Blockade of mTOR Signaling via Rapamycin Combined with Immunotherapy Augments Antiglioma Cytotoxic and Memory T-Cell Functions

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Abstract

The success of immunotherapeutic approaches targeting glioblastoma multiforme (GBM) demands a robust antiglioma T-cell cytotoxic and memory response. Recent evidence suggests that rapamycin regulates T-cell differentiation. Herein, we tested whether administration of rapamycin could enhance the efficacy of immunotherapy utilizing Fms-like tyrosine kinase 3 ligand (Ad-Flt3L) and thymidine kinase/ganciclovir (Ad-TK/GCV). Using the refractory rat RG2 glioma model, we demonstrate that administration of rapamycin with Ad-Flt3L + Ad-TK/GCV immunotherapy enhanced the cytotoxic activity of antitumor CD8⁺ T cells. Rats treated with rapamycin + Ad-Flt3L + Ad-TK/GCV exhibited massive reduction in the tumor volume and extended survival. Rapamycin administration also prolonged the survival of Ad-Flt3L + Ad-TK/GCV–treated GL26 tumor-bearing mice, associated with an increase in the frequency of tumor-specific and IFNγ⁺ CD8⁺ T cells. More importantly, rapamycin administration, even for a short interval, elicited a potent long-lasting central memory CD8⁺ T-cell response. The enhanced memory response translated to an increased frequency of tumor-specific CD8⁺ T cells within the tumor and IFNγ release, providing the mice with long-term survival advantage in response to tumor rechallenge. Our data, therefore, point to rapamycin as an attractive adjuvant to be used in combination with immunotherapy in a phase I clinical trial for GBM. Mol Cancer Ther; 13(12); 3024–36. ©2014 AACR.

Introduction

Malignant cancers of the central nervous system, i.e., glioblastoma multiforme grade 4 (GBM), are characterized by a high degree of immune suppression, mediated by high levels of TGFβ and/or IL10, which leads to an increase in T regulatory (Treg) cells, lack of lymphatic drainage, presence of the blood–brain barrier, and a paucity of antigen-presenting cells such as dendritic cells (DC) within the brain parenchyma (1–3). Taking into account these features, we developed a gene therapy–mediated immune-stimulatory approach for GBM, which relies on the expression of thymidine kinase (TK) that phosphor-ylates and activates the prodrug ganciclovir (GCV) inducing the death of actively proliferating tumor cells with the concomitant release of the endogenous TLR ligand, High-mobility group B1 protein (HMGB1); and Fms-like tyrosine kinase 3 ligand (Flt3L) that increases the recruitment of DCs to the tumor microenvironment (4–7). HMGB1 induces the maturation of DCs with the upregulation of costimulatory ligands and MHC II molecules and release of inflammatory cytokines such as IL12 and IFNγ, polarizing them toward an activated phenotype (8). The activated DCs subsequently prime antitumor T-cell responses in the draining lymph nodes (dLN). Cytotoxic CD8⁺ T cells are well recognized to mediate effective antitumor immunity (9). In addition, TH1 skewed CD4⁺ T cells also play an important role in enhancing the CD8⁺ T cell–mediated antitumor immunity. DC vaccination strategies tested in glioma patients in phase I clinical trials demonstrated significant cytotoxic T cell and memory T-cell infiltration in areas of intracranial tumor (10, 11). Efforts have also been made to enhance the potency and selectivity of antiglioma immune responses by adoptive immune therapy utilizing tumor-infiltrating lymphocytes (12). A 50% reduction in tumor size was observed in 2 out of 5 patients who were administered cytotoxic T lymphocytes generated by treatment with IL2 and autologous glioma cells (13). Clinical trials have also tested the potency of cytotoxic cells generated by stimulation with phytohaemagglutinin, anti-CD3 antibodies,
and IL2 or through the stimulation of peripheral blood lymphocytes with IFNγ, IL2, anti-CD3 antibodies, and IL16 (14, 15). Our own data have shown that modulating the T-cell infiltration into the tumor microenvironment and enhancing T-cell effector functions through the overexpression of IFNγ or IL2 or inhibition of NF-κB signaling extended the median survival of RG2 tumor-bearing rats as compared with Ad-Flt3L + Ad-TK/GCV–treated group (16). Overexpression of IFNγ resulted in upregulation of MHC I expression on tumor cells potentially increasing tumor antigen presentation to T cells. Addition of Ad-IL-2 to the Ad-Flt3L + Ad-TK/GCV immunotherapy enhanced the ratio of cytotoxic T lymphocytes/Tregs resulting in augmented antitumor CD8⁺ T-cell responses. Similar effects were obtained by the inhibition of NF-κB signaling that resulted in reduced Foxp3 expression in CD4⁺ T cells and enhanced IFNγ release by CD8⁺ T cells (17).

