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TITLE: Intraventricular Delivery of Engineered Oncolytic Herpes Simplex Virotherapy to Treat Localized and Metastatic Pediatric Brain Tumors

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1. INTRODUCTION

Effective, less-toxic therapies for malignant pediatric brain tumors represent an unmet need. The significant morbidity and mortality associated with childhood brain cancer is compounded by neurotoxicity for the developing brain caused by current therapies. Oncolytic HSV (oHSV) offers an inventive, targeted, less-toxic approach for children with brain tumors. Introduced mutations in the engineered virus prevent a productive infection in normal cells in the brain while maintaining the virus' oncolytic activity against cancer cells. One key limitation of oHSV to treat brain tumors is the current necessity for intratumoral inoculation of the virus. Intraventricular (IVT) delivery would overcome critical delivery barriers and provide significant advantages for pediatric brain tumor patients by obviating the need for invasive neurosurgical procedures and enabling repeat targeting of both intracranial and spinal metastatic disease. However, further research is required because of expected toxicity with this route. We hypothesized that the toxicity from IVT oHSV inoculation is immune mediated and can be prevented with administration of an immune modulator to reduce the host inflammatory reaction to the virus. Furthermore, we hypothesized that IVT delivery of oHSV will prolong survival in a murine model of disseminated medulloblastoma (MB). We proposed 3 specific aims to test these hypotheses: 1) to determine the underlying mechanism of IVT oHSV toxicity in immunocompetent and immunodeficient murine models; 2) to develop and test strategies to block underlying mechanism(s) of IVT oHSV toxicity in an immunocompetent murine model; and 3) to establish the efficacy of IVT oHSV in a murine model of disseminated MB. Lastly, as a career development award, the goal of this proposed research and career development plan was to provide Dr. Friedman with every opportunity to become a pre-eminent pediatric brain tumor translational researcher. The key components included: 1) frequent face-to-face interaction with mentors; 2) establishment of a mentoring committee; 3) continued education and advancement of clinical expertise; 4) development of both local and national collaborations; and 5) hands-on training for obtaining regulatory approval (FDA, IRB, etc.) necessary to translate a therapeutic agent to clinical trials.

2. KEYWORDS

Virotherapy; herpes virus; HSV; oncolytic; intraventricular delivery; immunotherapy; medulloblastoma; pediatric brain tumors.

3. ACCOMPLISHMENTS

The following includes the goals, timeline, and milestones for my training and educational development in pediatric brain cancer research:

Major Task 1: Training and educational development in pediatric brain cancer research	Months
Subtask 1: Participate in the monthly Pediatric Brain Tumor Board	1-36
Subtask 2: Participate in the Clinical and Translational Science Training Program	6-12
Subtask 3: Attend a yearly national scientific meeting in relevant scientific field	1-36
Subtask 4: Attend COG meeting annually	1-36

Subtask 5: Obtain regulatory approvals to conduct trial of intratumoral oHSV in children with recurrent brain tumors at UAB	1-12
Subtask 6: Write scientific manuscripts	12-36
Subtask 7: Apply for NIH funding (e.g. R01, R21)	18-36
<i>Milestone(s) Achieved: Presentation of project data at a national meeting; Open oHSV trial for children with recurrent brain tumors at UAB; Publish data in a top tier scientific journal; Obtain NIH funding</i>	12-36

I have made great progress towards this major task and have achieved many of the milestones as outlined below.

Subtask 1: Over the past year (Months 1-12), I served as Pediatric Brain Tumor Board Activity Director and led our monthly Pediatric Brain Tumor Board. This meeting consisted of pediatric neuro-oncologists, neurologists, neurosurgeons, neuropathologists, radiologists, fellows, residents, nurse practitioners, and rehabilitation physicians and therapists. This meeting has greatly enhanced my clinical expertise as all newly diagnosed and recurrent patient cases were discussed including patient presentation, imaging results, surgical approach, pathological diagnosis, and treatment options.

Subtask 2: I participated in the Clinical Investigator Training Program course which provided a practical and pragmatic approach for investigators. This program provided didactic sessions on the roles and responsibilities of investigators; how to navigate the IRB before, during and after a study; understanding study feasibility; principal investigator oversight during a study; study management; data management; and working together as a team. Panel discussions allowed for interactive question and answering sessions. This course was highly valuable to my training as a translational investigator actively translating oncolytic HSV from the lab to the clinics in an investigator-initiated clinical trial designed to test the safety and tolerability of this agent in children.

Subtask 3: In June 2015, I attended the 9th International Conference on Oncolytic Virus Therapeutics in Boston, MA. This was an outstanding meeting where I was able to confer with leading experts in oncolytic virotherapy from across the world. This year, my goal was to attend the 17th International Symposium on Pediatric Neuro-Oncology Liverpool, UK. Unfortunately, the meeting corresponded with a prior family commitment, and I was unable to attend; however I did submit an abstract to the meeting based on this research and it was presented by Eric Ring, MD, pediatric hematology/oncology fellow who started to work in my lab in July 2015. I am actively searching for another meeting I can attend later this year or early next year with a focus in pediatric neuro-oncology or oncolytic virotherapy.

Subtask 4: I attended the annual COG meeting in Dallas TX October 7-9th. This allowed me to network with other leading pediatric neuro-oncologists across the country.

Subtask 5: I obtained all necessary regulatory approvals needed to conduct the investigator-initiated phase I study of oHSV G207 in children with recurrent or progressive supratentorial brain tumors (Month 1-12). This included FDA Investigational New Drug, NIH DNA Recombinant Advisory Committee, UAB Institutional Biosafety Committee, and UAB IRB approval. We recently opened the study and are waiting to accrue the first patient (<https://clinicaltrials.gov/ct2/show/NCT02457845>).

Subtask 6: Over the next year, I anticipate working on a scientific manuscript directly related to this project. I am currently gaining experience writing a scientific manuscript on lab data not specifically related to this project.

Subtask 7: I applied for and was awarded an R01 grant from the FDA Orphan Product Development Program for the pediatric HSV trial. Applying for this grant was a very valuable experience in improving my grant writing skills and navigating an NIH grant application. I plan to apply for a laboratory based R01 or R21 during the time period outlined in the table above.

An additional activity I participated in over the past year that has been important for my training and educational development in pediatric neuro-oncology is grant reviews. I reviewed grant applications for two private foundations: The St. Baldrick's Foundation and the Rally Foundation for Childhood Cancer Research. This has provided me with first-hand exposure to grant reviews at the national level and has shown me what it takes to write a successful grant.

Lastly, I met with my mentors regularly (Dr. Gillespie weekly, Dr. Markert every other week) to discuss the research, review the data, and consider alternative approaches and future directions. These meetings have been invaluable for my professional development and for me obtaining greater proficiency in the lab.

The first year of funding has been very productive and has led to important new information regarding the toxicity of IVT oHSV and the ability to target MB with IVT oHSV. The following includes the goals, timeline and milestones of Major Task 1.

