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Chemotherapy Can Synergize With Adoptive Immunotherapy to Inhibit Medulloblastoma Growth

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Abstract. *Background/Aim: In the age of ever-increasing developments in targeted cancer treatments, new immune-based approaches for brain tumor therapy represent an attractive avenue. Despite encouraging pre-clinical data, results in patients have been sub-optimal, likely due to tumor-induced immune suppression and intrinsic resistance to immune attack. Chemotherapy and biologic agents may be able to disrupt these mechanisms and restore tumor sensitivity to immune attack. In this study, we explore whether a combination of gemcitabine and rapamycin can sensitize medulloblastoma cells to immunotherapy in vitro and in vivo. Materials and Methods: With the commercial medulloblastoma cell line, Daoy, we explored the concentrations of combinations of Gemcitabine with rapamycin needed to induce cytotoxicity. Next, we used flow cytometry to assess the cytotoxicity of chemotherapy-treated Daoy cells with the addition of anti-tumor T-cells, generated from naive T-cells stimulated in the presence of Daoy lysate-pulsed dendritic cells. Then, we examined the efficacy of chemotherapy alone versus chemotherapy plus immunotherapy in tumor growth inhibition of subcutaneous medulloblastoma xenografts. Results: Rapamycin alone at <1,000 nM had moderate activity against Daoy cells in vitro and IC_{50} was >1,000 nM. Gemcitabine had a 3-day IC_{50} alone of 10 nM but in combination with 100 nM rapamycin, it decreased to 1 nM, suggesting increased cytotoxicity with combined therapy. Stimulated T-cells mediated in-vitro cytotoxicity, although background cytotoxicity of unstimulated "naïve" T-cells was also significant. Finally, established subcutaneous Daoy cell xenografts in SCID mice were treated with chemotherapy alone or chemotherapy plus adoptive immunotherapy (stimulated and non-stimulated). Gemcitabine and rapamycin alone significantly slowed tumor growth, but the addition of immunotherapy further augmented inhibition. Conclusion: Combining immunotherapy and chemo-biologic therapy inhibit medulloblastoma cell and xenograft growth, and may offer an effective treatment for patients with medulloblastoma.*

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Brain tumors represent the most common solid tumor in the pediatric population and the second most common pediatric malignancy, accounting for approximately 20-25% of all primary childhood tumors and are associated with significant morbidity and mortality (1, 2). While treatments have improved over the past several decades, the prognosis for many of these cases is quite poor, and the current chemotherapy regimens are associated with significant side effects. At present, children who are older than three years of age typically receive some combination of surgery, radiation, and chemotherapy (2).

Immunotherapy, or the up-regulation of an individual's immune system to combat a malignancy, has recently regained a great deal of interest on the research front, since it potentially offers a targeted and less toxic modality with which to treat cancers (3). Immunotherapy alone has shown poor response rates in numerous clinical trials for a variety of different cancers (4). Several mechanisms of tumor evasion of cell-mediated immunity exist. One mechanism involves a tumor-induced immunosuppressive milieu; for example, TGF-beta secretion, T-regulatory cell recruitment, and myeloid-derived suppressor cell infiltration foster immunosuppression (5). Two other mechanisms include down-regulation of antigen presentation and evasion of apoptosis, which is normally triggered by cytotoxic T-cells (6). A fourth potential mechanism involves a population of cells isolated from human tumors, termed brain tumor-initiating cells (BTICs), which is thought to underlie both the initiation and persistence of brain tumors. This specific cell population has been shown to be resistant to both chemotherapy and radiation therapy due to up-regulation of multidrug resistance genes, DNA damage response pathways, and other mechanisms (7), suggesting that novel approaches are needed to eliminate these cells.

Various studies have analyzed the combination of chemotherapeutic treatments and immunotherapy with improved outcomes, compared with either therapy alone, both *in vitro* and *in vivo* (8-10). The addition of immunotherapy confers the added benefit of long-term immunologic memory

(9). Chemotherapy may synergize with immunotherapy *via* a number of different mechanisms such as reduction of immunosuppressive cell types (T-regulatory cells, myeloid suppressor cells), reduction of non-specific host T-cells that may compete for stimulatory cytokines or sensitization of tumor cells to immune mediated destruction (8).

In this study, we explore the utility of a combined chemotherapeutic and immunotherapeutic approach. We selected two chemotherapeutic compounds that have separately been shown to augment immunotherapy in various pre-clinical models: a cytotoxic chemotherapy agent, gemcitabine, and a mammalian target of rapamycin (mTOR) inhibitor, rapamycin. The nucleoside analog, gemcitabine, has been shown to synergize with immunotherapy in animal models as well as in clinical trials *via* promotion of T-cell mediated anti-tumor immune activity (11). Also, the mTOR pathway is associated with cancer cell growth and immunosuppression, and its inhibition with rapamycin has been shown to augment immunotherapy in various pre-clinical models (12-13). Targeted kinase inhibitors, starting with the widely used therapy, imatinib, have become standard treatments for numerous malignancies, including CML, GIST, and breast cancer. However, their activity as single agents in malignant brain tumors has been very limited. The PI3 kinase/mTOR pathway is well known to contribute to the pathogenesis of a variety of malignancies. Inhibition of mTOR with rapamycin sensitizes a variety of tumors to chemotherapy and radiation (12-13). More recently, the PI3K pathway has been shown to be involved with tumor immunoresistance *via* sensitization of the TNF-related apoptosis-inducing ligand (TRAIL) induced apoptotic pathways (14) as well as up-regulation of B7-H1 protein expression; B7-H1 protein binds programmed death-1 (PD-1) receptor, which is believed to underlie its role in tumor evasion of the host's immune system (15).

