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

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CONTRACTING ORGANIZATION: Duke University

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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. W<u>e hypothesize</u> 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

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

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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.

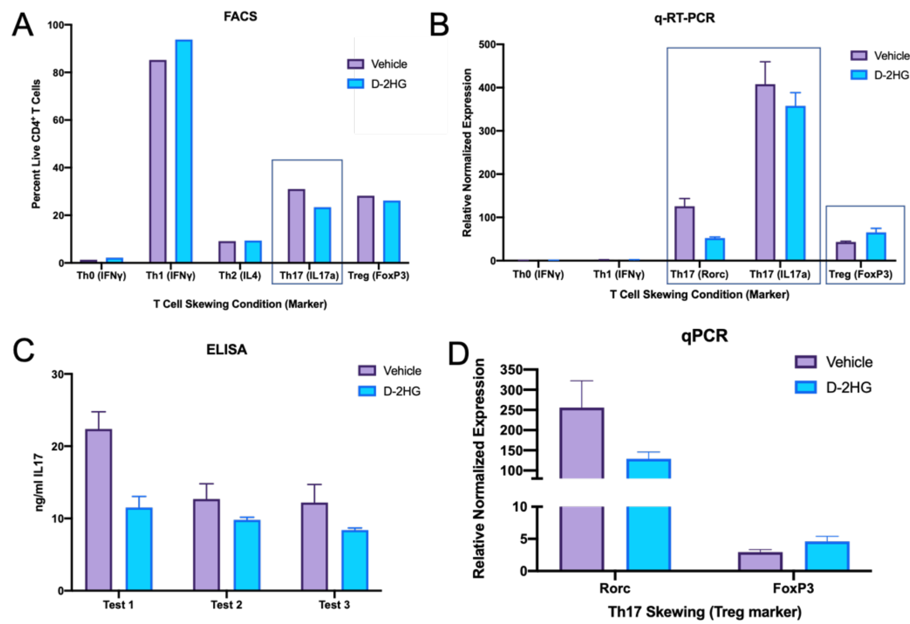
Subtask	Timeline	Status	Notes and pertinent data
1. IACUC review for animal experiments pertinent to CA190598	July 2020-Aug 2020	Complete (March 2020)	This subtask was completed in accordance with the proposed timeline.
2. Regulatory review and approval by USAMRMC ACURO	July 2020-Oct 2020	Complete (Aug 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 analsyis. 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**). 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	In progress	Metabolism is critical for the proper functioning and differentiation of effector T cells. With mutant IDH1s role in metabolism, together 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. 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 (**Figure 3A-B**). In contrast, D-2HG induces a slight reduction in maximal respiration of T cells skewed towards Tregs at D3, but by D5 induces a more robust depression to maximal respiration (**Figure 3C-D**). 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. These experiments are continuing to be pursued, however, due to some unforeseen changes in the core facility which houses the Seahorse XFe96 Analyzer, the remaining experiments had to be postponed. Please see "Section 5-Changes/Problems" below.
5. Determine metabolic effects of D-2HG on T cell activation	Jan 2021-Sept 2021	In progress	Please see "Section 5-Changes/Problems" below.
6. Identify mechanism underlying D-2HG mediated Th17 suppression.	Dec 2020-April 2021	Delayed progress	Please see "Section 5-Changes/Problems" below.
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 similar 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

