AWARD NUMBER: W81XWH-20-1-0501

TITLE: Modulating Th17 Cells in IDH1-Mutant Glioma to Promote Antitumor Immunity

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CONTRACTING ORGANIZATION: Duke University

REPORT DATE: JULY 2022

TYPE OF REPORT: Annual Progress Report

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
JULY 2022	Annual	7/1/2021 – 6/30/2022

4. TITLE AND SUBTITLE
Modulating Th17 Cells in IDH1-Mutant Glioma to Promote Antitumor Immunity

5a. CONTRACT NUMBER
W81XWH-20-1-0501

5b. GRANT NUMBER
GRANT12934335

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER
CA190598

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Duke University
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Gliomas are the most common primary malignant brain tumor of the central nervous system. In its most aggressive form, glioblastoma (GBM), patients are met with a dismal median survival of approximately 15 months and endure a grueling standard of care therapy. The burgeoning field of immunotherapy holds great promise with much progress being made in understanding how mutations in brain tumors impact the tumor-immune microenvironment to promote or suppress immune activity.

We suspect that the most frequent mutation identified in gliomas, the R132H hotspot mutation in *isocitrate dehydrogenase I* (*IDH1*), which produces the oncometabolite D-2-hydroxyglutarate (D-2HG), profoundly alters the tumor-immune microenvironment, promoting an immunosuppressive, and thus pro-tumor milieu. We hypothesize that D-2HG alters the metabolism of the tumor infiltrating lymphocytes, skewing their differentiation and propensity for activation and response. We predict that this is mediated by mutant IDH1 and D-2HGs effects on Hif1α. With the promise of immunotherapy, the identification of the IDH1 mutation present in as high as 80% of low grade glioma patients, and the effects mutant IDH1 is having on the tumor-immune microenvironment, we suspect that we can manipulate the tumor-immune microenvironment via modulation of D-2HG, promote infiltration of various immune cells into the tumor, and promote an enduring immune response resulting in improved survival in these patients.

15. SUBJECT TERMS
Glioma, Th17, D-2HG, brain cancer, tumor microenvironment

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
U	U	U	UU	18	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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REPORT

1. **INTRODUCTION:**

   - Gliomas are the most common primary malignant brain tumor of the central nervous system with several studies reporting a higher incidence of disease among military personnel. In its most aggressive form, glioblastoma (GBM), patients are met with a dismal median survival of approximately 15 months and usually endure a grueling standard of care therapy in the form of radiochemotherapy. The burgeoning field of immunotherapy holds great promise for patients and families afflicted by brain tumors with much progress being made in understanding how mutations in brain tumors impact the tumor-immune microenvironment to promote or suppress immune activity.

     We suspect that the most frequent mutation identified in gliomas, the R132H hotspot mutation in *isocitrate dehydrogenase I* (*IDH1*), which produces the oncometabolite D-2-hydroxyglutarate (D-2HG), profoundly alters the tumor-immune microenvironment, promoting an immunosuppressive, and thus pro-tumor milieu. Specifically, we hypothesize that D-2HG alters the metabolism of the tumor infiltrating lymphocytes, skewing their differentiation and propensity for activation and response. We predict that this is mediated by mutant IDH1 and D-2HGs effects on Hif1α. Additionally, we suspect that with the promise of immunotherapy, the identification of the IDH1 mutation present in as high as 80% of low grade glioma patients, and the effects mutant IDH1 is having on the tumor-immune microenvironment, that we can indeed manipulate the tumor-immune microenvironment via modulation of D-2HG, promote infiltration of various immune cells into the tumor, and promote an enduring immune response resulting in improved survival in these patients, a long-term outcome pertinent to the aspirations of the Defense Health Program.

2. **KEYWORDS:**

   - Glioma, Immunotherapy, Th17 cells, Microenvironment, D-2-hydroxyglutarate (D-2HG)

3. **ACCOMPLISHMENTS:**

   - **What were the major goals of the project?**

   Mutations in *IDH1* have been identified in the majority of low-grade gliomas. When mutated, IDH1 produces an oncometabolite that affects the cells in both an autonomous and non-autonomous fashion. The subtasks that comprise this first year of experiments for this project are aimed at elucidating those non-autonomous effects that D-2HG exhibits, specifically on cells of the immune system. To this end, the major goals were to determine how D-2HG impacts $CD4^+$ T cell differentiation, $CD8^+$ T cell activation, to assess the metabolic impact D-2HG plays in these differences and to also determine the underlying mechanisms for these differences. In the next year, the mechanism underlying these differences will be continued and the studies will be expanded into an *in vivo* model of IDH1-mutated glioma to determine the specific role that D-2HG plays in the Th17 lineage, and whether that can be exploited for therapeutic purposes.

   The specific goals of the project, the proposed timeline, the status of the subtasks, and any detailed notes or data are described in the next section.

   - **What was accomplished under these goals?**

     - *For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.*

Below is a table describing the progress and respective data as they relate to the major goals of the project to date. The timeline stated is what was proposed in our original Statement of Work. While several subtasks were completed ahead of schedule, or are anticipated to be completed ahead of schedule, two subtasks in particular have not followed the proposed timeline exactly. For those subtasks where we are behind schedule, further explanations are given under Section 5: Changes/Problems. While we have experienced delays in these areas, we do not anticipate the final data to be at all negatively impacted and we expect to adhere to a modified timeline that will have these milestones achieved in advance of the expiration of funds. Please note that Figures 1, 2, and 5 below are identical to the previous progress report as those portions of the project were complete. All other figures have been changed from the previous report.