Major Task 1: Characterize the effects of IVT delivery of oHSVs (G207 and M002), inactivated virus, HSV vehicle and saline in CBA/J mice (estimated 5 groups x 10 mice x 2 experiments = 100 mice)	Months
Subtask 1: Obtain ACURO approval for all proposed animal studies	1-4
Subtask 2: Determine expression of HSV entry receptors in ependymal cells and survey brain and spine tissue for HSV infection by IHC	4-6
Subtask 3: Characterize the nature and extent of the inflammatory response by IHC (T cell markers CD4, CD8, and $\gamma\delta$; B cells marker CD19; natural killer cell marker CD335 (NKp46); and macrophage marker Ly-71 (F4/80)).	4-8
Subtask 4: Analyze peripheral blood for changes in total white blood cell count and subpopulations of immune effector cells by hemocytometer counting, blood smear examination, and FACS analysis (Total percentages and ratios of CD4:CD8 T cells, Th1(CD4+IFN- γ +IL-4-):Th2(CD4+IFN- γ -IL-4+) cells, and B cells (CD19)	4-8
Subtask 5: Calculate median survival times and generate and compare Kaplan-Meier survival curves	4-8
<i>Milestone(s) Achieved: Determination of the cause (virus, virus antigens, vehicle) of toxicity from IVT oHSV</i>	

Progress to date:

Subtask 1: ACURO approval was obtained for all proposed animal studies on 9/9/2016.

Immunocompetent CBA/J mice were injected intraventricularly with 10 μ L 1x10⁷ plaque-forming units (PFU) of HSV G207, HSV M002, or inactivated M002, or 10 μ L of saline or 10% glycerol (vehicle). Mice in the inactivated M002, saline, and 10% glycerol groups developed no toxicity; however 30% of the G207 mice and 100% of the M002 became morbid requiring euthanasia on day 4 after the injection (median survival 4 days for M002) suggesting that toxicity with intraventricular HSV is unrelated to the vehicle or viral antigens alone. This difference in the toxicity between the two viruses was an interesting finding because the viruses are similar except for two differences: 1) G207 replicates less efficiently due to an additional mutation that disables expression of ribonucleotide reductase, and 2) M002 produces interleukin-12 (IL-12) which stimulates a cytotoxic T cell and NK cell response.

To characterize the nature of the toxicity and the extent of the inflammatory response, we repeated the study with the viruses and sacrificed mice on day 1, 2, 3, and 4 and then sacrificed surviving mice on day 7, 9, 16, 23 and 30. The ependyma in the brain and spine appeared normal on day 1; however by day 2, there was focal disruption of the ependymal lining with some nuclear shrinkage, fragmentation, and cell death (**Fig 1**). The ependymal cells stained positive for HSV-1 on day 2 indicating the engineered virus was able to enter the ependymal cells. By day 3, there was more diffuse disruption of the ependymal lining and edema, and by day 4, there was diffuse involvement, disruption of the ependymal lining with minimal to no normal lining near the injection site, and reactive ependymal proliferation due to damage. Scattered CD19+ B cells and CD8+ cytotoxic T cells (**Fig 2**), and occasional CD4+ T cells were seen on day 3 and 4. The pathology was qualitatively similar in G207 and M002-treated mice; however only G207 mice survived past day 4.

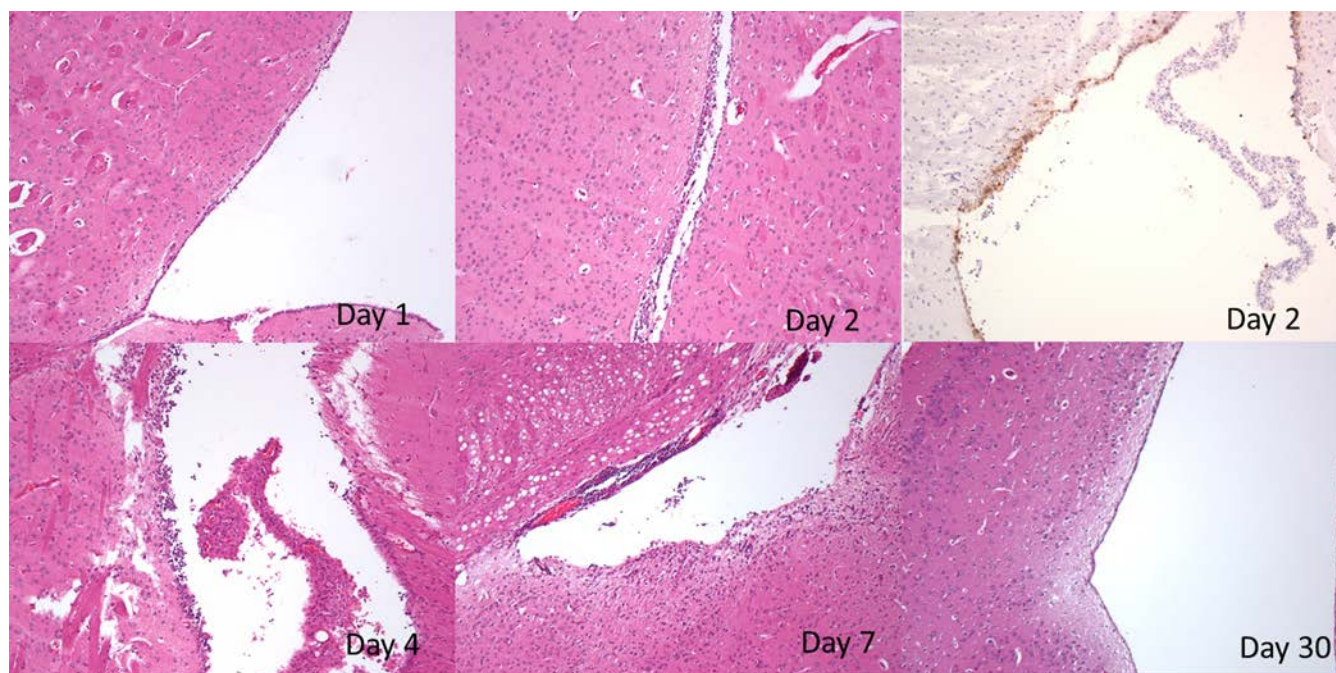


Fig 1. H&E and IHC staining of the ventricular lining of CBA/J mice after G207 inoculation demonstrated focal disruption of ependymal lining at day 2 with evidence of HSV-1 staining in ependymal cells, diffuse disruption and influx of immune effector cells by day 4, and subsequent slow repair of ependymal lining from day 7-30.

In surviving mice, the inflammatory response was decreased by day 7 and repair to the ependymal lining was seen over the next 3 weeks, although the lining was not completely normal in all areas (**Fig 1**). These findings are critically important as they highlight the time course of the inflammatory process that

occurs (maximum at day 4, resolving by day 7) and indicate the damage to the lining is largely repaired over several weeks.

We have achieved the milestone of Major Task 1 by determining the toxicity is due to virus; however there is more for us to learn based on the differences in toxicity between G207 and M002. While we know G207 and M002 entered the ependymal cells based on the HSV-1 staining on day 2, we do not yet know whether the infection was productive (i.e. the virus replicated and lysed the cells) or if the cell death was due to apoptosis. Furthermore, it is still unclear what role the immune response to the virus and the ependymal cell damage played in the morbidity of the mice. We hypothesize the ependymal cell death is due to apoptosis, and we are in the process of testing that hypothesis by performing IHC looking for expression of gD, a late virus protein that would be present if the infection was productive, and annexin V, which is associated with apoptosis. If cell death is due to apoptosis, then the increased toxicity seen in M002-treated mice is likely secondary to a more robust immune response from IL-12.