Additionally, with the recognition of BTICs, we hypothesized that T-cells activated in the presence of peptides specifically expressed on stem cells and stem cell-like populations, could represent a target for immunotherapy against specific brain tumors. Specifically, Bmi-1, SOX2, and maternal embryonic leucine zipper kinase (MELK) are three proteins that are commonly expressed on rapidly dividing cells and promote rapid cell growth and division (16-18). Interleukin-2 (IL-2) has been shown to augment the immune response to cancer cells when given alone, but especially when given in the presence of cancer cell specific stimulated T-cells (19, 20). Besides therapies that stimulate acquired immunity, activation of the innate immune system, *via* Toll-like receptor 4 (TLR4) signaling *via* lipopolysaccharide (LPS), represents another mechanism that promotes strengthened immunity, which may be harnessed in cancer therapy (21).

In this study, we combine Rapamycin and Gemcitabine therapy with adoptive immunotherapy directed against BTICs and LPS-induced innate immunity stimulation to test the hypothesis that combination of these modalities will more effectively control tumor growth than any of the treatment modalities alone.

Materials and Methods

Cell lines. Daoy cells, a commercially available human-derived pediatric medulloblastoma cell line, were purchased from American Type Culture Collection (ATCC number HTB-186; Manassas, VA, USA). The cell line was grown in Dulbecco's Modified Eagle Medium (DMEM) medium with glutamax (Gibco; Life Technologies) and 10% FBS (Sigma, St. Louis, MO, USA) and incubated at 37˚C in humidified 5% CO_2.

Cell viability assays. Analysis of Daoy cell viability was performed using the CellTiter 96® AQueous One Solution Cell Proliferation Assay system (Promega, Madison, WI, USA). Cells were seeded at 10,000 cells per well in 96-well plates and incubated at 37˚C in a humidified chamber with 5% CO_2 for 24 h prior to treatment. Then, cells were treated with Gemcitabine concentrations ranging from 0 nM to 100 nM for one day and Rapamycin concentrations ranging from 0 nM to 1,000 nM for three days and were subsequently analyzed with an MTT assay (MTT 98%, ACROS Organic, Thermo Fisher Scientific, NJ, USA). Rapamycin was purchased from LC Laboratories (R-5000, Woburn, MA, USA) and gemcitabine (Gemzar, Lilly USA, LLC, Indianapolis, IN, USA) was obtained from the Harbor-UCLA hospital pharmacy. After 72 h following treatment with various drug combinations, 20 μl of CellTiter 96® AQueous One Solution reagent was added to each well. Absorbance was recorded at 490 nm after 2 h. Background wells containing neither cells nor drug were used for subtraction and final absorbance calculations. Twelve wells per condition were used to calculate means and standard errors for combinations of treatments.

Analysis of mRNA expression by real-time polymerase chain reaction (RT-PCR). For the RT-PCR expression studies, drugs were incubated with the cells for 24 h. There were three 6-well plates employed, with each well containing 50,000 cells, with treatment groups of 1) none, 2) 50 nM Gemcitabine, 3) 500 nM Gemcitabine, 4) 100 nM Rapamycin, 5) 100 nM Rapamycin plus 50 nM Gemcitabine, and 6) 100 nM Rapamycin plus 500 nM Gemcitabine. RNA was then collected, and RT-PCR performed. Messenger RNA from β-Actin was used as an internal standard for the total amount of cDNA. Total RNA was extracted from cultured cells (RNeasy Mini Kit, Qiagen, Hilden, Germany). One μg of total RNA was used for first strand cDNA synthesis, using M-MLV Reverse Transcriptase (Fisher BioReagents, Fair Lawn, NJ, USA, Lot #093045) and Random Primers (Promega Corporation, Madison, WI, USA, Lots #28320207 & #294007). The synthesized cDNA was used as a template for RT-PCR (Fast SYBR Green I Master Mix, Applied Biosystems, Foster City, CA, USA). The random dNTP mix was purchased from Chicago Research (Chicago, IL, USA) and Taq DNA polymerase was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Primers for cDNA amplification were purchased from Sigma-Genosys (St. Louis, MO, USA) and were as follows: Bmi-1-sense: 5'-GGAGACCAGCAAGTATTGTCCT-3', Bmi-1-anti-sense: 5'-CATTGCTGCTGGGCATCGTAAG-3', SOX2-sense: 5'-ACCGGC GGCAACCAGAAGAACAG-3', SOX2-anti-sense: 5'-GCGCCGCGG CCGGTATTTAT-3', MELK-sense: 5'-CTTGGATCAGAGGCAGAT GTTTGGAG-3', MELK-anti-sense: 5'-GTTGTAATCTTGCATGATC CAGG-3', and β-Actin-sense: 5'-GATGAGATTGGCATGGCTTT-3', β-Actin-anti-sense: 5'-CACC TTCACCGTTCCAGTTT-3'.

Table I. *HLA-A201+ T-cells from HLA-A201+ donor PBMCs were either unstimulated, representing the control naïve T-cell group, or stimulated with a combination of six different peptides.*

Predicted HLA-A*0201 binding epitopes from stem cell related genes		
Bmi-1	SOX2	MELK
TLQDIVYKL [Bmi1(1)]	SMYLPGAEV [SOX2(1)]	WSMGILLYV [MELK(1)]
CLPSPSTPV [Bmi1(2)]	LLAPGGNSM [SOX2(2)]	VLMCGFLPF [MELK(2)]

Thermal cycling consisted of 30 cycles of denaturation for 30 s at 94˚C, annealing for 60 s at 60˚C, and extension for 60 s at 72˚C. 4% agarose gels were prepared, and a Full Scale DNA Ladder (Chicago Research) was used for scale. Semi-quantitative analysis was generated using the comparative cycle threshold (CT) method by normalizing levels of target mRNA CT to levels of β-Actin mRNA.

