Adaptive Immune Response to and Survival Effect of Temozolomide- and Valproic Acid-induced Autophagy in Glioblastoma

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Abstract. *Background/Aim: The combination of radiotherapy, temozolomide and valproic acid (VPA) has shown some promise in retrospective analyses of patients with glioblastoma, although their mechanisms of action remain unknown. Materials and Methods: We investigated the in vitro and in vivo effects of pretreating glioma cells with temozolomide and VPA as an immunization strategy to boost an adaptive immune response in a syngeneic mouse model. Results: Temozolomide and VPA induced autophagy in GL261 glioma cells, and caused tumor antigen-specific T-cells to become activated effector T-cells. Mice with a pre-existing glioma showed no improvement in clinical outcome when immunized with temozolomide- and VPA-treated glioma cells. Conclusion: Although temozolomide and VPA treatment of glioma cells can boost the adaptive immune response, in the context of a vaccine therapy, additional factors are necessary to eradicate the tumor and improve survival.*

Glioblastoma is a devastating type of brain tumor with a dismal prognosis, leaving only 27% of patients alive after 2 years (1). Adding the alkylating chemotherapy drug temozolomide to surgery and radiotherapy has significantly improved survival and is considered the standard of care (2). Retrospective analysis of the beneficial effect of adding temozolomide to the treatment of patients with glioblastoma surprisingly also showed that the antiepileptic drug valproic acid (VPA) modulates the efficacy of temozolomide plus radiotherapy, increasing patient survival (3-5). The discussion of the underlying biology of this effect of VPA has focused on the enhancement of the bioavailability of temozolomide because VPA can block hepatic enzyme activity required for breakdown of temozolomide (6). However, the modulating effect of VPA on temozolomide in combination with radiotherapy might also be due to the ability of VPA to induce autophagy (7, 8), a type of programmed cell death.

Chemotherapy is no longer considered to be immunologically silent (9), and the concept of immunogenic tumor cell death is becoming more important in the treatment of cancer (10). Immunogenic cell death involves changes in the composition of the tumor cell surface and the release of soluble mediators which signal through dendritic cells (DCs) to stimulate the presentation of tumor antigens to T-cells (11). Autophagy is considered crucial in the process of tumor cell death, allowing DCs to capture, process, and present tumor antigens (12), and autophagy has already been observed during VPA or temozolomide treatment of glioma cells *in vitro* (8, 13).

Several DC vaccines using a patient's own tumor cells are currently being tested (14), including in glioblastoma (DCVax®-L 2015). The motivation for this strategy is that DCs can efficiently process tumor cell antigen and present it to T-cells, leading to T-cell activation and effector function. However, the factors determining DC vaccine efficacy remain poorly understood, and pre-conditioning the vaccination site with a potent recall antigen prior to the vaccine improves outcome in glioblastoma (15), suggesting a role for the patient's own DCs in the immune response

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Key Words: Temozolomide, valproic acid, autophagy, glioblastoma, dendritic cells, CD8 T-cells.

against the tumor. Taken together with the evidence that autophagic cell death enhances tumor antigen presentation by DCs, we asked whether a combination of temozolomide and VPA pretreatment of tumor cells, within the context of a vaccine, could induce an adaptive immune response to pre-existing tumor cells in a glioma mouse model. Specifically, we wanted to investigate whether immunization with temozolomide- and VPA-treated glioma cells prolongs survival in mice with glioblastoma, explaining in part the clinical survival benefit of combining these drugs (3).