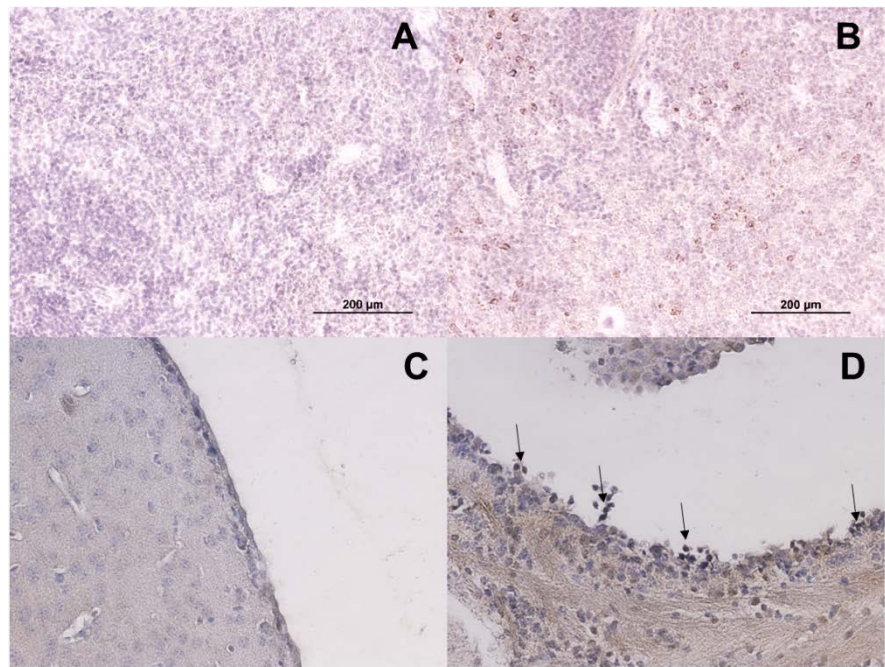


Fig 2. IHC staining for CD8+ cytotoxic T cells. **A)** Negative control spleen. **B)** positive control spleen. **C)** UV inactivated M002 day 3 demonstrated no CD8+ cells. **D)** M002 day 3 demonstrating scattered CD8+ cells (black arrow).

We next wanted to determine the effect of lowering the virus dose. Immunocompetent CBA/J mice were injected with 1×10^6 PFU of G207 or M002 in 10 μ L intraventricularly. Importantly, mice had no observable toxicity and appeared healthy throughout the experiment. Mice were sacrificed weekly starting at day 10 and brain and spinal tissue is being prepared for IHC to look for evidence of ependymal damage and/or inflammation. Because we were able to lower the dose of the virus without observable toxicity, we next elected to explore Major Task 5 to characterize the sensitivity of disseminated pediatric medulloblastoma (MB) to oHSV. Patient derived MB xenograft D341-luc, which is luciferase enabled, was used for the experiment. Athymic nude mice received 500,000 D341-luc cells intraventricularly. Five days later, the mice received 1×10^6 PFU of G207 or 10% glycerol in 10 μ L. Bioluminescence imaging was performed on day 4 and 7, and mice were euthanized when they became morbid. The single G207 dose significantly prolonged survival (**Fig 3**) and reduced spinal metastases (**Fig 4**). Because D341-luc is such an aggressive tumor and resulted in death of the control mice starting at day 11, we repeated the experiment with 250,000 cells and added a second dose of G207 one week after the first to establish safety and efficacy of repeat dosing. Importantly, this approach was safe and significantly prolonged median survival from 19 to 29 days ($p < 0.001$) (**Fig 5**). Mice in the control group developed large primary and metastatic tumors, whereas treated mice developed smaller primary tumors (**Fig 6**). Moreover, significantly more spinal metastases were seen in the control mice at day 14 and 18 than the G207 mice, and no spinal metastases developed in the treated mice past day 25. These findings suggest

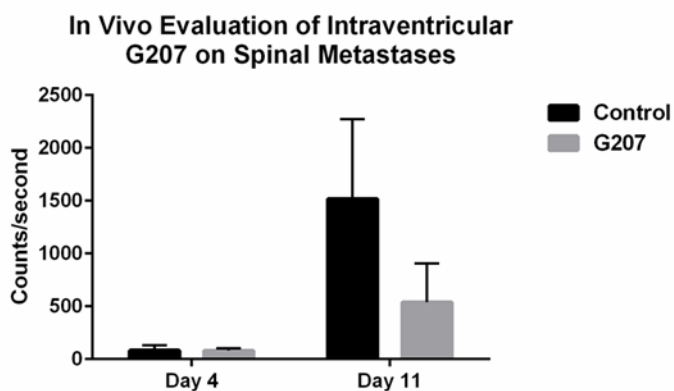


Fig. 4. Bioluminescence of spinal D341luc cells in athymic nude mice. Mice received intraventricular injection of 5×10^5 D341luc cells. Five days after tumor implantation, mice received a single intraventricular injection of 10 μ L of saline with 10% glycerol or 1×10^6 plaque-forming units of G207. Imaging was performed on day 4 and day 11.

that intraventricular G207 can be given safely, prolong survival, and decrease spinal metastases at a lower dose than is typically used in intratumoral injections.

Over the next year, in addition to determining the cause of ependymal cell death (infection or apoptosis), we will explore whether the toxicity seen at 1×10^7 PFU is mitigated in other mouse models (**Table 1**) which lack various immune effector cells. This will enable us to further pinpoint what role the influx of immune effector cells (cytotoxic T cells, T helper cells and B cells) has on the toxicity. We will confirm the results of the studies in the immunodeficient murine models by performing in vivo antibody blocking studies of the immune effector cell(s) thought to be contributing to toxicity. We will also begin to explore combining oHSV with immune modulators to see if we can block the toxicity seen at 1×10^7 PFU.

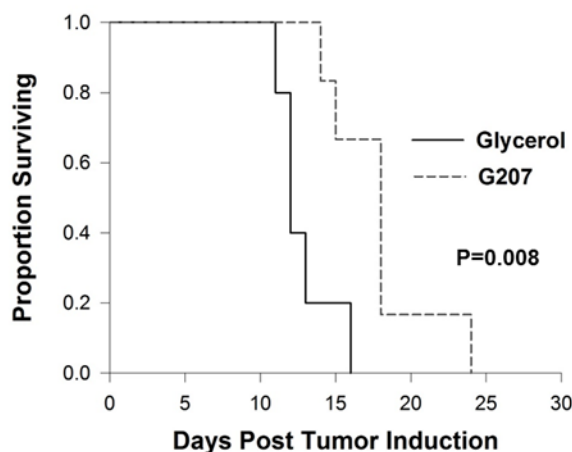


Fig. 3. Kaplan-Meier survival plots of athymic nude mice after intraventricular injection of 5×10^5 D341luc cells. Five days after tumor implantation, mice received a single intraventricular injection of 10 μ L of saline with 10% glycerol or 1×10^6 pfu of G207. Median survival time was significantly increased from 12.0 ± 0.5 to 18.0 ± 0.9 days with G207 treatment.