T-cell cytotoxicity studies. HLA-A201+ T-cells were generated from HLA-A201+ donor PBMCs and stimulated by peptide-pulsed dendritic cells (DCs) or T2 cells purchased from the American Type Culture Collection as CRL 1992. The DCs were generated from peripheral blood drawn from healthy volunteers by venipuncture with purification of leukocytes by Ficoll (Pharmacia, Piscataway, NJ, USA) gradient separation. Purified leukocytes were suspended in RPMI164, 5% heat-inactivated human AB serum, L-glutamine, and Penicillin/Streptomycin antibiotics. Incubation for 2 h at 37˚C in a humidified CO_2 incubator induced adherence of macrophages, DC progenitors, and monocytes to the flask walls and the non-adherent cells were washed following incubation. RPMI/serum medium with 800 U/ml of GM-CSF and 500 U/ml of IL-4 were added to the flask and incubated with the cells for 5 days to create immature DCs from monocytes. After the treatment, all medium was removed and pooled, and the cells were examined by morphology and phenotyping to confirm that they were DCs. In 1 ml of X-VIVO-15 with Gentamicin and Phenol Red (Lonza Walkersville, Inc., Walkersville, MD, USA), we suspended isolated DCs and incubated them with the same six peptides listed in Table I, derived from Bmi-1, SOX2, and MELK, at a total concentration of 5 μg/ml. The peptides were purchased from and confirmed by Biosynthesis Inc. (Lewisville, TX, USA, lots #T4111-1 through T4111-6).

T-cells were then either allowed to remain naïve or were stimulated with these peptide-pulsed dendritic cells (DCs), plated in a 24 well plate with IL-7 at a concentration of 2.5 ng/ml, and incubated overnight at 37˚C. The stimulated T-cells were harvested by resuspension in 90% X-VIVO-10 (Lonza Walkersville, Inc., Walkersville, MD, USA) and 10% human serum – type AB (Mediatech, Inc., Manassas, VA, USA), followed by spinning down, then resuspension in 1 ml 90% X-VIVO-10 with 10% human serum. The treated T-cells were termed peptide-pulsed T-cells (PP-T-cells). Target Daoy cells were marked with a fluorescent label (Dioc), purchased from Invitrogen (Carlsbad, CA, USA). Daoy cells were mixed with either the naïve T-cells or the PP-T-cells. These mixtures were performed in three different ratios: 1:10, 1:50, and 1:100. All reaction tubes were treated with propidium iodide, which labels dying or dead cells, purchased from Fisher Scientific (Chicago, IL, USA). The mixed populations were analyzed *via* flow-cytometry to determine the percentage of target cells killed by the effector cells.

Animals. All mouse experiments were in compliance with Los Angeles Biomedical Research Institute IACUC guidelines. SCID mice with heterotopic subcutaneous xenografts with DAOY cells were used for the study [(22); Jackson Laboratory, Bar Harbor, ME, USA; all males, 6 weeks old at the time of tumor transplantation]. Tumor cells (1×10^7) were injected under the skin in the right flank.

Mouse trial. Three weeks post-xenograft transplantation, mice were divided according to groupings as follows: Group 1 – n=5: Control – Phosphate buffered saline (PBS), Group 2 – n=4: Immunotherapy – Peptide generated T-cells + IL-2, Group 3 – n=5: Chemotherapy – Gemcitabine and Rapamycin treatment + IL-2, Group 4 – n=5: Immunotherapy + Chemotherapy – Gemcitabine/Rapamycin + Peptide generated T-cells + IL-2, and Group 5 – n=5: Immunotherapy + Chemotherapy + lipopolysaccharide (LPS) – Gemcitabine/Rapamycin + Peptide generated T-cells + IL-2 + LPS. There were five animals per group, except for group 2, in which one of the five mice died on day 1 of treatment.

The treatments were carried out as depicted in Figure 1. For groups 3, 4, and 5, gemcitabine in phosphate buffered saline (2 mg/kg IP) and rapamycin in DMSO (1 mg/kg IP) were given for five consecutive days for two cycles. Additionally, groups 2, 4, and 5 received peptide-generated T-cells, as a model of adoptive immunity. The mice in group 1 received PBS on the dates that mice in the other groups received either chemotherapy or IL-2; the volumes of PBS given on those dates were identical to the volume of treatment received by the experimental groups.

As in the T-cell cytotoxicity studies listed above, DC cells were generated, pulsed with the 6 peptides derived from Bmi-1, SOX2, and MELK, and incubated with naïve T-cells to generate activated T-cells targeting the Bmi-1, SOX2, and MELK peptides. Isolated, activated T-cells were administered in two doses to groups 2, 4, and 5. For groups 4 and 5, the T-cells were given following each Gemcitabine/Rapamycin cycle (1×10^7 and 5×10^6 cells, respectively). For groups 2-5, recombinant IL-2 (Pierce, Rockford, IL, USA) was given for three days in 5,000 unit I.P. injections, at a dilution of 30U/ml, following each T-cell infusion. Lastly, for group 5, LPS, purchased from Fisher Scientific, was given at a dose of 1 mg/kg for three days following the first IL-2 dose. Tumors were measured with digital calipers 3 times weekly. To estimate tumor volumes, an ellipsoid volume formula was utilized ($1/2 \times \text{length} \times \text{width}^2$) (23, 24).

Statistical methods. Baseline tumor volumes were compared among the five groups and were statistically different at that time point using analysis of variance (ANOVA). As such, baseline tumor volume at day zero was used as a covariate in the model for subsequent analysis in the ANCOVA. The mixed model was used to compare tumor volume among five groups at each day of treatment, and outcome was defined as the log of the tumor volume. A log transformation was applied to tumor volume for the better model fit as well as the model assumptions. In the mixed model, the independent variables were defined as 1) Group, considered as a between subject factor, 2) Day, considered as a within subject factor, and 3) Group by Time interaction. This final term was also added in the model to determine if the group effect changed over time. The two main effects and Group by Time interaction effect were tested. A ratio of Least Square Means (LS-means) of two groups was computed and tested both overall and at a given day. A Tukey method was used for multiple comparison adjustment for the *p*-values and confidence intervals. The residual analysis was conducted to check the model assumptions

