AWARD NUMBER: W81XWH-19-1-0349

TITLE: Oncolytic Viral Therapy to Potentiate Immune Checkpoint Blockade in Immunologically Cold Brain Tumors

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REPORT DATE: July 2022

TYPE OF REPORT: Annual

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	01Jul2021-30Jun2022

4. TITLE AND SUBTITLE

Oncolytic Viral Therapy to Potentiate Immune Checkpoint Blockade in Immunologically Cold Brain Tumors

5a. CONTRACT NUMBER
W81XWH-19-1-0349

5b. GRANT NUMBER
GRANT12715662

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of California, San Francisco

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

In year 3 of this project's funding period, we have made significant advances in mouse studies, multi-omics profiling of clinical and murine specimens, and bioinformatics analysis of the resulting data. We have completed the projected prospective collections of blood and CSF from the PNOC MV-NIS clinical trial and met our project's targets. The genomics profiling of these tissues, according to our SOW, is also complete. Likewise, we have completed profiling all archival frozen-tumor and blood specimens from the PNOC trial, as well as all archival and prospective frozen tumor tissues from UCSF. The resulting data have been preprocessed, subjected to quality control, and are the subject of ongoing analysis. We have made significant progress in our mouse studies, having performed the control and monotherapy arms for the Toca-511 virus and MV-NIS oncolytic virus treatment. The mouse studies included single-cell profiling of tumor tissue from responders and non-responders, as well as RNA-seq of murine blood specimens, which are complete. Preprocessing of the murine data and comparison to the human data is ongoing. Combination therapy arms of the murine studies are ongoing.

15. SUBJECT TERMS
medulloblastoma; oncolytic virus, immunotherapy; combination therapies; biomarker

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
Unclassified	Unclassified	Unclassified	Unclassified	20	**19b. TELEPHONE NUMBER** *(include area code)*

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1. Introduction

This research will determine if a pre-existing immune response will help or hinder oncolytic-virus (OV) therapy in medulloblastoma (MB), the most common malignant pediatric brain tumor. A positive answer to this question provides a rationale for OV combination therapy with immune-checkpoint blockade (ICB). Additionally, this research will identify predictive biomarkers for response to existing OV therapies for MB and advance our scientific understanding of the immune interactions with OV. To address this question, we are performing multi-omics profiling of tumor, blood and cerebral-spinal fluid (CSF) specimens from an ongoing clinical trial of measles virus encoding the human thyroidal sodium iodide symporter (MV-NIS) in recurrent medulloblastoma (NCT02962167). We perform complementary profiling of an immunocompetent intracranial model of MV-NIS and Toca 511 + Toca FC OV therapy for MB, as monotherapies and in combination with ICBs. The expected outcomes of this work will be: 1) a time-series map of the local and peripheral immune response during the course of MV-NIS therapy in the human disease, MV-NIS and Toca 511 + Toca FC with or without ICB in murine models; 2) predictive biomarkers for response to OV therapy; and, 3) targets to enhance OV efficacy. The significance of this work is that this project will provide a rationale for the use of OV in combination with immune checkpoint inhibitors, identify predictive biomarkers for response to OV and targets to enhance OV in MB.

2. Keywords

medulloblastoma; oncolytic virus, immunotherapy; combination therapies; biomarker

3. Accomplishments

Major goals of the project:

Specific Aim 1 Compare local and peripheral immune activation between MV-NIS responders and non-responders using human MB specimens obtained pre- and post-therapy.	**Timeline**	**Site**	**Percent Complete**
Major Task 0: HRPO review and approval	1-3	Drs. Diaz and Müller	100%
Major Task 1: Analysis of tumor tissues from the MV-NIS clinical trial	Months		
Subtask 1 Collection of fresh tissue.	4-48	Dr. Müller	100%
Subtask 2 Single-cell mRNA-sequencing, imaging mass-cytometry of fresh, frozen and FFPE tissues.	4-48	Dr. Diaz	90%
Subtask 3 Bioinformatics analysis of ‘omics data, including data pre-processing, principal component analysis, clustering, differential expression/ANOVA and regression modeling, providing information on intra-tumor immune composition correlates of survival and therapy response.	4-48	Dr. Diaz	75%
Milestone(s) Achieved	1		
Preliminary determination of MV-NIS treatment effect on the tumor microenvironment and local immune response.	27		90%

Major Task 2: Analysis of CSF, blood and postmortem specimens			
Subtask 1 Collection of CSF, blood and postmortem tissue.	4-48	Dr. Müller	100%
Subtask 2 Antibody arrays of CSF/blood to measure cytokine levels.	4-48	Dr. Diaz	0%
Subtask 3 RNA-seq of blood lukocytes and postmortem specimens to assess immune cell composition and phenotype.	4-48	Dr. Diaz	90%
Subtask 4 Bioinformatics analysis of 'omics and antibody array data, including data pre-processing, principal component analysis, clustering, differential expression/ANOVA and regression modeling, providing information on peripheral immune response correlates of survival and therapy response.	4-48	Dr. Diaz	90%
Milestone(s) Achieved:	2		
Preliminary determination of MV-NIS treatment effect on the peripheral immune response.	27		75%
Assessment of intratumor and peripheral immune responses during MV-NIS therapy, compared between responders and non-responders, testing the hypothesis that a robust, endogenous anti-tumor immune response attenuates MV-NIS killing efficiency.	48		75%
Major Task 3: Target prioritization and validation			
Subtask 1 Integration of 'omics data and target selection: integration of 'omics data will be performed by correlating blood and CSF leukocyte composition with cytokine levels in plasma and CSF and with the expression of cytokines/chemokines by the tumor and the cellular composition of the tumor. For target selection we will compare immune signatures to overall and progression-free survival. Dividing patients into above-median and below-median survival cohorts we will use differential expression and co-expression analyses to identify differences in the pre-treatment immune response that are predictive of response to MV-NIS, as well as pathways that are activated in cases of MV-NIS resistance. Targets will be prioritized based on target expression level and prevalence, gene-pathway analysis, and cross-referencing databases of FDA approved drugs (e.g. DGIdb - A resource for mining the druggable genome).	16-39	Dr. Diaz	50%
Subtask 2 Target validation: validation will be done in an immunocompetent murine model of MV-NIS therapy in MB using a genetic-knockdown approach via CRISPR (with shRNA as a backup strategy). 4 mice per group will be used for each of 3 groups: A) target knockdown, B) untreated, and C) treated with a non-specific guide-RNA/shRNA.	13-48	Dr. Kasahara	0%
Milestone(s) Achieved:	2		
Identification of at least 5 candidate targets	27		50%
In vivo target validation completed for initial candidates, providing a pre-clinical rationale for novel clinical trials of combination therapies.	39		0%
Specific Aim 2 Assess the effect of MV and Toca 511 + Toca FC therapies on tumor-infiltrating immune cells in MB.			