An antitumor immune response that elicits tumor regression and long-term survival requires the recruitment and expansion of powerful antitumor cytotoxic T lymphocytes. In addition, generation of antitumor memory T-cell response is required for averting tumor relapse, and it has been suggested that memory CD8⁺ T cells may determine the efficacy of antitumor therapies (18, 19). An agent that has recently been shown to regulate the magnitude and quality of T-cell responses is rapamycin (20). Rapamycin is a prototypic mTOR inhibitor that has been widely used clinically to prevent renal allograft rejection and it has been suggested that memory CD8⁺ T cells and enhanced IFNγ release by CD8⁺ T cells.

Promotes the development of CD8⁺ memory T-cell response. Rapamycin administration for a short duration results in eliciting a vigorous long-lasting antitumor memory response. Consequently, tumor-bearing animals treated with immunotherapy in the presence of rapamycin show better survival and respond more strongly to a second tumor challenge.

Materials and Methods

Syngeneic glioma models

All animal experiments were performed in accordance with protocols approved by the University Committee on Use and Care of Animals (UCUCA) at the University of Michigan. RG2 cells (20,000 in 3 μL DMEM) were stereotactically implanted in the right striatum of syngeneic Fisher rats (220–250 g; Harlan Laboratories Inc.) 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 (16, 31). GL26 cells expressing ovalbumin were prepared as before (32). Of note, 60,000 GL26-OVA cells in 1 μL DMEM were stereotactically implanted in the right striatum of syngeneic C57BL/6 mice (5–7 week; Harlan Laboratories Inc.) at the coordinates of 0.5 mm anterior and 2.1 mm lateral from the bregma and 3.2 mm ventral from the dura. Five days after tumor implantation, Fisher rats received an intratumoral injection of 3 μL of Ad vectors stereotactically using the same burr hole in four locations at 7.0 mm, 6.0 mm, 5.0 mm, and 4.0 mm ventral from the dura for the RG2 glioma model. C57BL/6 mice received 1.5 μL of Ad vectors in three locations at 3.8 mm, 3.5 mm, and 3.2 mm ventral from the dura at 14 days after tumor implantation. Animals were treated with 5 × 10⁶ pfu of Ad-Flt3L or 1 × 10⁸ pfu of Ad-TK or both Ad-Flt3L and Ad-TK. Animals treated with Ad-TK also received 25 mg/kg GCV twice daily for 7 days (mice) or 10 days (rats) starting the day after vector injection.

Rapamycin administration

Rapamycin (1.5 mg/kg; LC Laboratories) was administered intraperitoneally (i.p.) to the animals daily.

Quantification of DC maturation status

Antibodies for flow cytometry were obtained from BD Biosciences unless indicated otherwise. List of antibody clones used are indicated in Supplementary Table S1. Flt3L-derived DCs (1 × 10⁶) were incubated for 48 hours in media only or in the presence of 50 ng/mL of LPS (Invitrogen) or 10 ng/mL rapamycin + 50 ng/mL LPS. Forty-eight hours later, DC maturation markers CD80, CD86, MHC II, CD40, and CD25 were analyzed by flow cytometry. BMDCs were stained in FACS buffer (2% FBS in PBS) with anti-rat antibodies CD80, CD86, RT1B (MHC type II), CD40 (eBioscience), and CD25 for 30 minutes at 4°C. Data were acquired using a FACSscan flow cytometer and analyzed with Summit software v4.3. Live cells (50,000) were recorded for each sample. Release of IL10, IFNγ and IL12 from BMDC was determined in culture supernatant by ELISA (IL10 and IFNγ, R&D Systems;
IL12, Invitrogen). Absorbance was read on Spectramax Plus (Molecular Device) plate reader.

**Antigen-specific effector and memory T-cell functions**

Memory T cells in RG2 tumor–bearing rats were analyzed at day 33 after tumor implantation. Single-cell suspensions from spleen and dLNs were prepared as before followed by staining with CD3, CD8, CD45RC, and CD62L antibodies (7). Memory responses were visualized by staining for CD45RC⁺ CD62L⁺ high cells among the CD3⁺ CD8⁺ T cells in the spleen. At least 50,000 live cells (determined by FSC/SSC) were acquired for each spleen and LN sample. For GL26-OVA model, 24 days or 89 days after tumor implantation or 5 days after the second tumor challenge at 90 days (day 95), brains, spleens, and dLNs were harvested from mice. Tumor-infiltrating immune cells were isolated as described in Supplementary Methods. Cells were counted and labeled with CD8 and CD3 antibodies. Stained cells were incubated with SIINFEKL-H2Kb-tetramer (Beckman coulter) for 30 minutes at room temperature after which cells were washed and analyzed by flow cytometry. Intracellular IFNγ staining was performed using the BD Cytofix/Cytoperm kit (BD Biosciences). Antigen-specific memory responses were visualized by staining for CD44⁺ high CD62L⁺ high cells among the tetramer⁺ CD3⁺ CD8⁺ T cells in the spleen and dLNs of immunized mice. Data were acquired using a FACScan (Beckton Dickinson) flow cytometer and analyzed using Summit software v4.3. At least 20,000 tetramer⁺ CD8⁺ T cells were acquired for each sample.

