Cancer Therapy: Preclinical

Temozolomide Does Not Impair Gene Therapy-Mediated Antitumor Immunity in Syngeneic Brain Tumor Models

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Abstract

Purpose: Glioblastoma multiforme is the most common primary brain cancer in adults. Chemotherapy with temozolomide (TMZ) significantly prolongs the survival of patients with glioblastoma multiforme. However, the three-year survival is still approximately 5%. Herein, we combined intratumoral administration of an adenoviral vector expressing Flt3L (Ad-Flt3L) with systemic temozolomide to assess its impact on therapeutic efficacy.

Experimental Design: Wild-type or immunodeficient mice bearing intracranial glioblastoma multiforme or metastatic melanoma were treated with an intratumoral injection of Ad-Flt3L alone or in combination with the conditionally cytotoxic enzyme thymidine kinase (Ad-TK), followed by systemic administration of ganciclovir and temozolomide. We monitored survival and measured the tumor-infiltrating immune cells.

Results: Although treatment with temozolomide alone led to a small improvement in median survival, when used in combination with gene therapy-mediated immunotherapy, it significantly increased the survival of tumor-bearing mice. The antitumor effect was further enhanced by concomitant intratumoral administration of Ad-TK, leading to 50% to 70% long-term survival in all tumor models. Although temozolomide reduced the content of T cells in the tumor, this did not affect the therapeutic efficacy. The antitumor effect of Ad-Flt3L+Ad-TK+TMZ required an intact immune system because the treatment failed when administered to knock out mice that lacked lymphocytes or dendritic cells.

Conclusions: Our results challenge the notion that chemotherapy leads to a state of immune-suppression which impairs the ability of the immune system to mount an effective antitumor response. Our work indicates that temozolomide does not inhibit antitumor immunity and supports its clinical implementation in combination with immune-mediated therapies. *Clin Cancer Res; 20(6); 1555–65. ©2014 AACR.*

Introduction

Glioblastoma multiforme World Health Organization grade 4 is the most aggressive and frequent primary brain tumor (1), with an incidence of 4 to 5 per 100,000 inhabitants per year in industrialized countries. The current standard treatment for glioblastoma multiforme is surgical resection followed by radiotherapy and chemotherapy with temozolomide (TMZ; ref. 2). Temozolomide is an alkylating agent, which upon spontaneous conversion to its active metabolite 3-methyl-(triazen-1-yl) imidazole-4-carboxamide, methylates DNA leading to the death of proliferating cells. Temozolomide has a higher efficacy and better toxicology profile in patients with glioblastoma multiforme than other chemotherapeutic agents. Temozolomide exhibits adequate penetration of the blood–brain barrier, which could not be achieved by previous generation chemotherapeutics without dose-limiting systemic side effects (3). Because glioblastoma multiforme cells may express O6-methylguanine methyltransferase (MGMT), a DNA-repairing enzyme that repairs temozolomide-induced DNA damage, glioblastoma multiforme can be resistant to temozolomide-induced apoptosis. However, in 25% to 50% of patients with glioblastoma multiforme, MGMT promoter is spontaneously methylated and thus, temozolomide-induced DNA damage cannot be repaired. It has been shown that, in patients with glioblastoma multiforme with methylated MGMT promoter, temozolomide has significantly higher therapeutic benefit than in those

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Translational Relevance

As a prelude to a phase I clinical trial in patients with glioblastoma multiforme, we evaluated whether concomitant administration of the alkylating drug, temozolomide, would affect the antiglioblastoma multiforme immune mechanisms and efficacy of immune-stimulatory gene therapy. Our findings indicate that, despite the well known immune-suppressive effects of temozolomide, its administration does not impair gene therapy-mediated antitumor immunity. These results support the concomitant administration of temozolomide and immunotherapeutic approaches in patients undergoing phase I clinical trials for glioblastoma multiforme.

patients with active MGMT (4). Nevertheless, 3 years postdiagnosis, less than 5% of patients remain alive; this compares unfavorably with other cancers such as breast, lung, and prostate, and therefore new treatments are urgently needed.

Our laboratory has previously shown the antitumor effect of combined adenovirus (Ad)-mediated gene therapy in several rat and mouse brain tumor models. Combination of adenovirus vectors that encode the conditionally cytotoxic herpes simplex virus type 1–thymidine kinase (Ad-TK) and the immune stimulatory fms-like tyrosine kinase-3 ligand (Ad-Flt3L) induces an antitumor immune response that leads to tumor regression and long-term survival in mice and rats bearing intracranial glioblastoma multiforme (5–7). This therapeutic approach also shows a very safe neurotoxic profile when compared with other proapoptotic agents or immune stimulant molecules (5,8).

Our results in preclinical models have shown that the antitumor effect of Ad-TK+Ad-Flt3L is dependent on the immune system (6). Considering that chemotherapeutic agents may have a strong immunosuppressive effect, it is important to investigate the impact of concomitant chemotherapy with temozolomide on the efficacy of Ad-TK+Ad-Flt3L treatment. We evaluated the survival of mice bearing intracranial GL26 (9) and GL261 (10) glioblastoma multiforme, as well as intracranial B16 melanoma tumors after treatment with intratumoral delivery of Ad-TK+Ad-Flt3L (+GCV) followed by systemic administration of temozolomide. We also assessed the effect of these treatments on the expansion and infiltration of immune cell populations into the tumor microenvironment and the spleen. The efficacy of the combined therapies was also evaluated in KO mice that lack specific immune cell populations. Our results indicate that concomitant treatment with temozolomide does not impair the antitumor effect of Ad-TK+Ad-Flt3L and supports the clinical implementation of immunotherapeutic approaches in patients with glioblastoma multiforme receiving chemotherapy with temozolomide.

Materials and Methods

Adenoviral vectors

Adenovirus vectors used are based on adenovirus type 5 (Ad5), in which the left end of E1 and a portion of the E3 regions are deleted (E1-/E3-), and a cassette containing a recombinant exogenous gene and promoter is inserted in place of the E1 deletion. Two vectors were used: Ad-TK (expresses HSV1-thymidine kinase under the control of the hCMV promoter; ref. 11) and Ad-Flt3L (expresses human soluble fms-like tyrosine kinase ligand under the control of the hCMV promoter; refs. 5,11). Adenoviruses were grown and purified as previously described (12).

Animals and cell lines

Wild-type C57BL/6, CD4$^{-/-}$, CD8$^{-/-}$, Rag1$^{-/-}$, Igh-6$^{-/-}$, and IFNγ$^{-/-}$ mice, all on a C57BL/6 background, were purchased from Jackson Laboratories. CD11c knockout mice were bred in-house as previously described (13, 14). GL26 and GL261 cells were obtained from the National Cancer Institute (NCI; Bethesda, MD) repository (http://www.dtp.nci.nih.gov/branches/btb/tumor-catalog.pdf) in 2005 and 2007, respectively, whereas B16-F10 cells were obtained from American Type Culture Collection in 2007. Cells were grown in Dulbecco's Modified Eagle medium (DMEM; CellGro), supplemented with 10% fetal calf serum, 1% L-glutamine, 1% penicillin-streptomycin, and 1% non-essential amino acids, and passaged routinely. The tumorigenicity of the three cell lines was authenticated by histologic characterization of the intracranial tumors grown in syngeneic C57BL/6 mice. The day of surgery, cells were trypsinized, resuspended in DMEM without supplements, and kept on ice for up to 4 hours.