Major Task 0: ACURO review and approval	1-3	Dr. Kasahara	100%
Major Task 1: Profile a murine MB model in a time series during MV-NIS therapy			
Subtask 1 Preparation and validation of CSCG cell lines (a murine model of MB), reagents and 48 hCD46TgIC mice (Raffel lab), injection of mice, treatment administration, BLI imaging harvesting of tumor tissue/CSF/blood. We will randomize mice into 4 groups. Groups A and B will be injected with MV, C and D with heat-inactivated virus or PBS respectively. We will use 12 mice per group.	4-9	Dr. Kasahara	100%
Subtask 2 RNA-seq, scRNA-seq imaging mass-cytometry assays, including sequencing and data processing on the murine specimens collected in subtask 1 to assess immune cell composition and phenotype.	6-15	Dr. Diaz	50%
Subtask 4 Antibody array assays on CSF/blood on the murine specimens collected in subtask 1 to measure cytokine levels.	9-15	Dr. Diaz	0%
Subtask 5 Data analysis: bioinformatics analysis of 'omics data and array data, including data pre-processing, principal component analysis, clustering, differential expression/ANOVA and time-series regression modeling, providing information on differences in peripheral and local immune reactivity under MV-NIS therapy in treatment responders and non-responders.	9-21	Dr. Diaz	50%
Milestone(s) Achieved	1		
Integration of omics and protein-level assay data: correlating blood and CSF leukocyte composition with cytokine levels in plasma and CSF and with the expression of cytokines/chemokines in the tumor and cellular composition of the tumor, in a time series during MV-NIS therapy.	15		25%
Assessment of intratumor and peripheral immune responses during MV-NIS therapy, compared between responders and non-responders, testing the hypothesis that a robust, endogenous anti-tumor immune response attenuates MV-NIS killing efficiency.	21		25%
Major Task 2: Profile a murine MB model in a time series during Toca 511 + Toca FC therapy			
Subtask 1 Preparation and validation of Tu-2449 murine cell lines, reagents and 48 B6C3F1/J mice, injection of mice, treatment administration, BLI imaging, harvesting of tumor tissue/CSF/blood. We will randomize mice into 4 groups. Groups A and B will be injected with MV, C and D with heat-inactivated virus or PBS respectively. We will use 12 mice per group.	9-15	Dr. Kasahara	100%
Subtask 2 RNA-seq, scRNA-seq imaging mass-cytometry assays, including sequencing and data processing on the murine specimens collected in subtask 1, to assess immune composition and phenotype.	12-27	Dr. Diaz	75%
Subtask 3 Antibody arrays on CSF/blood on the murine specimens collected in subtask 1 to measure cytokine levels.	15-21	Dr. Diaz	0%
Subtask 4 Data analysis: bioinformatics analysis of 'omics data and array data, including data pre-processing, principal component analysis, clustering, differential expression/ANOVA and time-series regression modeling, providing information on	15-27	Dr. Diaz	50%

differences in peripheral and local immune reactivity under Toca 511 therapy in treatment responders and non-responders.			
Milestone(s) Achieved	1		
Integration of omics and protein-level assay data correlating blood and CSF leukocyte composition with cytokine levels in plasma and CSF and with the expression of cytokines/chemokines in the tumor and cellular composition of the tumor, in a time series during Toca 511 therapy.	27		50%
Assessment of intratumor and peripheral immune responses during Toca 511 therapy, compared between responders and non-responders, testing the hypothesis that a robust, endogenous anti-tumor immune response attenuates Toca 511 killing efficiency.	27		50%
Specific Aim 3 Assess the combinatorial effect of immune-checkpoint blockade with MV-NIS or Toca 511 + Toca FC therapy in MB.			
Major Task 1: Survival study of MV-NIS + immune-checkpoint inhibitors			
Subtask 1 Preparation and validation of CSCG murine cell lines, reagents and hCD46TgIC mice (Raffel lab), injection of mice, treatment administration, BLI imaging. 11 groups of 12 mice will be used. Mice will be randomized into one of 9 treatment groups: MV-NIS, anti-CTLA4, anti-PD-1, anti-PD-L1, anti-IDO1, MV-NIS+anit-CTLA4, MV-NIS+anti-PD-1, MV-NIS+anti-PD-L1, MV-NIS+anti-IDO1, and 2 control groups: heat-inactivated virus or PBS.	16-21	Dr. Kasahara	50%
Subtask 2 Kaplan-Meier analysis	21-23	Dr. Diaz	0%
Major Task 2: Survival study of Toca 511/Toca FC + immune-checkpoint inhibitors			
Subtask 1 Preparation and validation of Tu-2449 murine cell lines, reagents and B6C3F1/J mice, treatment administration, BLI imaging. 11 groups of 12 mice will be used. Mice will be randomized into one of 9 treatment groups: Toca 511, anti-CTLA4, anti-PD-1, anti-PD-L1, anti-IDO1, Toca 511+anit-CTLA4, Toca 511+anti-PD-1, Toca 511+anti-PD-L1, Toca 511+anti-IDO1, and 2 control groups: heat-inactivated virus or PBS.	22-27	Dr. Kasahara	50%
Subtask 2 Kaplan-Meier analysis	27-29	Dr. Diaz	0%
Major Task 3: Profile murine MB model in a time series during MV-NIS combination therapy			
Subtask 1 Preparation and validation of CSCG murine cell lines, reagents and hCD46TgIC mice (Raffel lab), injection of mice, treatment administration, BLI imaging, harvesting of tumor tissue/CSF/blood. 20 groups of 12 mice will be used. Mice will be randomized into one of 18 treatment groups, in 9 of the treatment groups mice will be sacrificed when the tumor regresses more than 30% or 20 days, whichever is sooner. In the other nine treatment groups mice will be sacrificed when requiring euthanasia (criterion described in Aim 2) or after 150 days, whichever is sooner. Both cohorts of 9 treatment groups will receive the following treatments: MV-NIS, anti-CTLA4, anti-PD-1, anti-PD-L1, anti-IDO1, MV-NIS+anit-CTLA4, MV-NIS+anti-PD-1, MV-NIS+anti-PD-L1, MV-NIS+anti-IDO1. There	28-33	Dr. Kasahara	50%

