-B (NF-κB) is an important regulator of Foxp3 expression in CD4+ T cells; the p65 (RelA) subunit of NF-κB binds to the Foxp3 promoter, thereby activating Foxp3 expression and regulating Treg development [33–36]. NF-κB inhibition by IκBα has been shown to decrease Foxp3 expression on CD4+ T cells and increase IFN-γ, and IL-2 secretion from regulatory T cells [31].

Herein, we tested the hypothesis that modulating T-cell recruitment into the tumor microenvironment, T-cell expansion, and enhancing anti-tumor effector T-cell function could lead to enhanced levels of Flt3L/TK-mediated therapeutic efficacy and anti-GBM immunity in the RG2 orthotopic tumor model. This model has been shown to be resistant to most treatment modalities including radiation, chemotherapy, and immunotherapy [37, 38]. Our results indicate that overexpression of IFN-γ or IL-2, or inhibition of the NF-κB signaling pathway within the tumor microenvironment (i.e., overexpression of IκBα or p65RHD) enhanced CTL-mediated immune responses and successfully extended the median survival of rats bearing intracranial RG2 GBM when combined with Ad-Flt3L + Ad-TK/GCV immune-mediated gene therapy. These findings indicate that enhancement of tumor-specific T-cell responses constitutes a critical therapeutic target to overcome immune evasion and enhance therapeutic efficacy of immunotherapies for brain cancer.

Methods

Adenoviral Vectors

First-generation adenoviral vectors (Ad) encoding human fms-like tyrosine kinase 3 ligand (Ad-Flt3L), herpes simplex virus type I-thymidine kinase (Ad-TK), the inhibitors of NF-κB alpha (Ad-IκBα), and p65RHD (Ad-p65RHD), interferon-gamma (Ad-IFN-γ), and CD40 ligand (Ad-CD40L), IL-12 (Ad-IL-12), murine Flt3L fused to murine immunoglobulin (IgG)2a (Ad-Flt3L-IgG), the TK mutant clone SR39 (Ad-SR39), murine interleukin-2 (Ad-IL-2), murine C-X-C motif ligand 10 Chemokine (CXCL-10) (Ad-CXCL-10), murine CCL2 (Ad-CCL2), and murine CCL3 (Ad-CCL3) were used in this study. Details regarding the rescue, generation, purification, and characterization of adenoviral vectors are described in the "Supplemental Materials and Methods", and are shown in Supplemental Figures 1 and 2.

Growth Profile of RG2 Glioma Cells: *In Vitro* and *In Vivo*

The *in vitro* growth kinetics of RG2 cells is described in the "Supplemental Materials and Methods". The tumor volumes at indicated days after implantation of RG2 cells in the striatum (n=3 per time point) were estimated using unbiased stereological techniques. Tumor mass within the brain sections was visualized with Nissl staining, and tumor volume was calculated using Stereo Investigator software (version 8.00.0; Microbrightfield, Inc., Williston, Vermont, as previously described [25].

Orthotopic RG2 Rat Glioma Model

All animal experiments were performed as previously described in accordance with protocols approved by the Cedars-Sinai Medical Center's Institutional Animal Care and Use Committee (IACUC) and conformed to the policies and procedures of the Comparative Medicine Department. RG2 cells (20,000 in 3 μl Dulbecco's Modified Eagle Medium) were stereotactically implanted in the right striatum of syngeneic Fisher rats (220 to 250 g; Harlan Laboratories Inc., Indianapolis, IN) at the coordinates of 1.0 mm anterior and 3.2 mm lateral from the bregma and 6.0-mm ventral from the dura, as previously described [25]. The generation of the RG2 cell line expressing the fluorescent protein citrine (RG2-citrine) is described in the "Supplemental Materials and Methods" (see Supplemental Fig. 3).

Stereotactic Intratumoral Ad Vector Injection

Five days after tumor implantation, rats received an intratumoral injection of Ad vectors using the same burr hole in 4 locations at 7.0 mm, 6.0 mm, 5.0 mm, and 4.0 mm ventral from the dura. Animals were treated with the following adenoviral vectors as described: Ad-Flt3L (3×10^8 pfu), Ad-Flt3L-IgG2a (3×10^8 pfu), Ad-TK (1×10^8 pfu), Ad-TKSR39 (Ad-SR39, 1×10^8 pfu), Ad-IL-2 (5×10^8 pfu), Ad-IFN-γ (5×10^8 pfu), Ad-CD40L (5×10^8 pfu), Ad-IL-12 (5×10^8 pfu), Ad-IκBα (5×10^8 pfu) or Ad-p65RHD (5×10^8 pfu) in a volume of 3 ul.

Immunohistochemistry

Free-floating immunohistochemistry (IHC) or Nissl staining was performed in serial coronal sections (60 μm) as described in the "Supplemental Materials and Methods" [26, 39].

Major Histocompatibility Complex (MHC) I and MHC II Expression on RG2 Cells

Expression of MHC I and MHC II *in vitro* and *in vivo* was analyzed as previously described [25] and in the "Supplemental Materials and Methods".

Cell Death Assay by Flow Cytometry

Cell death was assessed by AnnexinV and Propidium Iodide staining (Annexin V/PI) staining followed by flow cytometry as previously described [26] and in the "Supplemental Materials and Methods".

CTL Assay

Tumor specific cytotoxicic T-lymphocytes were assessed by flow cytometry, as previously described [26] and in the "Supplemental Materials and Methods".

Characterization of Immune Cells Infiltrating in the Tumor Microenvironment

Animals were perfused 7 days after treatment, tumor-infiltrating lymphocytes (TIL) were isolated, labeled, and analyzed by flow cytometry, as previously described [26] and in the "Supplemental Materials and Methods". Intracellular staining of IFN-γ in CD8+ T cells was performed after incubating TIL for 18 h with cell stimulation cocktail plus protein transport inhibitors. Immune cells were stained with CD3 and CD8α followed by fixation, permeabilization, and intracellular staining with IFN-γ. Note that the gate for the IFN-γ expressing T cells is the same. CD8+ central memory T cells were counted by staining the TIL with CD62L, CD45RC, CD8α, and CD3. The proportion of Foxp3+ regulatory T cells among TIL were measured by staining the TIL with Foxp3, CD25, and CD4. For immunohistochemistry to analyze CD8+ immune cell infiltration into the brain tumor, fixed brain sections were stained with anti-CD8α antibody followed by nickel-enhanced diaminobenzidine in sodium acetate staining.

Statistical Analyses

Kaplan Meier survival curves were analyzed using the Mantel log rank test using Prism GraphPad software (version 3.03; GraphPad Software, Inc., La Jolla, California, USA). *In vitro* and *in vivo* tumor growth rates were determined by nonlinear regression analysis using GraphPad software, Inc., Number Cruncher Statistical System (NCSS) statistical and power analysis software was used for the statistical analysis for tumor size and *in vitro* cell growth using randomization test. The flow cytometry and enzyme-linked immunosorbent assay (ELISA) data were analyzed by 1-way analysis of variance (ANOVA) followed by Tukey's post-hoc test or 2-way ANOVA followed by Turkey-Kramer post-hoc test using NCSS statistical and power analysis software. Differences between groups were considered significant at $p<0.05$.

Results

Therapeutic Efficacy of Ad-Flt3L + Ad-TK in the RG2 Glioma Model

Several vaccine trials for GBM have demonstrated cellular and humoral anti-tumor immune responses, and albeit safe, their clinical efficacy remains limited [5, 18, 40]. To more accurately model clinical outcomes (i.e., most immunotherapies display limited efficacy), we used a refractory GBM model (RG2), which has been reported to be resistant to most treatment modalities [37, 41–44], as well as immunotherapy [38]. *In vitro*, RG2 cells exhibited a doubling time of 83 days (Supplemental Fig. 4a). Tumor growth *in vivo* was analyzed at 3, 6, and 9 days after tumor implantation using Nissl staining (Figs. 1a–1b). Tumors were macroscopically evident at day 3, and the doubling time was 1.6 days. We evaluated the therapeutic efficacy of Ad-Flt3L + Ad-TK/GCV in this refractory GBM model (Fig. 1c). Rats treated with Ad-TK/GCV alone exhibited a median survival of 31.5 days, which was significantly longer than the 18-day survival of saline-treated rats (median survival ratio, 1.8; 95 % confidence interval, 1.2-3.5) (Fig. 1d). Although Ad-Flt3L alone did not improve survival, combination with Ad-TK/GCV led to a significant extension of the median survival to 41 days as compared with rats that received Ad-TK treatment (median survival, 32 days; median survival ratio, 1.3; 95 % confidence interval, 1.0-1.6) or saline-treated rats (median survival, 18 days; median survival ratio, 2.3; 95 % confidence interval, 1.3-7.5) (Fig. 1d). Although no animals survived longer than 45 days, brain tumors were macroscopically undetectable at 14 days after Ad-Flt3L/TK treatment, suggesting that Flt3L/TK effectively mediates brain tumor regression (Fig. 1e).