Materials and Methods

Reagents and cell culture. The H-2b-specific GL261 mouse glioma cell line was kindly provided by Oliver Grauer, Muenster, Germany. This cell line expresses a membrane-bound ovalbumin and was used in all experiments under the name GL261mOVA (16). The cells were maintained in Dulbecco's modified Eagle's medium (Sigma Aldrich, Munich, Germany) supplemented with 10% fetal calf serum (Biochrome, Berlin, Germany), 2 mmol L-glutamine, penicillin (100 IU/ml), streptomycin (100 μg/ml), G418 (200 μg/ml), vitamin supplement, and Minimun Essential Medium non-essential amino acids obtained from Sigma Aldrich. Temozolomide was from Merck, Darmstadt, Germany, VPA was purchased from Sigma Aldrich, and bafilomycin was from Invivogene (Toulouse, France). Microtubule-associated protein light chain 3 (LC3) antibody was from Novus Biologicals (Littleton, CO, USA); glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was from Santa Cruz, Santa Cruz, CA, USA; the mouse-specific cluster of differentiation 8 R-phycoerythrin-cyanine7 (CD8-PE-Cy7), CD45.1 Allophycocyanin (APC), and interferon gamma-PE (IFNγ) antibodies were purchased together with carboxyfluorescein succinimidyl ester (CFSE) from eBioscience (Frankfurt, Germany). Biotinylated IFNγ and horseradish peroxidase (HRP)-conjugated IFNγ were from Mabtech (Nacka Strand, Sweden). In order to generate murine DCs, bone marrow cells isolated from the femur and tibia were cultivated for 6 days in 10 ng/ml granulocyte macrophage colony-stimulating factor (GM). For maturation of DCs, we used 80 ng/ml tumor necrosis factor alpha (TNFα). GM and TNFα were both from PeproTech (Hamburg, Germany). The immunodominant H-2Kb-restricted epitope SIINFEKL was purchased from Iba (Goettingen, Germany) and pulsed on mature DCs at 10 μM for 60 min.

Female C57BL/6-J mice (CD45.2+) aged 6-12 weeks (approximately 20 g) were purchased from Charles River (Sulzfeld, Germany) and used for survival experiments. OT1 mice on the genetic CD45.1 background were a kind gift from Philipp Renner, Department of Surgery, Regensburg, Germany.

Immunoblot analysis. For detection of cellular levels of LC3-I and LC3-II, adherent GL261mOVA tumor cells were treated with 50 μM temozolomide, 2.5 mM valproic acid (VPA), or a combination of both for 24 h. During the final h of incubation, bafilomycin A1 at 100 nM was added to disrupt autophagic flux. Lysates of the treated cells were analyzed by immunoblot using 30 μg of protein per lane on 12% sodium dodecyl sulfate-polyacrylamide gels. After transfer to a nitrocellulose membrane, the blots were blocked in Tris-buffered saline containing 5% nonfat dried milk and 0.1% Tween 20 and incubated overnight at 4˚C with antibody to LC3. Visualization of protein bands was accomplished using horseradish peroxidase-conjugated secondary antibody from Advansta (Menlo Park, CA, USA) and enhanced chemiluminescence from Biozym (Hessisch Oldendorf, Germany). As a loading control, GAPDH levels were assessed using a specific antibody. Chemiluminescence signals were detected with an Imagequant LAS 4000 instrument from GE Healthcare (Munich, Germany).

Immunizations and CD8 T-cell IFNγ fluorescence cytometry with enhanced precursor frequency. To measure IFNγ levels from CD8 T-cells in mice with an enhanced tumor antigen-specific CD8 T-cell precursor frequency, GL261mOVA cells were treated for 24 hours with 50 μM temozolomide, 2.5 mM VPA, or a combination of both for 24 hours. Before injection, cells were washed extensively, resuspended at 10^7/ml, and 100 μl was injected using the intradermal (*i.d.*) immunization route into groups of three naive mice (no treatment, temozolomide, VPA, and both). In parallel, 10^7 CFSE-labeled H-2K^b SIINFEKL-specific OT1 cells were injected intravenously. After 5 days, draining lymph nodes were harvested and subjected to intracellular IFNγ fluorescence cytometric analysis using antibodies against CD8a and CD45.1 to identify transferred OT1 cells. Fluorescence cytometric analysis of CFSE dilution and intracellular IFNγ staining were performed on an LSR II instrument from Becton Dickenson (Heidelberg, Germany). Data were transferred as fcs files and analyzed with Kaluza software from Beckman Coulter Inc. (Krefeld, Germany).

CD8 T-cell IFNγ ELISPOT from the naïve repertoire GL261mOVA cells were pretreated as described above and used to immunize three naïve mice per group. After 12 days, spleens were harvested and CD8 T-cells purified using the magnetic cell seperation (MACS) strategy of Miltenyi Biotech (Bergisch Gladbach, Germany). Of the CD8 T-cells, 2×10^5 were added to 6.7×10^3 purified major histocompatibility complex class II (MHCII)^{high} DCs peptide-pulsed with SIINFEKL to give a T-cell:DC ratio of 30: 1. The cultures were incubated in 96-well plates pre-coated with a capture antibody for IFNγ. After 24-36 h, cells were washed out with mild detergent, and a biotin-conjugated IFNγ monoclonal antibody, followed by the HRP-based detection reagents from the Vectastain Elite Kit (Vector Laboratories, Burlingame, CA, USA) were added. Colored spots indicated the SIINFEKL-specific cells that had released IFNγ, and data are reported as spot-forming cells/10^6 CD8 T-cells. The ELISPOT plate was analyzed by an automated ELISPOT reader from AID Diagnostika GmbH (Strassberg, Germany). Triplicate wells were averaged, and the mean data are reported.