will be 2 control groups treated with heat-inactivated virus or PBS.			
Subtask 3 RNA-seq, scRNA-seq imaging mass-cytometry assays, including sequencing and data processing, on the murine specimens collected in subtask 1, to assess immune composition and phenotype.	30-39	Dr. Diaz	0%
Subtask 4 Antibody array assays on CSF/blood on the murine specimens collected in subtask 1, to assess cytokine levels.	33-39	Dr. Diaz	0%
Subtask 5 Data analysis: bioinformatics analysis of 'omics data and array data, including data pre-processing, principal component analysis, clustering, differential expression/ANOVA and time-series regression modeling, providing information on differences in peripheral and local immune reactivity under MV-NIS combination therapies in treatment responders and non-responders.	33-45	Dr. Diaz	0%
Milestone(s) Achieved	1		
Integration of 'omics and protein-level assay data: correlating blood and CSF leukocyte composition with cytokine levels in plasma and CSF and with the expression of cytokines/chemokines and inhibitor target-pathways in the tumor and cellular composition of the tumor, in a time series during therapy.	39		0%
Assessment of intratumor and peripheral immune responses during MV-NIS combination therapies, compared between responders and non-responders, testing the hypothesis that MV-NIS killing efficiency is accelerated by immune checkpoint inhibition.	45		0%
Major Task 4: Profile murine MB model in a time series during Toca 511 + Toca FC combination therapy			
Subtask 1 Preparation and validation of Tu-2449 murine cell lines, reagents and B6C3F1/J mice, treatment administration, BLI imaging, harvesting of tumor tissue/CSF/blood. 20 groups of 12 mice will be used. . Mice will be randomized into one of 18 treatment groups, in 9 of the treatment groups mice will be sacrificed when the tumor regresses more than 30% or 20 days, whichever is sooner. In the other 9 treatment groups mice will be sacrificed when requiring euthanasia (criterion described in Aim 2) or after 150 days, whichever is sooner. Both cohorts of 9 treatment groups will receive the following treatments: Toca 511, anti-CTLA4, anti-PD-1, anti-PD-L1, anti-IDO1, Toca 511+anit-CTLA4, Toca 511+anti-PD-1, Toca 511+anti-PD-L1, Toca 511+anti-IDO1. There will be 2 control groups treated with heat-inactivated virus or PBS.	33-39	Dr. Kasahara	50%
Subtask 3 RNA-seq, scRNA-seq imaging mass-cytometry assays, including sequencing and data processing on the murine specimens collected in subtask 1, to assess immune composition and phenotype.	36-48	Dr. Diaz	0%
Subtask 4 Perform antibody arrays on CSF/blood on the murine specimens collected in subtask 1, to assess cytokine levels.	39-45	Dr. Diaz	0%
Subtask 5 Data analysis: bioinformatics analysis of 'omics data and array data, including data pre-processing, principal component analysis, clustering, differential expression/ANOVA and time-series regression modeling, providing information on differences in peripheral and local immune reactivity under Toca	43-48	Dr. Diaz	0%

511 combination therapies in treatment responders and non-responders.			
Milestone(s) Achieved	1		
Integration of omics and protein-level assay data: correlating blood and CSF leukocyte composition with cytokine levels in plasma and CSF and with the expression of cytokines/chemokines and inhibitor target-pathways in the tumor and cellular composition of the tumor, in a time series during therapy.	45		0%
Assessment of intratumor and peripheral immune responses during Toca 511 combination therapies, compared between responders and non-responders, testing the hypothesis that Toca 511 killing efficiency is accelerated by immune checkpoint inhibition.	48		0%

Goals accomplished*:*

In year three of the project's period we have completed the acquisition of clinical specimens. Almost all the multi-omics profiling of clinical specimens has also been completed. We have made significant progress in the bioinformatics analysis and interpretation of the resulting data. Additionally, we have made considerable progress in the murine studies. This includes completion of the murine studies described in Aim 2. This progress is despite not receiving HRPO approval until month 8 of the project period, not receiving ACURO approval until year 2 of the project period, and delays due to the Covid-19 shutdown and slowdown.

We document each aim and task of the approved SOW and our corresponding progress to date:

Specific Aim 1: Compare local and peripheral immune activation between MV-NIS responders and non-responders using human MB specimens obtained pre- and post-therapy.

Major Task 0: HRPO review and approval. This activity is complete. However, instead of the original three months planned, this activity took seven months to complete due to the speed of the HRPO review process. This delay was outside of the PIs' control.

Major Task 1: Analysis of tumor tissue from the MV-NIS clinical trial (and controls). Completion of this task involves obtaining archival tumor specimens from the Children's Brain Tumor Tissue Consortium (CBTTC) and the UCSF Neurosurgery Tissue Core archives, the collection of prospective fresh tumor specimens, performance of 'omics profiling activities, bioinformatics preprocessing, bioinformatics analysis and data integration with Major Task 2 and Specific Aim 2-3 results. As of year 3, we have completed most of the collection, 'omics profiling, and data preprocessing activities. We summarize progress for each sample cohort in Table 1.

Table 1: progress summary for the analysis of clinical specimens.

		PNOC Trial		UCSF Core	
		Projected	Status	Projected	Status
Archival	Frozen tumor	6	6 snRNA-seq libraries prepped and sequened, data preprocesing complete, quality nominal, tissue retained for subsequent scATAC-seq assays	10	Neurosurgery tissue core screening of archival frozen specimens completed, 10 samples identified, distributed and profiled via snRNA-seq. Data preprocessing complete.

	FFPE tumor	14	5 RNA extractions performed from FFPE tissue, all show nominal concentrations	16	UCSF Neurosurgery tissue core has completed screening of archival FFPE specimens. Spatial profiling has begun and is ongoing.	
	CSF pre-treat.	3	Samples received at Diaz lab and pending processing. Pilot study of CSF-derived exosomes underway.			
	CSF post-treat.	19	Samples received at Diaz lab and pending processing. Pilot study of CSF-derived exosomes underway.			
	Blood pre-treat.	24	24 samples have been collected and QC'd. 22/24 samples had acceptable RNA quality have been profiled via RNA-seq. Data preprocessing is complete and analysis is ongoing.			
	Blood post-treat.	59	59 samples have now been profiled via RNA-seq. Data preprocessing is complete and analysis is ongoing.			
Prospective	Tumor	4	None collected.	6	6 samples collected, distributed, and profiled via snRNA-seq. Via PI supplemental funds 3 cases have been profiled via snATAC-seq and snCUT&Tag. Data preprocessing complete and analysis.	
	CSF pre-treat.	4	4 pretreatment samples from 4 patients collected, fractionated and shipped to Diaz lab. QC nominal.			
	CSF post-treat.	8	8 pretreatment samples from 4 patients collected, fractionated and shipped to Diaz lab. QC nominal.			
	Blood pre-treat.	4	4 pretreatment samples from 4 patients collected, fractionated, profiled via RNA-seq. Data preprocessing is complete and analysis is ongoing.	6	6 samples collected, distributed and profiled via RNA-seq. Data preprocessing complete and analysis is ongoing.	
	Blood post-treat.	8	8 pretreatment samples from 8 patients collected, fractionated, profiled via RNA-seq. Data preprocessing is complete and analysis is ongoing.			
Post mortem	Frozen tumor	2	None collected.			
	FFPE tumor	2	None collected.			

We have completed all necessary material-transfer agreements and tissue request documentation. All archival tumor specimens from the PNOC trial have been transferred to the Diaz lab. Screening of archival specimens from the UCSF Neurosurgery Core by the core's pathologist is also complete. Suitable frozen specimens from the UCSF Core have been identified, distributed and profiled via snRNA-seq by the Diaz lab. Screening of formalin-fixed paraffin embedded (FFPE) specimens at the UCSF Core is complete. FFPE

specimens have been identified and distributed and are pending profiling via spatial transcriptomics/proteomics. Collection of prospective specimens, as well as single-cell and bulk 'omics profiling of those specimens is complete.