To determine whether the lack of efficacy of Ad.TK + Ad.Flt3L in RG2 tumor-bearing rats was related to a limited transduction of Ad vectors in this model, we studied the transduction efficiency and therapeutic efficacy of Ad.Flt3L and Ad.TK in RG2 tumors in comparison with CNS-1 tumors. Allthough tallying (Supplemental Fig. 4b and 4c) with our previous reports, treatment with Ad.TK + Ad.Flt3L in CNS-1 tumor-bearing rats led to tumor erradication and long-term survival in 70 % of the animals ([39, 45, 46]), RG2 bearing rats succumbed to tumor burden by days 30 to 45 (Fig. 1 and Supplemental Figs. 5A–5B). However, the lower therapeutic efficacy observed in the RG2 model when compared to the CNS1 model did not seem to be related to a limited efficiency of transduction of Ad vectors in this model. Our results indicate that Ad vectors had a higher transduction rate in RG2 cells than in CNS-1 cells, both *in vitro* and *in vivo* (Supplemental Figs. 5C-5E). RG2 cells infected with Ad.hCMV.Flt3L released ~4 times more Flt3L in culture than CNS-1 cells (Supplemental Fig. 5C). *In vivo*, a similar pattern was observed: intratumor administration of Ad.hCMV.Flt3L in RG2 tumor-bearing rats led to circulating levels of Flt3L that were ~4 times higher than in CNS-1 tumor-bearing rats (Supplemental Fig. 5C). In addition, the sensitivity Ad.TK + GCV-induced cell death was very similar in RG2 and CNS-1 cells (Supplemental Fig. 5E).

Therapeutic Efficacy of SR39 Mutant TK and Flt3L-IgG Fusion Protein

To improve the therapeutic efficacy of Ad-Flt3L + Ad-TK treatment, we first aimed to optimize cytotoxicity of HSV1-TK and bioavailability of Flt3L. Black et al. [47] developed HSV1-TK mutants and showed that SR39 mutant TK, and had superior tumor killing in the presence of Ganciclovir

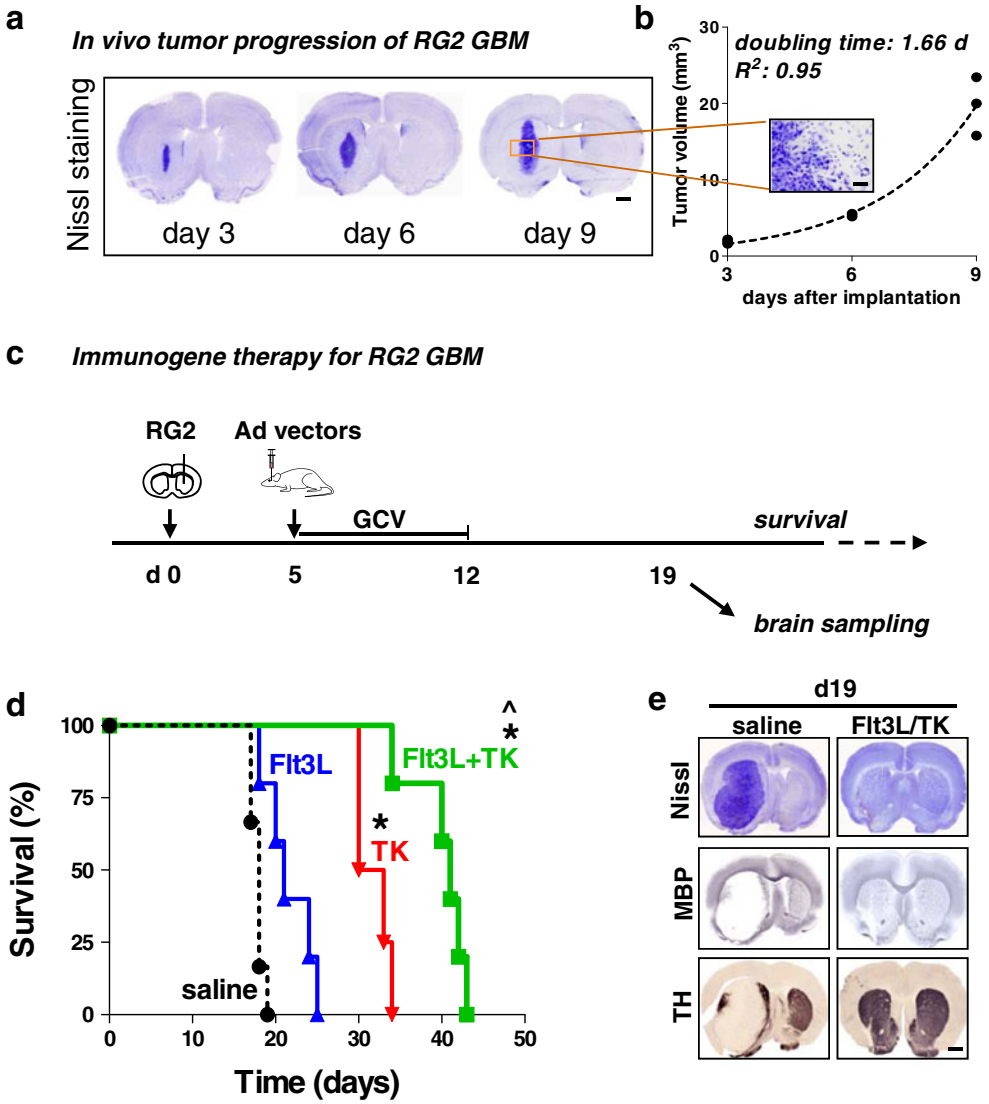

Fig. 1 *In vivo* tumor growth of RG2 glioma and therapeutic efficacy of Ad-Flt3L + Ad-TK for the tumor. (a-b), *In vivo* tumor growth rates of intracranial RG2 glioma in rats. Fisher rats were implanted in the striatum with 20,000 syngeneic RG2 cells. Brains were processed for stereology to determine tumor volume 3, 6, and 9 days after tumor implantation. Microphotographs show the appearance of representative brain sections stained with Nissl. (a) Scale bar, 2 mm. Graph shows tumor growth rate. Doubling time and regression coefficient (R^2) are indicated. (b) Scale bar, 100 μm. (c) Experimental design to assess therapeutic efficacy of immunogene therapy using adenoviral vectors expressing Flt3 ligand (Ad-Flt3L) and thymidine kinase (Ad-TK) in an orthotopic RG2 glioma model. Fisher rats were implanted with 20,000 RG2 glioma cells in the striatum. After 5 days, rats were treated with Ad vectors followed by ganciclovir (GCV) administration for 7 days. (d) Kaplan-Meier survival curve of rats treated with Ad-TK + Ad-Flt3L (n=5), Ad-TK (n=4), Ad-Flt3L (n=5), or saline (n=6). *$p<0.05$ *versus* saline; ^$p<0.05$ *versus* Ad-TK; Mantel log-rank test. (e) Micrographs show the neuropathology of rats bearing RG2 tumors treated with Ad-Flt3L + Ad-TK or saline. Rats were perfused 19 days after tumor implantation and brain sections were stained using Nissl, and immunocytochemistry was done using antibodies specific for myelin basic protein (MBP) and tyrosine hydroxylase (TH). Scale bar, 2 mm

(GCV). Thus, we compared SR39 and wild-type TK for its cytotoxicity and therapeutic efficacy in RG2 glioma model. Transgene expression of both SR39 and wild-type TK was confirmed on RG2 cells *in vitro* (Supplemental Fig. 4c); Ad-SR39 + GCV killed RG2 tumor cells more efficiently than Ad-TK + GCV (Fig. 2a). Transgene expression of SR39 and TK was also confirmed *in vivo* (Supplemental Fig. 6a). In spite of higher cytotoxic activity of SR39 *in vitro*, therapeutic efficacy of Ad-SR39 *in vivo* was not significantly different from that of Ad-TK. Median survival of rats treated with Ad-SR39 alone or Ad-Flt3L + Ad-SR39 were 26 days and 38.5 days, respectively (Fig. 2b).