Subtask	Timeline	Status	Notes and pertinent data
1. IACUC review for animal experiments pertinent to CA190598	July 2020-Aug 2020	Complete (March 2020)	This subtask was completed in accordance with the proposed timeline.
2. Regulatory review and approval by USAMRMC ACURO	July 2020-Oct 2020	Complete (Aug 2020)	This subtask was completed in accordance with the proposed timeline.
3. Assess impact of D-2HG on CD4 T cell differentiation *in vitro*.	Oct 2020-Jan 2021	Complete	To assess the non-autonomous impact of mutant IDH1 and its oncometabolite, D-2HG, we have performed a series of T cell differentiation experiments. Specifically, we investigated the impact of cell-permeable octyl-D-2HG (D-2HG) on the ability of $CD4^+$ T cells to differentiate. We skewed primary murine splenocytes derived from either OTII transgenic animals (enriched for $CD4^+$ T cells), or from C57Bl/6 animals followed purification with the EasySep Mouse $CD4^+$ T cell isolation kit from Stem Cell Technologies, into Th1, Th2, Th17, or Regulatory T cells (Treg) in the presence or absence of D-2HG. Following culturing of the cells under skewing conditions, FACS analysis, q-RT-PCR, and ELISA was performed to ensure the differentiations were successful as well as to observe any D-2HG-mediated differences (**Figure 1A and B**). Th0 and Th1 was assessed using IFN**γ** as a marker of induction. No significant differences were observed between D-2HG and vehicle treated conditions. Similarly, no differences were observed under Th2 skewing conditions as was indicated through a lack of difference using the marker IL4. Consistenly throughout our studies, we observe a reduction of IL17 production, an indicator of Th17 induction, following treatment with D-2HG. In addition to intracellular staining with IL17a (**Figure 1A**), we also observed reductions in the master transcriptional regulator of the Th17 lineage, Rorc (**Figure 1B**), and in the ability of these cells to produce and secrete IL17, as indicated through an IL17 ELISA (**Figure 1C**). Interestingly, under Th17 skewing conditions, D-2HG reduced IL17 production and Rorc expression, however, there was a modest increase in expression of FoxP3, the master regulator of the Treg lineage (**Figure 1D**). This phenotype is more readily apparent through

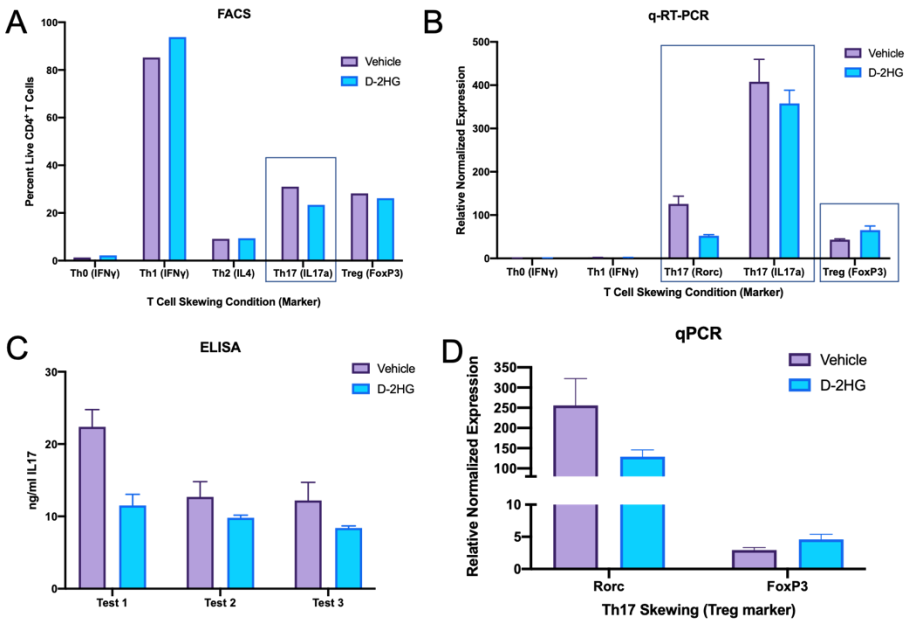
			q-PCR and is more difficult to observe through FACS analysis. As the Th17/Treg axis is tightly regulated and is dependent on a series of transcription factors. Our findings are in line with a hypothesis that D-2HG is promoting a Treg induction at the expense of Th17 cells. To further clarify this, we will be performing q-PCR using additional markers of both the Th17 and Treg lineage. Subtask 6 will also be used to further clarify and determine the mechanism underlying this phenotype and axis-shift. In addition to differentiation, we are also interested in the ability of T cells to become activated in the presence of D-2HG. We hypothesized that D-2HG would inhibit the ability of T cells to become activated as it confers an overall immunosuppressive role in the human glioma context. To address this, we harvested OTI and OTII splenocytes, enriched for $CD8^+$ and $CD4^+$ T cells, respectively, and treated them with their cognate peptide to promote activation. This was performed in the presence and absence of D-2HG. Following stimulation with their cognate peptide, the T cells will undergo rapid proliferation, forming clusters observed *in vitro*, and secreting IFN**γ**. Using our IncuCyte S3 Live Cell Imager, we were able to quantify the extent of cluster formation in these various contexts (**Figure 2A**). While there was no significant impact on cluster formation comparing $CD8^+$ T cells cultured in the presence or absence of D-2HG, there was a moderate reduction in IFN**γ** production as indicated by ELISA (**Figure 2B**). The $CD4^+$ T cells were unable to be activated using the cognate peptide as indicated by both an inability to undergo cluster formation as well as an inability to produce IFN**γ** (**Figure 2A-B**).
4. Determine metabolic effects of D-2HG on differentiated T cells	Dec 2020-Aug 2021	Complete	Metabolism is critical for the proper functioning and differentiation of effector T cells. With mutant IDH1s role in metabolism, together the with observed mutant IDH1-induced differences in T cell differentiation, we sought to determine whether D-2HG produced by mutant IDH1 is capable of perturbing T cell metabolism and whether that can contribute to D-2HG-induced differences in differentiation. Initial metabolic tests included the mitochondrial stress test to measure OCR (indicative of oxidative phosphorylation) and ECAR (indicative of glycolysis) in $CD4^+$ T cells under Th17 or Treg inducing conditions in the presence or absence of D-2HG. Our previous data showed an initial induction into Th17 (day 3 of differentiation (D3)), shows that D-2HG leads to a slightly higher basal rate of mitochondrial respiration and a greater maximal respiration rate compared to vehicle treated cells. By D5, this difference is restored to normal. Interestingly, acute treatment with D-2HG (D-2HG is spiked in at D5), shows that there is a significant reduction to the maximal respiration, indicating that acute exposure to D-2HG confers a negative impact on the overall fitness