We have made significant progress in our analysis of human tumor specimens. We have subjected all frozen tumor specimens to single-nucleus RNA-sequencing (snRNA-seq). All samples resulted in successful captures, generating approximately 2,000 cells per sample and over 2,500 genes detected per cell. These and other quality metrics were nominal. We've performed preprocessing using our previously described approaches (Wang et al., 2019, 2020). Briefly, this included quality control of sequenced reads, alignment and gene quantification, filtering of low-quality/doublet capture events, calling somatic mutations and filtering neoplastic cells based on those results, clustering, and cell-type classification for non-neoplastic cells. We have constructed a database to aggregate non-identifying patient meta-data alongside genomics readouts, and a system to backup the data we generate in this project to a secure cloud service. We have pre-processed published single-cell 'omics data (Hovestadt et al., 2019; Ocasio et al., 2019) through our pipeline to use as additional controls. We separated neoplastic cells from non-malignant infiltrates, identified glia, endothelial cells, as well as monocytic-lineage cells. In total, we have profiled specimens from all molecular subtypes of medulloblastoma, from cases ages ranged from less than one year to 60 years old, and from tissue derived from the posterior fossa, cerebellum, as well as the fourth ventricle (Figure 1). As described in Table 1, this cohort contains both cases from the PNOC MV-NIS clinical trial as well as untreated cases not in the trial as controls. In summary, Major Task 1 is largely complete, with some spatial profiling of FFPE specimens still pending.

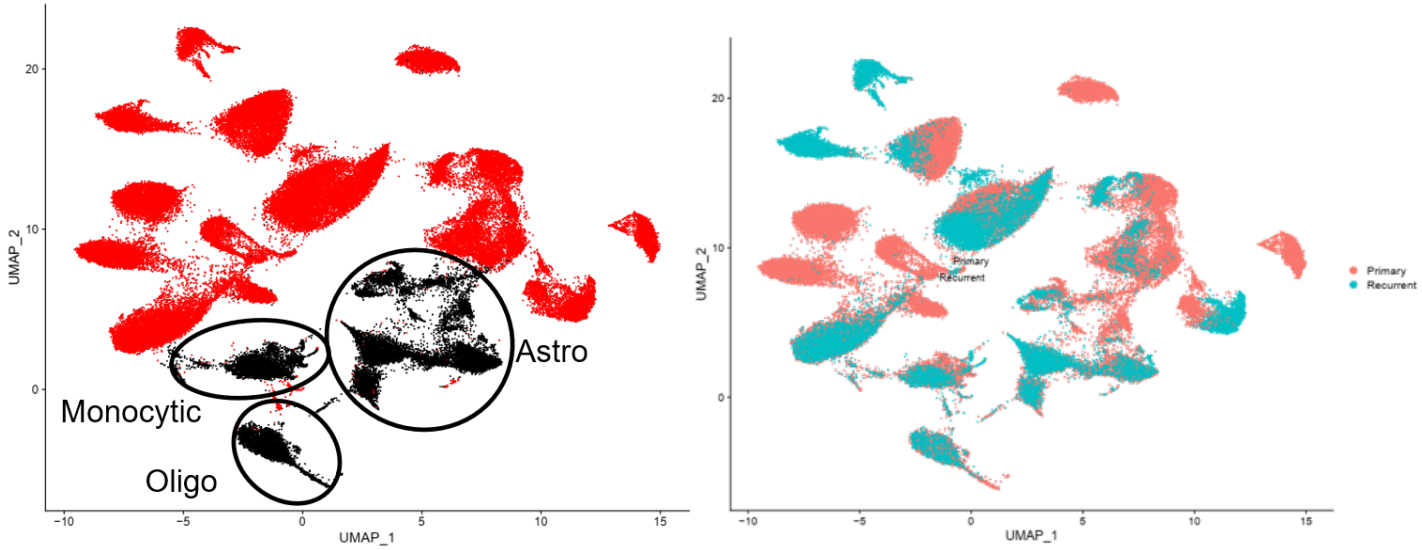


Figure 1: Summary of the snRNA-seq of tumor clinical specimens, visualized via a UMAP dimensionality reduction. Each point represents a cell. Left: 75,825 cells profiled, including monocytic-lineage cells, neoplastic cells, and non-malignant glia. Putative neoplastic cells, cells harboring clonal mutations found in the tumor, are annotated in red. Right: Cells labeled by primary vs. recurrent tumor specimen source.

In year 3 we performed significant additional analyses of the MB single-cell data. We've genotyped and classified cell types found in these tumors (Figure 2). In comparing treatment naïve and treated specimens, we found an upregulation of the DNA-damage response, RNA translation, WNT and NOTCH signaling in recurrent specimens. The percentages of stem-like cells increased by over two-fold at recurrence. We found that