Survival studies in vivo. Four groups of 10 mice (treated with temozolomide, VPA, both or neither treatment) were anesthetized before all intracranial procedures and placed in a stereotaxic fixation device (Stoelting, Wood Dale, IL, USA). A burr hole of 0.5 mm was drilled in the skull 1 mm lateral and 2 mm to the front of the bregma. The needle of a Hamilton syringe (Hamilton, Darmstadt, Germany) was introduced to a depth of 3 mm. A total of 5×10^4 GL261mOVA cells resuspended in 2 μl Phosphate-buffered saline (PBS) were slowly injected into the brain. The mice were observed and weighed daily and euthanized upon development of neurological symptoms or progressive weight loss greater than 20%. The experiments were performed according to the German Animal Protection law and registered under the number 54-2532.1-21/13.

Statistical analysis. Overall survival (OS) was determined in days from the date of tumor cell implantation. Kaplan–Meier curves were

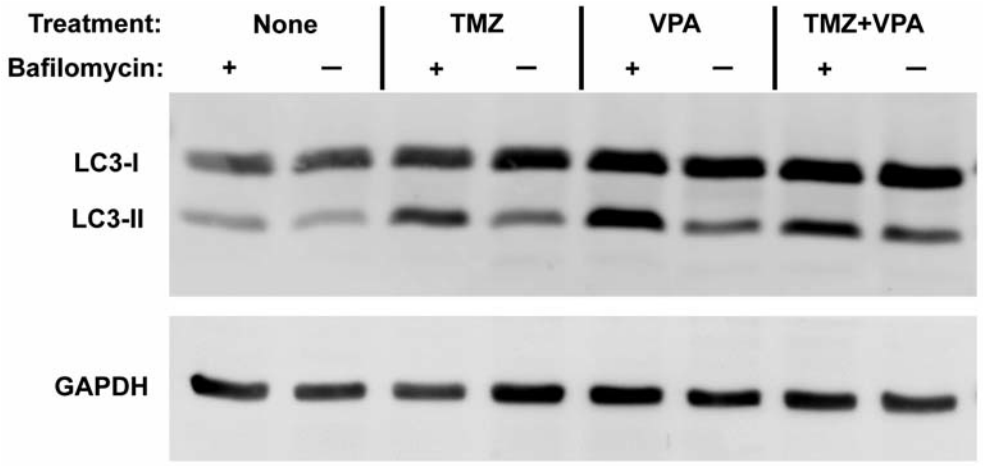

Figure 1. *Temozolomide (TMZ) and valproic acid (VPA) treatment induces autophagy in vitro. GL261 cells were treated in vitro with 50 μM TMZ, 2.5 mM VPA, or a combination of both (TMZ+VPA). After 24 hours, the cells were subjected to western blot analysis of autophagic activity by LC3-I/II antibody staining. Bafilomycin treatment to disrupt autophagosomal degradation during the last hour of treatment is indicated with + and no treatment with −. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as the loading control.*

plotted to estimate OS as a function of treatment. Group differences were assessed by the log-rank method. Statistical analysis was carried out using the Statistical Package for the Social Sciences 12.0 software (IBM, Armonck, NY, USA) and *p*-values of less than 0.05 were considered statistically significant.