Brain tumor models

Intracranial tumor models were generated as previously described (6, 9). For a brief description of the tumor implantation procedure, see Supplementary Materials and Methods. Fourteen days later, saline or gene therapy vectors were administered into the tumor using the following doses: Ad-TK: 0.35 × 10^8 pfu/site and Ad-Flt3L: 0.65 × 10^8 pfu/site. Twenty-four hours later, groups of animals were treated intraperitoneally (i.p.) with temozolomide (100 mg/kg of body weight/day; Selleck chemicals) for 2 weeks and/or Ganciclovir (GCV; 25 mg/kg/day) i.p. for 7 days.

Animals were allowed to recover and their health status was closely monitored. Mice were euthanized at specific time points or when their health status reached criteria established by the guidelines of the Institutional Animal Care and Use Committee at The University of Michigan School of Medicine (Ann Arbor, MI). Groups of mice were euthanized at specific time points and blood, spleen and tumors were collected immediately and processed for flow-cytometric analysis or ELISA.

Isolation and characterization of immune cells in blood, spleen, bone marrow, and tumor

Immune cells were purified from tumors and lymphoid organs and analyzed by flow cytometry as described before

(6, 15). For a brief description of this procedure, see Supplementary Materials and Methods.

HMGB1 ELISA

HMGB1 release was determined in mouse serum using a specific anti-HMGB1 ELISA (IBL-Transatlantic LLC) following the manufacturer's protocol. For a brief description of this technique, see Supplementary Materials and Methods.

Statistical analysis

Kaplan–Meier survival curves were analyzed using the Mantel log-rank test (GraphPad Prism version 3.00; Graph-Pad Software). Levels of HMGB1 and immune cell numbers were analyzed by one-way ANOVA followed by the Tukey test (Number Cruncher Statistical System [NCSS]) or by the Student t test. The Pearson test was used to determine r between HMGB1 release and survival rates (GraphPad Prism). P values of <0.05 were used to determine the null hypothesis to be invalid.

Results

Temozolomide improves the survival of brain tumor-bearing mice treated with Ad-Flt3L

We first assessed the efficacy of temozolomide alone or in combination with Ad-Flt3L in immune-competent mouse models of brain cancer. We implanted GL26 glioblastoma multiforme cells in the brain of C57BL/6 mice and 14 days later, they received an intratumoral injection of saline or Ad-Flt3L. A group of mice received intraperitoneal injections of temozolomide (100 mg/kg/d) once a day for 14 days (Fig. 1A). We found that although Ad-Flt3L alone did not modify the median survival of tumor-bearing mice (saline: 31 days; Ad-Flt3L: 43 days), temozolomide led to only a small improvement in the median survival (temozolomide: 52.5 days, $P < 0.05$ vs. saline, log-rank test; Fig. 1B). However, combination of intratumoral Ad-Flt3L and systemic temozolomide induced tumor regression and long-term survival in approximately 20% of the mice (3/16 mice, $P < 0.05$ vs. saline, vs. Ad-Flt3L alone, and vs. temozolomide alone, log-rank test).

Considering that soluble HMGB1 seems to be required for the activation of tumor-infiltrating antigen-presenting cells (APC) recruited by Ad-Flt3L, we evaluated whether this treatment affects the metabolism of nuclear HMBG1. We found that treatment with temozolomide alone did not affect circulating levels of HMGB1 (Fig. 1B). However, combined treatment with intratumoral Ad-Flt3L and systemic temozolomide increased circulating levels of HMGB1 approximately 3-fold 7 days after the treatment (Fig. 1B, $P < 0.05$ vs. saline and vs. temozolomide, one-way ANOVA). Using GL26 cells that express a cytoplasmic yellow fluorescent protein (YFP) and in which HMGB1 is fused to the red fluorescent protein cherry (citrine-GL26-Cherry-HMGB1 cells), we evaluated the cytoplasmic translocation of HMGB1 in response to temozolomide alone or in combination with Ad-Flt3L (Fig. 1C). Supplementary Figure S1 shows citrine-GL26-Cherry-HMGB1 cells treated *in vitro* with temozolomide, Ad-TK+GCV, or both. In healthy cells,

citrine fluorescence is very intense and yields a green signal, whereas HMGB1 is visualized in red, as it is fused to Cherry, and is confined to the nucleus, colocalizing with 4′, -dia-midino2-phenylindole (DAPI). When these cells undergo apoptotic cell death, citrine seems to undergo rapid degradation, whereas HMGB1 is translocated to the cytoplasm and then released from the cell. In Supplementary Figure S1, the top panels show citrine (green) and HMGB1 (red). When citrine is degrading, the fluorescence intensity is lower, and when it colocalizes with HMGB1, it yields a yellow signal. Bottom panels in Supplementary Fig. S1 show HMGB1 (red) and DAPI. Although temozolomide treatment induced HMGB1 translocation in a few cells, most of the cells treated with Ad-TK+GCV in the presence or absence of temozolomide exhibited cytoplasmic HMGB1. Similar results were obtained *in vivo* (Fig. 1C). Mice bearing intracranial citrine-GL26-Cherry-HMGB1 tumors were treated with systemic temozolomide and local Ad-Flt3L. Confocal images show loss of citrine staining and cytoplasmic HMGB1 localization in the tumors treated with Ad-Flt3L and systemic temozolomide (Fig. 1C). Again tumor-bearing mice treated with temozolomide, exhibited only a few cells with cytoplasmic HMGB1. These findings correlated with the circulating levels of HMGB1 measured in these mice. Although temozolomide treatment increased slightly serum HMGB1 levels, combination with Ad-Flt3L treatment substantially raised the circulating levels of HMGB1 (Fig. 1B).

To evaluate whether the efficacy of this treatment was dependent on the immune system, we implanted GL26 tumors in the brain of KO mice that lack specific immune cell populations and treated them with an intratumoral injection of Ad-Flt3L and systemic temozolomide for 14 days (Fig. 2). The treatment with Ad-Flt3L and temozolomide did not lead to long-term survival in mice that lacked T lymphocytes (Rag1 KO, CD4 KO, and CD8 KO) or that were unable to produce IFN-γ (IFN-γ KO; Fig. 2). In mice that lack dendritic cells (DCs; CD11c KO), the combined treatment improved the median survival of GL26 tumor-bearing mice (saline: 33.5 days, TMZ+Ad-Flt3L: 54.5 days, $P < 0.05$, log-rank test). However, although three of 17 GL26 tumor-bearing wild-type mice treated with Ad-Flt3L+TMZ survived for over 100 days, all the mice lacking CD11c cells succumbed to tumor burden (Fig. 2). These observations are in agreement with our previous reports that indicate that TLR2 signaling in DCs is required for the efficacy of our immunotherapy (6).