The estimated half-life of Flt3L is ~5 h [48]; it has been reported that the Fc fragment of IgG confers the fusion molecule longer serum half-life and therapeutic efficacy of such fusion proteins has been demonstrated in preclinical models of melanoma, allergic rhinitis, or Lupus [49–51], or rheumatoid arthritis in the clinic [52]. Therefore, we incorporated a soluble

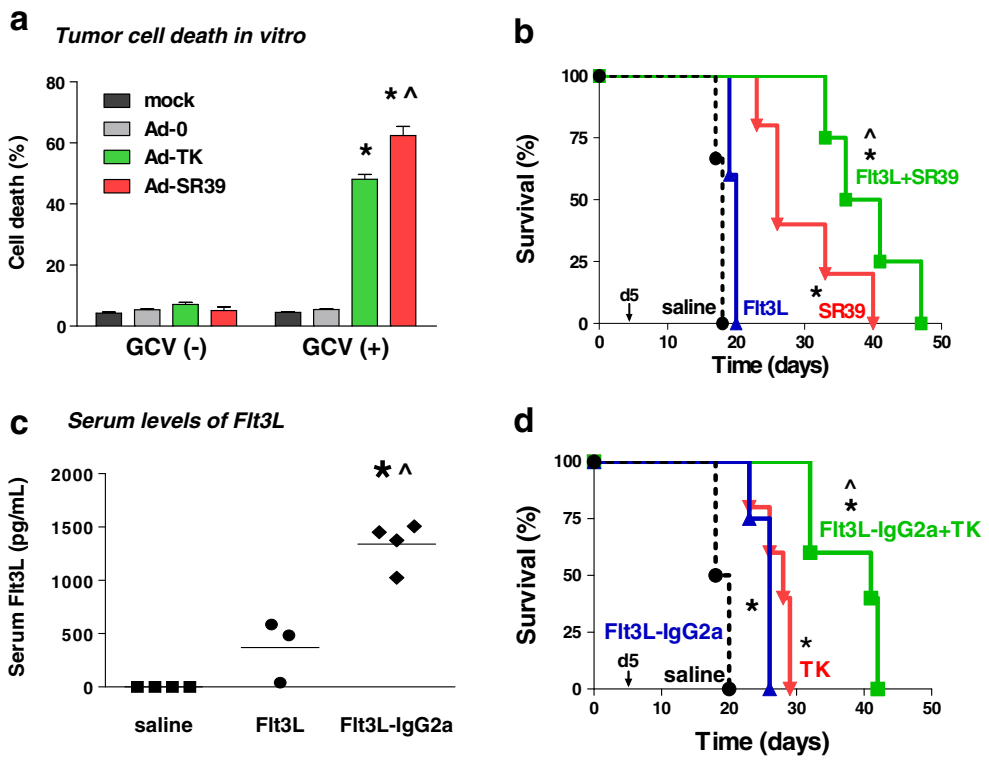

Fig. 2 SR39 is more cytotoxic than TK and Flt3L-IgG fusion protein has longer serum half-life than Flt3L, but they do not improve the therapeutic efficacy of Ad-Flt3L + Ad-TK. (a) Cytotoxicity of SR39, a mutant form of TK, is higher than TK *in vitro*. RG2 cells were treated with Ad-0 (adenoviral vector without transgene), Ad-TK, or Ad-SR39 in the presence or absence of gancyclovir (GCV). Cell death in RG2 cells was assessed by Fluorescein Isothiocyanate (FITC)-annexin V and propidium iodide (PI). Cells positive for Annexin-V and/or PI were considered to be dead. The proportion of dead cells is shown. *$p<0.05$ *versus* mock; ^$p<0.05$ *versus* Ad-TK; 2-way analysis of variance (ANOVA) followed by Turkey's test. (b) Kaplan-Meier survival curve of rats treated with Ad-SR39+Ad-Flt3L (n=5), Ad-SR39 (n=5), Ad-Flt3L (n=5) or saline (n=5). *$p<0.05$ *versus* saline; ^$p<0.05$ *versus* Ad-SR39; Mantel log-rank test. (c) Transgene expression of Ad-Flt3L and Ad-Flt3L-IgG2a *in vivo*. Fisher rats were implanted with 20,000 RG2 cells, and 5 days later they were treated with Ad-Flt3L or Ad-Flt3L-IgG2a. Four days after vector injection, the serum was collected and Flt3L levels were measured by enzyme-linked immunosorbent assay. *$p<0.05$ *versus* saline, ^$p<0.05$ *versus* Ad-Flt3L; 1-way analysis of variance followed by Tukey's test. (d) Kaplan-Meier survival curve of rats treated with Ad-TK + Ad-Flt3L-IgG2a (n=5), Ad-TK (n=5), Ad-Flt3L-IgG2a (n=5), or saline (n=5). *$p<0.05$ *versus* saline; ^$p<0.05$ *versus* Ad-TK; Mantel log-rank test

form of murine Flt3L fused with Fc portion of murine IgG2a (Flt3L-IgG2a) into an adenoviral vector to assess whether it would elicit enhanced therapeutic efficacy. Comparable levels of Flt3L were released into the supernatant of RG2 cells infected with Ad-Flt3L or Ad-Flt3L-IgG2a (Supplemental Fig. 4b); in addition, transduction efficiency was comparable between Flt3L and Flt3L-IgG2a both *in vitro* and *in vivo* (Supplemental Fig. 4c and Supplemental Fig. 6b, respectively). Flt3L levels in the serum were significantly higher in RG2 bearing rats treated with Ad-Flt3L-IgG2a than those treated with Ad-Flt3L (Fig. 2c), suggesting that Flt3L-IgG2a in the serum has longer half-life and is more stable than Flt3L. Consistent with this, Ad-Flt3L-IgG2a alone prolonged the survival of rats bearing RG2 tumors with a median survival of 26 days (Fig. 2d), whereas Ad-Flt3L alone did not improve the survival *versus* saline-treated control rats (Fig. 1d). However, median survival of rats bearing RG2 tumors treated with Ad-Flt3L-IgG2a + Ad-TK was 41 days (Fig. 2d), very similar to the median survival of Ad-Flt3L + Ad-TK (43 days, Fig. 1d).

Cytokines that Induce Migration and Activation of DCs, When Used in Combination with Ad-Flt3L + Ad-TK Do Not Enhance Therapeutic Efficacy

DCs that preferentially induce Th1 polarizing immune responses are characterized by secretion of high levels of IL-12 or CXCL-10 [53]. Secretion of IL-12 and CXCL-10 from DCs is strongly induced by CD40L [53, 54]. Thus, we tested whether co-administration of Ad-CD40L, Ad-IL-12 or CXCL-10 would improve therapeutic efficacy of Ad-Flt3L + Ad-TK (Fig. 3a). None of the combination treatments prolonged the survival of rats bearing RG2 tumor when compared to Ad-Flt3L + Ad-TK treatment (Fig. 3b–3d).

It has been shown that CCL2 (MCP-1) or CCL3 (MIP-1α) strongly enhance migration of DCs [55–57], whereas Flt3L mainly enhances proliferation of DCs. Thus, we tested the hypothesis that enhancing DC migration into the tumor microenvironment would improve Flt3L/TK-mediated therapeutic efficacy. The results shown in Figure 3 (panels E and F)