			of the cells. We have performed this assay at several timepoints to assess D-2HGs impact on metabolism at different points in differentiation. Here, we find that the phenotype we described persists and begins to abate by D4 and 5. Here, we show that on D2, there is the maximum difference between the OCR rate of D-2HG treated Th17-skewed CD4 T cells, and that D-2HG leads to a metabolic state more indicative of oxidative phosphorylation compared to the preferential metabolic state of Th17 cells of glycolysis (**Figure 3A, B**). In contrast, D-2HG induces a more subtle impact on maximal respiration of T cells skewed towards Tregs at D3, but by D5 induces a more robust depression to maximal respiration. Tregs preferential metabolic state is that of oxidative phosphorylation which is indicated in our data. Our recent data shows that there is a differential impact on the metabolic activity of T cells skewed to the Treg lineage, with a more obvious impact at later timepoints (D4 and 5) (**Figure 3A, B**). Acute treatment with D-2HG under Treg skewing conditions confers a comparable effect on maximal respiration, suggesting that D-2HG likely impacts Th17 and Treg cells differently, though impacts the oxidative phosphorylation state. ATP Rate Assay was also performed on the aforementioned T cells, showing again that D-2HG causes an enhanced oxidative phosphorylation of T cells that are skewed toward both the Th17 and Treg lineage (**Figure 3C-E**).Lastly, we show that when compared D2 and D4 for D-2HGs effects on metabolism, that in all cases, D-2HG promotes oxidative phosphorylation (though, it is more subtle among the Treg lineage, particularly at D2) (**Figure 3F**).
5. Determine metabolic effects of D-2HG on T cell activation	Jan 2021-Sept 2021	Delayed progress	Due to the previously mentioned issue using the core facility which houses the Seahorse instrument, this particular task has been delayed. Due to the slight reduction observed in IFN**γ** production via ELISA following our previous T cell activation studies (Figure 2A and B), we will follow a similar protocol as this, however, due to the quick process of activation following stimulation with cognate peptide, we will spike in D-2HG to assess its impact specifically on T cell activation. Additionally, based on data from Figure 2A and B, we will not run this experiment using OTII/CD4 T cells as we were unable to stimulate with cognate peptide. We expect this to be complete by December 2022.
6. Identify mechanism underlying D-2HG mediated Th17 suppression.	Dec 2020-April 2021	Delayed progress	As mentioned in a previous report, we encountered issues propagating high titer virus that were to be used to retrovirally transduce primary T cells and report on specific activity (FoxP3, IL17a, Stat3, and Hif1a). As a result, we have modified our approach to instead include inhibitors of pathways that we suspect are mediating the mutant IDH1/D-2HG-mediated suppression of the Th17 lineage. Specifically, we will be targeting Hif1a and the mTOR pathway to see if inhibition of their pathways will rescue the D-2HG