To evaluate the efficacy of this treatment in alternative tumor models, we used mice bearing intracranial GL261 glioblastoma multiforme. Although GL26 and GL261 have a similar origin, they are two different cell lines (16). Both tumors were induced in C57BL/6 mice by methylcholan-threne injection, GL261 tumor was developed by Seligman and Shear (17), and GL26 tumor, by Sugiura (18). Both tumors were propagated by serial subcutaneous transplantation in C57BL/6 mice (16, 19) until *in vitro* growing cell cultures were established (16). The average of the median survival times of animals with GL26 and GL261 tumors implanted in the brain is very similar (∼28–35 days). GL26
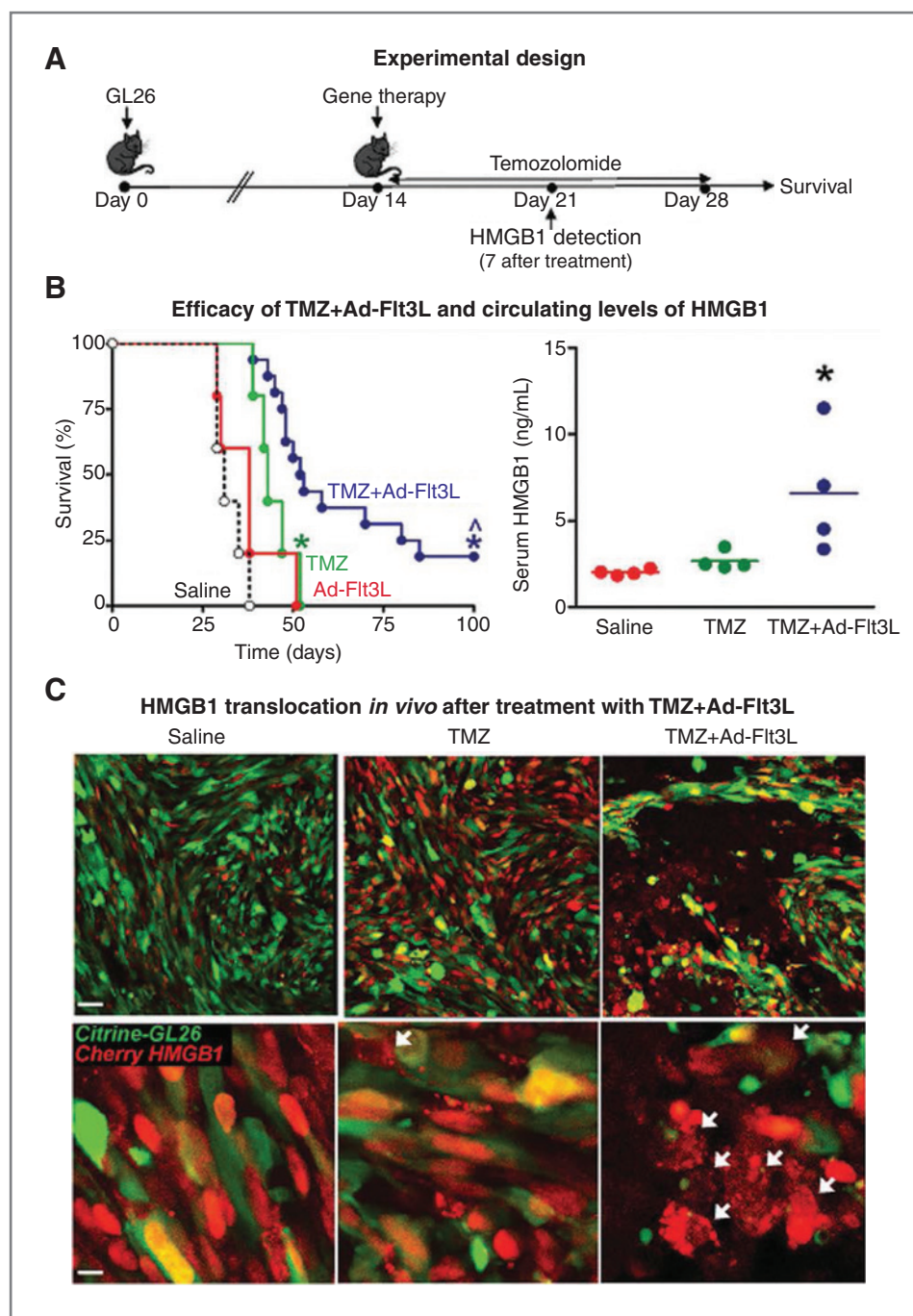
Figure 1. Efficacy of temozolomide (TMZ) in combination with Ad-Flt3L in GL26 tumor model. A, C57BL/6 mice were implanted in the striatum with GL26 glioma cells. Fourteen days later, mice ($n = 5$–16/group) were treated by intratumoral injections of Ad-Flt3L, or saline followed by daily administration of temozolomide. B, Kaplan–Meier survival curves. *, $P < 0.05$ versus saline, ^, $P < 0.05$versus temozolomide alone (Mantel log-rank test). This experiment has been performed three times. In a set of mice, the serum levels of the proinflammatory nuclear protein HMGB1 were assessed by ELISA 7 days after the treatment. *, $P < 0.05$ versus saline (one-way ANOVA followed by the Tukey test). This experiment has been performed twice. C, C57BL/6 mice were implanted in the striatum with citrine-GL26-Cherry-HMGB1, which were stably transfected to express the YFP citrine and HMGB1 fused to red fluorescent protein cherry. Fourteen days later, they were treated with saline, TMZ, or Ad-Flt3L+TMZ. Five days after treatment, the cellular location of cherry-HMGB1 in these cells was assessed by confocal microscopy. Arrows, tumor cells (green) with cytoplasmic HMGB1 (red).

and GL261 tumors are histopathologically similar, but GL26 tumors are more hemorrhagic and necrotic than GL261 (16, 20). These two tumor cell lines have been used side by side in preclinical neuro-oncology research (6, 21, 22). Alternatively, we used mice implanted with B16 melanoma cells in the brain as a model of brain metastasis. We treated GL261 and B16 tumor-bearing mice with temozolomide alone or in combination with Ad-Flt3L (Supplementary Fig. S2A). We found that temozolomide alone increased 40% to 50% the median survival of tumor-

bearing mice (GL261: saline: 31.5 days; temozolomide: 43.5; B16: saline: 27, temozolomide: 41; $P < 0.05$, log-rank test), but did not lead to long-term survival (Supplementary Fig. S2B). However, administration of temozolomide in combination with Ad-Flt3L injection led to 30% long-term survivals in GL261 tumor-bearing mice (3/10 mice, median survival: 58 days; $P < 0.05$, log-rank test).