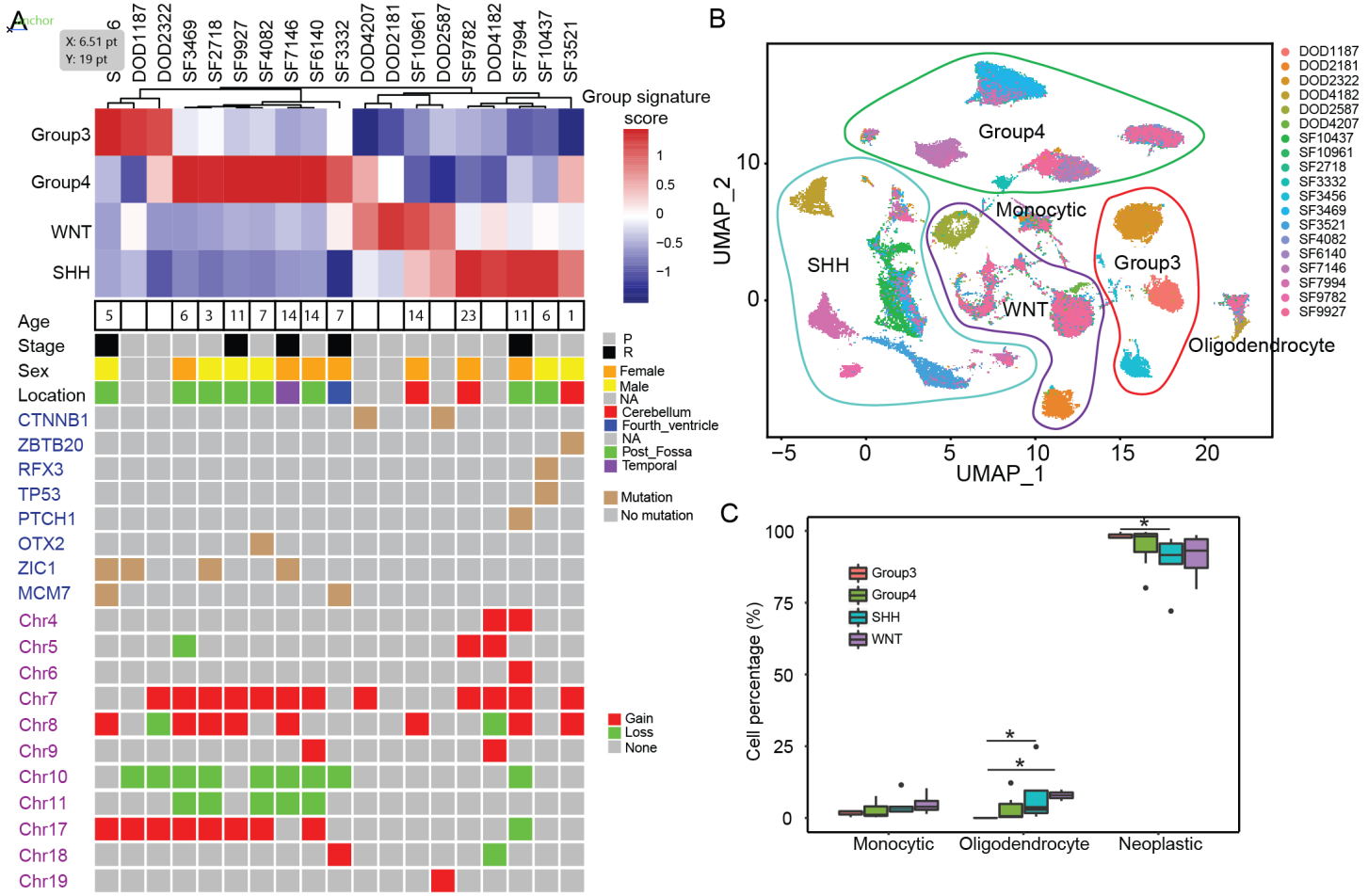


Figure 2: A) Summary of genotypes by sample. B) Scatter-plot of cells, grouped by molecular subtype and cell type. C) The percentages of neoplastic and non-malignant cell types observed, grouped by MB molecular subtype.

microglia and oligodendrocyte-lineage cells were the most abundant non-malignant tumor-associated cell types, representing 2%-10% of cells profiled. Microglia abundances were relatively stable across molecular subtypes, and when comparing primary to recurrent tumors (Figure 3). There was a moderate, but statistically significant, increase in oligodendrocyte abundance in SSH and WNT tumors, compared to Group 3/4 tumors (Figure 2C). Lastly, MBs are of cerebellar origin and the cell of origin for Group 3/4 tumors has not been elucidated. To determine the Group 3/4 cell of origin, as well as potentially learn novel therapeutic targets, we compared our human MB snRNA-seq data to published snRNA-seq from the developing cerebellum. Using a machine learning approach, we projected the developmental data onto our Group 3/4 MB data. We found that human Group 3/4 MBs have a lineage hierarchy that parallels the glutamatergic neuronal lineage of the developing cerebellum (Figure 4). This analysis implicated rhombic-lip stem cells as the putative Group 3/4 cell of origin, a novel and impactful discovery if validated.

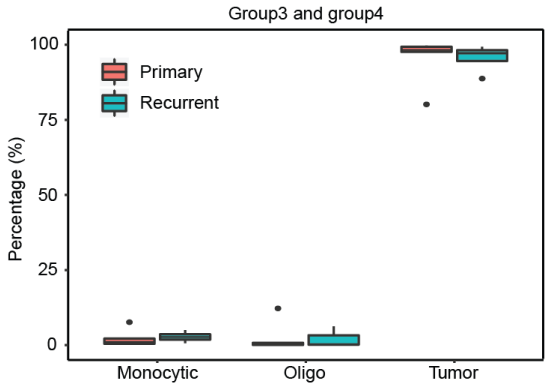


Figure 3: Percentages of cell types observed, compared between treatment naïve and treated specimens.

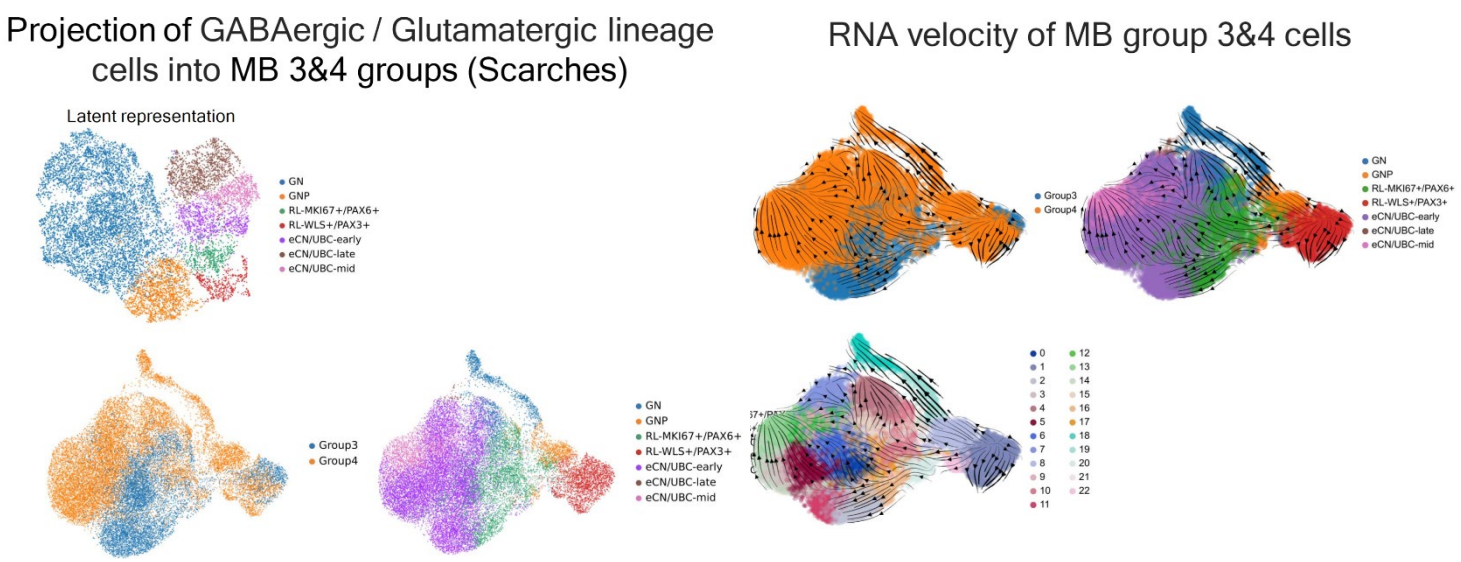


Figure 4: (Left) Latent space representation of snRNA-seq from human fetal cerebellum above, a projection of cell-type labels from embryonic cerebellar neuroglia onto Group 3/4 MB cells, showing a recapitulation of the cellular hierarchy observed in the developing brain in MB. (Right) RNA-velocity analysis estimates lineage trajectories in MB data, demonstrating a bifurcation structure that parallels the developing brain, rooted in a rhombic-lip neural stem cell-like population.