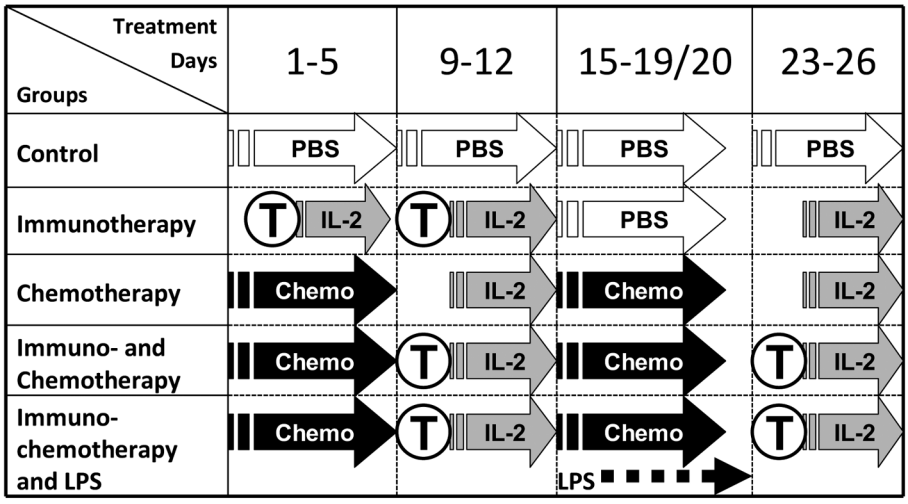

Figure 1. *Treatment schedule for the mouse study. Note that the volume of PBS given was matched to that of the treatments given to mice in the experimental groups. PBS: Phosphate buffered saline; IL-2: Interleukin 2; T: T cells; Chemo: chemotherapy with Gemcitabine and Rapamycin; LPS: lipopolysaccharide.*

using Student's *t*-test was used for comparison of the means of two groups. All data analyses were conducted using SAS 9.4 (Cary, NC, USA), and the Proc Mixed procedure was used for the mixed model. *p*-Values of <0.05 were considered statistically significant.

Results

Gemcitabine and rapamycin show activity against Daoy cells. As depicted in Figure 2, rapamycin and gemcitabine demonstrated cytotoxic effects on treated Daoy cells. Estimated IC_{50} values were calculated. Rapamycin treatment in concentrations from 0-1,000 nM (Figure 2A) resulted in a mild degree of cytotoxicity but did not achieve an IC_{50} [approximated IC_{50} of Rapamycin monotherapy against Daoy cell culture in literature is also closer to ≥1,000 nM (25)]. Gemcitabine resulted in an IC_{50} at a concentration of 7.1 nM (Figure 2B). The combination of 100 nM rapamycin and gemcitabine demonstrated an IC_{50} of 0.9 nM. Our study showed that the combination of gemcitabine and rapamycin results in greater cytotoxicity than either medication alone.

PP-T-cells generate cytotoxic T-cell responses that were significantly better than naïve T-cells. As demonstrated in Figure 3 and Figure 4, induction of adoptive immunity *via* pretreatment of naïve T-cells with peptides derived from Bmi-1, SOX2, and MELK resulted in an increased level of Daoy cell death compared to controls at the same dilution. For this study, the "target cells" are the Daoy cells, and the "effector cells" are the T-cells, naïve or peptide-pulsed. Three different preparations of the two cell populations were prepared. For the 1:10 target:effector dilution, PP-T-cells resulted in a Daoy cell cytotoxicity of 57.9% (SD=0.31), while naïve T-cells induced a Daoy cell cytotoxicity of 47.5% (SD=2.07). In the 1:50 target:effector dilution, PP-T-cells caused a Daoy cell cytotoxicity of 58.7% (SD=1.81) and the naïve T-cells induced a Daoy cell cytotoxicity was 52.6% (SD=2.81). Lastly, in the 1:100 target:effector dilution, Daoy cell cytotoxicity with PP-T-cell treatment was 60.7% (SD=1.05), whereas Daoy cells incubated with naïve T-cell had a cytotoxicity of 52.1% (SD=1.17). The absolute differences between the Daoy cell cytotoxicities treated with PP-T-cells and naïve T-cells in the 1:10, 1:50, and 1:100 target:effector dilutions were 10.4%, 6.08%, and 8.62%, respectively. The ratios of Daoy cell cytotoxicity incubated with PP-T-cells and naïve T-cells in the 1:10, 1:50, and 1:100 groups were 1.22, 1.12, and 1.17, respectively. Thus, even with the relatively high cytotoxicity demonstrated with the naïve T-cell treatments, there was a statistically significant increase in cytotoxicity among the treatments including the PP-T-cells. The 1:10 and 1:50 dilutions were significant at $p<0.05$, and the 1:100 dilution showed significance at $p<0.01$.

Gemcitabine and rapamycin treatments impact expression of stem-cell related genes in Daoy cells. Daoy cells were treated with various concentrations of gemcitabine and rapamycin, and resultant mRNA expression levels are depicted in Figure 5. Bmi-1 expression levels increased with gemcitabine treatment. Solitary rapamycin treatment slightly decreased Bmi-1 expression. In dual-treated cells, Bmi-1 expression increased as the concentrations of gemcitabine rose, though these Bmi-1 levels were lower when compared with the gemcitabine-treated, rapamycin-free cells. However, the differences in Bmi-1 expression between the cells treated with