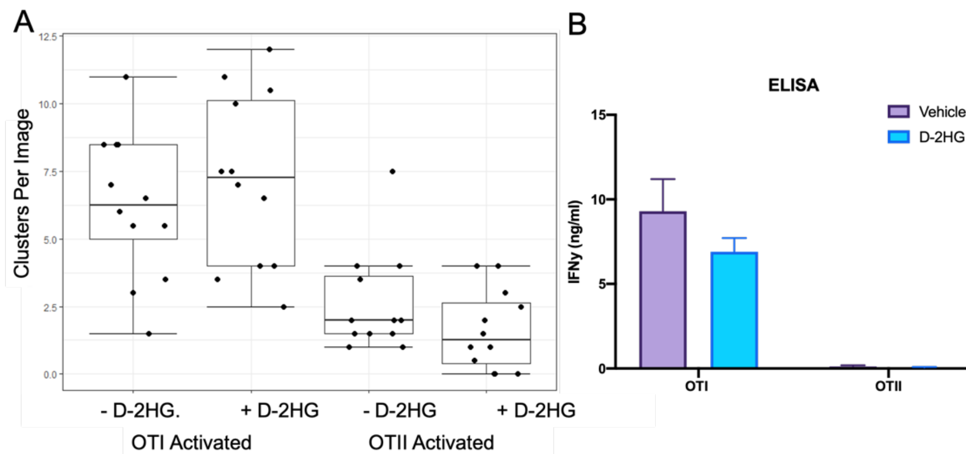
			inhibition on immune cells in the brain. Additionally, we will be testing the functionality of these Tregs.
8. Determine optimal dose for *in vivo* D-2HG suppression via mutant IDH1inhibitors.	Dec 2020-Oct 2021	Ahead of schedule	As one component to these studies is to investigate the impact of mutant 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	Upcoming	We have begun planning these experiments and they will be underway in the coming weeks. We are on schedule to complete this subtask according to the originally proposed timeline.
10. Therapeutic benefit of adoptively transferred restored Th17 cells.	July 2021-April 2022	Upcoming	We have begun planning these experiments and they will be underway in the coming weeks. We are on schedule to complete this subtask according to the originally proposed timeline.

Figure 1:



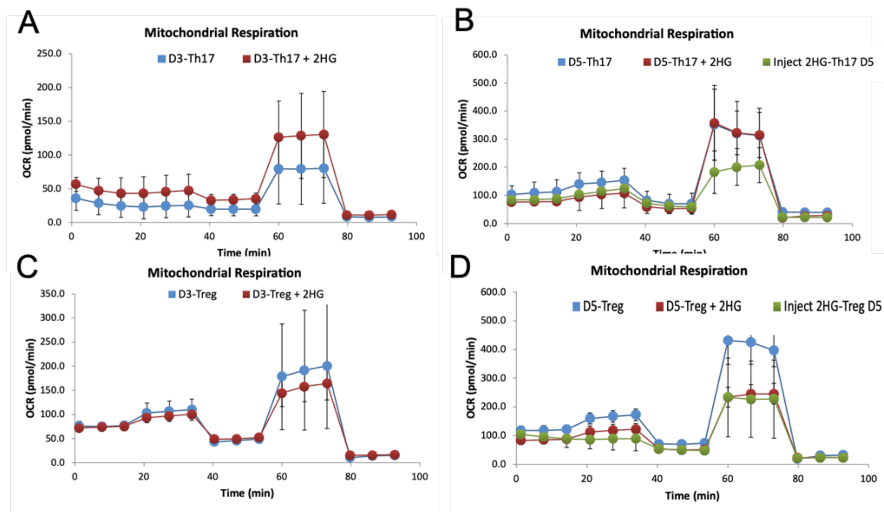
Assessing D-2HGs role in T cell differentiation. CD4+ T cells were skewed through the Th1, Th2, Th17, or Treg lineage in the presence or absence of D-2HG. Experimental readouts include A. FACS analysis with markers specific for the different lineages including IFN**γ** for Th1, IL4 for Th2, IL17a for 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:



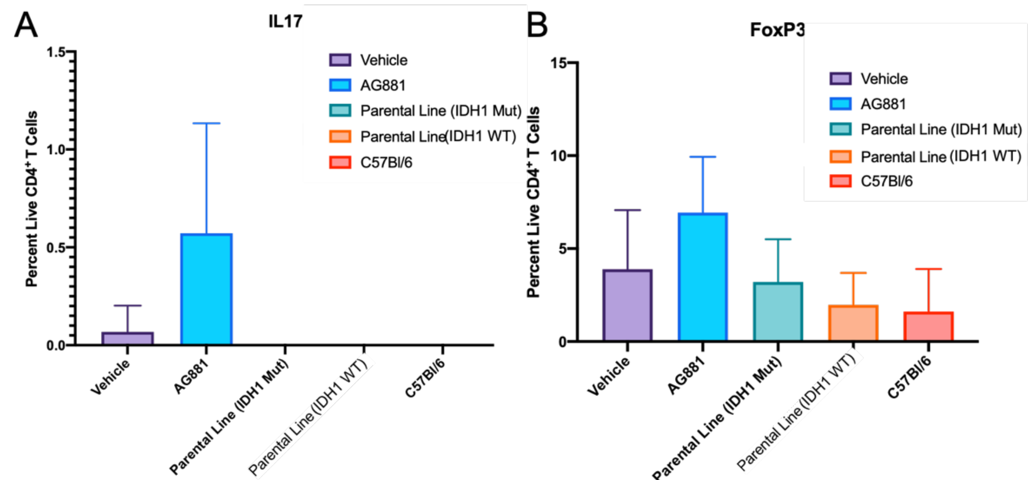
Assessing D-2HGs role in T cell activation. OTI and OTII splenocytes were harvested and stimulated with cognate peptide in the presence or absence of D-2HG. A. Activation was assessed through image analysis (IncuCyte S3) of cluster formation and through B. IFN**γ** secretion assayed through ELISA.