Major Task 2: Analysis of CSF, blood and postmortem specimens. This task has similar components to Major Task 1: sample acquisition, QC, genomics assays and bioinformatics analysis. Additionally, for this task, protocols for the collection and snap-freezing of tumor tissue, for the collection of CSF and blood, for their fractionation and the freezing of derived leukocytes and supernatant were drafted to ensure optimal and consistent sample acquisition across centers. These protocols were distributed and discussed with all centers.

We have completed the projected prospective collections of blood and CSF from the PNOC MV-NIS clinical trial and met our project's targets. These samples have been fractionated for downstream assays on the leukocytes and supernatant separately. We will continue to collect blood and CSF specimens from the PNOC MV-NIS trial as they become available, exceeding our original projections.

All archival specimens have been transferred to the Diaz lab. We assessed QC metrics for all archival specimens and found it to them to be nominal, as described in our previous report. We therefore proceeded to perform RNA-seq (Figure 5). We performed clustering of gene expression by patient across the time series of collection points. We found corelated increases in gene expression in days 4 and 7 post MV-NIS treatment. Although we are still interpreting the genes observed in these clusters and what this means for therapy, at least some of these changes can be attributed to changes in the composition of leukocytes in patients' blood. In particular, when we deconvolved the bulk RNA-seq data to infer its composition we found increases in the content of natural killer cells in days 4 and 7, T-regulatory lymphocytes in day 4 as well as increased macrophages and dendritic cells in day 7. In summary, Major Task 2 is mostly complete with some analysis of CSF and postmortem specimens pending.

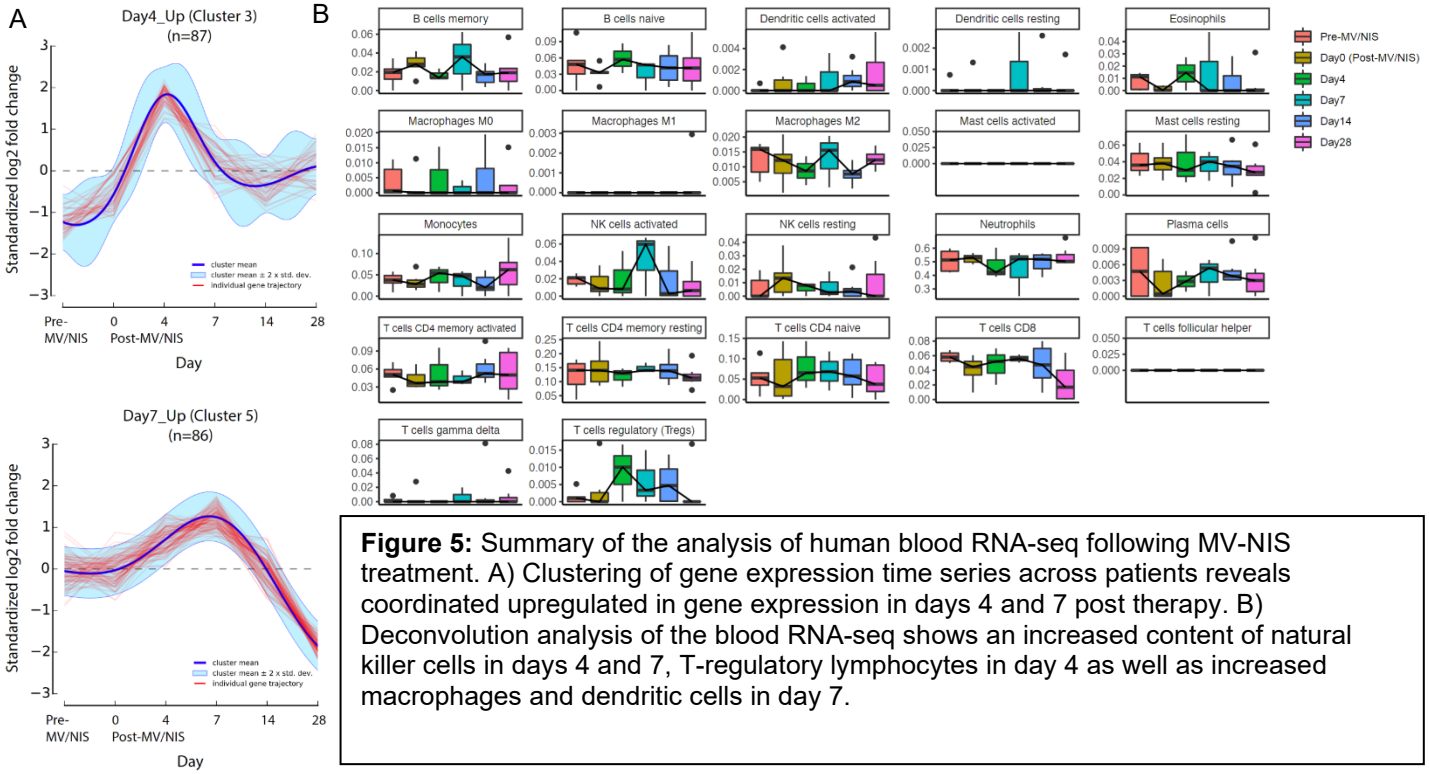


Figure 5: Summary of the analysis of human blood RNA-seq following MV-NIS treatment. A) Clustering of gene expression time series across patients reveals coordinated upregulated in gene expression in days 4 and 7 post therapy. B) Deconvolution analysis of the blood RNA-seq shows an increased content of natural killer cells in days 4 and 7, T-regulatory lymphocytes in day 4 as well as increased macrophages and dendritic cells in day 7.

Major Task 3: Target prioritization and validation. This task involves integrated bioinformatics analysis of the clinical data and the prediction of gene targets to enhance OV direct killing and/or indirect anti-tumor immune responses. Subsequently, targets will be validated genetically in vivo. We have taken the first step toward the end by aggregating the samples and public data, performing genomics assays, sequencing and bioinformatics analysis. Data analysis and interpretation is ongoing.

Specific Aim 2 Assess the effect of MV and Toca 511 + Toca FC therapies on tumor-infiltrating immune cells in MB.