Table 1: Murine mouse models to characterize immune effector cells responsible for IVT oHSV toxicity

Mouse model	Mature B cells	Mature T cells	Natural Killer cells	Macrophages
NOD scid gamma	Absent	Absent	Absent	Defective
Rag1	Absent	Absent	Present	Present
Athymic nude	Present	Absent	Present	Present

Metastatic D341 MB vs Intraventricular G207

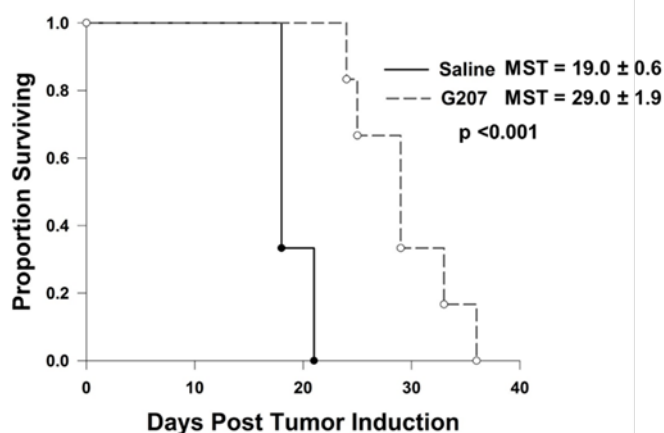


Fig 5. Kaplan-Meier survival plots of athymic nude mice after intraventricular injection of 2.5×10^5 D341luc cells. Five days and 12 days after tumor implantation, mice received an intraventricular injection of 10 μ L of saline with 10% glycerol or 1×10^6 pfu of G207.

We will also follow-up on our exciting data indicating that intraventricular oHSV can target disseminated MB by testing the ability of G207 and M002 to kill another patient-derived pediatric group

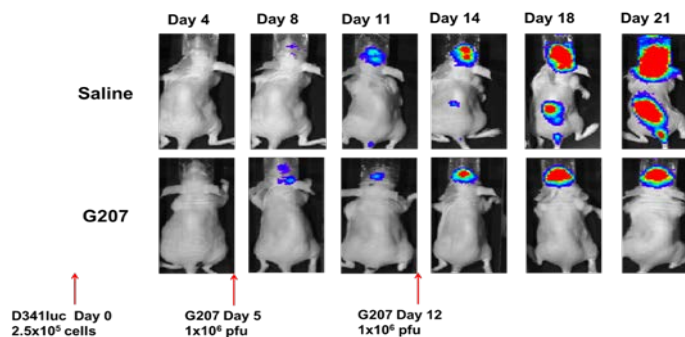


Fig. 6. Example of characteristic bioluminescence imaging of athymic nude mice after intraventricular injection of 2.5×10^5 D341luc cells. Mice were treated with saline or G207 at post-tumor inoculation day 5 and 12. Control mice developed larger primary tumors and metastatic spinal disease compared to treated mice.

3 MB xenograft D425-luc. Additionally, we will begin to test two murine medulloblastoma models that we obtained this year as planned: 1) subgroup 3 (non-WNT/non-SHH) and 2) sonic hedgehog (Trp53^{-/-} Ptch^{-/+}).

Review of other Subtasks for Major Task 1:

Subtask 2: We have encountered difficulty staining ependymal cells for HSV entry molecules because of a lack of effective murine antibodies. We have successfully utilized flow cytometry for murine CD111, an adhesion molecule that is the most efficient entry molecule for HSV, and are in the process of trying to optimize the antibody for IHC. Nevertheless, the HSV-1 staining seen at day 2 indirectly indicates that HSV entry receptors are present on ependymal cells because without entry receptors the virus would not be able to enter the cell. The next step is to determine whether the infection is productive or not.

Subtask 3: We have assessed T cells and B cell markers (**Fig 8**). The abcam antibody to F4/80 demonstrated weak staining and the NKp46 was suboptimal in control tissue. We are exploring suitable alternative antibodies.

Subtask 4: We have successfully utilized flow cytometry to determine CD4, CD8 and CD19 cell populations from blood in mice (**Fig 9**). We can accurately determine CD4:CD8 ratio. We have also calculated Th1 and Th2 percentages and the Th1:Th2 ratio (**Fig 10**). We are planning a repeat experiment to evaluate changes in these various immune effector cell populations over time after intraventricular injection of saline, M002 and G207.

Subtask 5: This has been completed. Median survival was 4 days for M002.

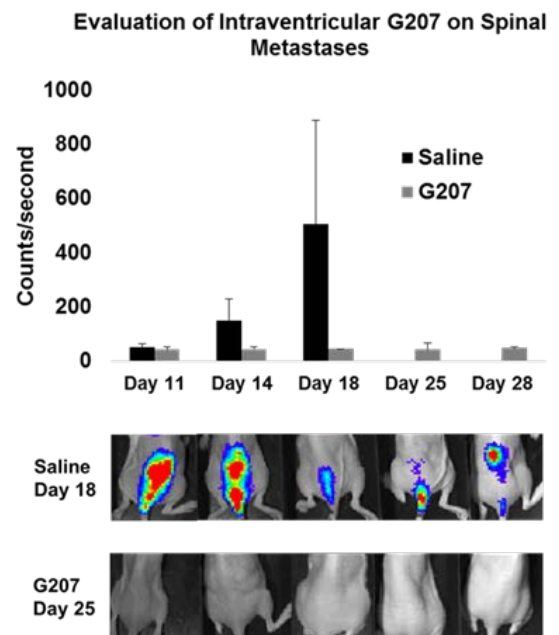


Fig 7. Bioluminescence of spinal D341luc cells in athymic nude mice. Mice received intraventricular injection of 2.5×10^5 D341luc cells. Five and 12 days after tumor implantation, mice received a single intraventricular 10 μ L injection of saline with 10% glycerol or 1×10^6 plaque-forming units of G207. Bioluminescence was measured twice weekly starting at day 11. Pictures show control mice at day 18 and G207-treated mice at day 25.

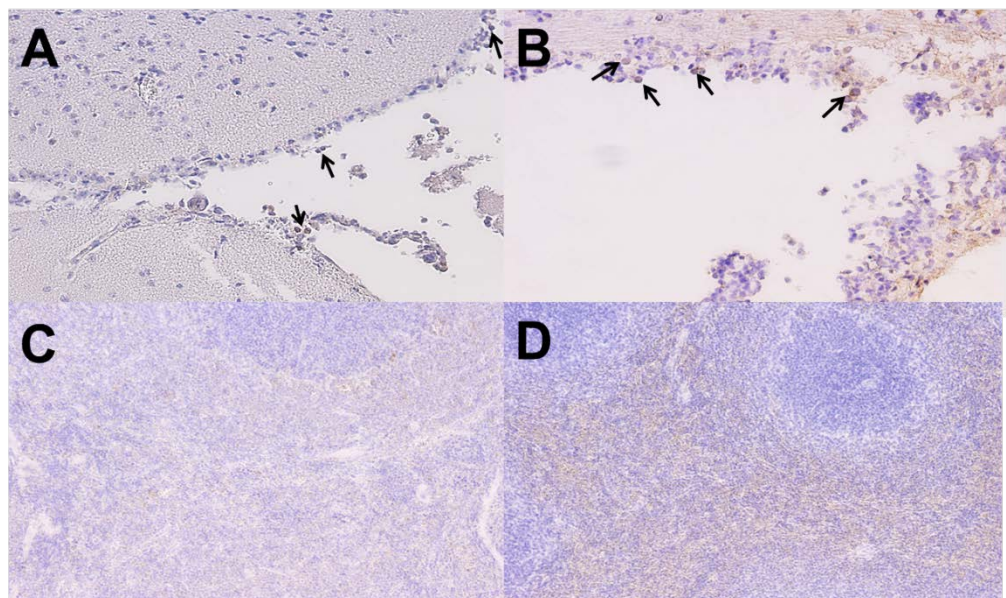


Fig 8. IHC staining for immune effector cells. **A)** Ventricular lining on day 4 after treatment with M002 demonstrated occasional CD4⁺ T cells (arrows) and **B)** scattered CD19⁺ B cells (arrows). **C)** Spleen tissue positive control for NK cell antibody demonstrated inadequate staining at 1:100 and 1:200 indicative of a suboptimal antibody. **D)** Spleen tissue positive control of macrophage demonstrated weak staining.