Although this treatment did not lead to long-term survival in B16 tumor-bearing mice, it significantly improved the median survival (46 days, $P < 0.05$, log-

Figure 2. Efficacy of temozolomide (TMZ) and Ad-Flt3L in immunosuppressed mice bearing GL26 tumors. GL26 glioma cells were implanted in the brain of immune-deficient mice lacking DCs (CD11c$^{-/-}$), T and B cells (Rag1$^{-/-}$), CD4 or CD8 T cells (CD4$^{-/-}$, CD8$^{-/-}$), IFN-γ producing cells (IFN$\gamma^{-/-}$), or B cells (Igh6$^{-/-}$). Fourteen days after tumor implantation, mice were treated by intratumoral injection of Ad-Flt3L followed by daily administration of temozolomide. *, $P < 0.05$ versus saline (Mantel log-rank test). This experiment has been performed twice.
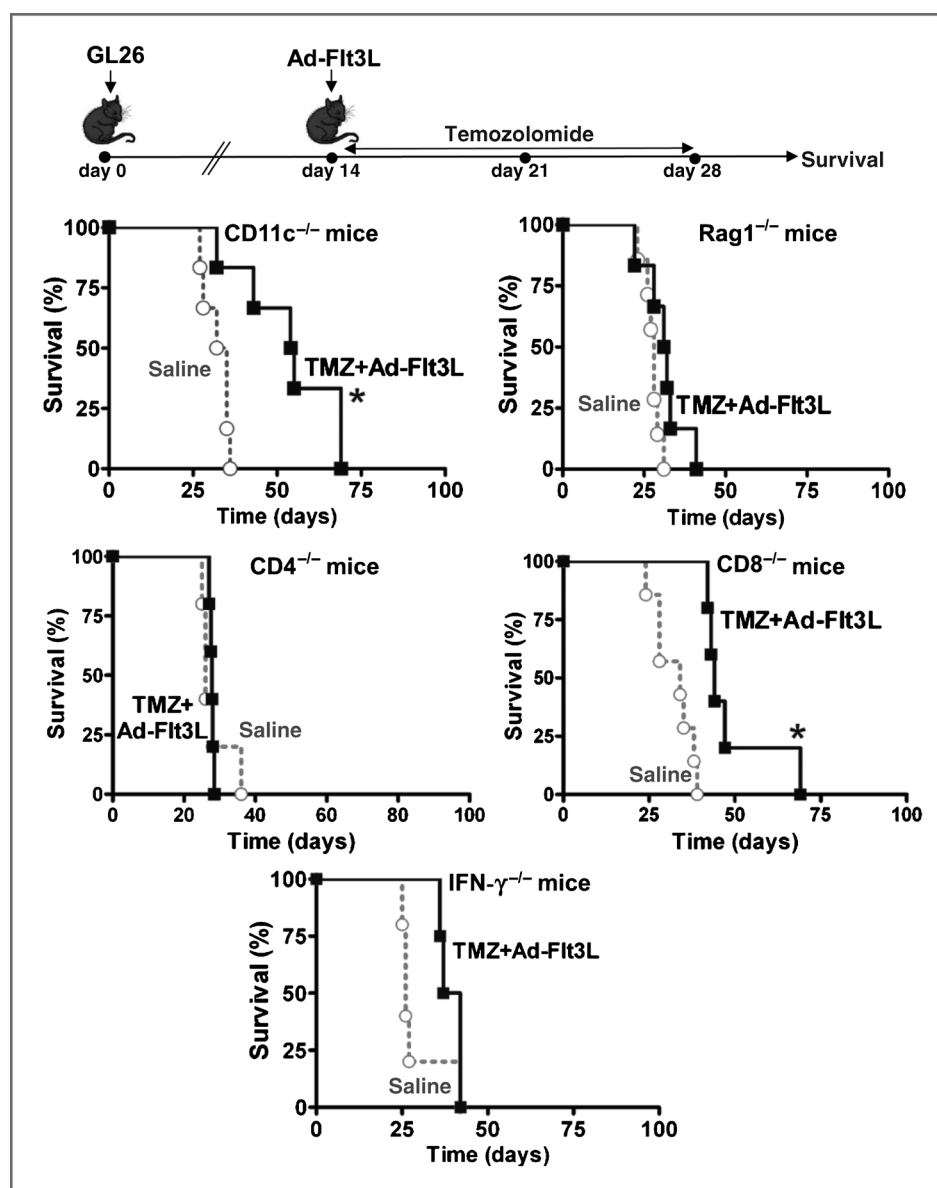
rank test) when compared with temozolomide treatment alone (Supplementary Fig. S2B). In a set of mice euthanized 7 days after the treatment, combined treatment but not temozolomide alone increased the circulating levels of HMGB1 in both tumor models (Supplementary Fig. S2C, $P < 0.05$ vs. saline and temozolomide alone, one-way ANOVA).

Temozolomide does not reduce the efficacy of combined conditional cytotoxic/immune stimulatory gene therapy in brain tumor-bearing mice

We next evaluated whether addition of systemic temozolomide affects the efficacy of immunotherapy with Ad-TK+Ad-Flt3L. Tallying with our previous results (6, 15), treatment with Ad-TK in combination with Ad-Flt3L induced long-term survival in approximately 40% of GL26

(Fig. 3A; $P < 0.05$ vs. saline, log-rank test). The efficacy of this treatment was not inhibited by the concomitant administration of temozolomide.

When we evaluated soluble HMGB1 in serum, we found that temozolomide chemotherapy did not affect the circulating levels of HMGB1 induced by Ad-TK+Ad-Flt3L treatment (Fig. 3A). We found similar results when we evaluated the cytoplasmic translocation of HMGB1 in citrine-GL26-cherry-HMGB1 tumors (Fig. 3B). Ad-TK+Ad-Flt3L-treated tumors exhibited loss of tumor structure with massive cell death and cytoplasmic HMGB1 translocation (Fig. 3B). A similar pattern was observed when mice were administered systemic temozolomide in addition to Ad-TK+Ad-Flt3L treatment (Fig. 3B)