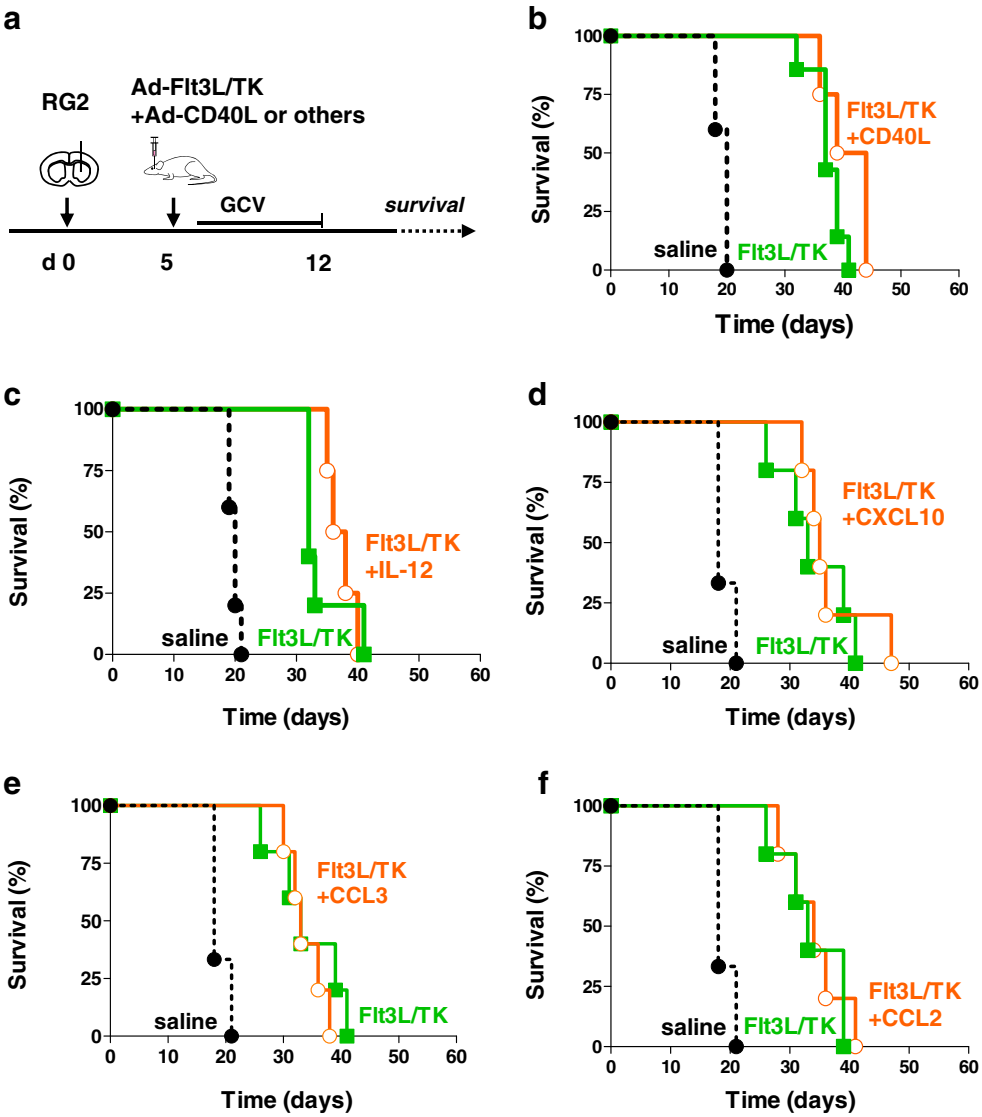

Fig. 3 Manipulation of Dendritic Cell (DC) functions in combination with Ad-Flt3L + Ad-TK. (a) Fisher rats were implanted with 20,000 RG2 glioma cells in the striatum. After 5 days, rats were treated with Ad vectors followed by gancyclovir (GCV) administration for 7 days. (b-f) Kaplan-Meier survival curve of rats treated with Ad-Flt3L/TK + Ad-CD40L. (b) Ad-Flt3/TK + Ad-IL-12. (c) Ad-Flt3/TK + Ad-CXCL-10. (d) Ad-Flt3L/TK + Ad-CCL3. (e) and Ad-Flt3/TK + Ad-CCL2. (f) Compared with Ad-Flt3L/TK or saline (n=4-5 for each group) are shown

indicate that neither Ad-CCL2 nor Ad-CCL3 enhanced the therapeutic efficacy of Ad-Flt3L + Ad-TK. Taken together, these findings suggest that supplementing intratumoral Flt3L/TK gene therapy with therapeutic genes that can enhance the activation status or levels of DCs would not provide added therapeutic benefits.

Ad-IFN-γ Enhanced Expression of MHC I on RG2 Cells and Improved the Therapeutic Efficacy of Ad-Flt3L + Ad-TK

IFN-γ is known to increase MHC I expression on tumor cells, thus potentially enhancing the presentation of tumor-associated antigens [38]. Because RG2 cells have been reported to display low expression of MHC I [38], we analyzed the effect of IFN-γ on MHC I and MHC II expression. We found that IFN-γ increased the expression of MHC I, and had no effect of expression of MHC II on RG2 cells *in vitro* (Fig. 4a). We also found expression of MHC I and lack of expression of MHC II on RG2-citrine cells *in vivo* (Fig. 4b, Supplemental Fig. 7). Because increased MHC I expression levels have been correlated with increased immune-mediated recognition of tumor cells [38], we tested the therapeutic efficacy of Ad-IFN-γ in combination with Ad-Flt3L + Ad-TK. Co-administration of Ad-IFN-γ combined with Ad-Flt3L + Ad-TK treatment significantly prolonged the median survival (41 days) of tumor-bearing animals compared to Ad-Flt3L + Ad-TK (30 days) (median survival ratio, 1.4; 95 % confidence interval, 1.0-2.3) (Fig. 4c).

Ad-IL-2 Enhances Anti-Tumor T-Cell Responses Induced by Ad-Flt3L/TK and Prolongs the Survival of Rats Bearing RG2 Glioma

IL-2, as well as IFN-γ, can enhance anti-tumor immunity by increasing T-cell proliferation and function [58, 59]. Thus, we examined anti-tumor immunity and therapeutic efficacy

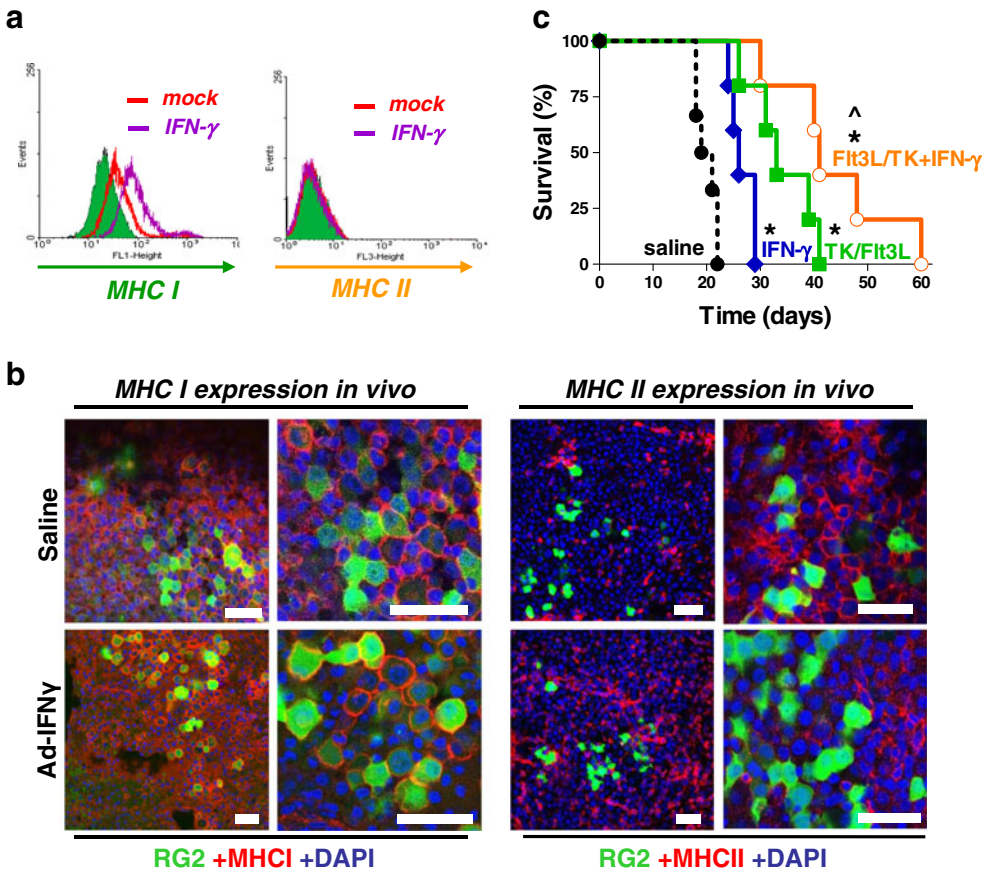

Fig. 4 Interferon-gamma (IFN-γ) increases the expression of Major histocompatibility complex (MHC) class I on RG2 glioma cells and enhances the therapeutic efficacy of Ad-Flt3L +Ad-TK. (a) Effect of IFN-γ on the expression of MHC class I and class II complexes on RG2 cells *in vitro*. RG2 cells were incubated with 10 ng/mL recombinant rat IFN-γ or mock for 48 h. Overlays show the fluorescence intensity of RG2 cells labeled with FITC-MHC I (left panel) or PerCP-MHC II (right panel). Green area represents isotype control. (b) Effect of IFN-γ on the expression of MHC I and MHC II on RG2 cells *in vivo*. Fisher rats were implanted with 200,000 RG2-citrine cells in the brain, and 5 days later they were treated with Ad-IFN-γ or saline. Four days after the treatment, brains were processed for IHC. Confocal micrographs show the expression of MHC I (red) and MHC II (red) on RG2-citrine cells (green). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (blue); scale bar, 40 μm. (c) Fisher rats were implanted with 20,000 RG2 cells in the striatum, and 5 days later they were treated with Ad vectors. Kaplan-Meier survival curve of rats treated with Ad-Flt3L/TK + Ad-IFN-γ (n=5), Ad-Flt3L/TK (n=5), Ad-IFN-γ (n=5), or saline (n=5) are shown. *$p<0.05$ *versus* saline, ^$p<0.05$ *versus* Ad-Flt3L/TK; Mantel log rank test

induced by co-delivery of Ad-IL-2 in combination with Ad-Flt3L + Ad-TK. Samples from brain and cervical lymph nodes were collected at 7 and 28 days after the treatment (Fig. 5a). Immunohistochemical analyses of brains 7 days post-treatment showed that most CD8+ immune cells were localized at the margins of the brain tumor in saline-treated animals (Fig. 5b). Ad-Flt3L + Ad-TK treatment enhanced infiltration of CD8+ immune cells within the tumor microenvironment and co-administration of Ad-IL-2 with Ad-Flt3L + Ad-TK further induced the infiltration CD8+ immune cells throughout the brain tumor mass. To analyze the functionality of CD8+ T cells in the brain tumor microenvironment, we measured the IFN-γ production of brain tumor infiltrating CD8+ T cells by flow cytometry. Ad-Flt3L + Ad-TK increased IFN-γ production by tumor infiltrating CD8+ T cells compared to controls. Treatment with Ad-IL-2 in combination with Ad-Flt3L + Ad-TK further enhanced the IFN-γ production by tumor infiltrating CD8+ T cells (Fig. 5c). Considering that IL-2 may have effects on Tregs, we also assessed the frequency of tumor infiltrating Tregs after the treatment with Ad.IL-2 (Fig. 5c). We found that administration of Ad.IL-2 in combination with Ad.TK + Ad.Flt3L led to a reduction in the number of Tregs infiltrating the brain tumor mass. We then analyzed cytolytic function of T cells collected from the cervical draining lymph nodes of Adv-treated tumor-bearing mice. Flow cytometry-based CTL assay showed that co-administration of Ad-IL-2 enhanced the cytolytic activity of T cells when compared to Ad-Flt3L + Ad-TK (Fig. 5d).