**Statistical analysis**

All statistical analysis was carried out using the GraphPad Prism software (version 5.0a). Kaplan–Meier survival curves were analyzed using the Mantel log-rank test. Costimulatory ligand expression and cytokine release from DCs was analyzed by one-way ANOVA followed by Bonferroni post-test. Antigen-specific cytokine and memory CD8⁺ T-cell responses were analyzed using one-way ANOVA followed by Bonferroni post-test. *P* values of <0.05 were considered to be significant.

**Results**

**Rapamycin enhances therapeutic efficacy of Ad-Flt3L + Ad-TK/GCV–mediated gene therapy in the RG2 intracranial glioma model**

Rapamycin and its analogs have exhibited clinical benefits against tumors such as endometrial and renal cancer either through a direct growth-inhibitory effect on cancer cells or through its ability to determine T-cell fate (33). To test whether rapamycin could further enhance the antitumor immunity elicited by Ad-TK/GCV or Ad-TK/GCV + Ad-Flt3L gene therapy, rats were implanted with RG2 tumors, and 5 days after tumor implantation, Ad-TK/GCV alone or the combination Ad-Flt3L + Ad-TK/GCV immune-mediated gene therapy was initiated. Rats were also treated with rapamycin beginning 5 days after tumor implantation until day 40 (Fig. 1A). Administration of Ad-TK/GCV gene therapy to the tumor-bearing rats resulted in increase in their median survival period of 19.5 days (saline treated) to 32 days (*P* <0.01, Fig. 1B). The median survival time of the animals treated with the Ad-Flt3L + Ad-TK/GCV immunotherapy was also significantly enhanced from 19.5 days (saline treated) to 36 days (*P* <0.01, Fig. 1D). In addition, combining rapamycin administration with Ad-Flt3L + Ad-TK/GCV immunotherapy resulted in an additional increase in the median survival time of tumor-bearing rats to 47 days compared with 36 days for the Ad-Flt3L + Ad-TK/GCV immunotherapy alone–treated group (*P* <0.001, Fig. 1D). In fact, approximately 89% ±10% of the RG2 tumor–bearing rats treated with rapamycin and immunotherapy survived beyond day 42 when all tumor-bearing rats treated with immunotherapy alone had perished (Fig. 1D). Consistent with the increased survival, rats treated with Ad-Flt3L + Ad-TK/GCV therapy or rapamycin in combination with Ad-Flt3L + Ad-TK/GCV showed a drastic reduction in the tumor volume at day 12 as compared with the saline-treated group (*P* <0.01, Fig. 1E). The difference in tumor volume was even more apparent at day 33 when the average tumor volume for Ad-Flt3L + Ad-TK/GCV–treated animals was 77.41 ± 26.01 mm³, whereas rats treated with rapamycin + Ad-Flt3L + Ad-TK/GCV showed an average tumor volume of 3.1 ± 0.58 mm³. In contrast, rapamycin administration during Ad-TK/GCV cytotoxic gene therapy failed to further increase the survival of Ad-TK/GCV–only–treated mice, suggesting that rapamycin potentially modulates the antitumor immune-surveillance mechanisms mediated by Flt3L immunotherapy (Fig. 1B). Animals treated with rapamycin alone also showed a significant increase in their survival period (24 days) compared with saline-administered rats (19.5 days), indicating a direct effect of rapamycin on tumor growth (*P* <0.01, Fig. 1B and D). To examine the effect of rapamycin on tumor cells, RG2 cells were treated with a combination of rapamycin (0–100 nmol/L) and Ad-TK (MOI = 0, 20, 200) and 24 hours later, incubated with 25 μmol/L GCV for an additional 48 hours. Cell viability was assessed by annexin V/PI staining. As positive controls for annexin V and PI staining, cells treated with staurosporine or cells subjected to freeze-thaw cycles were used respectively. Treatment with staurosporine resulted in an increase in annexin V⁻ cells (apoptosis), multiple cycles of freeze-thawing caused an increase in PI⁺ cells (necrosis/late apoptosis). Annexin V/PI double-positive cells were increased under both the treatments (Supplementary Fig. S1). Both Ad-TK/GCV and rapamycin treatment of RG2 cells led to a progressive increase in the percentage of apoptotic cells (annexin V⁺–positive cells) in a dose-dependent manner. In the absence of Ad-TK, rapamycin treatment of RG2 cells resulted in approximately 57% reduction in cell viability (*P* <0.001 vs. 0 nmol/L rapamycin, Fig. 1C). At the same time, while Ad-TK/GCV
Rapamycin significantly reduced RG2 cell viability, rapamycin treatment resulted in a further decrease in RG2 cell viability (P < 0.001, vs. 0 nmol/L rapamycin at 20 MOI Ad-TK, Fig. 1C). We also performed Western blot analysis on rapamycin-treated RG2 cells. The two most characterized downstream regulators activated by mTOR signaling are the eIF4E binding proteins (4E-BP) and the ribosomal proteins p70S6 kinase. Tumor cells were first starved of growth factors and then treated with rapamycin before stimulation with 20% FCS. Similar to what has been shown before, whereas rapamycin's inhibition led to the dephosphorylation of p70S6 kinase, it did not target 4E-BP1 phosphorylation status (Supplementary Fig. S2; ref. 34).