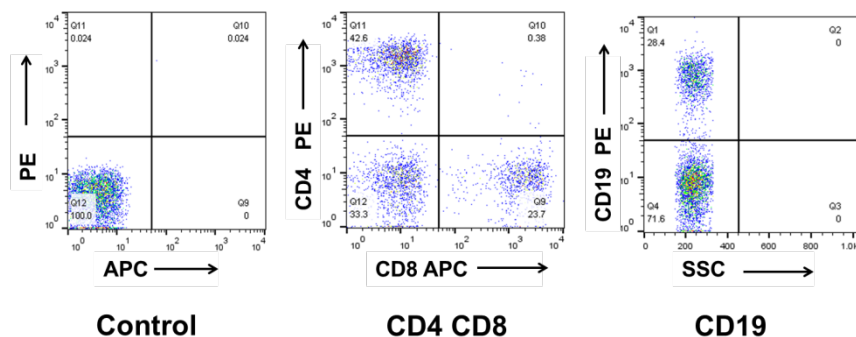


Fig 9. Flow cytometry results of peripheral blood in CBA/J mouse. CD4+ helper T cells represented 42.6% of cells, CD8+ cytotoxic T cells represented 23.7%, and CD19 B cells were 28.4% of the white blood cells. CD4:CD8 ratio was 1.8.

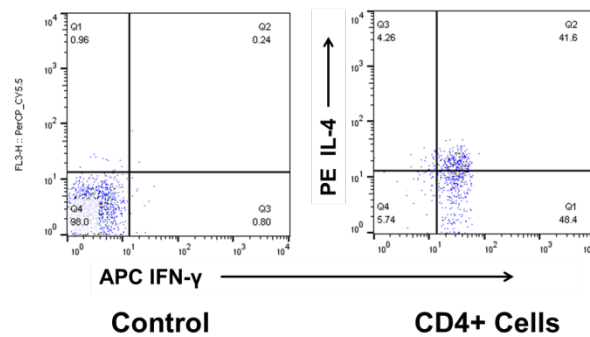


Fig 10. Percent of Th1 (INF- γ /IL-4-) and Th2 (INF- γ /IL-4+) CD4+ cells in the peripheral blood of CBA/J mice was determined by flow cytometry. Th1 cells represented 48.4% and Th2 cells were 4.3% of the CD4+ population. Th1:Th2 ratio was 11.4

The following includes the goals, timeline and milestones of Major Task 2:

Major Task 2: Characterize the effects of IVT delivery of oHSVs (G207 and M002), inactivated virus, HSV vehicle and saline in immunodeficient murine models (NOD scid gamma, Rag1, athymic nude; estimated 5 groups x 10 mice x 2 experiments = 100 mice/model)	
Subtask 1: Survey brain and spine tissue for HSV infection by IHC	8-14
Subtask 2: Characterize the nature and extent of the inflammatory response by IHC as described above	8-14
Subtask 3: Analyze peripheral blood as described above	8-14
Subtask 4: Calculate median survival times and generate and compare Kaplan-Meier survival curves	8-14
<i>Milestone(s) Achieved: Determination of the mediator(s) of toxicity from IVT oHSV</i>	

Thus far we have performed experiments in athymic nude mice and demonstrated that similar to CBA/J mice, intraventricular virus did not cause morbidity at 1×10^6 . We plan to expand these studies to 1×10^7 PFU and explore toxicity in SCID gamma and Rag1 models as well if we see toxicity in athymic nude mice at 1×10^7 . However, if M002 and G207 do not cause morbidity in athymic nude mice like they do in immunocompetent CBA/J mice, then that will implicate T cells and we will proceed with T cell subset blocking studies in Major Task 3.

The experiments described below in Major Task 3 and Major Task 4 will be performed over the next year and are part of the future direction of this research. We anticipate initiating and completing these studies as planned during year 2 and 3.

Major Task 3: Characterize the effects of IVT injection of saline and either vehicle, live virus or inactivated virus after depletion of various immune effector cells (CD4, CD8 T cells; B cells; natural killer cells; and macrophages) in CBA/J mice (estimated 5 groups each with a control x 2 of 10 mice x 2 experiments = 200 mice)	
Subtask 1: Confirm successful blocking of cell of interest by FACS analysis	14-20

Subtask 2: Survey brain and spine tissue for HSV infection by IHC	14-20
Subtask 3: Characterize the nature and extent of the inflammatory response by IHC as described above	14-20
Subtask 4: Analyze peripheral blood as described above	14-20
Subtask 5: Calculate median survival times and generate and compare Kaplan-Meier survival curves	14-20
<i>Milestone(s) Achieved: Determination/confirmation of the mediator(s) of toxicity from IVT oHSV</i>	

Major Task 4: Characterize the effects of intraventricular delivery of oHSV or saline in combination with various immune modulators in CBA/J mice (estimated 10 time points x 3 drugs x 3 mice = 90 + 3 drugs x 2 groups x 10 mice x 2 experiments = 120 or total of 210 mice)	
Subtask 1: Determine the ideal time to give the immune modulator (from 48 hours prior to virus inoculation to 48 hours after inoculation)	20-22
Subtask 2: Survey brain and spine tissue for HSV infection by IHC	22-28
Subtask 3: Characterize the nature and extent of the inflammatory response by IHC as described above	22-28
Subtask 4: Analyze peripheral blood as described above	22-28
Subtask 5: Calculate median survival times and generate and compare Kaplan-Meier survival curves	22-28
<i>Milestone(s) Achieved: Determination of effective toxicity mitigating strategies (agents, timing)</i>	

The following includes the goals, timeline and milestones of Major Task 5:

Major Task 5: Characterize the effects of IVT G207 and M002 in CBA/J mice bearing disseminated MB from pediatric MB xenografts D425 and D341 (no identifiable information will be accessible to the research team by any means) and in $Trp^{53-/-} Ptch^{-/+}$ mice with MB (estimated 3 groups x 2 tumors x 10 mice x 2 experiments = 120 athymic nude mice and 3 groups x 10 mice x 2 experiments = 60 $Trp53^{-/-} Ptch^{-/+}$ mice)	
Subtask 1: Maintain and passage pediatric MB xenografts D425 and D341 in athymic nude mice to provide cells for oHSV sensitivity testing (2 mice x 6 months x 2 tumors + 3 mice x 2 tumors for tissue for studies = 30)	28-34
Subtask 2: Analyze tumor response by bioluminescence imaging	28-36
Subtask 3: Survey brain and spine tissue for HSV infection by IHC	28-36
Subtask 4: Characterize the nature and extent of the inflammatory response by IHC as described above	28-36

Subtask 5: Calculate median survival times and generate and compare Kaplan-Meier survival curves	28-36
<i>Milestone(s) Achieved: Characterization of the sensitivity of disseminated pediatric medulloblastoma to oHSV</i>	28-36

We anticipated initiating and completing these studies in year 3; however since decreasing the virus dose by a log proved safe and a critical component of this grant is to determine whether intraventricular oHSV can target pediatric MB so that this approach can be translated from the lab to the clinics to hopefully benefit patients in need, we elected to begin exploring this task in year 1. Thus far we have partially achieved the milestone of Major Task 5 by determining, for the first time, that a disseminated pediatric MB model is sensitive to oHSV.