Results

Temozolomide and VPA induce autophagy in glioma cells. To characterize the cellular response of our experimental system with clinically relevant doses of temozolomide and VPA, we first treated the syngeneic murine GL261 cell line alone or in combination with these drugs. We quantified the autophagic activity under these conditions by monitoring LC3, a standard marker of autophagy. LC3-I is converted to LC3-II, which correlates with the number of autophagosomes. With 50 μM temozolomide or 2.5 mM VPA (17), we detected an enhanced LC3-II isoform band in comparison to untreated cells at 24 h (Figure 1). This effect was time-dependent, with a maximum at 24 h of treatment (measurements were made at 12, 24, and 48 h; data not shown). Adding bafilomycin in order to block autophagosomal degradation further enhanced this effect (Figure 1). There was neither an additive nor a synergistic effect of a combination therapy of temozolomide plus VPA in terms of autophagic activity (Figure 1). In accordance with published results, we were unable to detect visual signs of toxicity, and cell lysates subjected to cleaved caspase 3 immunoblot analysis confirmed the absence of apoptotic cell death for up to 48 h (data not shown). In summary, these results confirmed that treatment with temozolomide or VPA induced autophagy in GL261 cells.

Immunization with glioma cells pretreated with temozolomide and VPA induces proliferation and IFNγ production of adoptively transferred tumor antigen-specific CD8 T-cells. After observing glioma cell autophagy induced by temozolomide and VPA *in vitro*, we next asked if such treated glioma cells would still be detected by the immune system *in vivo*. We, therefore, performed *i.d.* immunizations with 10^6 pretreated GL261 cells expressing a membrane-bound form of full-length ovalbumin as antigen (GL261mOVA). We also adoptively transferred 10^7 CFSE-labeled transgenic CD8 T-cells (OT1) specific for the immunodominant H-$2K^b$ ovalbumin epitope SIINFEKL. To investigate whether professional antigen-presenting cells could take up the cell-bound antigen, process it and present it to immune effector cells, CD8 T-cell proliferation and IFNγ production were measured from the draining lymph nodes at day 5. Both temozolomide- and VPA-pretreated GL261mOVA cells elicited robust CD8 T-cell proliferation, demonstrating that tumor-bound antigen had been processed and presented (Figure 2 A-D). We also observed intracellular IFNγ production with an increasing number of cell divisions, demonstrating the effectiveness of antigen-specific T-cell priming (Figure 2 E-H). Quantification of intracellular IFNγ did not demonstrate its significant increase in either the single or combination treatment arm of temozolomide and VPA (Figure 2I). In summary, this experiment revealed that glioma cells treated with temozolomide and VPA were still captured by antigen-presenting cells, which were capable of processing and presenting antigen to induce a cytotoxic T-cell immune response.