Rapamycin affects cell surface markers and cytokine profiles of Flt3L-induced DCs

Rapamycin has previously been shown to exert immunosuppressive effects on DCs, and bone marrow–derived DCs generated in its presence show reduced production of proinflammatory cytokines and impaired ability to induce allogeneic T cell responses (35, 36). In contrast, several studies have shown that rapamycin promotes proinflammatory cytokine release by DCs and enhances...
Rapamycin enhances effector and memory CD8+ T-cell function induced by Ad-Flt3L + Ad-TK/GCV

Because rapamycin promoted an increase in T<sub>H1</sub> cytokine release from DCs, which fosters the development of cytotoxic T lymphocyte–mediated immune response against pathogens and tumors, we examined the impact of rapamycin administration on T-cell activation in rapamycin + Ad-Flt3L + Ad-TK/GCV–treated brain tumor-bearing rats. CD8<sup>+</sup> T cells from the dLN<sub>s</sub> of rapamycin + Ad-Flt3L + Ad-TK/GCV–treated tumor-bearing animals were harvested on day 12 and tested for their cytolytic activity toward RG2 cells (Fig. 3A). At day 12, the tumor volumes in Ad-Flt3L + Ad-TK/GCV only group are similar to the tumor volumes of rats treated with the combination therapy. Cytotoxicity of T cells isolated from animals treated with gene therapy in the presence of rapamycin was observed to be significantly higher (P < 0.05, 61.27% ± 1.5% target cell lysis at 20:1 E:T ratio) when compared with the gene therapy alone–treated group (51.07% ± 1.02% target cell lysis), indicating that rapamycin enhanced the cytotoxic activity of tumor-infiltrating T cells (Fig. 3B). In addition, rapamycin elicited an increase in the percentage of central memory CD8<sup>+</sup> T cells in the spleen of tumor-bearing rats treated with Ad-TK/GCV + Ad-Flt3L immunotherapy compared with the immunotherapy alone group (Fig. 3C).

Rapamycin is required at an early stage to enhance therapeutic efficacy of Ad-Flt3L-Ad-TK/GCV immunotherapy

The duration and timing of rapamycin administration has been shown to influence the development of CD8<sup>+</sup> T-cell memory response such that a short duration of high-dose rapamycin promoted CD8<sup>+</sup> T-cell persistence, whereas a prolonged high-dose regimen abolished memory responses (27, 39). Therefore, to facilitate the development of a therapeutic regimen that effectively supports memory CD8<sup>+</sup> T-cell responses; we next characterized the impact of the timing of rapamycin administration in our system. We administered rapamycin to Ad-Flt3L + Ad-TK/GCV–treated rats at two different time points after tumor implantation (Fig. 4A). Interestingly, we observed that rapamycin could enhance the therapeutic efficacy of the Ad-Flt3L + Ad-TK/GCV immunotherapy only when it was administered at the initiation of the therapy (Fig. 4B). Rapamycin administration during the initiation of immunotherapy (between days 5 and 20 after tumor implantation) enhanced the survival of Ad-Flt3L + Ad-TK/GCV–treated rats from 35 to 60 days (P < 0.001, Fig. 4B). In contrast, rapamycin administration after the initiation of immunotherapy (between days 20 and 40) failed to potentiate the efficacy of the immunogene therapy (P > 0.05 vs. Ad-Flt3L + Ad-TK/GCV, Fig. 4C). Previous data from our laboratory have shown that the generation of T-cell response following immunogene therapy peaks within 7 days after gene therapy administration; the peak could be prolonged by rapamycin administration and the presence of rapamycin could enhance the Flt3L-mediated recruitment and activation of DCs and priming of the anti-GBM T-cell response. To examine the effect of rapamycin on DC activation, expression of costimulatory molecules, and cytokine release from rapamycin-treated Flt3L-derived bone marrow–derived DCs was monitored in the presence of LPS. Generation of plasmacytoid DCs (pDC) and conventional DCs (cDC) in mouse bone marrow–derived DCs was monitored by staining for B220 and CD11c markers on the cells obtained 7 days after Flt3L stimulation (Supplementary Fig. S3). However, due to the lack of a definitive marker for rat pDCs, FSC/SSC profiles were used to identify total DCs. LPS stimulation upregulated the expression of CD86, CD80, and CD40 on DCs compared with the media-treated group (P < 0.001, Fig. 2A). The expression levels of CD86, CD80, and CD40 were slightly enhanced in the presence of rapamycin by 1.5, 1.3, and 1.2-fold, respectively, compared with the LPS-treated group (P < 0.001 vs. LPS, Fig. 2A). In contrast, rapamycin stimulation did not enhance LPS-induced CD25 and MHC II expression on DCs (Fig. 2A). Because cytokines produced by DCs are crucial for the polarization of CD4<sup>+</sup> T cells and the subsequent generation of CD8<sup>+</sup> T–mediated adaptive immunity, we next examined the effect of rapamycin on the proinflammatory (IFN<sub>γ</sub> and IL12) and immune-suppressive (IL10) cytokine release by DCs. Whereas rapamycin + LPS stimulation resulted in a marginal decrease in IFN<sub>γ</sub> secretion (1.1 fold vs. LPS, P < 0.001), rapamycin + LPS treatment boosted the levels of IL12 (1.2 fold vs. LPS, P < 0.001) and decreased IL10 release (2 fold vs. LPS, P < 0.001) by the DCs (Fig. 2B). Overall, the presence of rapamycin during stimulation of DCs with LPS, therefore, strongly enhanced the T<sub>H1</sub>/T<sub>H2</sub> cytokine ratio (P < 0.001, vs. LPS), indicating a bias toward immune-stimulatory cytokine release. These results prompted us to test whether rapamycin could also enhance the therapeutic efficacy of antigenic glioma DC vaccine. Animals vaccinated with DCs loaded with Ad-TK/GCV–treated tumor cell lysate before tumor cell implantation (Supplementary Fig. S4A) exhibited increased circulating anti-RG2 antibodies (~4-fold increase in relative fluorescence vs. saline, P < 0.001, Supplementary Fig. S4B) and increased cytotoxic activity against tumor cells (42.90% ± 1.25% target cell lysis) when compared with the saline-vaccinated controls (32.67% ± 0.24% target cell lysis, P < 0.01, Supplementary Fig. S4C). However, DC vaccination failed to improve the survival of tumor-bearing rats (Supplementary Fig. S4D). Interestingly and in agreement with the effect of rapamycin on DCs observed in vitro, animals immunized with the DC vaccine in the presence of rapamycin (Supplementary Fig. S4E) showed a significant increase in their median survival time (26 days vs. 21 days for saline, P < 0.001, Supplementary Fig. S4F).