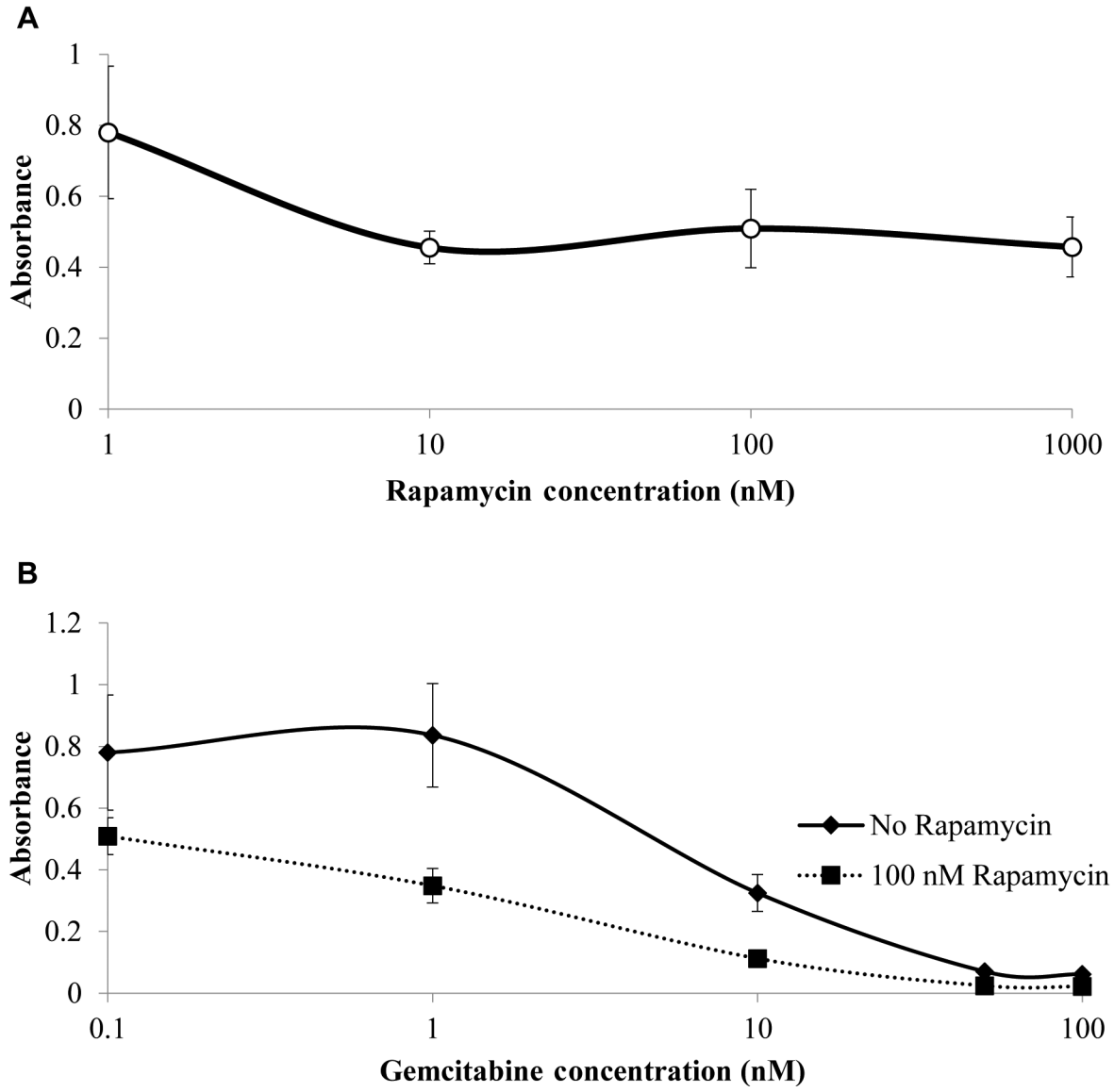

Figure 2. *Gemcitabine IC_{50} against Daoy decreases 10-fold with addition of Rapamycin. A. Rapamycin treatment in concentrations from 0-1000 nM. It resulted in a mild degree of cytotoxicity but did not achieve an IC_{50}. B. Decreasing cell viability of Daoy cells treated with increasing concentrations of gemcitabine. Points represent mean of twelve values with standard deviation. Gemcitabine-treated Daoy cells demonstrated an IC_{50} of approximately 10 nM. Daoy cells treated with 100 nM Rapamycin and increasing concentrations of Gemcitabine demonstrated an IC_{50} of about 1 nM.*

gemcitabine or both gemcitabine and rapamycin were not statistically significant, based on the student's *t*-test. MELK expression decreased upon treatment with gemcitabine alone. Sole rapamycin treatment greatly decreased MELK expression. However, dual-treatment increased MELK expression, and the expression was positively correlated with gemcitabine concentrations. SOX2 was expressed in all cell groups but levels were not significantly different between any of the treatment groups. Neither MELK nor SOX2 expression levels showed statistically significant differences with the student's t test between Daoy cells treated with gemcitabine alone or both chemotherapeutic agents.

Adoptive infusion of T-cells stimulated with peptide-pulsed DCs significantly augments inhibition of tumor growth by combined rapamycin/gemcitabine therapy, and this effect is further increased by addition of lipopolysaccharide (LPS). Bmi-1, MELK, and SOX2 are all stem cell-related genes that are expressed in BTICs. Daoy cells also express these proteins, as demonstrated by the prior study. Figure 6 depicts the tumor