Figure 3:



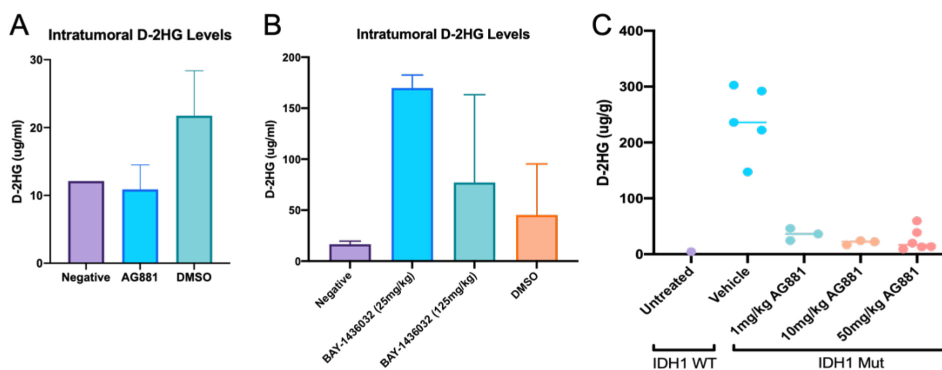
D-2HGs impact on mitochondrial respiration in Th17 and Treg skewed CD4+ T cells. OTII splenocytes were harvested and skewed into Th17 (A, B) or Treg (C, D) in the presence or absence of D-2HG. OCR was assayed through the Seahorse XFe96 Analyzer and metabolic fitness assessed at D3 (A, C) and D5 (B, D).

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).

- **What opportunities for training and professional development has the project provided?**
  - The team available for this project includes a postdoctoral associate and a research technician. In both cases, significant training opportunities have been made available and both individuals have mastered the protocols and techniques required for these proposed studies. 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 technician also regularly attend informational webinars and trainings regarding spectral flow cytometry and Seahorse metabolic analysis.

- **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 regarding this data by November 2021.

- **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 year, 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 reporter constructs that we are actively propagating. Additionally, our next cohort of experiments involves expansion of Th17 cells and assessing their potential as an anti-tumor therapeutic. To this end, we have optimized our Th17 inductions to be able to culture and expand Th17 cells for their eventual use in adoptive transfer. 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 plan to investigate whether this axis can be manipulated and whether we can promote anti-tumor activity by inducing Th17 induction and suppressing the Treg population. 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 encountered two hurdles as we are fulfilling the aims of the proposed studies. The first involves our 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. We anticipate being able to use the machine by mid-August. 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. We expect both subtasks to be completed by November 2021.

    - Additionally, we have encountered another 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 begun initial optimizations using their control plasmids and once complete, they will aid in propagating high-titer virus for the use of transducing primary murine splenocytes. While Subtask 6 is delayed, we do anticipate being able to complete this task by December 2021.

  - **Changes that had a significant impact on expenditures**

        - As mentioned above, the Seahorse XFe96 Analyzer has moved to a different core facility and modifications to the usage and fee structure for the instrument have been implemented. Initially, $230 per plate was budgeted and all reagents and plates were accounted for. In light of the recent changes, users of the instrument must purchase their own reagents and plates. In addition to that, a $200 fee per run will also be implemented. In total, this results in a greater cost overall than what was initially expected. 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 a manuscript by November 2021.

        - **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.

    - **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:	***Kristen Brooks-Roso***
Project Role:	*Research Technician, III*
Nearest person month worked:	*4.8*
Contribution to Project:	*Ms. Brooks-Roso 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:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc. Reminder: Pages shall be consecutively numbered throughout the report.* ***DO NOT RENUMBER PAGES IN THE APPENDICES.***