Ad-Flt3L + Ad-TK treatment of RG2 brain tumors leads to almost complete tumor regression within 14 days, but intracranial RG2 tumors always relapse and kill the host.

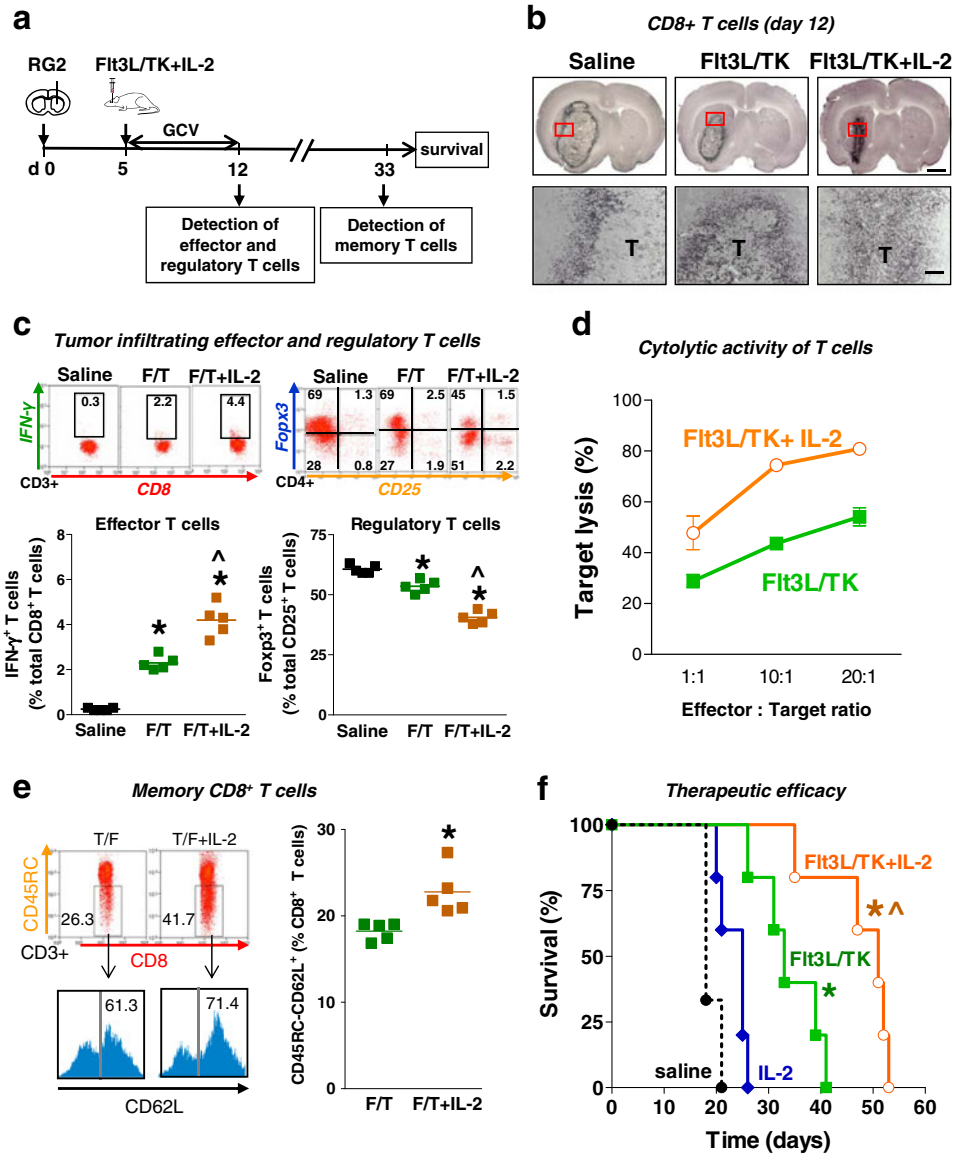

Fig. 5 Ad-interleukin (IL)-2 enhances anti-tumor T-cell responses induced by Ad-Flt3L/TK and prolongs the survival of rats bearing RG2 glioma. (a) Experimental design to assess the therapeutic efficacy and anti-tumor immune responses of Ad-IL-2 in combination with Ad-Flt3L/TK. (b) CD8+-immune cell infiltration into the tumor. Seven days after the treatment (12 days after tumor implantation), brains were processed and stained with CD8α. Lower panels are high magnification images of area defined in the red box in the images above. The tumor mass is indicated with a (T). Scale bar, 2 mm (upper panels) and 0.2 mm (lower panels). (c) Flow cytometry analysis of effector and regulatory T cells. Seven days after the treatment with Ad-Flt3L/TK + Ad-IL-2 (F/T+IL-2), Ad-Flt3L/TK (F/T) or saline, immune cells were isolated from brain tumor and incubated for 18 h with PMA, ionomycin, blefedrin A, and monensin. Immune cells were then stained with APC-CD3 and PE-CD8α followed by fixation, permeabilization, and intracellular staining with FITC-IFN-γ. Labeled cells were analyzed by flow cytometry; representative dot plots, and corresponding percentage of CD8+ CD3+ cells producing IFN-γ are shown. For the detection of Tregs, immune cells were isolated from brain tumor and stained with eFluor450-Foxp3, APC-CD4, and PE-CD25. Representative dot plots and corresponding percentage of each quadrant are shown. The column scatter graph shows a percentage of CD4+ CD25+ cells that express Foxp3 for Ad-Flt3L/TK treated rats (F/T) and Ad-Flt3L/TK + Ad-IL-2 (F/T+IL-2). *$p<0.5$ *versus* saline, ^$p<0.05$ *versus* F/T; one-way analysis of variance (ANOVA) followed by Tukey's test. (d) Cytotoxic T-lymphocyte (CTL) assay. RG2 cells stained with VivoTag-680 were incubated for 5 h at 37 °C with T cells isolated from tumor bearing rats 7 days after the treatment with Ad-Flt3L/TK + Ad-IL-2 or Ad-Flt3L/TK at the indicated ratios. Tumor cell death was assessed by Annexin-V and propidium iodide (PI) followed by flow cytometry. Differences between treatment groups were evaluated by 2-way ANOVA followed by Tukey's post test. (e) Effect of Ad-IL-2 on the levels of central memory CD8+ T cell. Upper panels show representative dot plots and corresponding percentage of memory T cells (CD45RC- CD8+ CD3+) in the spleen at 28 days after immunogene therapy by F/T or F/T+IL-2. Memory T cells were then gated for the expression of CD62 (lower histograms). The number is the percentage of CD62Lhigh population. The column scatter graph shows the percentage of central memory CD8+ T-cell population (CD62Lhigh CD45RC+) among CD8+ T cells (CD8+ CD3+) for each group. *$p<0.05$ *versus* F/T; Student *t* test. (f) Kaplan-Meier survival curve of rats treated with Ad-Flt3L/TK + Ad-IL-2 (n=5), Ad-Flt3L/TK (n=5), Ad-IL-2 (n=5), or saline (n=5) are shown. *$p<0.05$ *versus* saline, ^$p<0.05$ *versus* Ad-Flt3L/TK; Mantel log rank test

The refractory nature of RG2 GBM, therefore, could be caused by the suppression of anti-tumor memory T cells in the tumor microenvironment. Thus, we tested the possibility that IL-2 could enhance the function of CD8+ memory T cells. As CD62L + central memory CD8+ T cells, rather than CD62L-effector memory CD8+ T cells, play a role in long-term anti-tumor immunity [31]; we analyzed the central memory CD8+ T-cell population in the spleen at 28 days after treatment. Ad-IL-2 increased the percentage of CD45RC-memory T-cell population among CD8+ T cells. Furthermore, IL-2 induced elevated levels of CD62L expression on these memory CD8+ T cells (Fig. 5e). The percentage of central memory population (CD45RC-CD62L+) among CD8+ T cells in the spleen was significantly higher in rats treated with Ad-IL-2 + Ad-Flt3L + Ad-TK than those treated with Ad-Flt3L + Ad-TK alone (Fig. 5e). Compatible with these findings, Ad-IL-2, in combination with Ad-Flt3L + Ad-TK, significantly prolonged the median survival (51 days) of rats bearing RG2 GBM compared to Ad-Flt3L + Ad-TK alone (33 days; median survival ratio, 1.6; 95 % confidence interval, 1.1-2.8) (Fig. 5f). We also tested whether Ad-IL-2 showed anti-tumor effects when administered 14 days after Ad-Flt3L + Ad-TK treatment. Ad-IL-2 injection 14 days after Ad-Flt3L + Ad-TK treatment did not improve survival of tumor bearing animals (Supplemental Fig. 8). Taken together, these findings demonstrate that co-delivery of Ad-IL-2 enhances anti-RG2 effector and memory CD8+ T-cell function induced by Ad-Flt3L + Ad-TK/GCV and improves the therapeutic efficacy of Ad-Flt3L + Ad-TK in a refractory RG2 glioma model.