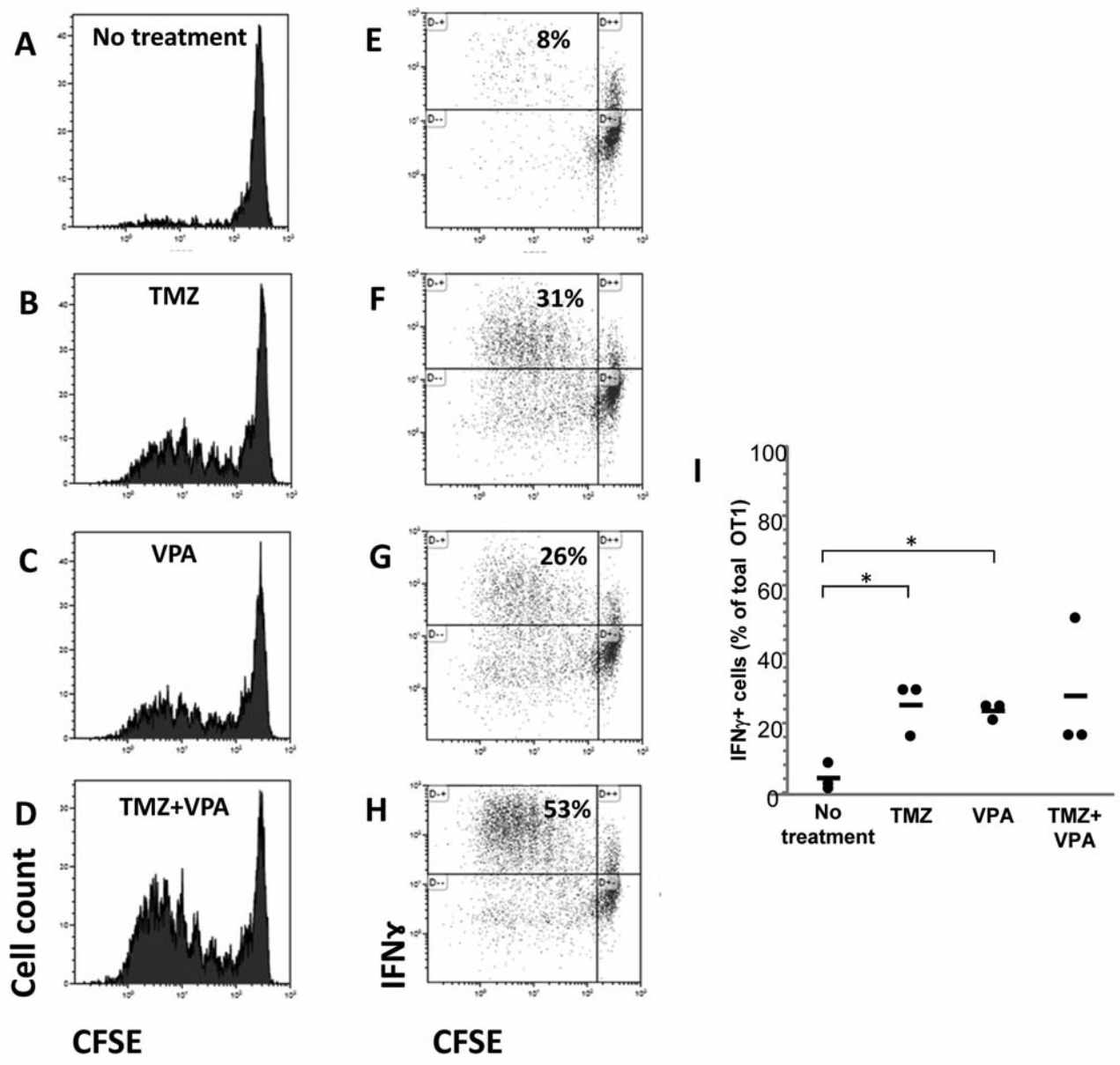

Figure 2. *Temozolomide (TMZ)- and valproic acid (VPA)-treated glioma cells induce proliferation and interferon gamma (IFNγ) production of antigen-specific CD8 T-cells in vivo. GL261mOVA cells were treated for 24 hours with TMZ, VPA or both (TMZ+VPA) and then injected intradermally into C57BL/6 mice harboring Carboxyfluorescein succinimidyl ester (CFSE)-labeled SIINFEKL-specific transgenic CD8 T-cells (OT1). After 5 days, draining lymph nodes were harvested, and adoptively transferred OT1 cells identified by fluorescence cytometry analysis (CD8 and CD45.1 positive cells). Panels A-D show CFSE dilutions reflecting divisions of transferred OT1 cells. Panels E to H show intracellular IFNγ staining. The divided and IFNγ-positive OT1 cells as a percentage of total OT1 cells are indicated. In I, individual mice of one experiment are represented by circles, and means are represented by horizontal bars. A representative of three independent experiments is shown. *Statistically significant at $p<0.05$ (t-test).*

Temozolomide- and VPA-treated glioma cells induce priming of tumor antigen-specific CD8 T-cells from the naïve repertoire. Given the non-physiological setup of the previous experiment with an enhanced precursor frequency of adoptively transferred transgenic CD8 T-cells, we asked whether we would observe similar results in a naïve, wild-type T-cell repertoire. We, therefore, pretreated GL261mOVA cells with temozolomide, VPA, or both, and injected them *i.d.* into naïve mice. After 12 days, we measured the frequency of splenic CD8 T-cells specific for tumor antigen by ELISPOT. We were unable to detect significant differences in the pretreatment arms with temozolomide, VPA, or both (Figure