their T-cell activation potential (37, 38). The increase in therapeutic efficacy observed after rapamycin coadministration with Ad-Flt3L + Ad-TK/GCV treatment of RG2 intracranial glioma suggested that rapamycin could enhance the Flt3L-mediated recruitment and activation of DCs and priming of the anti-GBM T-cell response. To examine the effect of rapamycin on DC activation, expression of costimulatory molecules, and cytokine release from rapamycin-treated Flt3L-derived bone marrow–derived DCs was monitored in the presence of LPS. Generation of plasmacytoid DCs (pDC) and conventional DCs (cDC) in mouse bone marrow–derived DCs was monitored by staining for B220 and CD11c markers on the cells obtained 7 days after Flt3L stimulation (Supplementary Fig. S3). However, due to the lack of a definitive marker for rat pDCs, FSC/SSC profiles were used to identify total DCs. LPS stimulation upregulated the expression of CD86, CD80, and CD40 on DCs compared with the media-treated group (P < 0.001, Fig. 2A). The expression levels of CD86, CD80, and CD40 were slightly enhanced in the presence of rapamycin by 1.5, 1.3, and 1.2-fold, respectively, compared with the LPS-treated group (P < 0.001 vs. LPS, Fig. 2A). In contrast, rapamycin stimulation did not enhance LPS-induced CD25 and MHC II expression on DCs (Fig. 2A). Because cytokines produced by DCs are crucial for the polarization of CD4<sup>+</sup> T cells and the subsequent generation of CD8<sup>+</sup> T–mediated adaptive immunity, we next examined the effect of rapamycin on the proinflammatory (IFN<sub>γ</sub> and IL12) and immune-suppressive (IL10) cytokine release by DCs. Whereas rapamycin + LPS stimulation resulted in a marginal decrease in IFN<sub>γ</sub> secretion (1.1 fold vs. LPS, P < 0.001), rapamycin + LPS treatment boosted the levels of IL12 (1.2 fold vs. LPS, P < 0.001) and decreased IL10 release (2 fold vs. LPS, P < 0.001) by the DCs (Fig. 2B). Overall, the presence of rapamycin during stimulation of DCs with LPS, therefore, strongly enhanced the T<sub>H1</sub>/T<sub>H2</sub> cytokine ratio (P < 0.001, vs. LPS), indicating a bias toward immune-stimulatory cytokine release. These results prompted us to test whether rapamycin could also enhance the therapeutic efficacy of antigenic glioma DC vaccine. Animals vaccinated with DCs loaded with Ad-TK/GCV–treated tumor cell lysate before tumor cell implantation (Supplementary Fig. S4A) exhibited increased circulating anti-RG2 antibodies (~4-fold increase in relative fluorescence vs. saline, P < 0.001, Supplementary Fig. S4B) and increased cytotoxic activity against tumor cells (42.90% ± 1.25% target cell lysis) when compared with the saline-vaccinated controls (32.67% ± 0.24% target cell lysis, P < 0.01, Supplementary Fig. S4C). However, DC vaccination failed to improve the survival of tumor-bearing rats (Supplementary Fig. S4D). Interestingly and in agreement with the effect of rapamycin on DCs observed in vitro, animals immunized with the DC vaccine in the presence of rapamycin (Supplementary Fig. S4E) showed a significant increase in their median survival time (26 days vs. 21 days for saline, P < 0.001, Supplementary Fig. S4F).
administration. Our data, therefore, suggested that rapamycin mediates its effects by modulating TK/Flt3L-mediated effector phase of the antitumor immune response and was crucially required during the expansion phase of the effector T cells.