			mediated suppression of the Th17 lineage, thereby solidifying these pathways in IDH1's impact on these immune cell subsets. We expect to begin these experiments in October 2022 and complete them in February 2023.
7. Impact of D-2HG on CD4 T cell lineages *in vivo.*	March 2021-Oct 2021	In progress	To assess the impact of D-2HG on T cell lineages *in vivo*, we are utilizing our optimized, genetically faithful and biologically relevant murine models of glioma. These models rely on orthotopic intracranial injection of neural stem cells engineered to harbor the most common mutations observed in human gliomas including expression of mutant IDH1 and deletion of *TP53*. Following orthotopic intracranial injection with the IDH1 mutant parental line, the animals were treated daily with either vehicle or the IDH1 mutant inhibitor, AG881. At the time of symptoms, animals were euthanized and the brain tumor tissue was dissociated and stained. Additional samples from separate animals were also harvested for controls, including animals injected with an IDH1 wildtype line, and normal brain tissue from a C57Bl/6 animal. We observe mutant IDH1 inhibition and reduction of intratumoral D-2HG via administration with AG881 yields an increase in IL17 producing $CD4^+$ T cells (**Figure 4A**). No IL17 producing $CD4^+$ T cells were observed in the IDH1 wildtype expressing tumors or the normal C57BL/6 brain. Interestingly, there were an increased number of Tregs in the AG881 treated animals (**Figure 4B**). We are currently further pursuing this and incorporating additional markers for various lineages and cell types to acquire a more comprehensive landscape of the impact of mutant IDH1 inhibition on immune cells in the brain. We have recently optimized our protocol for isolating tumor infiltrating lymphocytes and this will be applied to our animals that are actively being treated (expect to be completed by December 2022).
8. Determine optimal dose for *in vivo* D-2HG suppression via mutant IDH1inhibitors.	Dec 2020-Oct 2021	Complete	As one component to these studies is to investigate the impact of mutant IDH1 inhibition and restoration of intertumoral Th17 cells to the brain tumor, we investigated two mutant IDH1 inhibitors known to both cross the blood-brain-barrier and to reduce intratunoral D-2HG levels to non-tumor levels. Utilizing the aforementioned orthotopic intracranial injection model of IDH1-mutant murine glioma, we tested AG881 and BAY1436032 (**Figure 5A-B**) and their ability to reduce D-2HG. Animals were injected with an IDH1 wildtype line (negative control for D-2HG), or with the IDH1-mutant expressing lines. These were treated with either AG881, DMSO, or BAY1436032. AG881 was more effective at reducing D-2HG to non-tumor levels at a lower dose. Therefore, a more stringent dose response was established, showing that AG881 administered at doses of 1mg/kg daily is capable of reducing intratumoral D-2HG levels to non-tumor levels (**Figure 5C**). Subsequent studies will administer AG881 at a dose of 5mg/kg.

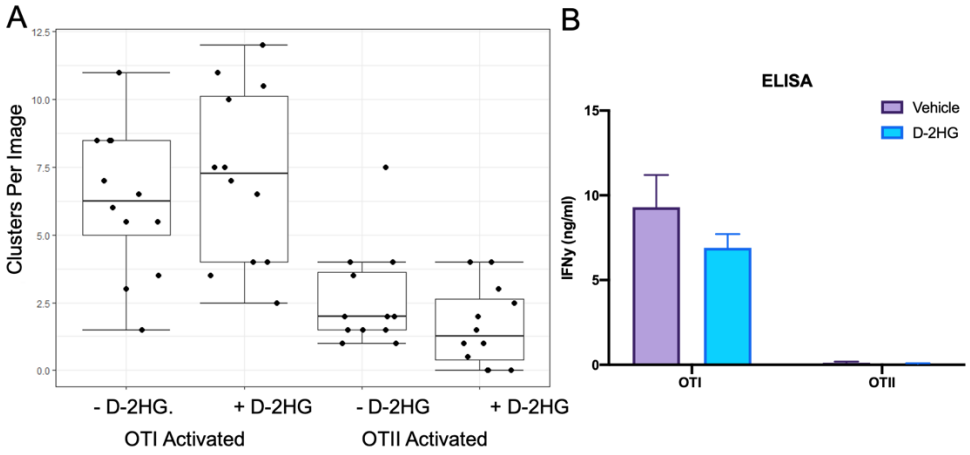
9. Inhibition of mutant IDH1 to reverse Th17 suppression *in vivo*.	July 2021-Dec 2021	Ongoing	Our previous data characterizing the differences in tumor gene expression showed several differences among IDH1 mutant and wildtype tumors. Applying our RNAseq data to various pathways, including Th17 (**Figure 6A**) and Treg (**Figure 6B**) gene sets, we show that mutant IDH1 confers an overall reduction in those genes implicated in the Th17 lineage. Interestingly, we see a similar reduction in Treg-related genes. We are currently inspecting these gene lists and finding those most differentially expressed genes for each of these to get a more thorough understanding of the impact on gene expression. As our previous data (shown) is limited by a low number of animals, we have another cohort that is ongoing (actively being treated). Samples will be applied to the following: 1. FACS analysis of these various lineages to assess the impact of mutant IDH inhibition on them; 2. BioLegend LegendPlex to assay cytokine production and assay the impact of mutant IDH1 inhibition on the glioma microenvironment; 3. RNAseq.
10. Therapeutic benefit of adoptively transferred Th17 cells.	July 2021-April 2022	Ongoing	We performed an initial experiment and are planning to repeat the experiment of investigating the therapeutic potential of adoptively transferring Th17 cells into glioma-bearing animals. **Figure 7A** shows the induction of IL17 that occurs during our T cell skewing, suggesting that our protocols are robust. These cells were adoptively transferred and survival curves are currently being established (not all animals have succumbed to disease yet). At the experimental endpoint, samples will be collected for H&E, assessment of cytotoxicity and profiling of the immune microenvironment both through IHC, FACS, and cytokine profiling via BioLegend LegendPlex. We anticipate this task to be completed by March 2023.