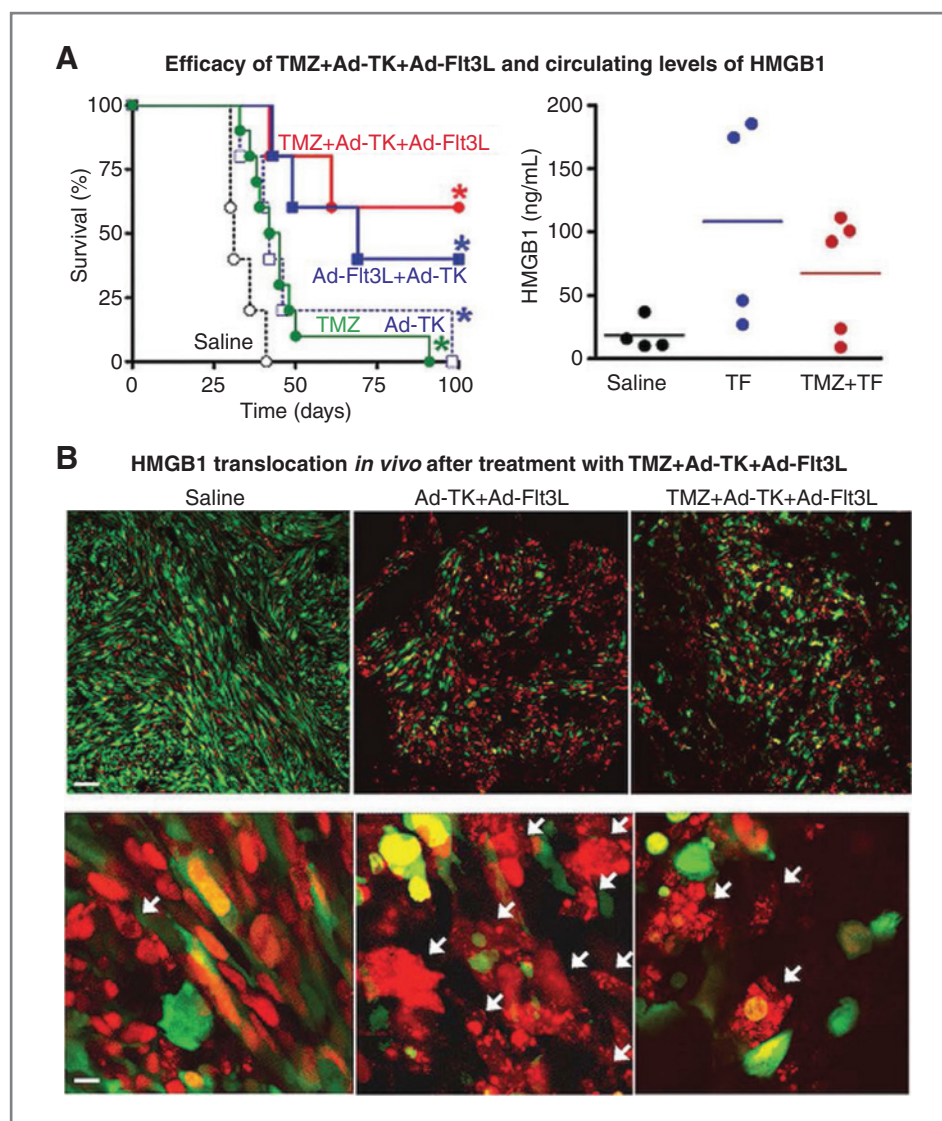To assess the translational implications of our findings, we assessed the effect of temozolomide treatment on the

A Efficacy of TMZ+Ad-TK+Ad-Flt3L and circulating levels of HMGB1

B HMGB1 translocation *in vivo* after treatment with TMZ+Ad-TK+Ad-Flt3L

Figure 3. Efficacy of temozolomide (TMZ) in combination with Ad-TK and Ad-Flt3L in GL26 tumor model. A, wild-type C57BL/6 mice were implanted in the striatum with GL26 glioma cells. Fourteen days after tumor implantation, mice were treated by intratumoral injection of Ad-TK alone or in combination with Ad-Flt3L followed by daily administration of GCV and temozolomide. *, $P <$ 0.05 versus saline (Mantel log-rank test). This experiment has been performed three times. In a set of mice (4–5/group), serum levels of the proinflammatory nuclear protein HMGB1 were assessed by ELISA 7 days posttreatment. This experiment has been performed twice. B, C57BL/6 mice were implanted in the striatum with citrine-GL26-Cherry-HMGB1, which were stably transfected to express the YFP citrine and HMGB1 fused to red fluorescent protein cherry. Fourteen days later, they were treated with saline, Ad-TK+Ad-Flt3L, or Ad-TK+Ad-Flt3L+TMZ. Five days after treatment, the cellular location of cherry-HMGB1 in these cells was assessed by confocal microscopy. Arrows, tumor cells (green) with cytoplasmic HMGB1 (red).

efficacy of Ad-TK+Ad-Flt3L in two additional brain tumor models. Systemic administration of temozolomide did not inhibit the antitumor immunity elicited by Ad-TK+Ad-Flt3L in mice bearing intracranial GL261 or B16 tumors (Supplementary Fig. S3A). We found that Ad-TK+Ad-Flt3L led to long-term survival of approximately 40% and approximately 25% of Gl261 and B16 tumor-bearing mice, respectively (Supplementary Fig. S3A), in accordance to our previous reports (6, 15). Treatment with Ad-TK+Ad-Flt3L significantly increased the circulating levels of soluble HMGB1 in both tumor models, and this effect was not affected by concomitant administration of temozolomide (Supplementary Fig. S3B). Considering that our findings indicate that only the treatment combinations that increased the circulating levels of HMGB1 led to therapeutic efficacy in the three models of brain cancer, we evaluated whether there was a correlation between the serum levels of HMGB1 and the median survival of tumor-bearing mice.

The correlation analysis between the circulating levels of HMGB1 (mean of each treatment group) and the median survival of each treatment group in the three different tumor models studied indicated that the efficacy of the treatment positively correlated (Pearson $r = 0.56$, $P < 0.05$) with the circulating levels of HMGB1 (Supplementary Fig. S4).

Effect of temozolomide on the infiltration of immune cells into the tumor mass induced by Ad-TK+Ad-Flt3L gene therapy

Treatment with Ad-TK+Ad-Flt3L stimulates the immune system to mount an antitumor immune response that detects and eradicates tumor cells spread throughout the brain. Considering that high doses of temozolomide could exert a lymphodepletive effect, we first evaluated the effect of temozolomide (100 mg/kg) administered for 7 and 14 days to GL26 tumor-bearing mice. Complete blood counts (CBC) and flow cytometry were performed to assess the levels of