**Rapamycin enhances antigen-specific cytotoxic T-cell activity induced by Ad-Flt3L + Ad-TK/GCV in a mouse syngeneic intracranial GBM model**

Our data with the RG2 glioma model indicated that rapamycin enhances the therapeutic efficacy of Ad-Flt3L + Ad-TK/GCV immune-mediated gene therapy by modulating the generation of effector and memory T-cell responses. To examine the antigen specificity of the cytotoxic and memory T-cell response in more detail, we made use of the GL26 syngeneic glioma model in mice. GL26-OVA cells expressing the surrogate tumor antigen ovalbumin were implanted in the striatum of wild-type C57BL/6 mice and treatment was initiated as indicated (Fig. 5A). The induction of antigen-specific cytotoxic CD8\(^+\) T-cell responses and immunologic memory were visualized using the ovalbumin-derived peptide epitope SIINFEKL-H2K\(^b\) tetramer. Flow cytometric analysis of tumor-infiltrating lymphocytes labeled with anti-CD8 antibodies and SIINFEKL-H2K\(^b\) tetramers revealed more ovalbumin-specific CD8\(^+\) T cells in the Ad-Flt3L + Ad-TK/GCV immunotherapy–treated mice (\(P < 0.001\) vs. saline-treated group, Fig. 5B). Although combining rapamycin with the immunogene therapy did not further increase the percentage of tetramer\(^+\) CD8\(^+\) T cells in the brain, a significant increase in the frequency of CD8\(^+\) T-producing IFN\(\gamma\) was observed in these mice (\(\approx 2\)-fold change vs. immunotherapy alone, \(P < 0.01\)) indicating an increase
in the activity of CD8<sup>+</sup> T cells (Fig. 5C). Consistent with our previous results, Ad-Flt3L + Ad-TK/GCV immunotherapy induced ovalbumin-specific CD8<sup>+</sup> T cells in the dLN and spleen, respectively. Additional rapamycin treatment further enhanced the percentage of ovalbumin-specific central memory CD8<sup>+</sup> T cells in the spleen of tumor-bearing mice by approximately 1.3 fold (P < 0.05 vs. immunotherapy alone, Fig. 5D). A similar trend was observed for cells from the dLNs (P > 0.05 vs. immunotherapy alone, Fig. 5E). Consistent with the increased cytotoxic T-cell activity against tumor, rapamycin coadministration prolonged the survival of GL26-OVA tumor-implanted mice, such that 62% of mice treated with rapamycin + Ad-Flt3L + Ad-TK/GCV survived beyond day 60 compared with 41% from the immunotherapy treatment alone group (P < 0.05, Fig. 5F).

**Rapamycin enhances antigen-specific memory T-cell response against glioma**

In most cases, GBM recurs after treatment (40); thus, development of immunologic memory response capable of recognizing brain tumor antigen, leading to the rejection of recurrent GBM becomes crucial for preventing relapse. Analysis of spleens and dLNs 10 days after tumor implantation showed that rapamycin administration induced higher percentage of antigen-specific memory CD8<sup>+</sup> T cells in the spleens of Ad-Flt3L + Ad-TK/GCV- treated GL26-OVA tumor-bearing mice (Fig. 5D). Rapamycin treatment alone failed to enhance the tumor-specific effector or memory T-cell responses and consequently provided no survival benefit.
memory response, mice that survived the first tumor were implanted with a second tumor in the contralateral hemisphere as indicated in the figure (Fig. 6A). One day before the second tumor challenge (day 89), spleen and dLNs of these mice were examined for the presence of antigen-specific memory CD8+ T cells. Mice treated with the combination of rapamycin + Ad-Flt3L + Ad-TK/GCV showed ~1.5-fold higher percentages of antigen-specific CD44high CD62Lhigh tetramer− CD8+ T cells in the spleen and dLNs (P < 0.05 vs. immunotherapy alone, Fig. 6B and C). When rechallenged with tumor, mice also showed increased percentages of tetramer− CD8+ T cells in the brain (~1.3 fold, P < 0.001 vs. immunotherapy alone, Fig. 6D). Furthermore, tumor-infiltrating CD8+ T cells from rapamycin + Ad-Flt3L + Ad-TK/GCV-treated mice produced IFNγ at a 3-fold higher frequency than those treated with immune gene therapy alone (P < 0.001, Fig. 6E).