Figure 1:



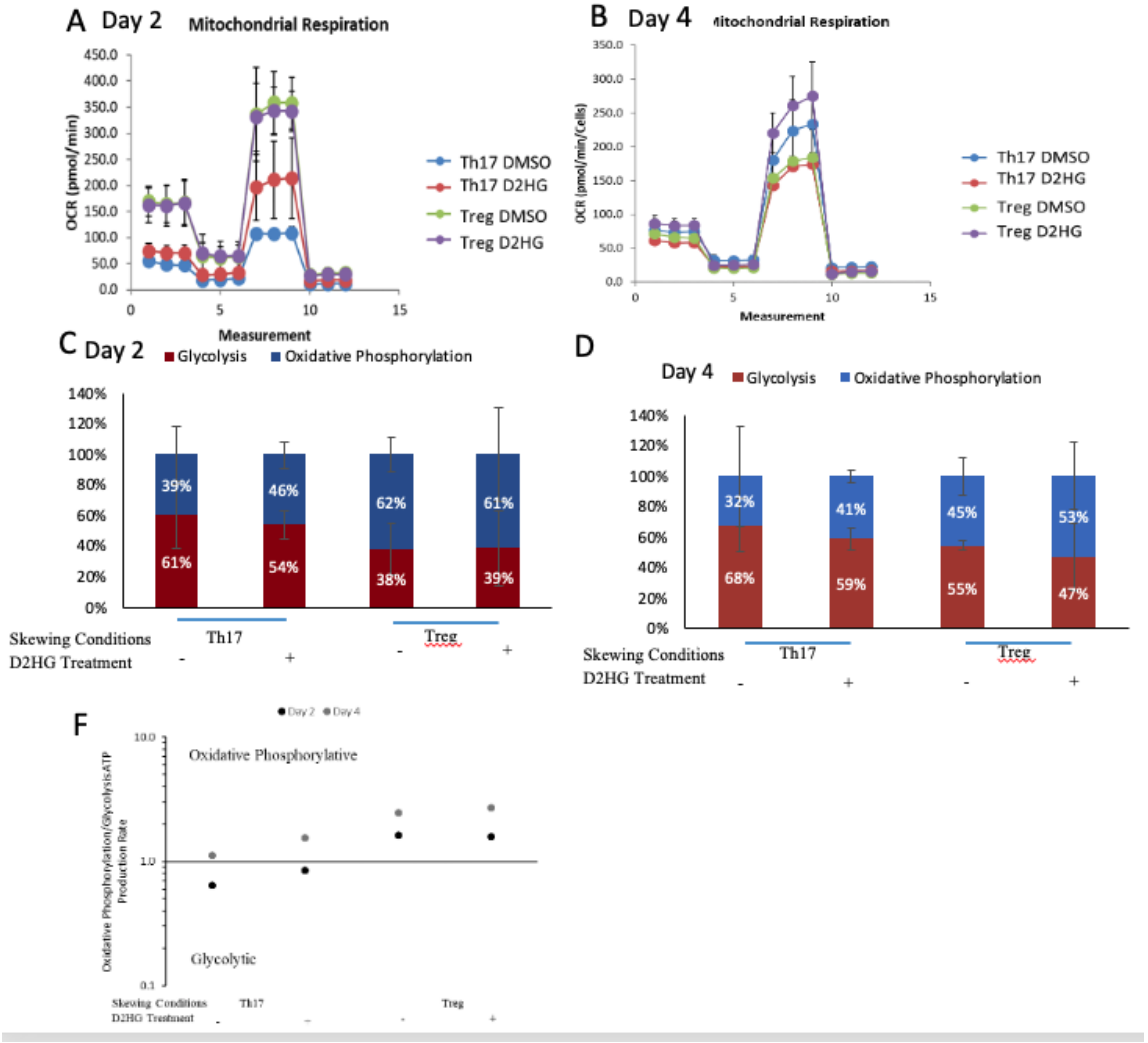
Assessing D-2HGs role in T cell differentiation. $CD4^+$ T cells were skewed through the Th1, Th2, Th17, or Treg lineage in the presence or absence of D-2HG. Experimental readouts include A. FACS analysis with markers specific for the different lineages including IFN**γ** for Th1, IL4 for Th2, IL17a for Th17, and FoxP3 for Treg and B. q-RT-PCR using IFN**γ** for Th1, Rorc and IL17a for Th17, and FoxP3 for Treg; C. IL17 ELISA was performed on T cells undergoing Th17 induction. D. q-RT-PCR of T cells undergoing Th17 induction using markers of Th17 and Treg.