Figure 4. Immune cell infiltration in GL26 brain tumors treated with temozolomide (TMZ) in combination with Ad-TK+Ad-Flt3L. Wild-type C57BL/6 mice were implanted in the striatum with GL26 glioma cells. Fourteen days after tumor implantation, mice were treated by intratumoral injection of Ad-Flt3L+Ad-TK followed by daily administration of GCV and temozolomide. Seven days later, tumor-infiltrating immune cells were purified and analyzed using flow cytometry. This experiment has been performed twice. A, scatter plots show the content of T cells in the tumor. Total immune cells were assessed by gating live cells with CD45, and then T cells were determined plotting against CD3, CD8, and CD4. B, scatter plots show the content of DCs in the tumor, which were assessed by live cells with CD45, then plotting against B220 and CD11c. pDC were identified as $CD11c^+$ $B220^+$ $CD45^+$ and cDC as $CD11c^+$ $B220^- CD45^+$. *, $P < 0.05$ versus saline; ^, $P < 0.05$ versus TF (one-way ANOVA followed by the Tukey test).
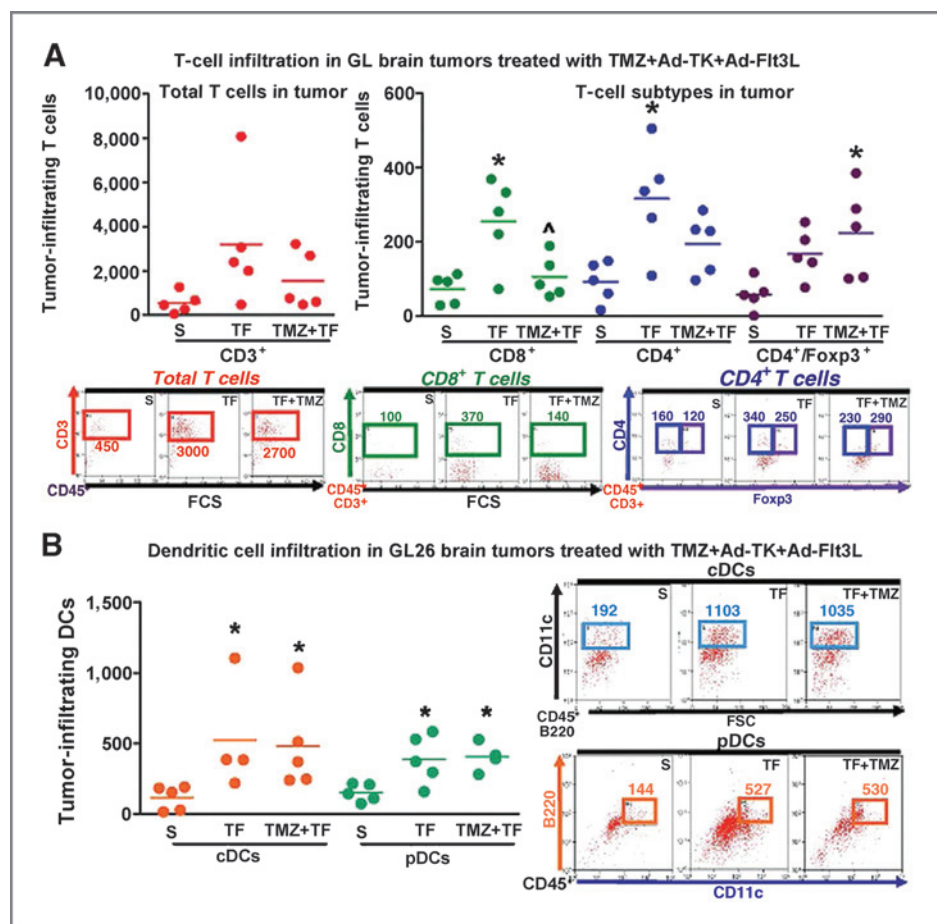
circulating lymphocytes and the degree of lymphodepletion. We found that temozolomide induced a profound lymphodepletion when administered for 7 or 14 days (Supplementary Fig. S5). CBC indicated that there was an approximately 95% reduction in circulating lymphocytes, and we determined by flow cytometry that the depletion affected both $CD4^+$ and $CD8^+$ T cells (Supplementary Fig. S5). We also observed a substantial reduction in the content of $CD4^+$ and $CD8^+$ T cells in the spleen and bone marrow of these mice (Supplementary Fig. S6). Our findings indicate that temozolomide exerts a lymphodepletive effect when administered for 7 or 14 days and they are in agreement with reports that show that high doses of temozolomide are lymphodepletive in humans and rodents (23, 24).

To evaluate the effect of temozolomide on the expansion and infiltration of immune cells induced by Ad-TK+Ad-Flt3L, we implanted GL26 cells in the brain of C57BL/6 mice and 14 days later, we treated them with an intratumoral injection of Ad-TK and Ad-Flt3L followed by systemic administration with temozolomide. Seven days later, immune cells were purified from the tumor and the spleen and analyzed by flow cytometry. We assessed the infiltration of T cells and DCs within the tumor mass. We found that Ad-TK+Ad-Flt3L treatment induced a significant increase in the number of tumor-infiltrating $CD8^+$ and $CD4^+$ T cells. Con-

comitant treatment with temozolomide reduced the infiltration of $CD8^+$ T cells and increased the infiltration of regulatory T cells (Fig. 4A). Temozolomide treatment did not affect the recruitment of plasmacytoid (pDCs) and conventional DCs (cDCs) induced by Ad-TK+Ad-Flt3L in the brain (Fig. 4B).

Mice treated with temozolomide in combination with Ad-TK+Ad-Flt3L exhibited an increased percentage of T cells in the spleen, specifically $CD8^+$ T cells, when compared with mice that received only gene therapy (Supplementary Fig. S7A and S7B). Addition of temozolomide to the treatment with Ad-TK+Ad-Flt3L did not affect the number of total B cells in the spleen (Supplementary Fig. S7D), but increased the content of Marginal Zone B cells (Supplementary Fig. S7E).