Rapamycin administration in combination with Ad-Flt3L + Ad-TK/GCV also conferred long-term survival advantage in response to the second tumor challenge; 100% of the mice in this treatment group survived beyond 90 days after the second tumor challenge compared with 60% of the mice from the immune gene therapy alone group (Fig. 6F).

Discussion

Because of the limited success of the current treatment modalities for patients with GBM, there is an urgent need to develop novel therapies; particularly those that can inhibit tumor relapse. We have previously shown that immune-mediated gene therapy utilizing adenovirus-expressing Flt3L and TK followed by GCV administration exhibits effective antitumor immunity and results in tumor regression in several mouse and rat glioma models (4, 6, 41). An important factor determining the success of these therapeutic approaches is the ability to harness the memory T-cell response. Recently, there has been considerable interest in the mTOR pathway as a determinant of T-cell fate. In particular, several studies have highlighted the use of the mTOR inhibitor rapamycin to modulate CD8+ T-cell memory phenotype. Herein, we provide evidence that rapamycin potentiates the therapeutic efficacy of the immune-mediated gene therapy in two syngeneic, intracranial glioma models, RG2 and GL26. We demonstrate that coadministration of rapamycin with Ad-Flt3L + Ad-TK/GCV enhances the tumor antigen–specific cytotoxic CD8+ T-cell responses. Furthermore, rapamycin strongly favors the generation of a robust memory CD8+ T-cell response that, in turn, boosts the adaptive immune response against a second tumor challenge and confers a survival advantage to the mice.