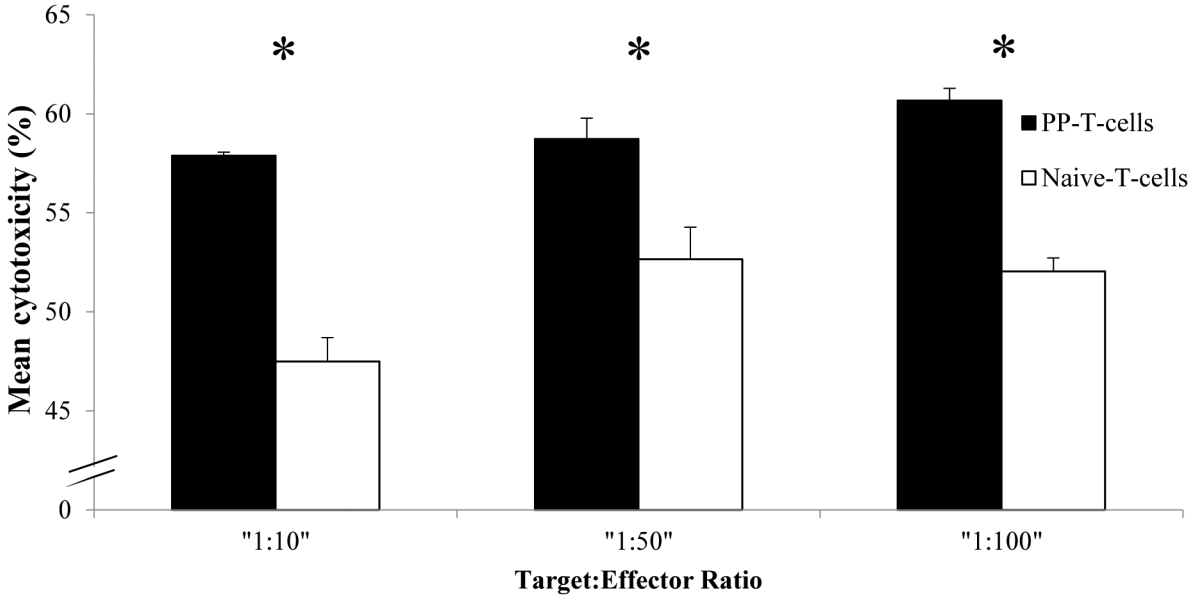

Figure 3. *PP-T-cells provide significantly higher cytotoxicity compared to Naïve T-cells. Target:effector ratios of approximately 1:10, 1:50, and 1:100 are included and represent different ratios of the two cell populations present: Daoy cells and PP-T-cells, respectively (n=3). Even with the higher cytotoxicity demonstrated in the Naïve T-cell treatments, there was a statistically significant increase in cytotoxicity among the treatments including the PP-T-cells, as determined by student's test. All differences are significant with p<0.05 shown with asterisk.*

growth of the Daoy cell xenografts in each of the treatment groups. As shown, the addition of PP-T-cells with concomitant IL-2 infusion did not result in a statistically different outcome in tumor volume compared with the outcome in the control group. However, groups 3 and 4 showed a relative decrease in tumor burden compared with controls. A greater treatment effect was noted in the mice receiving chemotherapy, IL-2, and peptide-stimulated T-cells (group 4) compared to the mice that received chemotherapy and IL-2 alone (group 2).

The mixed model analysis revealed that there is a significant group effect (*i.e.*, tumor volumes are different among groups overall, $p<0.0001$), a significant day effect (*i.e.*, tumor volumes are different between different days, $p<0.0001$), and a significant group by time interaction (*i.e.*, the group differences are changing over time, $p=0.0008$), after adjusting for the baseline difference. Pertinent and significant pair-wise group differences averaging over all days are shown in Figure 6. Of all of the different treatment groups, the greatest decrease in tumor growth was seen in the mice treated with chemotherapy, IL-2, peptide-stimulated T-cells, and LPS (group 5).

Discussion

In this study we have demonstrated the ability of peptide-pulsed dendritic cell stimulated T-cells to act together with chemo/biologic therapy to slow the growth of a brain tumor cell line in a subcutaneous xenograft model. The initial data presented show additive effects of combining a nucleoside analog gemcitabine with an mTOR inhibitor, rapamycin. Gemcitabine was chosen for this study in part due to pre-clinical evidence of synergy with immunotherapy but also due to its extensive use in patients with brain metastases from a variety of cancers, as well as being used for radiation sensitization for brain tumors (26). The exact mechanism of potential synergy between gemcitabine and rapamycin is beyond the scope of this paper, but likely involves interactions with several cell death pathways including those mediated by p53 and MDM2 (27). It was shown in a human leiomyosarcoma model, that rapamycin and gemcitabine synergize by reducing ERK1/2 activation and by eliminating rapamycin-induced simulation of Akt phosphorylation (28). For breast cancer cells, *in vitro* co-treatment with rapamycin and gemcitabine (or doxorubicin) resulted in an additive effect, whereas synergistic interactions were observed in combinations with paclitaxel, carboplatin, and vinorelbine (29).

Next, we confirmed that T-cells generated from *in vitro* stimulation with peptide-pulsed DCs can achieve cytotoxicity against our Daoy medulloblastoma model. The peptides we chose are epitopes from a variety of proteins, which have been associated with brain tumor stem cells (or brain tumor initiating cells). MELK is a kinase, but the other two genes are nuclear transcription factors, SOX2 and Bmi1. We did not confirm whether our T-cells were truly depleting or targeting a stem cell population in our Daoy model; however, we did