In view of our observations in tumor-infiltrating immune cells, we aimed to assess whether the antitumor effect of Ad-TK+Ad-Flt3L in combination with chemotherapy with temozolomide was dependent on the immune system. Thus, we implanted GL26 tumors in the brain of KO mice that lack specific immune cell populations and treated them with an intratumoral injection of Ad-TK+Ad-Flt3L and systemic temozolomide for 14 days (Fig. 5). The treatment with Ad-TK+Ad-Flt3L alone or in combination with temozolomide did not promote long-term survival in tumor-
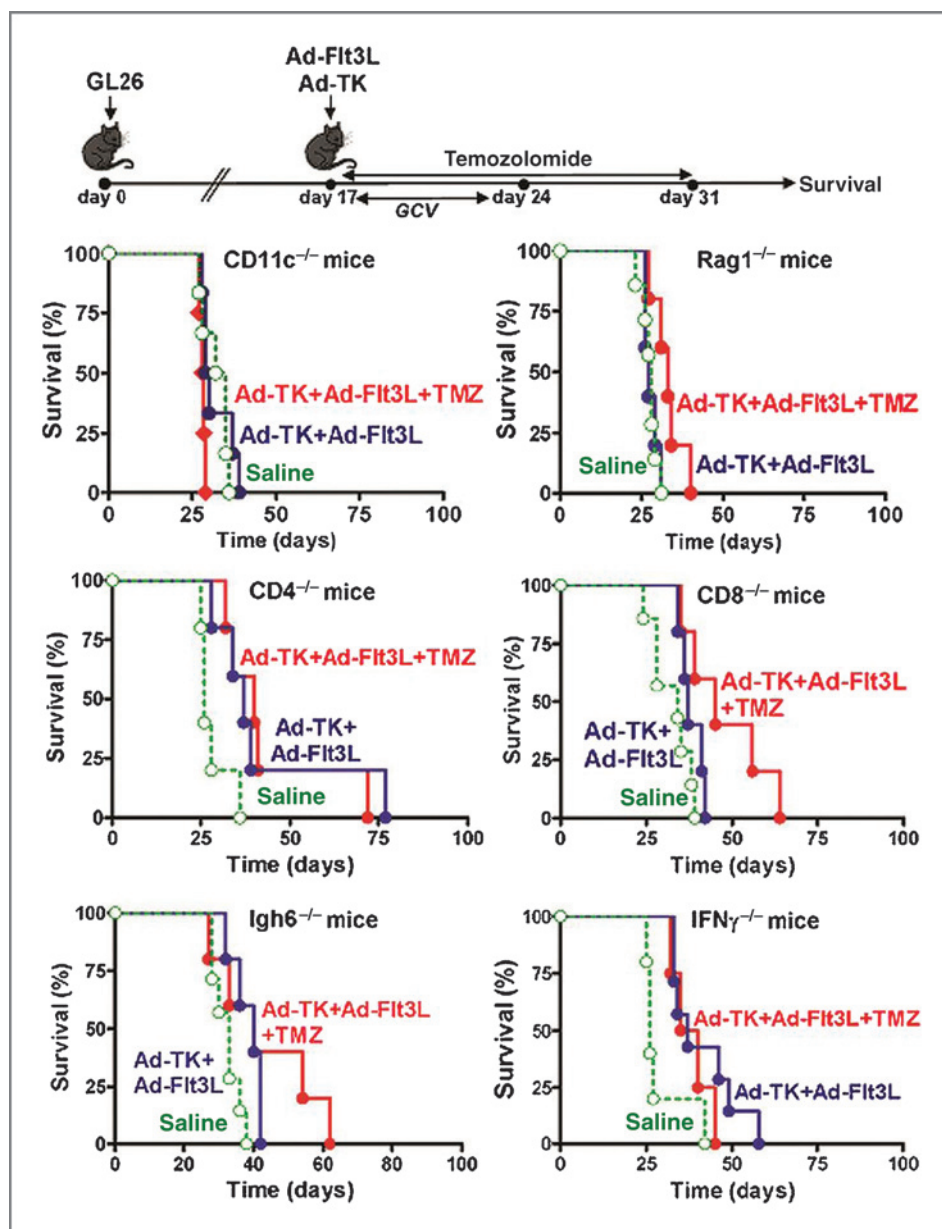
Figure 5. Role of immune cell populations in the antitumor effect of TMZ+Ad-TK+Ad-Flt3L. Kaplan–Meier curves show the survival of immune-deficient mice lacking DCs (CD11c$^{-/-}$), T and B cells (Rag1$^{-/-}$), CD4 or CD8 T cells (CD4$^{-/-}$, CD8$^{-/-}$), IFN-γ producing cells (IFN$\gamma^{-/-}$), or B cells (Igh6$^{-/-}$) that were implanted in the brain with GL26 tumors and were treated 14 days later Ad-Flt3L+Ad-TK and temozolomide (TMZ). This experiment has been performed twice.

bearing CD11c KO mice. The therapy with Ad-TK+Ad-Flt3L alone or in combination with temozolomide chemotherapy also failed to provide long-term survival in mice that lack T lymphocytes (Rag1 KO, CD4 KO and CD8 KO), B lymphocytes (Igh6 KO), or that were unable to produce IFN-γ (IFN-γ KO).

Thus, we conclude that lymphocytes and APCs are required for the antitumor effect of Ad-Flt3L+Ad-TK in combination with temozolomide. Despite its apparent immunosuppressive effect, temozolomide does not impair the efficacy of immunotherapy with Ad-Flt3L alone or in combination with Ad-TK. These findings suggest that immunotherapeutic approaches can be administered simultaneously with chemotherapy with temozolomide in patients with brain cancer.

Discussion

Although temozolomide has traditionally been ascribed with immunosuppressive activities, this notion has shifted, thus the use of immunotherapy in combination with chemotherapy may provide a boost to current therapeutic outcomes in patients with glioblastoma multiforme. Our aim was to assess whether chemotherapy, using temozolomide would impair the antitumor efficacy of immunotherapy with Ad-TK+Ad-Flt3L. Our results show that treatment with temozolomide does not reduce the efficacy of Ad-TK+Ad-Flt3L gene therapy in mouse models of brain cancer. Moreover, combination of temozolomide with intracranial administration of Ad-Flt3L significantly improved the median survival when compared with each treatment alone, leading to long-term

survival in 20% to 25% of mice bearing intracranial glioblastoma multiforme, whereas the mice that received temozolomide or Ad-Flt3L succumbed to tumor burden. Tallying with our results, it has been observed that chemotherapy with temozolomide enhances T-cell–dependent antitumor immunity induced by several immunotherapeutic strategies in murine models of brain cancer. Concomitant administration of temozolomide enhances the efficacy of tumor antigen-pulsed DC vaccines in mice bearing intracranial glioblastoma multiforme, whereas mice treated with either therapy alone fails (25, 26). Chemotherapy with temozolomide greatly enhanced the efficacy of CCL2 blockade using anti-CCL2 antibodies in GL261 glioma-bearing mice (27). Although single treatment with anti-CCL2 induced only a very modest increase in the median survival, addition of temozolomide treatment led to long-term survival in 80% of treated mice (27).

Although temozolomide seems to improve the efficacy of many immunotherapeutic approaches, it is well recognized that temozolomide induces lymphopenia in the majority of treated patients. Bone marrow cells seem to be particularly sensitive to temozolomide due to a low MGMT activity, which results in myelosuppression (25). Our findings indicate that administration of temozolomide may reduce the total number of $CD8^+$ T cells present within the tumor mass. A reduction in the circulating levels of $CD8^+$ T cells that was accompanied by a proportional increase in circulating regulatory T-cell (Treg) levels was also observed in patients with glioblastoma multiforme receiving systemic temozolomide treatment (28). Low-dose (0.2–5 mg/kg) chemotherapy treatment lowers the levels of tumor-infiltrating and splenic Tregs in rats bearing intracranial RG2 tumors, which was not observed with the standard treatment with temozolomide (10 mg/kg for 21 days or 30 mg/kg for 5 days; ref. 29). Our results show that treatment with higher doses of temozolomide did not inhibit the infiltration of Tregs, and in fact, concomitant treatment with temozolomide induced a slight increase in the intratumoral infiltration of Tregs. This has been also observed in patients with glioblastoma multiforme receiving dose-intensified treatment with temozolomide (100 mg/m^2 for 21 days; ref. 23). On the other hand, we found that temozolomide treatment did not affect the recruitment of cDCs and pDCs in the brain tumor induced by the immunotherapy and induced an expansion in the population of MZB cells in the spleen, which, as our previous results indicate, are required for the antitumor immunity induced by this treatment (15). Although temozolomide reduces the intratumor infiltration of $CD4^+$ and $CD8^+$ T cells induced by Ad-TK+Ad-Flt3L and increases the infiltration of Tregs, this chemotherapeutic agent did not abolish the therapeutic efficacy of the immunotherapy. Tallying with our findings, doses of 125 mg/kg in mice bearing intracranial melanoma metastasis were shown to lead to a profound reduction in circulating $CD4^+$ and $CD8^+$ T-cell (24). An increase in circulating levels of Tregs

was detected when brain metastasis-bearing mice were injected with high doses of temozolomide (30). However, an increase in serum levels of interleukin-2 that facilitated optimal T-cell expansion was also observed in these mice (24). It has been suggested that depletion of Tregs using neutralizing antibodies administered systemically may enhance the efficacy of temozolomide chemotherapy in combination with immunotherapeutic approaches (30).