Figure 2:



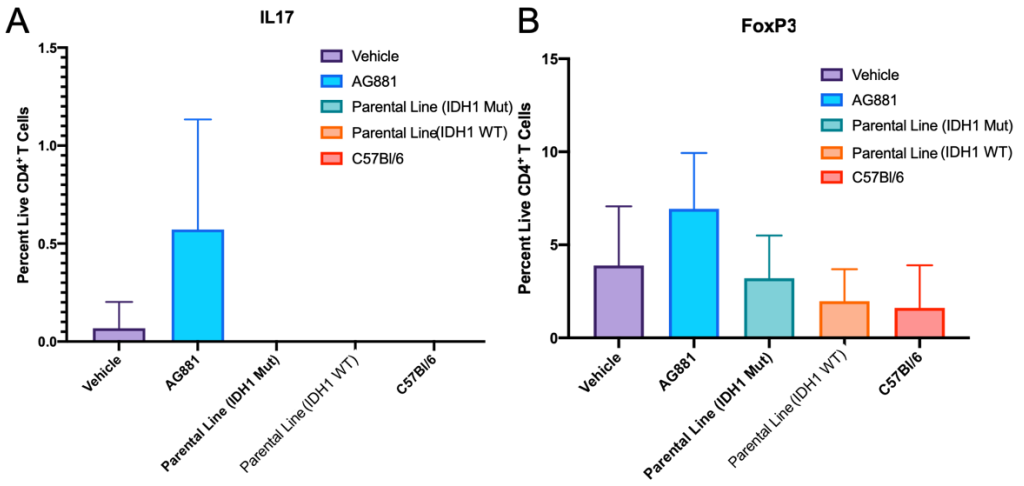
Assessing D-2HGs role in T cell activation. OTI and OTII splenocytes were harvested and stimulated with cognate peptide in the presence or absence of D-2HG. A. Activation was assessed through image analysis (IncuCyte S3) of cluster formation and through B. IFN**γ** secretion assayed through ELISA.

Figure 3:



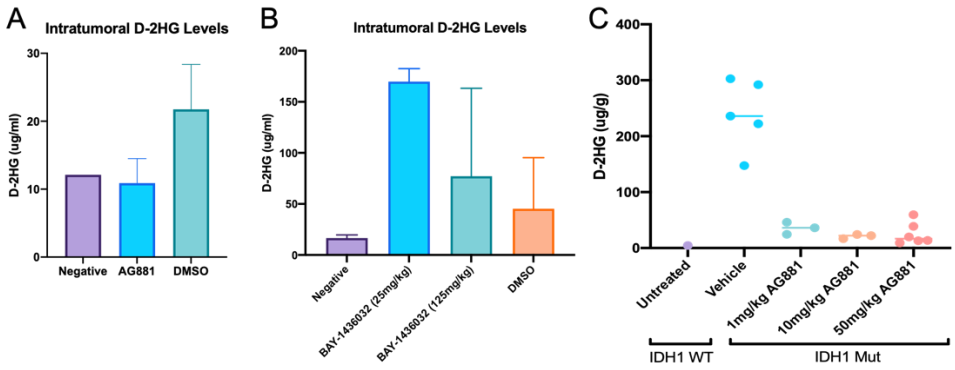
D-2HGs impact on mitochondrial respiration in Th17 and Treg skewed CD4⁺ T cells. C57Bl/6 splenocytes were harvested, CD4 T cells were enriched and skewed into Th17 or Treg cells. Mitochondrial Stress Test via Seahorse XFe96 Analyzer was used to assay oxygen consumption rate (OCR) on D2 (A) and D4 (B). D-2HG increases reliance on oxidative phosphorylation on D2 Th17 induction (C)and D4 for Th17 and Treg induction as shown in the ATP Rate Assay (D). E. The overall ratio of oxidative phosphorylation to glycolysis shows the metabolic shifts that occur under each skewing condition and timepoint.

Figure 4:



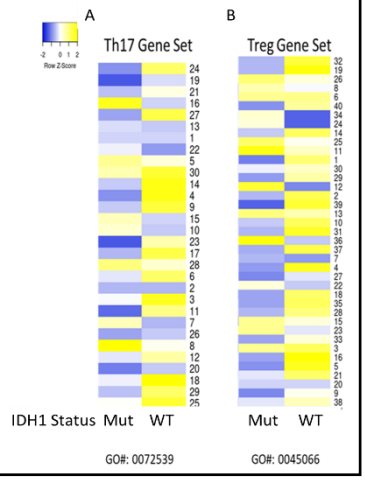
Mutant IDH1s impact on CD4 T cell lineages *in vivo*. Genetically engineered mouse neural stem cell lines harboring mutant IDH1 and P53 deletion were intracranially injected into C57Bl/6 animals. AG881 was administered daily. A wildtype IDH1 line as well as normal brain tissue was collected for control. Intratumoral IL17 (A) and FoxP3 (B) populations were assessed under the different conditions to determine mutant IDH1s role on Th17 and Tregs *in vivo*.

Figure 5:



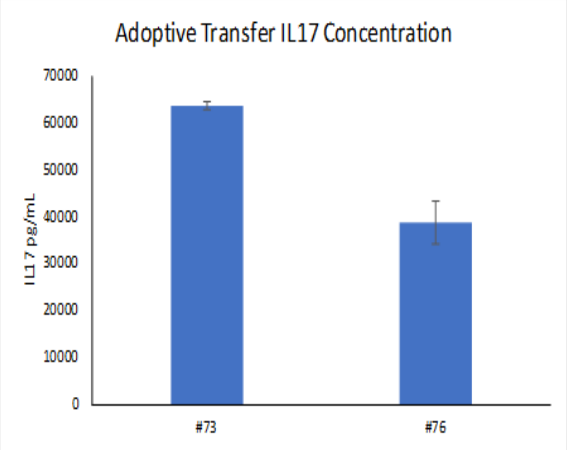
Abrogation of D-2HG through mutant IDH1 inhibition using AG881 or BAY1436032. Intratumoral D-2HG levels were assessed (LC-MS/MS) following intracranial injection of IDH1 wildtype (negative) or IDH1 mutant expressing lines. Animals were treated with AG881 or DMSO (A) or BAY1436032 (B). A dose response of the more effective drug was performed (C).