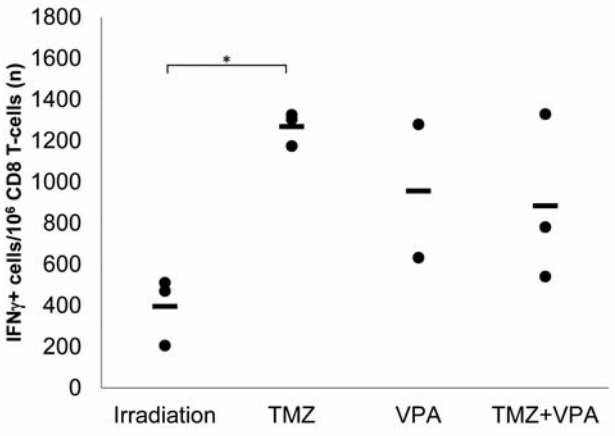

Figure 3. *Temozolomide (TMZ)- and valproic acid (VPA)-treated glioma cells induce interferon gamma (IFNγ)-positive effector cells in a naïve T-cell repertoire. GL261mOVA cells were pretreated as described in the Materials and Methods with TMZ, VPA or both (TMZ+VPA) before being injected intradermally into naïve C57BL/6 mice. After 12 days, CD8 T-cells isolated from the spleen were analyzed for IFNγ production in an ELISPOT assay. Each circle represents an individual mouse. The bars represent the mean of the group. As a control for apoptotic cell death, UVB-irradiated GL261 cells (irradiation) were used. A representative of two independent experiments is shown. *Statistically significant at p<0.05 (t-test).*

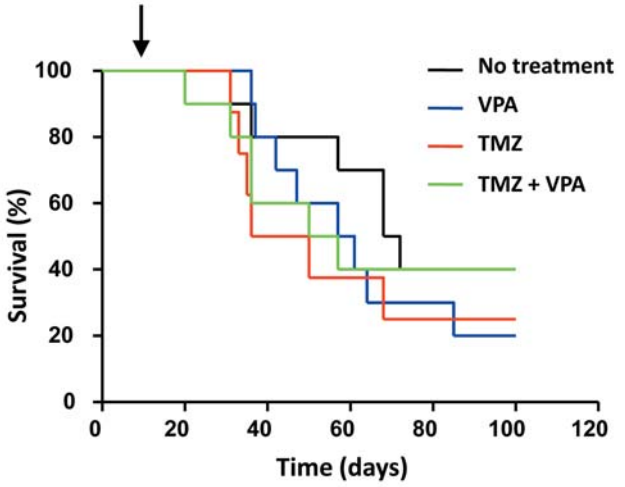

Figure 4. *Pretreatment of glioma cells with temozolomide (TMZ), valproic acid (VPA) or both had no significant survival benefit in an orthotopic, syngeneic glioma mouse vaccination model. Seven days after orthotopic implantation of 50,000 syngeneic GL261mOVA cells into C57BL/6 mice, GL261mOVA cells treated with TMZ, VPA or a combination of both were injected intradermally (arrow). Animals in groups of 10 were monitored daily for development of neurological symptoms and euthanized after demonstrating debilitating symptoms. Survival was recorded in a Kaplan–Meier curve. Statistical analysis revealed no significant differences between any of the treatments compared to no treatment. Log-rank test VPA: (HR)=0.56, 95% confidence interval (CI) =0.19-164, p=0.29; TMZ: HR-0.49, 95% CI=0.14-1.70, p=0.26; and VPA+TMZ: HR=0.75, 95% CI=0.23-2.47, p=0.64.*

3). Irradiation of GL261mOVA cells with UVB light, which induces apoptosis, was much weaker at eliciting an adaptive immune response (Figure 3), supporting the theory that antigen from apoptotic cells is not efficiently presented. Taken together, these results show that temozolomide, VPA and the combination treatment induce autophagy in glioma cells, and glioma antigen can subsequently be recognized and processed by the immune system to elicit a priming response from antigen-specific CD8 T-cells.

Pretreatment of glioma cells with temozolomide, VPA or both had no significant survival benefit in an orthotopic, syngeneic glioma mouse vaccination model. Finally, we asked whether we would observe a survival benefit with immunizations consisting of temozolomide- and VPA-pretreated glioma cells in a glioma mouse model. We stereotactically implanted 5×10^4 GL261mOVA cells into the brains of syngeneic mice. After 7 days, we i.d. vaccinated with GL261mOVA cells pretreated with temozolomide, VPA, or both, and recorded survival over time. Mice treated with an *i.d.* saline injection (no treatment) showed the best survival results, with a median survival of 70 days (Figure 4). We recorded a median survival of 43 days for mice vaccinated with glioma cells pretreated with temozolomide, 59 days for those pretreated with VPA and 53.5 days for those pretreated with temozolomide plus VPA (Figure 4). There was no significant difference of any treatment group compared to saline-treated animals as analyzed by log-rank test. These findings suggest that the combination of temozolomide and VPA treatment does not boost the immune response to glioma.