A proliferative response of lymphocytes following temozolomide-induced lymphopenia has been proposed to favor the development of adaptive antitumor immunity (24). It has been recently reported that administration of myeloablative doses of temozolomide (125 mg/kg) to brain tumor-bearing mice leads to a profound and sustained lymphopenia, but stimulates the expansion of antigen-specific $CD8^+$ T cells in response to an antitumor vaccine, leading to an improve in median survival (24). T cells from mice receiving myeloablative doses of temozolomide exhibited enhanced secretion of proinflammatory cytokines when compared with mice treated with a lower nonmyeloablative dose of temozolomide (60 mg/mL; ref. 24). It has been proposed that lymphopenia may amplify the immune response by decreasing competition at the surface of APCs, which improves cytokine availability, amplifying T-cell function (25). Lymphodepletion may also reset the host's immune system, reducing the tolerance toward endogenous antigens (25).

Temozolomide may enhance the antitumor effect of the immunotherapy not only through its direct effects on the immune system, but also through indirect mechanisms that involve changes in tumor cells. In recent years, the induction of immunogenic cell death by proapoptotic agents has been involved in the stimulation of antitumor immunity (31). It has been observed that temozolomide increases the expression of proinflammatory molecules, such as calreticulin, on the surface of glioblastoma multiforme cells (26). It has been proposed that temozolomide could enhance the immunogenicity of tumor cells by generating novel antigens due to its mutagenic properties (32).

Our results indicate that treatment with temozolomide in combination with intracranial injection with Ad-Flt3L led to cytoplasmic translocation of HMGB1 and increased circulating levels of HMGB1, which may affect the onset of the antitumor immune response. We have previously shown that TLR2 activation of DCs via soluble HMGB1 released from apoptotic tumor cells is required for the antitumor immune response triggered by Ad-TK+Ad-Flt3L treatment (6). In fact, we found a positive correlation between circulating HMGB1 and survival in the tumor models studied here. These findings support the use of HMGB1 circulating levels as a noninvasive surrogate marker of therapeutic efficacy of cytotoxic/immunestimulatory strategies.

Treatment with temozolomide has been shown to enhance antitumor immunity when combined with immunotherapeutic approaches not only in murine models of glioblastoma multiforme, but also in patients with

glioblastoma multiforme undergoing clinical trials. Cervical intranodal vaccination with DCs pulsed with tumor lysates following radiotherapy and temozolomide chemotherapy resulted in an increased frequency of IFN-γ–positive CD4$^+$ T cells that was associated with prolonged survival of patients with glioblastoma multiforme (28). In a phase I clinical trial, patients with glioblastoma multiforme that received temozolomide chemotherapy following the administration of Trp2-pulsed DC vaccines exhibited slower tumor progression and prolonged survival than those receiving either therapy alone (33). In a phase II clinical trial undertaken to assess the immunogenicity of an EGFRvIII-targeted peptide vaccine, concomitant administration of temozolomide did not impair antitumor immunity. Despite developing a grade 2 to 3 lymphopenia, all patients with glioblastoma multiforme treated with temozolomide developed EGFRvIII-specific humoral and cellular immunity upon administration of a peptide vaccine (23). Combination of proapoptotic gene therapy and chemotherapy with temozolomide is also a valuable therapeutic strategy for the treatment of patients with glioblastoma multiforme. Intracranial administration of Ad-TK followed by GCV administration can kill glioblastoma multiforme cells even if they harbor functional MGMT, which confers resistance to temozolomide and can be detected in approximately 50% of patients with glioblastoma multiforme (34). This vector has proved safe when administered in the brain tumor bed of patients with glioblastoma multiforme during surgery in combination with radiotherapy and chemotherapy with temozolomide (35). A synergistic effect of Ad-TK and temozolomide has been observed, which seems to be related to the inhibition of DNA repair enzymes by phosphorylated GCV (36).

Although the models tested here evaluate the efficacy of the immune stimulant treatment during the temozolomide consolidation phase and we are not assessing the effect of concomitant radiotherapy, clinical trials suggest that addition of radiotherapy favors rather than impairs the therapeutic benefit of immunotherapies in patients with glioblastoma multiforme. In a pilot study, five of eight patients with glioblastoma multiforme exhibited antitumor immunity after receiving intradermal vaccines of tumor lysate-loaded DC following radiotherapy and temozolomide (37). In 10 patients with glioblastoma multiforme, DC vaccination following radiation and temozolomide led to tumor-specific immunity that was associated with prolonged survival (38). Results from a phase II clinical trial indicate that patients with glioblastoma multiforme receiving DC vaccines after radiation and chemotherapy have an improved survival compared with patients receiving the standard treatment alone (39). Our previous preclinical results suggest that both temozolomide and radiation induce tumor cell death that leads to the release of HMGB1 that activates TLR2 in tumor-infiltrating DCs recruited by Ad-Flt3L, which in turn trigger the antitumor immune response that eradicates intracranial glioblastoma multiforme (6). In patients with

glioblastoma multiforme, gene therapy vectors are injected in the tumor bed after neurosurgery. Administration of Ad-Flt3L in the brain of these patients could lead to long-term expression of Flt3L, which would elicit recruitment of APCs and lymphocytes. When temozolomide is administered to these patients, it will induce apoptosis of tumor cells that remain in the brain, further promoting the release of tumor antigens and proinflammatory molecules, such as HMGB1. This will in turn boost the activation of immune cells recruited in response to expression of Flt3L within the tumor cavity.

In summary, our results indicate that concomitant administration of temozolomide chemotherapy does not inhibit the antitumor effect of Ad-TK+Ad-Flt3L in murine models of brain cancer. These findings suggest that chemotherapy with temozolomide would not impair the antitumor immune response triggered by immune-stimulatory strategies. Our results support the administration of immunotherapeutic approaches in patients with brain cancer undergoing chemotherapy with temozolomide.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: K. Yagiz, P.R. Lowenstein, M.G. Castro
Development of methodology: K. Yagiz
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Candolfi, K. Yagiz, M. Wibowo, G.H. Ahlzadeh, M. Puntel, H. Ghiasi, N. Kamran, C. Paran, P.R. Lowenstein, M.G. Castro
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Candolfi, K. Yagiz, M. Wibowo, N. Kamran, P.R. Lowenstein, M.G. Castro
Writing, review, and/or revision of the manuscript: M. Candolfi, K. Yagiz, N. Kamran, P.R. Lowenstein, M.G. Castro
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Yagiz, P.R. Lowenstein, M.G. Castro
Study supervision: K. Yagiz, P.R. Lowenstein, M.G. Castro

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Temozolomide Does Not Impair Gene Therapy-Mediated Antitumor Immunity in Syngeneic Brain Tumor Models

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