Inhibition of NF-κB Signaling Inhibits Foxp3 Expression on Regulatory T Cells and Enhances Anti-Tumor Immunity

Foxp3 not only plays a role for the development of CD4 + CD25+ regulatory T cells (Treg), but it also has the ability to inhibit IL-2 and IFN-γ production by CD4+ helper T cells [33]. As it has been shown that preclinical GBM models and also human GBMs are heavily infiltrated by Tregs, which mediate powerful immune suppression, we hypothesized that down modulation of Foxp3 would also enhance the therapeutic efficacy of Ad-Flt3L + Ad-TK. NF-κB regulates Foxp3 expression in CD4 T cells (i.e., NF-κB subunit p65 binds to the promoter of Foxp3) [34–36]. Thus, we delivered Ad-IκBα, an inhibitor of NF-κB transcription factor, into the tumor mass to suppress Foxp3 expression on CD4 T cells in the tumor microenvironment. To test the effect of NF-κB inhibition on the expression of Foxp3 on CD4+ T cells *in vivo*, Fisher rats were implanted in the striatum with RG2 cells, treated with Ad-Flt3L + Ad-TK, or Ad-IκBα + Ad-Flt3L + Ad-TK, and were perfused 7 days after the treatment. As shown in Figure 6a, Ad-IκBα elicited a reduction in Foxp3 expression on tumor infiltrating CD4 T cells. Further the percentage of tumor infiltrating CD25+ CD4+ Tregs that are positive for Foxp3 was significantly lower in rats treated with Ad-IκBα + Ad-Flt3L + Ad-TK compared to those treated with Ad-Flt3L + Ad-TK alone. In accordance, IFN-γ production from tumor infiltrating CD4 T cells was significantly higher in rats treated with Ad-IκBα + Ad-Flt3L + Ad-TK compared to those treated with Ad-Flt3L + Ad-TK alone (Fig. 6b). Then we examined anti-tumor immune responses (i.e., IFN-γ production by CD8 T cells and CTL activity). IFN-γ production by tumor infiltrating CD8+ T cells was markedly enhanced by co-administration of Ad-IκBα with Ad-Flt3L + Ad-TK (Fig. 6c). Cytolytic T-lymphocyte activity was also enhanced by Ad-IκBα in combination with Ad-Flt3L + Ad-TK (Fig. 6d). In addition, Ad-IκBα treatment in combination with Ad-Flt3L + Ad-TK prolonged the median survival (50 days) of rats bearing RG2 glioma compared to Ad-Flt3L + Ad-TK (31 days), and elicited long-term survival in 20 % of the treated, tumor-bearing rats (median survival ratio, 1.6; 95 % confidence interval, 1.1-2.8) (Fig. 6e). Adenoviral vectors expressing a dominant negative C-terminal truncation mutant of p65/RelA (p65RHD) that interfere with NF-κB-mediated transactivation also improved the therapeutic efficacy of Ad-Flt3L + Ad-TK for RG2 glioma (median survival, 41 days) (median survival ratio, 1.3; 95 % confidence interval, 1.0-2.1) (Fig. 6f). On the other hand, Ad-IκBα did not affect cytotoxicity of Ad-TK (Supplemental Fig. 9). Taken together, these findings demonstrate that NF-κB inhibition decreases the number of Foxp3+ regulatory T cells, increases IFN-γ production from Th1 CD4 T cells, and enhances the therapeutic efficacy and anti-tumor immunity induced by Ad-Flt3L + Ad-TK.

Discussion

RG2 is a chemically induced rat glioma cell line that is syngeneic with Fisher rats. Histopathological examination demonstrated that RG2 glioma displays a highly infiltrative growth pattern, microvascular proliferation, and central necrosis [60]. RG2 cells are nonimmunogenic in syngeneic hosts and have low levels MHC class I expression as compared with C6 and 9 L glioma [38]. In addition, similar to human GBM, RG2 glioma is refractory to most therapeutic modalities, including radiation, chemotherapy, and immunotherapy [37, 38], thus, RG2 is an attractive preclinical model for testing novel GBM therapeutics. Examination of the gene expression profile demonstrated that RG2 cells have increased expression of growth factors, such as *PDGF-β*, *IGF-β*, and the *Ras* oncogene along with an increase in *EGFR*, *cyclin D1*, and *cyclin D2* expression, showing a similarities with human GBM [60, 61]. RG2