Figure 6:



RNAseq of 3 IDH1 mutant and 3 IDH1 wildtype murine tumors. Normalized FPKM values for three individual IDH1 mutant and wildtype tumors were averaged. Gene lists for A. Th17 and B. Treg cells were acquired (GO # 0072539 and 0045066, respectively), and heatmaps were generated. Gene expression of Th17 and Treg-specific genes were downregulated in mutant tumors.

Figure 7:



Preparation for Th17 adoptive transfer. Two individual C57Bl/6 animals (#73 and #76) were vaccinated and boosted with 25-mer mutant IDH1 specific peptide followed by euthanasia and CD4 T cell purification from splenocytes. Cells were put under Th17 skewing conditions in the presence or absence of cognate peptide and after 4 days, supernatant was collected for ELISA. ELISA shows robust IL17 production, suggesting protocol was efficient. Cells were subsequently intravenously injected into tumor-bearing animals. Experiments are underway to assess therapeutic efficacy via survival and anti-tumor response as indicated through H&E, IHC, and cytokine profiling.

- **What opportunities for training and professional development has the project provided?**
  - The team available for this project includes a postdoctoral associate and a laboratory research analyst. In both cases, significant training opportunities have been made available and both individuals have mastered the protocols and techniques required for these studies. The research analyst continues to be trained by both members of the laboratory as well as the staff at Duke (including those in the Division of Laboratory Animal Research). Neither of these individuals had experience in mouse handling, immune cell culture, or FACS analysis in advance of these studies. In terms of professional development, the postdoctoral associate regularly attends Duke University's Postdoc Association's events which are catered to professional development. Both the postdoctoral associate and the research analyst regularly attend informational webinars and trainings regarding spectral flow cytometry and Seahorse metabolic analysis, as well as join lab meetings for our colleagues (Dr. Yiping He, Associate Professor in the Department of Pathology, Dr. Matthew Waitkus, Assistant Professor in the Department of Neurosurgery, and Dr. David Ashley, Director of the Preston Robert Tisch Brain Tumor Center). In all cases, we present and receive valuable feedback regarding our experiments as they relate to this research.

- **How were the results disseminated to communities of interest?**
  - The results to date for the proposed studies have not yet been disseminated in publication format. However, the PI Christopher Pirozzi has presented these findings in oral presentation and discussion format at an annual scientific convening that he attends as part of being associated with the Hope Funds for Cancer Research. It is anticipated that the data to date in complement with the data expected to result in the coming weeks will be able to comprise a publication. The goal is to submit the first publication funded by this grant followed by a second characterizing the impact of mutant IDH1 inhibition to the Th17/Treg axes in vivo by December 2022. We hope to present this work at the annual AACR meeting in April 2023.

- **What do you plan to do during the next reporting period to accomplish the goals?**
  - As is laid out in the chart above, several subtasks remain to be completed. We will be strategically tackling these subtasks to ensure they are completed within the timelines and in advance of completion of this funding period. In most instances, we have adhered to the proposed timeline and in those cases where we are unable to do so, we have explained in Section 5, below. Specifically, over the next several months, our focus will be on elucidating the mechanism underlying mutant IDH1 and D-2HGs role in suppression of the Th17 lineage. This will be performed through a variety of inhibitors specific to known Th17-specific pathways including mTOR and Hif1a. We have begun our adoptive transfer and will continue to investigate the therapeutic efficacy of adoptive transfer of Th17 cells. We have optimized our Th17 inductions to be able to culture and expand Th17 cells and have performed our first adoptive transfer (ongoing). These two portions of the proposed studies are slated to be completed within the next 9 months.

4. **IMPACT:**

- **What was the impact on the development of the principal discipline(s) of the project?**

  - Our studies are indicating that mutant IDH1 and its production of D-2HG may be impacting the Th17/Treg axis that exists. Specifically, D-2HG may be inhibiting Th17 induction, while promoting Treg induction. Through the use of AG881, a mutant IDH1 inhibitor, we are investigating whether this axis can be manipulated and whether we can promote anti-tumor activity by inducing Th17 induction and suppressing the Treg population. Our first RNAseq datasets are being analyzed and we have another cohort of animals that are currently being aged for their eventual euthanasia and use for FACS, RNAseq, and cytokine profiling via BioLegend LegendPlex. Shifting of the axis through modulating the tumor-immune microenvironment will be an appealing and unique therapeutic approach toward treating or sensitizing IDH1-mutant expressing gliomas.

- **What was the impact on other disciplines?**

  - Nothing to report.

- **What was the impact on technology transfer?**

  - Nothing to report.

- **What was the impact on society beyond science and technology?**

  - Nothing to report.