Although rapamycin has traditionally been used as an immunosuppressive agent, reports have shown that it can enhance the effect of vaccines targeting bacterial and viral infections (27, 29). We observed that rapamycin upregulated the LPS-induced expression of costimulatory ligands CD80 and CD86 on Flt3L-derived DCs. At the same time, rapamycin stimulation promoted the release
Figure 5. Rapamycin (rapa) enhances antigen-specific effector T-cell function in GL26-induced glioma in mice. A, experimental design to assess the effect of rapamycin on antigen-specific effector and memory T-cell function in GL26-induced glioma. C57BL/6 mice were implanted with 20,000 GL26-OVA in the right striatum of the brain and, 14 days later, animals received Ad-Flt3L + Ad-TK/GCV treatment in the presence (indicated as F/T + rapa) or absence (indicated as F/T) of rapamycin administration for 28 days. GL26-OVA tumor-bearing mice were also treated with saline or rapamycin only as controls. B and C, flow cytometric analysis measuring OVA-specific CD8+ T cells (B) and SIINFEKL-induced IFN-γ production by CD8+ T (C) cells in brain tumor 24 days after tumor implantation. D and E, OVA-specific central memory CD8+ T (CD44+ CD62Lhigh/CD3+ tetramer+ ) cells in spleen (D) and dLN (E) at day 24. Data in B–E were analyzed using one-way ANOVA, mean ± SEM are indicated; **, P < 0.01; ***, P < 0.005, ns, not significant. F, Kaplan–Meier survival curves of mice bearing tumors treated with saline or rapamycin or Ad-Flt3L + Ad-TK (Flt3L/TK) or Ad-Flt3L + Ad-TK + rapamycin (Flt3L/TK + rapa), n = 5 animals per group. Survival curves were compared using the Mantel–Cox test; *, P < 0.05; **, P < 0.005.
Figure 6. Rapamycin (rapa) enhances antigen-specific memory T-cell function in GL26-induced glioma in mice. A, Ad-Flt3L + Ad-TK/GCV–treated mice from Fig. 5 that survived to 90 days were given a second tumor challenge in the left striatum. Brains, lymph nodes, and spleens were collected at indicated time points. B and C, percentage of CD44+CD62Lhigh population among tet+CD8+ T cells in spleen (B) and dLNs (C) at day 89 are shown. D and E, OVA-specific CD8+ T cells (D) and SIINFEKL–induced IFNγ production by CD8+ T cells (E) in brain tumors 5 days after the second challenge (at day 95). Data were compared using an unpaired two-tailed Student t test. Mean ± SEM is indicated; *, P < 0.05; **, P < 0.01; and ***, P < 0.005. F, survivors were rechallenged at day 90 without further treatment. Survival curves were compared using the Mantel–Cox test.
of Th1-polarizing cytokines by LPS-stimulated DCs, indicating that rapamycin favors the development of the Th1 phenotype. Consistent with the effect of rapamycin on DCs, we observed that administration of rapamycin at the time of DC vaccination prolonged the survival of RG2 tumor-bearing rats. In addition, rapamycin has been shown to enhance the life span of activated DCs, thus increasing the duration of their interaction with T cells. The shortened life span of activated DCs is one of the factors responsible for the modest success of DC vaccination in clinics and could be why DC vaccination alone failed to improve the survival of RG2 tumor-bearing rats in the absence of rapamycin in our system (26). Apart from its effects on DC maturation and kinetics, rapamycin has been shown to influence CD4\(^{+}\) T-cell fate and memory CD8\(^{+}\) T-cell development (19, 20, 27, 42). We thus tested whether rapamycin could enhance the therapeutic efficacy of the Flt3L/TK immunogene therapy, which relies on the recruitment of antigen-presenting cells to the tumor environment and priming of T-cell responses (5, 7). We observed that combining rapamycin administration with Ad-Flt3L + Ad-TK/GCV gene therapy led to a remarkable increase in the survival of tumor-bearing animals in both RG2 (rat) and GL26 (mice) glioma models. Rapamycin by itself can induce apoptosis and, consistent with previous studies, it increased the sensitivity of RG2 cells to Ad-TK/GCV and showed a marginal increase in the survival of tumor-bearing animals as compared with the saline-treated group. However, rapamycin-induced tumor cell apoptosis does not appear to be the primary mechanism contributing to tumor regression as no significant difference in survival between Ad-TK/GCV and Ad-TK/GCV + rapamycin–administered animals was observed. Instead, our data suggest that rapamycin mediates its effects by potentiating the TK/Flt3L-mediated antglioma immune response. The observation that the effect of rapamycin is abolished if its administration is delayed would support the hypothesis that it is crucially required during the antigen presentation and the subsequent expansion phase of antitumor T cells. Accordingly, we observed enhanced cytotoxic T-cell responses as indicated by the increased lysis of RG2 tumor cells by activated T cells isolated from the dLN of tumor-bearing animals. GL26-OVA tumor–implanted mice also showed elevated percentages of tumor-specific and IFN-\(\gamma\)-producing CD8\(^{+}\) T cells. Of note, minimal contraction of antigen-specific CD8\(^{+}\) T cells was observed at day 89 compared with the frequency of antigen-specific CD8\(^{+}\) T cells at day 24 in the rapamycin-treated group. Because no significant differences in the percentage of antigen-specific CD8\(^{+}\) T cells in the presence or absence of rapamycin were observed at day 24, it would suggest that rapamycin modulates the function and quality of T-cell responses rather than their proliferation. Our data are in accordance with the observations by Araki and colleagues who showed that rapamycin administration during the effector to memory transition phase was crucial to the generation of improved memory T-cell responses (27). Thus, in our experiments, initiating rapamycin treatment after day 20 failed to affect the survival of tumor-bearing animals. Rapamycin treatment also accelerated memory T-cell development as indicated by the increased percentages of CD4\(^{high}\) CD62L\(^{high}\) CD8\(^{+}\) T cells. This would also imply the generation of a robust central memory response, as CD62L expression is high on central memory T cells. Memory T cells with high CD62L expression are also associated with enhanced proliferative capacity (43). Of note, the effects of rapamycin administration were long lasting as higher numbers of memory T cells were observed at day 89 along with an increased frequency of tumor-specific IFN-\(\gamma\)\(^{+}\) CD8\(^{+}\) T cells at day 95, even though the treatment with rapamycin was suspended at day 42. Indeed, this enhanced immunologic memory was associated with prolonged survival when the mice were challenged with a second tumor implantation. Taken together, our data demonstrate that rapamycin enhances the therapeutic efficacy of Ad-Flt3L + Ad-TK/GCV gene therapy in a 2-fold manner: (i) by enhancing the functional quality of the antglioma cytotoxic CD8\(^{+}\) T-cell response and promoting the maintenance of tumor antigen–specific CD8\(^{+}\) T cells, and (ii) eliciting a potent CD8\(^{+}\) memory phenotype resulting in heightened antitumor effector CD8\(^{+}\) T-cell response when rechallenged with the tumor. Moreover, we provide evidence that rapamycin administration for a short duration is sufficient to boost antitumor CD8\(^{+}\) memory T-cell response that provides long-term survival advantage in the event of tumor rechallenge. Because relapse is a common occurrence in patients with GBM, our studies point to rapamycin administration as a novel therapeutic target to be used in combination with TK/Flt3L immune-gene therapy in a phase I clinical trial for GBM.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: Y. Mineharu, M.G. Castro
Development of methodology: Y. Mineharu, N. Kamran, M.G. Castro
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Mineharu, N. Kamran, M.G. Castro
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Mineharu, N. Kamran, M.G. Castro
Writing, review, and/or revision of the manuscript: Y. Mineharu, N. Kamran, P.R. Lowenstein, M.G. Castro
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.G. Castro
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Molecular Cancer Therapeutics

Blockade of mTOR Signaling via Rapamycin Combined with Immunotherapy Augments Antiglioma Cytotoxic and Memory T-Cell Functions

Yohei Mineharu, Neha Kamran, Pedro R. Lowenstein, et al.


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