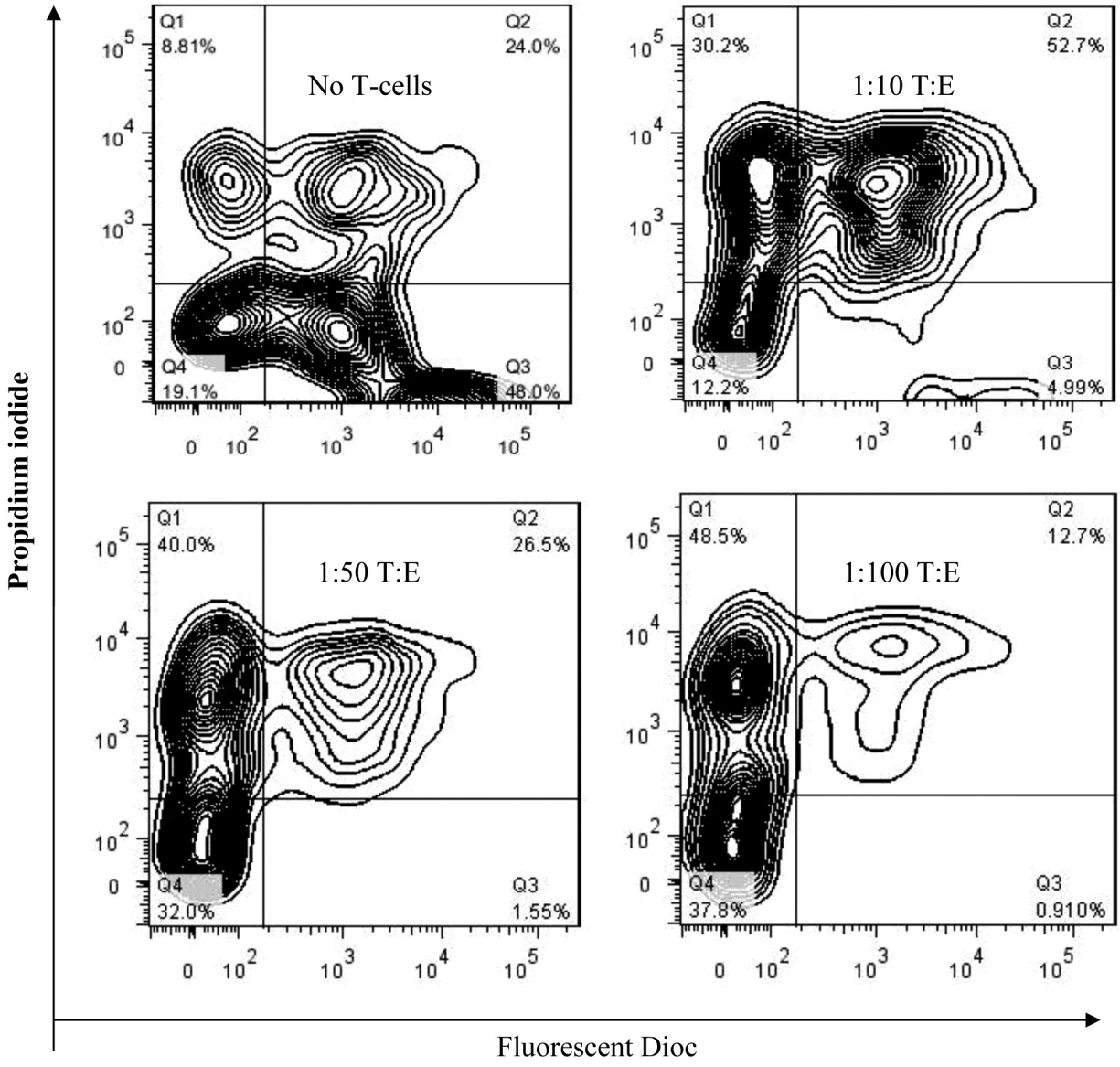

Figure 4. *Flow cytometry confirms the accuracy of the target:effector ratios in this study. The x-axis, indicating Dioc fluorescence separates two cell populations: high Dioc levels represent the Daoy cells and low Dioc levels are reflective of the PP-T-cells. The y-axis showing propidium iodide reflects cell death.*

show that these proteins are expressed at various levels in these cells. Future studies will be needed to determine whether there is indeed a "stem-like" population in malignant brain tumors that can be targeted with specific immuno-therapy. The studies do provide a proof of concept that nuclear protein epitopes can be targeted with immune based strategies, as evidenced by current active trials targeting H3.3 K27M mutant gliomas with a peptide vaccine (NCT02960230).

Finally, our study comparing combinations of chemo/biologic therapy with and without immuno-therapy shows that the combination of chemo/biologic therapy with immunotherapy has the greatest effects on tumor growth. This result is not entirely surprising, but again provides a proof of concept for future therapeutic approaches for malignant brain tumors in children and adults. In the past, we have attempted to combine conventional chemotherapeutic agents in intensive regimens, attempting to use agents that did not have overlapping toxicities. However, all these agents act through DNA-damage related mechanisms, and we have reached our limit in combining these agents without causing undue toxicities at the expense of sacrificing efficacy. Hopefully, by adding in another modality, immunotherapy, which has a completely different

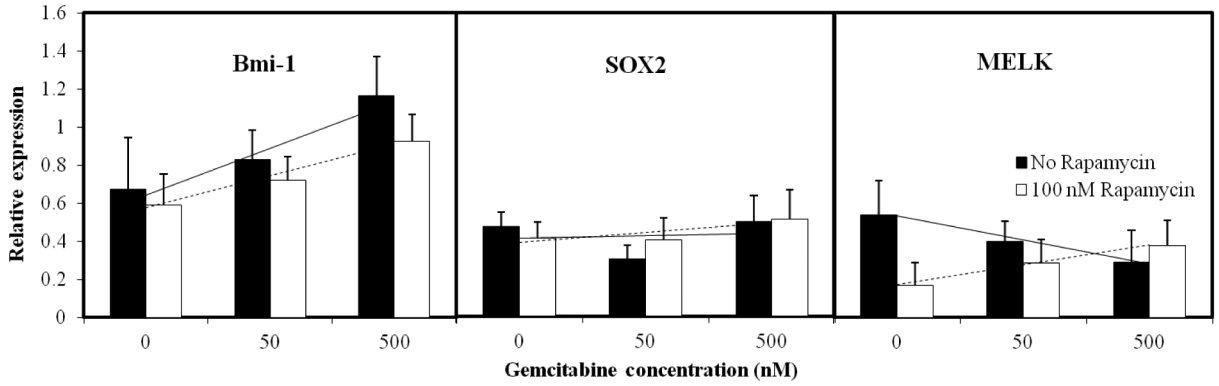

Figure 5. *Gemcitabine and rapamycin impact expression of stem cell related genes. Six different treatment groups were established and Daoy cells were incubated for 24 h with 1) no treatment, 2) 50 nM Gemcitabine, 3) 500 nM Gemcitabine, 4) 100 nM Rapamycin, 5) 100 nM Rapamycin and 50 nM Gemcitabine, and 6) 100 nM Rapamycin and 500 nM Gemcitabine. For each of the groups, RT-PCR was carried out and levels of Bmi-1, SOX2, MELK, and β-Actin mRNA were assessed. Ratios of the mRNA of interest to β-Actin mRNA were calculated and are depicted above with standard error bars included. Although changes are not statistically significant Bmi-1 expression rose with sole gemcitabine treatment and this rise was repeated, but at a lower level with the addition of 100 nM Rapamycin. SOX2 expression was unaltered by treatment. MELK expression decreased with increasing Gemcitabine, but with the addition of Rapamycin, expression levels slowly increased with rising Gemcitabine concentrations. The qPCR results are triplicates. Lines show "trends".*