5. **CHANGES/PROBLEMS:**

- **Changes in approach and reasons for change**

  - Nothing to report.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

  - We have previously encountered an issue with the metabolic studies and the arrangement that we had with the Cellular Metabolism and Analysis Core Facility at Duke University. In June 2021, the PI that ran the Core Facility and the Seahorse XFe96 Analyzer left Duke. As a result, the instrument has been moved to a new core facility. In addition to the required "down time" of the instrument as it is moved to its new location, there have also been modifications made to the usage and fee structure for the instrument. We have been in correspondence with the new leading PI and our members have begun training this July. While navigating the new system and structure was complicated, we have successfully been trained and have regular access to the instrument. Our experiments and research plan, as indicated by our data above, is back on track. While this does set back the progress of Subtask 4 and 5, we do not expect it to lead to significant delays in these portions of the project.

  - As mentioned in previous reports, we have encountered a problem in the form of propagating high-titer virus required for investigating the mechanism underlying D-2HG mediated Th17 suppression (Subtask 6). Our proposal utilizes reporter constructs for the main transcriptional regulators of the Th17 and Treg lineage. These reporters were acquired from SBI (System Biosciences) which have a disclaimer stating the difficulty in the use of these constructs. After a series of optimizations, we have generated virus capable of transducing cells with a 20% efficiency. Ideally, this would be greater than 20% in order to ensure we can acquire meaningful data. We have reached out to the Duke Viral Vector Core Facility that has extensive experience in virus propagation and transduction of primary cells. We have since changed our approach slightly such that instead of using viral reporters, we will be investigating the underlying mechanism of mutant IDH1-mediated Th17 suppression via

inhibitors of known pathways, specifically mTOR and Hif1a, and using FACS, BioLegend LegendPlex, and RNAseq as our alternative readouts.

- **Changes that had a significant impact on expenditures**
  - No other changes (other than the aforementioned issue with the Seahorse as described in the previous report) have lead to significant impact on the expenditures. We are currently working to optimize our plate usage to run several experiments at once and also identify any other cost-saving opportunities for other subtasks of this project.
- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
  - Nothing to report.
- **Significant changes in use or care of human subjects**
  - Nothing to report.
- **Significant changes in use or care of vertebrate animals.**
  - Nothing to report.
- **Significant changes in use of biohazards and/or select agents**
  - Nothing to report.

6. **PRODUCTS:**

- **Publications, conference papers, and presentations**
  - **Journal publications.** As mentioned above, combining the data we have acquired this past year together with the data we expect to acquire in the coming weeks, we anticipate being able to submit two manuscripts by December 2022. The first will detail the mouse models established and the second will describe the impact of the IDH1 mutation on the glioma microenvironment, how it is modulated through D-2HG inhibition via small molecule inhibition, and how the Th17 and Treg lineage is subsequently effected in this process. A third publication closer to June 2023 is expected detailing the effects of Th17 adoptive transfer into glioma bearing animals which is the final milestone of this funded award.
  - **Books or other non-periodical, one-time publications.** Nothing to report.
  - **Other publications, conference papers, and presentations.** Christopher Pirozzi, the PI to these studies presented his work at an annual scientific convening for the Hope Funds for Cancer Research both in July 2020 and July 2021. Christopher was previously funded as a postdoctoral fellow and is now an alumni of the Hope Funds for Cancer Research and is invited to their events to describe the research progress in the field of brain cancer. As part of this, Christopher did present the work currently funded by this award. Additionally, Christopher presented the highlights of this project at the inaugural Annual Retreat of the Preston Robert Tisch Brain Tumor Center at Duke.
- **Website(s) or other Internet site(s)**
  Nothing to report.
- **Technologies or techniques**
  Nothing to report.
- **Inventions, patent applications, and/or licenses**
  Nothing to report.

- **Other Products**
  Nothing to report.

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

- **What individuals have worked on the project?**

Name:	***Christopher J. Pirozzi, Ph.D.***
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	0000-0003-3507-1936
Nearest person month worked:	7.2
Contribution to Project:	*Dr. Pirozzi is the PI of this project. He is actively leading his team to ensure all subtasks are accomplished and milestones reached. Dr. Pirozzi designs the experiments, performs the experiments, analyzes the data, and will be preparing the manuscripts that result from the acquired data.*
Funding Support:	
Name:	***Nathan M. Reynolds, Ph.D.***
Project Role:	*Postdoctoral Associate*
Nearest person month worked:	*12*
Contribution to Project:	*Dr. Reynolds is responsible for performing the experiments outlined in the subtasks for this project. He is heavily involved in all aspects and will be leading the upcoming animal experiments involving adoptive transfer of the expanded Th17 cells.*
Funding Support:	
Name:	***Ashleigh Soedel***
Project Role:	*Laboratory Analyst, II*
Nearest person month worked:	*4.8*
Contribution to Project:	*Ms. Soedel is involved in animal maintenance and treatments. Additionally, she handles all portions of the project that involve RNA extractions, q-PCR, and ELISA assays.*
Funding Support:	
Name:	***Ms. Paula K. Greer***
Project Role:	*Senior Laboratory Analyst*

Nearest person month worked:	*2.4*
Contribution to Project:	*Ms. Greer serves as the laboratory manager for several labs. Ms. Greer's contributions to the Pirozzi lab include lab management and ordering, logistical planning, experimental design, editorial review of manuscripts, and navigator to the many Duke systems.*
Funding Support:	

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
  - Nothing to report.
- **What other organizations were involved as partners?**
  - Nothing to report.

8. **SPECIAL REPORTING REQUIREMENTS**
   - Nothing to report.
9. **APPENDICES: *N/A***