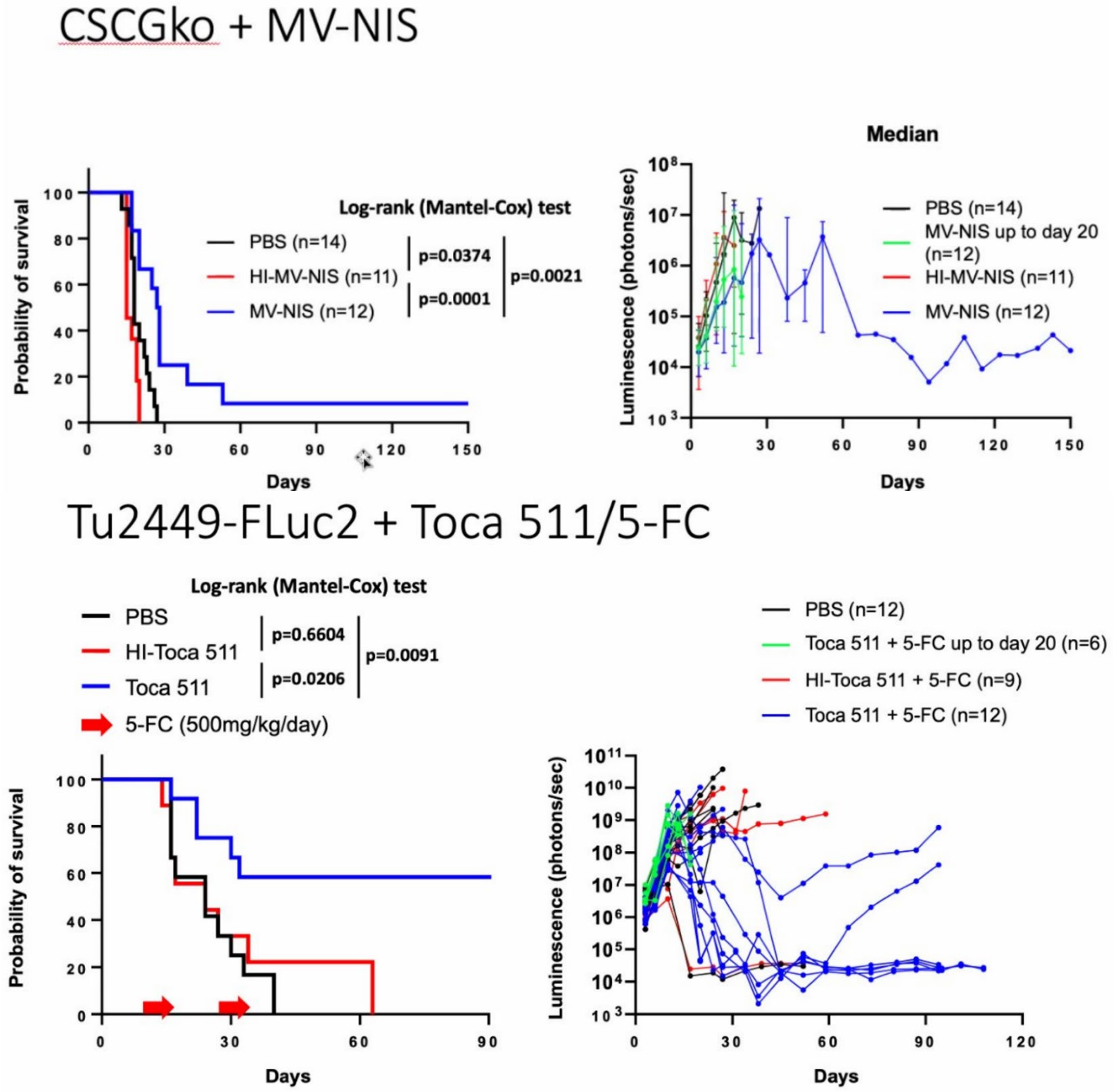
Major Task 0: ACURO review and approval. This activity is complete. We have begun the murine studies. Instead of the original three months planned, this activity took over 12 months to complete due to the speed of the HRPO review process. This delay was outside of the PIs' control.

Major Task 1: Profile a murine MB model in a time series during MV-NIS therapy. Activities for this task included injection of tumor cells into mouse brains, BLI imaging, injection of MV-NIS oncolytic virus and as well as mouse euthanasia and tissue harvesting. We harvested tumor tissue, blood and CSF from sacrificed mice and the majority of the tumor tissue was used for genomics profiling via snRNA-seq. Approximately 25% of the tumor tissue was retained for spatial profiling. Likewise, blood was subjected to RNA extraction and profiling via RNA-seq. We have largely completed these studies (Table 2), and are in a position to now preprocess these data, analyze them and integrate them with our human data to address our central hypothesis. Sequencing and data preprocessing for the tumor tissue-derived snRNA-seq data is complete, QC is nominal and data analysis and interpretation is ongoing.

Major Task 2: Profile a murine MB model in a time series during Toca 511 + Toca FC therapy. Activities for this task included injection of tumor cells into mouse brains, BLI imaging, injection of Toca-511 virus and

administration of the Toca-FC chemotherapy pro-drug, as well as mouse euthanasia and tissue harvesting. We harvested tumor tissue, blood and CSF from sacrificed mice and the majority of the tumor tissue was used for genomics profiling via snRNA-seq. Approximately 25% of the tumor tissue was retained for spatial profiling. Likewise blood was subjected to RNA extraction and profiling via RNA-seq. We have largely completed these

Figure 6: (Top) Survival curves and bioluminescence plots of the MV-NIS murine studies in Aim 2. (Bottom) Likewise, for Toca 511/5-FC.



studies (Table 2), and are in a position to now preprocess these data, analyze them and integrate them with our human data to address our central hypothesis. Sequencing and data preprocessing for the tumor tissue-derived snRNA-seq data is complete, QC is nominal and data analysis and interpretation is ongoing.

In summary, the murine studies for Aim 2 are largely complete (Figure 6). The genomics profiling and data analysis are ongoing.

Specific Aim 3 Assess the combinatorial effect of immune-checkpoint blockade with MV-NIS or Toca 511 + Toca FC therapy in MB.

Major Task 1: Survival study of MV-NIS + immune-checkpoint inhibitors. We have performed validation of the CSCG cell line, and we are taking a staggered approach to implementing this task due to the large number of groups and very large numbers of animals involved, the prolonged time course required for survival studies, and limitations in animal housing capacity and imaging facility capacity. Accordingly, we have started with survival studies of control groups for this task, which have been initiated. Otherwise, there is nothing to report.

Major Task 2: Survival study of Toca 511/Toca FC + immune-checkpoint inhibitors. We have performed validation of the Tu-2449 cell line, and we are taking a staggered approach to implementing this task due to the large number of groups and very large numbers of animals involved, the prolonged time course required for survival studies, and limitations in animal housing capacity and imaging facility capacity. Accordingly, we have started with survival studies of control groups for this task, which have been initiated. Otherwise, there is nothing to report.

Major Task 3: Profile murine MB model in a time series during MV-NIS combination therapy. We have performed validation of the CSCG cell line. As above, we are taking a staggered approach to implementing this task, and have started with time series studies of control groups, which have been initiated. Otherwise, there is nothing to report.

Major Task 4: Profile murine MB model in a time series during Toca 511 + Toca FC combination therapy. We have performed validation of the Tu-2449 cell line. As above, we are taking a staggered approach to implementing this task, and have started with time series studies of control groups, which have been initiated. Otherwise, there is nothing to report.

Opportunities for training and professional development

Nothing to report.in the

Dissemination to communities of interest

Nothing to report.