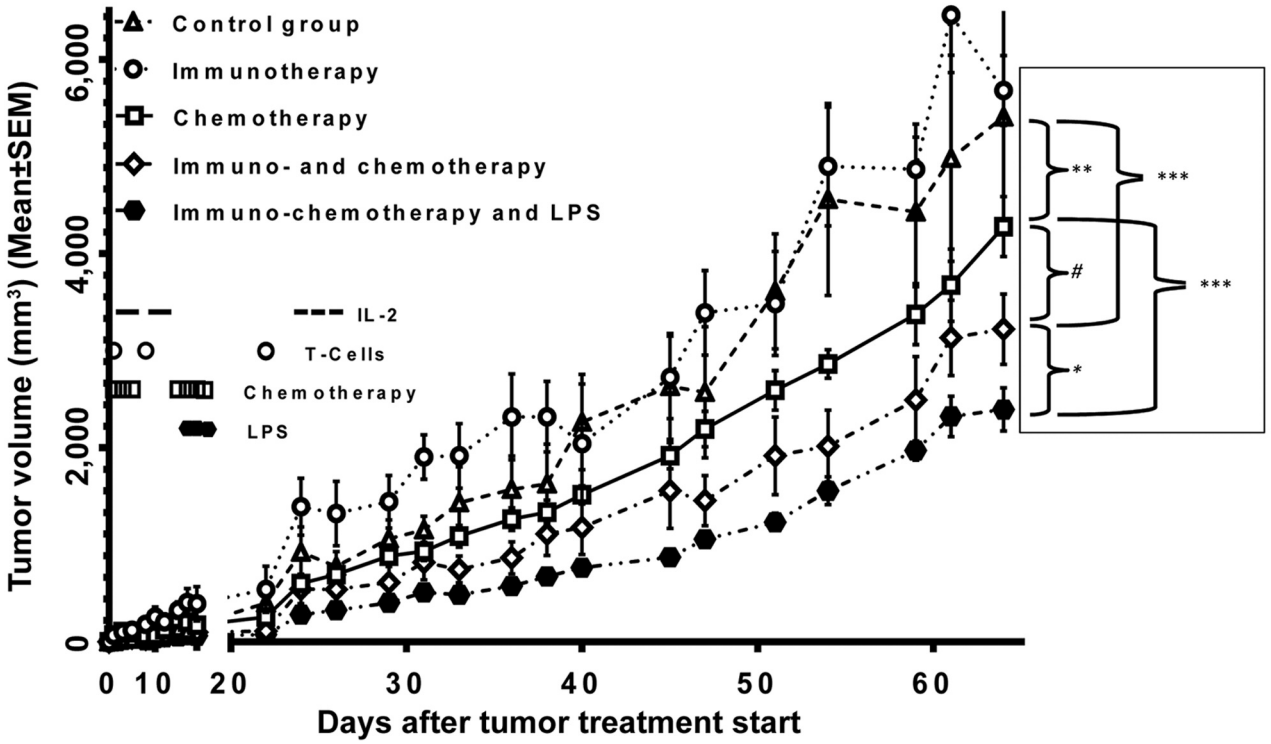

Figure 6. *Lipopolysaccharide increases the effect of adoptive T-cell immunotherapy, which significantly augments tumor inhibition by rapamycin and gemcitabine. Five different treatment groups were established using SCID mice. Group 1 (n=5) was the control group and received only PBS. Group 2 (n=4) received Peptide generated T-cells and IL-2. Group 3 (n=5) received Gemcitabine, Rapamycin, and IL-2. Group 4 (n=5) received Gemcitabine, Rapamycin, Peptide generated T-cells, and IL-2. Group 5 (n=5) received Gemcitabine, Rapamycin, Peptide generated T-cells, IL-2, and LPS. Groups 1 and 2 did not demonstrate significant differences in tumor growth. Group 3 showed decreased tumor growth relative to group 1, while Group 4 had a further decrease in tumor growth, compared with Group 3. Notably, the greatest decrease in tumor growth was demonstrated by the mice in Group 5. Pertinent results from ANCOVA for comparison of tumor volumes are shown in the box: *p=0.03, **p<0.01, ***p<0.001 and #p=0.262.*

toxicity profile we can develop novel combinations that will hopefully add efficacy and keep undesirable side effects to a minimum. Suggested modern approaches for pediatric medulloblastoma include conjunction of immunotherapy with chemotherapy (30), since studies alike have shown that simultaneous treatment with vaccination and chemotherapy improves antigen-specific T-cell activity (31, 32). In fact, mTOR inhibition used in our study can be potentially substituted with other small-molecules synergizing with DNA-damaging chemotherapies in medulloblastoma such cell cycle checkpoint kinase inhibitors (33).

In conclusion, our results suggest that a combination of chemo/biologic-therapy and immunotherapy can be used to achieve better tumor responses than either therapy alone. These results are based upon treated cell lines and mouse models with a subcutaneous medulloblastoma brain tumor cell line. Both models are limited in their applicability to mimic brain tumor responses to therapy. However, studies have demonstrated that immunotherapy can generate intracranial tumor responses (34-36), so this methodology might be translatable to a clinical trial. Furthermore, the additional LPS administration, which appeared to improve treatment response to chemotherapeutic and immunotherapeutic agents, suggests a role for more specific agonists of the TLR2 and TLR4 signaling pathways in immunotherapy protocols.

Conflicts of Interest

No relevant conflicts of interest to declare.

Authors' Contributions

Dr. Lasky provided the original conceptual design of this study. Yuntao Wang carried out the experiments and collected the data. Dr. Pak is the statistician, who also participated in interpretation of results and manuscript writing along with Drs. Lasky, Bradford and Panosyan.

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