Subtask 1: We are currently maintaining D431 MB xenograft in athymic nude mice for ongoing studies and plan to study the sensitivity of disseminated D425 in year 2.

Subtask 2: We have analyzed D341 response by bioluminescence imaging and are planning for similar studies with D425 and the murine MB models.

Subtask 3: Brain and spine tissue has been saved from the D341 experiment and will be analyzed for HSV infection. We will also plan to survey tumor tissue for evidence of HSV infection at various time points.

Subtask 4: Brain and spine tissue has been saved from the D341 experiment and we plan to characterize the nature and extent of inflammatory response in the tissue.

Subtask 5: Median survival times were calculated for D341 and Kaplan-Meier survival curves are included above.

Thus far, results were disseminated through 2 scientific abstracts presented at meetings and during a Pediatric Medical Grand Rounds lecture at UAB (see section 6 (Product) for specific information). The first meeting was at the National Conference on Undergraduate Research (NCUR). This was attended by a UAB premedical undergraduate student, Tina Etminan, who has been gaining research experience in my lab over the past 3 years. This national undergraduate research conference provided an opportunity to highlight our ongoing research activities and educate undergraduate students and potentially increase interest in scientific research and pediatric brain tumor research. The other meeting was the 17th International Symposium on Pediatric Neuro-Oncology. This meeting was attended by researchers and physicians from around the globe who are focused on pediatric brain cancer research. Dr. Eric Ring, a 2nd year pediatric hematology/oncology fellow at UAB who joined my lab in July 2016 as part of the research training component of his fellowship, attended the conference and presented the research. Working with Ms. Etminan and Dr. Ring on these abstracts provided me with an opportunity to enhance my skills as a developing mentor, and these meetings provided them with an opportunity to learn how to prepare an abstract and a poster and how to talk about the research with others who may not be familiar with oncolytic virotherapy and our research approach.

4. IMPACT

Oncolytic HSV (oHSV) has shown great promise in treating adult brain tumors and non-CNS solid tumors, as demonstrated by results from adult phase I trials conducted at UAB in glioblastoma and the recent first FDA approval of an oncolytic virus, engineered HSV Imlygic, to treat melanoma. One of the key limitations of oHSV to treat brain tumors is the current necessity for intratumoral inoculation of the virus which requires a neurosurgical procedure and limits the ability for multiple doses and the direct targeting of leptomeningeal disease and spinal metastases. Many pediatric brain tumors and some adult tumors spread extensively throughout the brain and spine. Intraventricular (IVT) delivery would overcome critical delivery barriers and provide significant advantages for pediatric brain tumor patients (and adult patients) by obviating the need for invasive neurosurgical procedures and enabling repeat targeting of both intracranial and spinal metastatic disease.

The critical first step in developing intraventricular delivery of oHSV for human use is determining the cause of the toxicity. In the first year of this grant, we have conclusively determined that toxicity is not mediated by virus antigens alone or the vehicle the virus is carried in. Only active virus caused toxicity and virus was seen in the ependymal lining. This is a novel finding. Over the next year, we anticipate sorting out if the virus is replicating in and lysing the ependymal cells or just causing apoptosis of the cells without production of late virus proteins. For the first time ever, we have demonstrated the timing of the inflammatory response post-injection of the virus intraventricularly with a peak response seen at day 4 and a decline seen by day 7. Importantly, we found that the ependymal lining regenerates over several weeks although not completely. Over the next year, we will further investigate the role of this secondary immune response in the toxicity by exploring whether specific immune effector cells are implicated in the toxicity along with the virus.

The discovery that we can reduce the virus dose by a log to decrease toxicity is extremely impactful in our pursuit to advance intraventricular delivery of oHSV to clinical studies. This finding is even more significant when paired with our results demonstrating for the first time that intraventricular oHSV can target both primary and metastatic disease in a pediatric medulloblastoma model. Remarkably, two treatments did not cause toxicity in the mice and eliminated the development and progression of spinal metastases in treated mice. This suggests that this approach may be feasible and efficacious. Our work over the next two years will continue to focus on the causes of virus toxicity when the virus is given at higher doses and ways we might be able to mitigate or block the toxicity, so that higher doses can be used which may result in even more profound responses.

Overall, these findings will expand scientific knowledge in the fields of virotherapy and immunotherapy, and will have an impact on the development of future viral vectors and future clinical trials. Ultimately, if these findings are used to translate this therapy to clinical trials, it could have an impact on the fields of pediatric hematology/oncology, pediatric neuro-oncology, and adult oncology and neuro-oncology.

5. CHANGES/PROBLEMS

There were no changes in the approach. There were no significant problems or delays in conducting the research. There were no changes that had a significant impact on expenditures. There were no significant

changes or deviations in the use or care of vertebrate animals or biohazards. Annual IACUC approval was renewed on 12/21/2015.

6. PRODUCTS

There have been two abstracts presented at conferences. Both poster presentations acknowledged federal support for the work.

1. Etminan T, Nan L, Moore BP, Ring EK, Gillespie GY, Friedman GK. Toxicity of Intraventricular Genetically-Engineered Oncolytic Herpes Simplex Virus Injection. Poster Presentation, 30th Annual National Conference on Undergraduate Research (NCUR) held in Asheville, North Carolina, April 7-9, 2016.

This abstract described the ependymal lining toxicity and inflammation seen with IVT HSV G207 and M002 treatment along with the recovery of the lining seen over time.

2. Etminan T, Ring EK, Nan L, Moore BP, Hjelmeland A, Li R, Gillespie GY, Markert JM, Friedman GK. Intraventricular oncolytic engineered herpes simplex virus prolongs survival and reduces spinal metastases in mice bearing human group 3 medulloblastoma. Abstracts from the 17th International Symposium on Pediatric Neuro-Oncology, June 12-15 2016 Liverpool, UK. Neuro Oncol 2016; 18 (suppl 3): iii106.

This abstract described the ability of IVT HSV G207 to prolong survival and reduce MB spinal metastases in mice.

I also was invited to give UAB Pediatric Medical Grand Rounds. This presentation on June 9, 2016 was entitled, "Pediatric Cancer Gone Viral: Clinical Application of Oncolytic Virotherapy", and included some of the data related to oHSV prolonging survival and reducing MB spinal metastases in mice. Federal support for this work was acknowledged.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name: Gregory Friedman, MD

Project Role: PI

Nearest person month worked: 6.6

Contribution to Project: He has been responsible for the research that has been conducted and has directed the effort of others working in his lab. He has analyzed the data and has reported the findings in the above abstracts and presentation. He has provided quality assurance for the research and has been responsible for all regulatory forms that are required. He has actively participated in the career development tasks outlined above. He has met on a regular basis with both of his mentors to review his progress and to plan future training endeavors as identified as needed.

Name: G Yancey Gillespie, PhD

Project Role: Mentor

Nearest person month worked: 1

Contribution to Project: Over the past year, Dr. Gillespie met with Dr. Friedman weekly for approximately 2 hours. In these lab meetings, Dr. Gillespie discussed current research activities with Dr. Friedman and helped him plan for future activities and identify problems impeding his progress. He has supported Dr. Friedman's research training by providing an intellectual atmosphere that values and rewards scholarly research, by providing opportunities for daily contact with other researchers in the neurosciences and by providing facilities in which this training program can be conducted.