Plans for the next reporting period

In the next reporting period the Kasahara lab will complete the murine studies in Specific Aim 3. This will include completion of the preparation and validation of CSCG murine cell lines, reagents and hCD46TgIC mice and beginning the injection of mice, treatment administration, BLI imaging. 11 groups of 12 mice will be used. Mice will be randomized into one of 9 treatment groups: MV-NIS, anti-CTLA4, anti-PD-1, anti-PD-L1, anti-IDO1, MV-NIS+anit-CTLA4, MV-NIS+anti-PD-1, MV-NIS+anti-PD-L1, MV-NIS+anti-IDO1, and 2 control groups: heat-inactivated virus or PBS.

The genomics activities on human specimens are largely complete. However, the spatial profiling of FFPE specimens and CSF protein array assays are still pending. The Diaz lab will complete these remaining studies

in the next project period. Bioinformatics analysis of the genomics data from human and murine analyses will be ongoing throughout the next period. This will include data pre-processing, principal component analysis (or T-SNE analysis), clustering, differential expression/ANOVA and time-series regression modeling, providing information on differences in peripheral and local immune reactivity under MV-NIS therapy in treatment responders and non-responders. For target selection we will compare immune signatures to overall and progression-free survival. Dividing patients into above-median and below-median survival cohorts we will use differential expression and co-expression analyses to identify differences in the pre-treatment immune response that are predictive of response to MV-NIS, as well as pathways that are activated in cases of MV-NIS resistance. Targets will be prioritized based on target expression level and prevalence, gene-pathway analysis, and cross-referencing databases of FDA approved drugs (e.g. DGIdb - A resource for mining the druggable genome.

4. Impact

Impact on the development of the principal disciplines of the project

We have assembled what is perhaps the largest database and single-cell atlas of human medulloblastoma to date. This atlas describes local and peripheral immune composition at unprecedented resolution. For our purposes, it will form a basis for assessing response to OV therapy. However, this resource has the potential for broad impact on the field of brain-tumor heterogeneity and medulloblastoma in particular.

Impact on other disciplines

Nothing to report.

Impact on technology transfer

Nothing to report.

Impact on society beyond science and technology

Nothing to report.

5. Changes/Problems

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

There have been two sets of problems, those due to delays in HRPO and ACURO review and approval which were outside of the PIs' control and delays due to the Covid-19 pandemic. ACURO approval for the murine studies was delayed for 12 months. Thus, the tasks in Specific Aims 2/3 have been delayed. We are experiencing a delay of about 12 months for the mouse studies due to delay in ACURO approval as well as delays due to Covid-19.

Lab activities have been impacted due to Covid-19 in the following ways:

1. We had three months of complete shutdown, during which library preps, sequencing and tissue processing was suspended. This includes the additional approved spatial and single-cell assays which we were unable to begin until the stop-work order was lifted.
2. During shutdown the UCSF Neurosurgery Tissue Core was restricted to essential activities and was not permitted to provide fresh tissue or blood specimens from prospective collections for research use, which were deemed non-essential activities. Likewise, the core facilities supporting the PNOC trial

faced similar constraints. Now that they have restarted operations, they have been constrained to work at limited capacity which has further slowed down collection and distribution activites.

3. Since reopening we have only been able to work at 25% and then 50% capacity, limiting our rate of progress.
4. Since reopening, there has been a run on multiple common laboratory reagents, including reagents for nuclei extraction and RNA quantification. Delays in acquiring needed reagents have slowed our work.
5. Our dedicated high-performance compute node, installed in the HDFCC Translational Informatics Cluster, died just before the stop-work order. We were not able to have this replaced until after the stop-work order was listed and the timeframe for having the replacement installed was elongated due to Covid-19 slowdown and associated complications. Having to develop codes for workarounds in the cloud and reliance on desktop/laptop computers slowed our bioinformatics progress considerably.

Changes that had a significant impact on expenditures

As noted above, UCSF and California regulations currently limit our laboratory operations to 50% capacity. Much of the work in the Diaz lab is bioinformatics analysis and now that our compute node has been restored there are no anticipated delays in that aspect of the project. However, Diaz-lab genomics assays and Kasahara-lab murine assays will be slowed until we are able to operate at 100% capacity. Likewise, the shutdown and subsequent limit on capacity has slowed our ability to obtain clinical specimens prospectively. We anticipate that this may lead to delays. We have been in contact with our SO contact Dr. Bunker and will address a possible request for an extension of the project period and associated personnel support, if necessary, toward the end of the project period, 6/30/23.

Pilot study of exosome-derived RNA from CSF fluid and blood plasma

Liquid biopsies from CSF fluid or blood have been shown to yield prognostic biomarkers for MB (e.g. Liu Cancer Cell 2021). We've partnered with the Francis lab at UCSF, which has developed a pipeline to isolate exosomes from CSF or blood and profile their genetic content. Exosomes are small vesicles that are expressed by cells and can contain RNA messages. We've applied this technology to a handful of the CSF and blood specimens derived from this trial to assess feasibility and utility. This work is ongoing.

6. Products

Presentations

a. "Myeloid cell interactions with emerging viral therapies". **Aaron Diaz**. Presentation at the Society for Neuro-Oncology Annual Meeting, Immunotherapy Promises and Challenges Think Tank, Nov. 2019.

b. "Virotherapy and Immunotherapy: Strategies for Synergy from Bench-to-Bedside and Back Again". **Noriyuki Kasahara**. Presentation at the Society for Neuro-Oncology Annual Meeting, Immunotherapy Promises and Challenges Think Tank, Nov. 2019.

Publications

a. P. Chuntova, **N. Kasahara**, **A. Diaz** et al. Unique challenges for glioblastoma immunotherapy—discussions across neuro-oncology and non-neuro-oncology experts in cancer immunology. Meeting Report from the 2019 SNO Immuno-Oncology Think Tank. **Neuro-Oncology**. 2021, 23(3).
b. L. Wang, J. Jung, H. Babikir, K. Shamardani, **N. Kasahara**, **S. Muller**, **A. Diaz**. MEDB-59. A draft atlas of medulloblastoma cellular evolution under therapy. **Neuro-Oncology**. 2022, 24.

Books

Nothing to report.

Websites

Nothing to report.

Technologies

Nothing to report.

Inventions/Patents

Nothing to report.

7. Participants & Other Collaborating Organizations

Individuals that have worked on the project

Name:	Noriyuki Kasahara, MD PhD
Project Role:	PI
Nearest person month worked:	0.6
Contribution to Project:	Dr. Kasahara supervised the in vivo studies using murine models.
Funding Support:	No changes to report.

Name:	Pavlina Chuntova, PhD
Project Role:	Specialist
Nearest person month worked:	2.4
Contribution to Project:	Dr. Chuntova maintained the transgenic animal colony and assisted in establishing murine models of medulloblastoma.
Funding Support:	No changes to report.

Name:	Akihito Inagaki, PhD
Project Role:	Specialist
Nearest person month worked:	3.0
Contribution to Project:	Dr. Inagaki established and conducted in vivo studies of murine models of medulloblastoma.
Funding Support:	No changes to report.

8. **Special Reporting Requirements:** None

9. **Appendices:** None