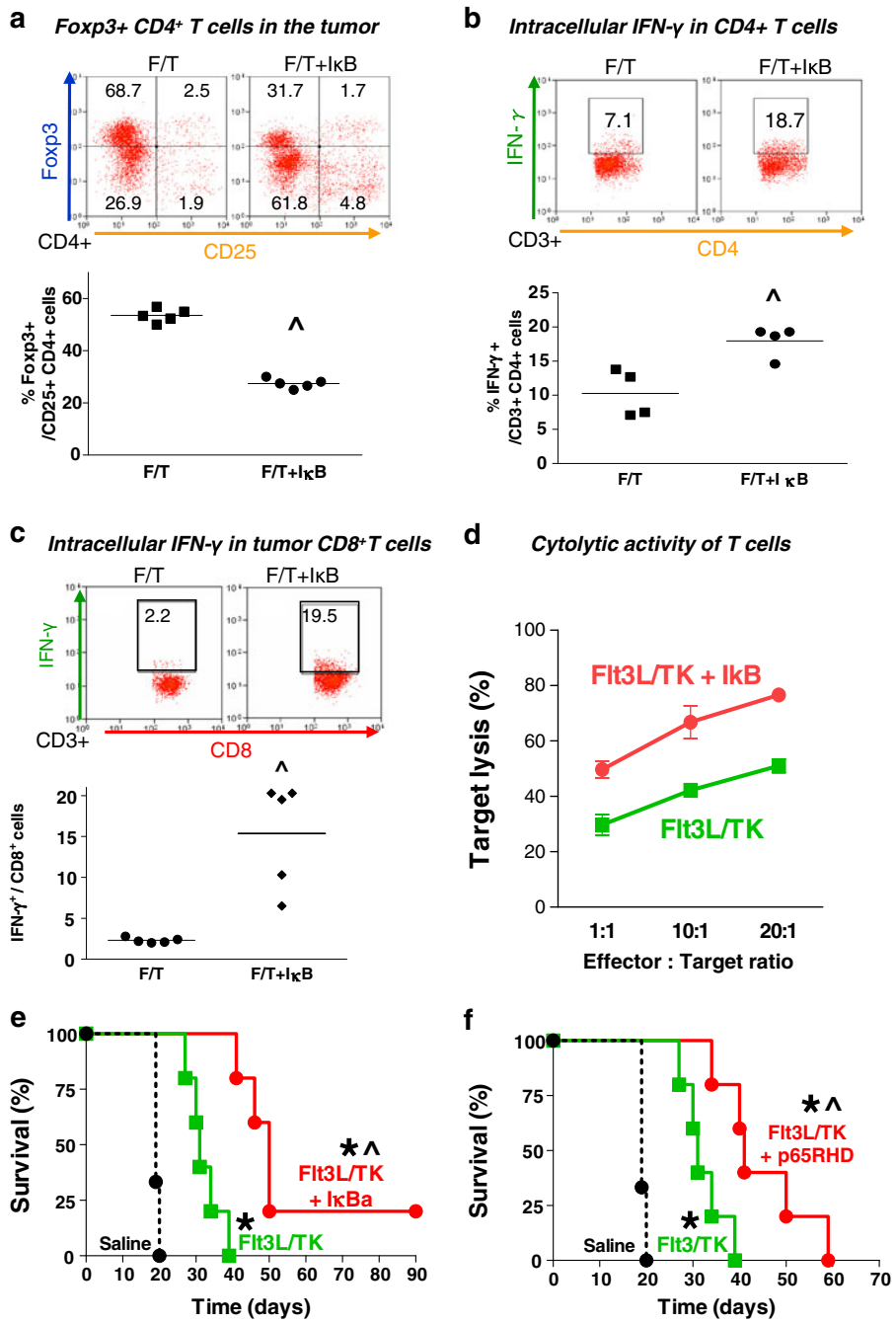

Fig. 6 Nuclear factor kappa-B (NF-κB) inhibition by Ad-IκBα reduces tumor infiltrating Foxp3+ regulatory T cells and enhances anti-tumor T-cell responses. (a) Effect of Ad-IκBα on Foxp3 expression of CD4+ T cells. Immune cells were isolated from brain tumor and stained with eFluor450-Foxp3, APC-CD4, and PE-CD25. Representative dot plots and corresponding percentage of each quadrant are shown. The column scatter graph shows percentage of CD4+ CD25+ cells that express Foxp3 for Ad-Flt3L/TK-treated rats (F/T) and Ad-Flt3L/TK + Ad-IκBα treated rats (F/T +IκB). ^$p<0.05$ *versus* F/T; Student's *t* test. (b, c) Flow cytometry analysis measuring interferon (IFN)-γ production by CD4+ T cells (b) and CD8+ T cells (c). Fisher rats were implanted with RG2 tumor cells, and 5 days later they were treated with Ad-Flt3L/TK (F/T) or Ad-FLt3L/TK + Ad- IκBα (F/T+ IκB). Seven days after the treatment, immune cells were isolated from the brain tumor and incubated for 18 h with Phorbol 12-myristate 13-acetate (PMA), ionomycin, blefedrin A, and monensin. Those immune cells were stained with APC-CD3 and PE-CD4 or PE-CD8α followed by fixation, permeabilization, and intracellular staining with FITC-IFN-γ. Representative dot plots and corresponding percentage of IFN-γ+ cells among CD4+ CD3+ cells (b) or CD8+ CD3+ cells (c) are shown. *$p<0.05$ *versus* saline; ^$p<0.05$ *versus* Ad-Flt3L/TK; Student's *t* test. (d) Cytolytic activity of T-lymphocytes from cervical draining lymph nodes. RG2 cells stained with VivoTag-680 were incubated with lymphocyte isolated from rats 7 days after the treatment with Ad-Flt3L/TK+Ad-IL-2 or Ad-Flt3L/TK for 5 h at the indicated ratios. Tumor cell death was assessed by flow cytometry with FITC-Annexin-V and Propidium Iodide (PI). The difference between the treatment groups was evaluated by 2-way analysis of variance followed by Turkey's post-hoc test. (e, f) Kaplan-Meier survival curve of rats treated with Ad-Flt3L/TK + Ad-IκBα (n=5), Ad-Flt3L/TK + Ad-p65RHD (n=5), Ad-Flt3L/TK (n=5), or saline (n=5) are shown. *$p<$ 0.05 *versus* saline; ^$p<0.05$ *versus* Ad-Flt3L/TK; Mantel log rank test

cells, as well as other rat glioma cell lines, upregulated *MDR2* gene, which plays a critical role in drug resistance [61]. Interestingly, among 4 rat glioma cell lines (C6, 9 L, F98, and RG2), the Rb tumor suppressor gene exhibited low expression in RG2 cells [61]. In agreement with these aggressive characteristics, Ad-Flt3L + Ad-TK/GCV immunogene therapy had limited efficacy for the RG2 glioma model compared to CNS1, F98, and 9L [25]. Therefore, optimization of the therapeutic efficacy in this refractory model is expected to provide a compelling rationale for the optimization of immune-mediated therapies for human GBM.

Our results indicate that the combination of Ad-Flt3L with Ad-TK/GCV elicited enhanced the therapeutic efficacy when compared to saline-treated tumor bearing animals or Ad-TK/GCV alone, but did not lead to long-term survival. Considering that the lack of therapeutic efficacy of Ad-Flt3L/TK treatment could be due to a limited transduction efficiency of the gene therapy vectors used, we assessed the transduction efficiency of Ad vectors in the RG2 tumor model and compared it to that of the CNS-1 tumor model, in which Ad.TK + Ad.Flt3L exerts a robust anti-tumor effect leading to more than 70 % long-term survival [39, 45, 46]. We found that Ad vectors had a very efficient transduction rate in RG2 tumors *in vitro* and *in vivo*. Transduction efficiency was higher in RG2 tumors than in CNS-1 tumors. Taken together, these results suggest that the lower therapeutic efficacy of Ad.TK + Ad.Flt3L in RG2 tumor-bearing rats is not related to a limited transduction efficiency of Ad vectors, and is more likely related to differences in the characteristics of the tumor microenvironment and the immune response of the host, which dampen the development of an effective anti-tumor immune response against RG2 tumors. In addition, a great body of evidence indicates that adenoviral vectors are very efficient for gene delivery to human GBM [62–69]. Results from our laboratory and others indicate that adenoviral vectors constitute an excellent tool to infect human GBM [64, 66–71]. Gene therapy clinical trials for GBM showed that Ads efficiently transduce such tumors and are clinically more effective when compared to retroviral vectors [64, 66–69]. In a landmark human GBM *in vivo* experiment, 10 % of glioma cells were transduced 2 days after intratumoral administration of Ads, a high transduction efficiency compared to the almost negligible levels seen for retroviral transduction of the same tumor type [64]. Gene therapy approaches using adenoviral vectors for the treatment of GBM have been tested in recent clinical trials and involve the delivery of conditionally cytotoxic genes [72, 73], soluble pro-apoptotic cytokines [74], or oncolytic adenoviral vectors [75, 76]. In addition, this approach has a powerful bystander effect which involves: 1) the release of Flt3L from infected tumor cells that reach the systemic circulation and stimulates the expansion and recruitment of immune cells involved in the anti-tumor immune response, 2) TK-phosphorylated GCV that can be transported from infected cells to neighboring tumor cells through gap junctions, amplifying the cytotoxic effect of Ad.TK, 3) the anti-tumor immune response triggered by this treatment can detect and kill tumor cells spread throughout the normal brain parenchyma. Thus, the proposed gene therapeutic strategy does not rely exclusively in the transduction of tumor cells.

We aimed to optimize the therapeutic efficacy of Ad-Flt3L + Ad-TK/GCV in this refractory glioma model by enhancing: 1) tumor killing, 2) DC migration into the tumor microenvironment and DC activation, and 3) tumor infiltrating T-cell functions. We have previously demonstrated that Ad-TK/GCV treatment is the most effective and safest tumor killing strategy when compared to other tumor killing approaches, such as Ad-TNF-α, Ad-TRAIL, and Ad-FasL [27]. We showed that Ad-TK/GCV increases both autophagy and apoptosis in tumor cells [26]. Immunogenicity of tumor cells treated with Ad-TK/GCV was higher than necrotic tumor cells and was comparable with those treated with temozolomide, which is a standard chemotherapeutic agent for human GBM. We also showed that dying tumor cells treated with Ad-TK/GCV release HMGB1, which induce maturation of DCs through TLR-2 [24]. DC maturation by HMGB1 is essential for tumor regression induced by Ad-Flt3L + Ad-TK/GCV [24]. We thus tested SR39 mutant TK that reportedly has a higher cytotoxic effect than wild-type TK in the refractory RG2 GBM model [47]. Ad-SR39/GCV showed a higher cytotoxic effect on RG2 cells *in vitro* but the therapeutic efficacy *in vivo* was comparable to wild-type TK. Although, at day 14 post Flt3L/TK treatment, tumor cells were macroscopically undetectable in the brain (Fig. 1e), all animals succumbed to tumor burden at <45 days. These results suggest that TK mediated conditional cytotoxicity in combination with Flt3L is insufficient to eradicate completely the primary tumor. Residual tumor cells are likely to remain in the surrounding brain parenchyma, which become resistant to immunotherapy, thus causing recurrence.

To increase the number of DCs in the tumor microenvironment, we used a soluble Flt3L-IgG chimeric protein [49]. The Fc fragment of IgG confers the fusion molecule longer serum half-life [50]. In a preclinical model of melanoma, B16 melanoma cell vaccines expressing Flt3L-IgG2a, in combination with CTLA-4 blockade, prevented the growth of B16-BL6 tumors more effectively than B16 vaccine expressing granulocyte macrophage colony-stimulating factor (GM-CSF) [49]. In the RG2 GBM model, Flt3L-IgG2a exhibited higher circulating levels and intratumoral injection of Ad-Flt3L-IgG2a showed enhanced therapeutic efficacy than Ad-Flt3L. Still, Ad-Flt3L-IgG2a combined with Ad-TK/GCV did not show significant improvement in the median survival compared to Ad-Flt3L + Ad-TK/GCV. CCL2 and CCL3 are chemokines that enhance migration of DC,

whereas Flt3L mainly induces DC differentiation and expansion. It has been reported that CCL2 and CCL3 facilitate migration of DCs generated by Flt3L [55]. When we tested the combination of these chemokines with Ad-Flt3L + Ad-TK/GCV, in the RG2 model, they did not elicit any additional survival benefit. As it has been shown that DCs lose responsiveness to CCL2 or CCL3 after being stimulated by TNF-α or CD40L [55], it is possible that DCs recruited into the tumor microenvironment in response to HGMB1 released from Ad-TK/GCV treated RG2 tumors might not be able to respond further to CCL2 or CCL3.