Name: James Markert, MD

Project Role: Mentor

Nearest person month worked: 1

Contribution to Project: Over the past year, Dr. Markert met with Dr. Friedman every other week for approximately 1 hours. In these lab meetings, Dr. Markert discussed the research with Dr. Friedman and identified future areas to pursue. He advised Dr. Friedman and provided insight through the regulatory process for the clinical trial as part of Dr. Friedman's career development.

Name: Li Nan

Project Role: Research assistant

Nearest person month worked: 2

Contribution to Project: Ms. Nan has assisted Dr. Friedman in performing the experiments outlined in the project.

Funding Support: Effort related to this project is supported by Dr. Friedman's startup funds. Ms. Nan is also supported through other projects including grants from the Rally Foundation and St. Baldrick's Foundation.

Name: Blake Moore

Project Role: Research assistant

Nearest person month worked: 2

Contribution to Project: Mr. Moore has assisted Dr. Friedman in performing the experiments outlined in the project.

Funding Support: Effort related to this project is supported by Dr. Friedman's startup funds. Mr. Moore is also supported through other projects including grants from the Rally Foundation and St. Baldrick's Foundation.

Name: Eric Ring, MD

Project Role: Pediatric Hematology/Oncology fellow

Nearest person month worked: 1

Contribution to Project: Dr. Ring is a fellow conducting research in Dr. Friedman's lab. His main project is unrelated to this project but as part of his training, he has participated in various experiments outlined in the project.

Funding Support: UAB Division of Pediatric Hematology/Oncology

Name: Tina Etminan

Project Role: Undergraduate student

Nearest person month worked: 1

Contribution to Project: Ms. Etminan is a premedical student gaining research experience in Dr. Friedman's lab. As part of her learning experience, she has participated in various experiments outlined in the project.

Funding Support: No support

Name: Rong Li, MD, PhD

Project Role: pathologist

Nearest person month worked: 1

Contribution to Project: Dr. Li has helped Dr. Friedman examine the immunohistochemistry slides and characterize the toxicity from oHSV IVT. She has assisted Dr. Friedman in taking pictures of the pathology.

Funding Support: No support

No other organizations were involved as partners.

The following are changes in the active other support for Gregory Friedman, MD:

Previously active grant that has closed:

Hyundai Hope on Wheels	Friedman, Gregory K (PI)	10/01/13-09/30/15	1.0 cal-mos
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Pending grant that is active:

St. Baldrick's Foundation	Friedman, Gregory K (PI)	07/01/16-06/30/17	1.2 cal-mos
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No scientific overlap.

Active grant that was renewed:

The Rally Foundation	Friedman, Gregory K (PI)	07/01/16-06/30/18	0.6 cal-mos
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The goal of this renewed grant is to examine checkpoint protein expression in medulloblastoma and how HSV modulates that expression. There is no scientific overlap.

New grant that was funded:

1R01FD005379-01, FDA	Friedman, Gregory K (PI)	04/01/16-03/31/19	1.8 cal-mos
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Ph1 of HSV G207 and Radiation to Treat Pediatric Brain Tumors IND16294

The goal of this phase I study is to determine the safety and tolerability of HSV G207 alone or combined with low dose radiation in children with progressive or recurrent malignant supratentorial brain tumors. There is no scientific overlap.

Pending grant:

Hyundai Hope on Wheels Friedman Gregory K (PI) 12/31/16-12/30/18 0.6 cal-mos

Enhancement of Immunovirotherapy with IDO Inhibition in Pediatric Medulloblastoma

The goal of this grant is to develop an immunovirotherapy by combining oncolytic HSV with IDO inhibition. There is no scientific overlap.

Aim 1: Characterize expression of IDO in pediatric MB and determine how HSV M002 modulates IDO expression.

Aim 2: Establish the safety and efficacy of combining IDO inhibition with M002 in murine MB models.

The following are changes in the active other support for James Markert, M.D.:

Previously active grant that has closed:

W81XWH-10-NFRP-IIRA NF 100157 Markert, James (PI) 09/01/2011-08/31/2015 1.2 cal-mos

Pending grant that is active:

Northwest Biotherapeutics Markert, James (site PI) 3/19/15-3/18/20 0.36 cal-mos

A Phase III Clinical Trial Evaluating DCVax-L, Autologous Dendritic Cells (DC) Pulsed with Tumor Lysate Antigen for the Treatment of Glioblastoma Multiforme (GBM)

New grant that was funded:

R01FD05379-01 Friedman, Gregory (PI) 04/01/16-03/31/19 0.36 cal-mos

Phase I of HSV G207 and Radiation to Treat Pediatric Brain Tumors

Role: Co-Investigator

Pending grant:

Hyundai Hope on Wheels PI: Friedman, Gregory 12/31/16-12/30/18 0.36 cal-mos

Enhancement of Immunovirotherapy with IDO Inhibition in Pediatric Medulloblastoma

Role: Co-Investigator

The following are changes in the active other support for G. Yancey Gillespie, Ph.D.:

Previously active grant that has closed:

1 R01 CA138517-03	Nozell, S.E., Ph.D. (PI)	07/01/2010 – 06/30/2015	0.6 cal-mos
5 P01-CA71933-15	Whitley, Richard J., M.D. (PI)	7/1/2009-6/30/2015	0.12 cal-mos
W81XWH-10-NFRP-IIRA-NF1001157	Markert, J.M., MD (PI)	09/01/2011 – 08/31/201	0.12 cal-mos

8. SPECIAL REPORTING REQUIREMENTS

Not applicable.

9. APPENDICES

Copy of abstract from the 17th International Symposium on Pediatric Neuro-Oncology, June 12-15 2016
Liverpool, UK. Neuro Oncol 2016; 18 (suppl 3): iii106.

and the prognosis. **CONCLUSION:** Combination of radiation and chemotherapy following gross total resection is an effective and tolerable treatment for pediatric medulloblastoma. The prognosis is much poorer in heightened risk pts. Searching the tumor markers combined with molecular diagnostics mode may improve the survival of pediatric medulloblastoma.

MB-41. INTRAVENTRICULAR ONCOLYTIC ENGINEERED HERPES SIMPLEX VIRUS PROLONGS SURVIVAL AND REDUCES SPINAL METASTASES IN MICE BEARING HUMAN GROUP 3 MEDULLOBLASTOMA

Tina Ertman, Eric Ring, Li Nan, Blake Moore, Anita Hjelmeland, Rong Li, G. Yancey Gillespie, and Gregory Friedman; University of Alabama at Birmingham, Birmingham, AL, USA

Outcomes are especially poor for children with group 3 medulloblastoma, and surviving patients often suffer from lifelong neurocognitive, psychosocial, and neuroendocrine impairments secondary to current therapies including surgery, chemotherapy and radiation. Oncolytic herpes simplex virus (oHSV) is a novel, promising approach for targeting pediatric medulloblastoma. Through engineered mutations (e.g. $\gamma_134.5$ neurovirulence gene deletions), oHSV replicates in and kills cancer cells while sparing normal cells. Our group and others have successfully utilized intratumoral inoculation of the virus; however this route of delivery does not directly target metastatic spinal disease frequently seen in medulloblastoma. We hypothesized that intraventricular oHSV could target metastatic medulloblastoma. Athymic nude mice were injected intraventricularly with 5×10^5 bioluminescently-enabled D341MED group 3 human medulloblastoma cells expressing luciferase. Five days later, 10 mice received 10 microliters of intraventricular 10% glycerol in saline or 1×10^6 plaque forming units (pfu) of G207, a $\gamma_134.5$ -deleted oHSV that is currently being used intratumorally in a phase I study in children with recurrent or progressive supratentorial brain tumors (clinicaltrials.gov NCT02457845). Bioluminescent imaging was performed weekly, and counts/second was measured in the neuraxis. Mice that developed neurologic morbidity were euthanized and survival times were measured. Median survival times were significantly increased from 12.0 ± 0.5 to 18.0 ± 0.9 days with a single G207 treatment ($p = 0.008$). Metastases quantified by bioluminescence were significantly decreased ($p = 0.02$) one week after G207 treatment. These data indicate that intraventricular oHSV can successfully target metastatic pediatric medulloblastoma in mice. Further studies examining the safety and efficacy of intraventricular oHSV are in progress