Discussion

The results presented here showed that the effects of temozolomide and VPA on glioma cell autophagy and the resulting adaptive immune response were similar to the combination treatment of temozolomide plus VPA. Although temozolomide and VPA induced an immunogenic tumor cell death, in a glioma mouse model, neither the singular pretreatments nor the combination pretreatment of glioma cells used in a vaccine were capable of providing survival benefit. These findings highlight the complex nature of glioma treatment and the adaptive immune response to tumors. Considering the importance of immunotherapy in the treatment of glioblastoma, it is compelling to speculate that the role of autophagy in brain tumors is a double-edged sword, that the location of a tumor vaccine might influence the survival outcome, and that VPA may not enhance existing treatment regimens for glioblastoma.

A growing body of evidence indicates that autophagy can either induce cancer cell death by programming the cell to kill itself, or promote cell survival by maintaining metabolic homeostasis in response to metabolic stress (18). Although autophagy has been perceived to be a mechanism protective against cancer development, after cancer is established and treatment is the focus of care, the role of autophagy might change. Indeed, autophagy was shown to limit the efficacy of combination radio- and chemotherapy, since blocking autophagy was somewhat beneficial for survival (19), although the sample size in that study was too small to be conclusive. This paradoxical effect could be due to the timing of autophagy induction, as well as the precise point in the autophagy pathway that is blocked.

Taking into account the rather complex nature of immunogenic cell death, we have to ask if autophagy is an inducer of or simply a step within this process. Whether other pharmacological inducers of autophagy which preferentially act at different sites within the autophagy machinery would perform equally well or better should be addressed scientifically. Our future studies will focus on calreticulin exposure, ATP release and the chromatin protein high-mobility group protein B1 (HMGB1), which could explain some of the differences in the published outcomes on autophagy-driven immunogenic cell death. Our own previous studies in this field also used a viral antigen (20), which does not easily translate into the current system with membrane-bound antigen. Adjuvants that mimic a type I IFN danger signal, such as imiquimod, would also be useful in teasing out the roles of autophagy in glioblastoma.

The possibility that tumor cells themselves induce immune tolerance can never be ruled out, but the robust induction of IFNγ-positive cells both in mice with adoptively transferred OT1 cells and mice with a naive T-cell repertoire makes this explanation rather unlikely here. However, homing and recruiting of T-cells is, in our opinion, a suitable candidate for explaining the absence of a survival benefit in the treatment groups. To generate a useful immune response, the effector cells not only have to expand but also reach the target site. This could explain why the immune responses we observed did not translate into a survival benefit. Previous reports have shown that crossing the endothelial barrier can be a problem, with T-cells killed in a CD95-dependent manner (21). The induction of an immune response not at the original tumor site does raise concerns for ongoing clinical trials using DCs loaded with tumor antigen lysates. Indeed, injection of cell-associated antigen directly into the brain has been shown to be more immunogenic than peripheral administration (22). It is, therefore, not surprising that the choice of how to immunize, where to immunize and which adjuvant to use is currently one of the most hotly discussed topics in clinical immunotherapy trials. It will also be necessary to correlate the expansion of effector cells in the periphery, where they can be easily be monitored, with the number of effector cells in the tumor bed.

Finally, the role of VPA as a beneficial modulator of survival in glioblastoma has been discussed extensively in the past, and although several groups reported a prolonged survival with VPA and temozolomide in clinical trials, there were caveats. These reports were retrospective in nature, and a prospective randomized clinical trial has yet to be performed (23). In addition, none of these studies were able to evaluate the exact dosing of and exposure time to VPA due to their retrospective nature, so we are still left with limited data to counsel patients about whether VPA should be added to the standard treatment of care. Further complicating the situation, at the time of writing, a new combined analysis of four large-scale clinical trials (CENTIRC, CORE, AVAGlio and RTOG 0825) showed that the survival effect from VPA was no longer present (24).

In summary, we showed that tumor cells undergoing autophagic cell death induced by temozolomide and VPA in the context of a vaccine could elicit a cytotoxic T-cell immune response which failed to translate into a survival benefit in an orthotopic glioma model. The effects of temozolomide and VPA together were neither additive nor synergistic on autophagy induction *in vitro* and the resulting antigen presentation and priming *in vivo*. The increased tumor antigen-specific immune response resulting from the temozolomide- and VPA-pretreated glioma cell vaccine was not sufficient to improve survival.

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Received December 22, 2015
Revised January 24, 2016
Accepted January 29, 2016