IL-12 and CXCL-10 are cytokines that are secreted from DCs and have the ability to skew Th1 dominant immunity. DCs that secrete high levels of IL-12 and CXCL-10 are called DC1, and they are capable of inducing better anti-tumor immunity than non-DC1 [53, 77]. CD40 is highly expressed on Flt3L induced DCs, and CD40 ligation by CD40L stimulates DCs to secrete IL-12 and CXCL-10 [78]. Combination of CD40L and Flt3L has been reported to have synergistic effect and suppressed growth of intradermally implanted B10.2 and 87 sarcoma cells more effectively than either therapy alone, presumably via IL-12 secretion from DCs [78]. Thus, we tested Ad-IL-12, Ad-CXCL-10 and Ad-CD40L in combination with Ad-Flt3L + Ad-TK/GCV. None of these combination treatments prolonged the survival of rats bearing intracranial RG2 tumors when compared with Ad-Flt3L + Ad-TK/GCV. Because HMGB1 released from Ad-TK/GCV treated dying tumor cells strongly enhances DC maturation via TLR-2 [24], it could be possible that HMGB1 might induce maximal secretion of IL-12 and CXCL-10 from DCs in this model. Although delivery of IL-12 or chemokines CXCL-10, CCL2 and CCL3 did not improve efficacy of our gene therapy approach, further optimization of each of these platforms in future studies could lead to significant benefit, and could lead to an understanding of why these therapies failed in the current therapeutic paradigm.

Recent evidence suggests that Th1-skewed immune responses mediate anti-tumor effects through multiple mechanisms [77]. CD8+ cytotoxic T cells are well recognized as important effector T cells that can mediate effective anti-tumor immunity, and Th1-skewed CD4+ T helper cells are known to support effective CD8+ T-cell effector functions [79]. In addition, IFN-γ secreted by Th1 CD4+ T cells can have a direct anti-tumor effect thorough upregulation of MHC type I expression on the tumor cell surface, which is associated with increased tumor antigen presentation and enhanced anti-tumor immunity [38]. In agreement with this, our data showed that IFN-γ treatment increased MHC I expression on RG2 cells. Co-administration of Ad-IFN-γ with Ad-Flt3L + Ad-TK/GCV prolonged the median survival (~1.4 times longer *vs* Ad-Flt3L + Ad-TK/GCV) of rats bearing RG2 GBM.

CD4+ T-cell help is essential for effector and memory CD8+ T-cell functions [80]. IL-2 is produced by naïve CD4 + T cells, and it mediates helper-dependent CD8+ T-cell responses [81]. In addition, IL-2 plays a role in effector and memory functions of CD8+ T cells [82–85]. During primary infection, IL-2 is critical for the development of CD8+ memory T cells that undergo robust expansion during secondary infection [83]. IL-2 has also been reported to play a role in the expansion and regulatory functions of Tregs [86–88]; in contrast others reported that IL-2 is not required for human Treg suppressor function under optimal stimulatory condition, and IL-2 consumption plays no role in Treg-mediated suppression [89]. In preclinical models and clinical settings, several studies demonstrated that IL-2 stimulates anti-tumor immune responses [90, 91]. Interestingly, vaccination with autologous acute myeloid leukemia cells expressing CD80 and IL-2 induced expansion of both cytotoxic CD8+ T cells and Foxp3+ Tregs, but cytolytic activity was enhanced in patients who received the vaccination [91]. Because the expansion of CD8+ T cells was much higher than Tregs, the authors speculated that the lower number of Tregs relative to CD8+ effector T cells prevented Tregs from exerting their suppressive activity. It has also been reported that IL-2 enhanced the therapeutic efficacy of autologous tumor lysate vaccination combined with continuous intratumoral GM-CSF infusion in 9 L rat glioma model [58, 59]. In line with this evidence, our model showed that co-administration of Ad-IL-2 enhanced T-cell-mediated immune responses illustrated by an increase IFN-γ production by CD8+ T cells and enhanced tumor specific cytolytic function of tumor infiltrating T cells. Ad.IL-2 in combination with Ad.TK + Ad.Flt3L also induced a reduction in the frequency of tumor-infiltrating Tregs. Considering that this treatment also increased the infiltration of effector T cells, addition of Ad.IL-2 to Ad.TK + Ad.Flt3L gene therapy greatly increases the ratio of effector T cells/Tregs, improving the anti-tumor immune response triggered by this treatment, as observed in the improved survival of animals treated with the 3 vectors combined. Ad-IL-2 also increased the number of CD8+ memory T cells and the expression of CD62L on these memory cells. CD62L is a receptor associated with homing to secondary lymphatic organs. Memory CD8+ T cells (CD45RC- CD8+ CD3+) with high expression levels of CD62 are central memory CD8+ T cells, which play a role in long-term anti-tumor immune responses rather than effector memory T cells that express low levels of CD62L [31]. In accordance with enhanced cytotoxicity and memory CD8 T-cell functions, co-administration of Ad-IL-2 with Ad-Flt3L + Ad-TK/GCV significantly prolonged the median survival (~1.6 times *vs* Ad-Flt3L + Ad-TK) of rats bearing RG2 tumors.

RG2 tumors always relapse after the initial regression induced by the combination Ad-Flt3L + Ad-TK/GCV gene

therapy, suggesting that anti-tumor effector T-cell functions may be suppressed in the tumor microenvironment. Tregs have potent suppressive effects on anti-tumor immunity. Foxp3 has been identified as a master regulator for the development and suppressive function of Tregs [31]. In addition, recent evidence demonstrated that Foxp3 plays a role in inhibiting Th1 cytokines, IL-2, and IFN-γ from helper T cells [33–36]. NF-κB regulates Foxp3 expression in CD4+ T cells (i.e., p65 subunit binds to the Foxp3 promoter and c-Rel binds to the Foxp3 enhancer to activate NF-κB signaling) [92, 93]; thus, we used Ad-IκBα and Ad-p65RHD (dominant negative C-terminal truncation mutant of p65) to inhibit Foxp3 transactivation. Our data indicate that intratumoral injection of Ad-IκBα suppressed Foxp3 expression in CD4+ T cells, including CD4+ CD25+ Tregs, and it also increased IFN-γ secretion from CD4+ helper T cells. In accordance with these findings, Ad-IκBα enhanced anti-tumor immune responses, as illustrated by increased IFN-γ production from CD8+ T cells, and enhanced cytolytic activity of T cells. Co-administration of Ad-IκBα with Ad-Flt3L + Ad-TK/GCV prolonged the median survival (~1.6 times *vs* Ad-Flt3L + Ad-TK/GCV) and achieved long-term survival in ~20 % of animals bearing RG2 GBM. Efficacy of NF-κB inhibition was confirmed by Ad-p65RHD, which also enhanced therapeutic efficacy of Ad-Flt3L + Ad-TK/GCV. Co-administration of these vectors in the tumor bed after surgical resection of the main tumor mass may serve as a powerful adjuvant in the treatment of GBM.

NF-κB inhibition has been shown to have a direct effect on tumor cells through sensitizing tumor cells to apoptotic stimuli. Tsuboi et al. [94] demonstrated that NF-κB inhibition by pitavastatin-induced autophagy in glioma cells and sensitized them to radiation therapy. Ad-IKKβdn (dominant-negative adenoviral IKKβ construct) was also tested as anti-cancer therapy and sensitized human prostate carcinoma cells, neuroblastoma cells, and lung cancer cells to TNF-related apoptosis-inducing ligand (TRAIL)- or TNF-induced apoptosis [95]. Adenovirus-mediated IκBα gene transfer also has been applied to improve the sensitivity of various tumors to anticancer drugs or radiation both *in vitro* and *in vivo* in pre-clinical models by increasing apoptosis. Our data did not show an effect of Ad-IκBα on the cytotoxic effects of Ad-TK/GCV. Given that improvement of cytotoxicy of TK by using SR39 mutant TK did not prolong the survival of rats bearing RG2 tumor, it is unlikely that the therapeutic benefit elicited by the combination of Ad-IκBα with Ad-Flt3L + Ad-TK/GCV is caused by effects on tumor cell death and/or inhibition of tumor growth.

In conclusion, the present study showed that Th1-skewed immunity induced by inhibition of Foxp3 via NF-κB inhibition and exogenous Th1 cytokines, such as IL-2 and IFN-γ enhanced therapeutic efficacy of Ad-Flt3L + Ad-TK/GCV. Considering that NF-κB inhibition in the tumor microenvironment can induce both Treg suppression and Th1 cytokine production, intratumoral delivery of Ad-IκBα can be a potential strategy to enhance GBM immunotherapeutic strategies. These findings indicate that enhancement of tumor specific T-cell responses should be considered as a critical adjuvant to circumvent immune evasion and improve efficacy of immune-mediated strategies currently under evaluation in human clinical trials.

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Required Author Forms Disclosure forms provided by the authors are available with the online version of this article.

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