MB-42. OPTIMIZATION OF CHIMERIC ANTIGEN RECEPTOR T-CELL THERAPY FOR MEDULLOBLASTOMA

Anandani Nellan^{1,2}, Christopher Rota¹, Katherine Warren¹, and Daniel Lee¹; ¹National Cancer Institute, National Institutes of Health, Bethesda, MD, USA; ²Johns Hopkins Hospital, Baltimore, MD, USA

BACKGROUND: Approximately 40% of medulloblastoma express HER2 and high-risk tumors are more likely to express HER2. The first generation HER2 chimeric antigen receptor (CAR) T-cell was delivered intratumorally in orthotopic medulloblastoma within the forebrain of SCID mice and resulted in the regression of tumors, but all tumors recurred. The primary hypothesis of this study is that second and third generation HER2 specific CAR T-cells (CD28, 41BB and CD3 ζ signaling domains) delivered intratumorally and intravenously will have anti-tumor effects in orthotopic medulloblastoma within the posterior fossa of NSG mice. Second and third generation CAR T-cells have increased survival, expansion, and cytokine production, resulting in improved tumor killing. **METHODS:** DAOY and D283 medulloblastoma cell lines were characterized with FACS analysis for HER2 expression. Cells were co-incubated with HER2 CAR T-cells compared to control T-cells in a chromium release assay and interferon-gamma release assay. Medulloblastoma cell lines were stably transduced with a luciferase gene to facilitate bioluminescent imaging. Orthotopic medulloblastoma cells were implanted in the posterior fossa of NSG mice and treated with intravenous or intratumoral HER2 CAR T-cells compared to control. **RESULTS:** DAOY and D283 cells express extracellular HER2 by FACS analysis. HER2 CAR T-cells demonstrate in-vitro killing and cytokine production that is specific to medulloblastoma cells expressing HER2 on the surface of the cell. In-vivo studies show complete tumor regression in NSG mice treated with intravenous or intratumoral second and third generation HER2 CAR T-cells compared to control. Repeat experiments are ongoing with a focus on T-cell trafficking and persistence.

MB-43. REDUCED DOSE CRANIOSPINAL IRRADIATION (CSI) IS FEASIBLE FOR STANDARD RISK ADULT MEDULLOBLASTOMA (MBL) PATIENTS SIMILARLY TO PEDIATRIC POPULATION

Maura Massimino¹, Marie Pierre Sunyach², Lorenza Gandola¹, Filippo Spreafico¹, Alice Bonneville Levard², Emilia Pecori¹, Barbara Diletto¹, Elisabetta Schiavello¹, Veronica Biassoni¹, and Didier Frappaz²; ¹Fondazione IRCCS Istituto Nazionale dei Tumori, Milano, Italy; ²Centre Leon Bérard, Lyon, France

MBL, the most common malignant pediatric brain tumor, represents 1% of adult brain tumors. Recent molecular classification suggests that MBL is not a similar disease in children and adults. For standard risk pediatric medulloblastomas current therapy includes CSI at reduced doses (23.4Gy) associated with chemotherapy. Most same stage adult patients still receive 36Gy of CSI. Hence the adoption of 23.4Gy together with chemotherapy (CT), also for older patients in some institutions, is worthwhile reporting. The series of adults, treated according to the HIT protocol generated a 73% 5-year PFS {Friedrich, Eur J Cancer 2013} that constitutes the best data. We gathered M0 medulloblastomas with no/minimal post-surgical residues and no biological negative factor, from 1999 to 2015. Thirty were adults (median age 26, 18-48 years; median f-up 48 months, 5-192) and 47 children (median age 9 years; median f-up 45 months, 57-110). All had received 23.4Gy CSI plus posterior fossa/tumor bed boost and chemotherapy: pre-RT carbo/VPx2 courses or 8-in-onex2 courses (10 adults) and post-RT CT (carbo/VPx2 courses in 11 and 8-in-onex2 courses in 2 adults; CDDP/VCR/CCNUx8 courses in children). 5-year PFS and OS were respectively $83 \pm 9\%$ and $94 \pm 6\%$ for adults, versus $92 \pm 5\%$ and 100% for children (p ns); Median progression time were 53 versus 40 months for older versus younger. Relapses were local (3), local + CSF (1) for adults versus local (1) and disseminated (2) for children. These combined series present comparable – or even better – results than those after high CSI doses both in adults and children highlighting the need for treatment redefinition in adults.

MB-44. SUBGROUP-SPECIFIC QUANTITATIVE PROTEOMIC ANALYSIS OF MEDULLOBLASTOMA

Ling San Lau¹, Mojca Stampar¹, Jerome Staal¹, Huizhen Zhang¹, Stefan Pfister², Paul Northcott³, Michael Taylor⁴, Yetrib Hathout^{1,5}, Javad Nazarian^{1,5}, Kristy Brown¹, and Brian Rood^{1,5}; ¹Children's National Medical Center, Washington, DC, USA; ²German Cancer Research Center and University Hospital, Heidelberg, Germany; ³St. Jude Children's Research Hospital, Memphis, TN, USA; ⁴Hospital for Sick Children, Toronto, ON, Canada; ⁵George Washington University, Washington, DC, USA

Despite extensive genomic characterization of medulloblastoma, very few actionable therapeutic targets have emerged. Given the discordance between gene and protein expression and because proteins are the functional components of the cell, understanding the proteome is integral to deciphering cancer cell biology. We used stable isotope labeling of amino acids in cell culture (SILAC) for accurate quantification of tumor tissue proteins. We created a SILAC reference atlas called the Labeled Atlas of Medulloblastoma Proteins (LAMP) from 8 primary and established medulloblastoma cell lines and spiked it equally into 38 medulloblastoma tumor tissue lysates from all subgroups. Mass spectrometry was used to quantitate proteins and statistical identification and quantification confidence was calculated at the level of the peptide. The correlation between gene methylation, gene expression and protein abundance was also analyzed. Accurate quantitation was achieved for an average of 1310 proteins per sample. Comparison of replicates yielded regression values of >0.95 . Supervised hierarchical clustering and statistical testing were used to identify proteins enriched in each tumor subgroup. For example, we identified 359 proteins that were significantly differentially abundant in group 3 and group 4 tumors compared to cerebellum. Comparing group 3 and 4 tumors, 205 proteins were differentially abundant. Using these proteins, we performed Ingenuity Pathway Analysis and identified the eIF2 protein translation, regulation of eIF4F, mTOR signaling, axonal guidance, oxidative phosphorylation and mitochondrial dysfunction pathways to be most highly represented. Quantitative proteomics is a powerful platform for discovery biology, providing insight into tumor cellular function and yielding potential therapeutic targets.