The epigenetic determinants of CAR T cell response to Diffuse Midline Glioma

SPECIFIC AIMS
Diffuse midline glioma (DMG) is an incurable childhood tumor with a median survival of approximately 1 year following standard of care radiation therapy. No new therapies have been approved in the last 25 years despite over 250 clinical trials, underlying the need for novel treatments. Recently, chimeric antigen receptor (CAR) T cell immunotherapy has emerged as a promising therapeutic modality in this malignancy, with a recent trial showing objective clinical responses to GD2-directed CAR T cells. Despite this promising initial activity, the factors which mediated eventual tumor progression in these patients are poorly understood and optimizing CAR T cell therapy will require a deeper understanding of these resistance mechanisms. Chromatin, the complex of DNA and histones, is known to be remodeled within tumor cells to adapt to immunotherapy. This can be by regulating target antigen expression or by altering expression of immunomodulatory molecules which act in an antigen independent manner. The specific epigenetic mechanisms employed by DMGs to resist CAR T killing are poorly understood. The central hypothesis of this proposal is that epigenetic mechanisms within tumor cells regulate sensitivity to CAR T cell killing through target antigen dependent and independent mechanisms. The goal of this proposal is to characterize the epigenetic mechanisms within tumor cells which regulate CAR T cell activity, which will ultimately permit novel combinatorial approaches to augment CAR T cell efficacy in this disease. The Foster Lab has optimized the development of mRNA based GD2 targeted CAR T cells for use in DMGs, which show efficient tumor cell clearance in vivo with well characterized kinetics. The Phillips lab has generated and optimized a chromatin focused CRISPR library, which has been successfully used to characterize epigenetic mechanisms of drug resistance in prior work. Here, leveraging the expertise of both labs, we will study the epigenetic determinants of CAR T cell activity through the following aims:

Specific Aim 1: Identify functional epigenetic regulators of the CAR T cell response using a high throughput CRISPR screen in vivo. Using a chromatin focused CRISPR library, we will perform a loss of function genetic screen in DMG xenografts in vivo treated with either GD2 directed CAR T cells or non-targeting CAR T cells. ‘Hits’ which either resist or augment CAR T activity, will be functionally validated in vivo and characterized mechanistically by assessing effect on GD2 expression, and determining chromatin occupancy and impact on gene expression using ChIP-seq and RNA-seq respectively.

Specific Aim 2: Characterize the epigenomic adaptation of DMG xenografts to CAR T cell treatment. We will characterize changes in chromatin accessibility and gene expression in DMG tissue we have harvested from mice treated with GD2 directed vs non-targeting CAR T cells, using ATAC-seq paired with RNA-seq. Differentially accessible chromatin regions will be subjected to motif analysis to identify candidate trans acting factors mediating changes in the DMG chromatin state associated with CAR T treatment.

IMPACT AND FUTURE WORK
Through successful completion of our 2 orthogonal aims, we will identify the epigenetic mechanisms employed by DMG cells which regulate the response to CAR T cells. We will nominate potential chromatin factors which can be targeted to enhance the efficacy of CAR T cells. Data from this work will form the basis of a joint NIH proposal from the Foster and Phillips with the ultimate goal to generate a novel clinical trial in DMG patients.

BACKGROUND AND SIGNIFICANCE
Diffuse midline glioma. DMG is a devastating pediatric brain and spinal cord tumor that is incurable. While radiation can prolong the life of a child with DMG briefly, tumor location makes surgery impossible, and the median survival of a child diagnosed with DMG is 11 to 15 months [1]. Decades of chemotherapy trials have failed to show any benefit [2] and these tumors remain in dire need of innovative therapy. DMGs are characterized by recurrent mutations in histone H3[3], highlighting epigenetic dysregulation as a central driver of tumorigenesis in this disease.

GD2-directed CAR T cells represent a promising therapy for DMG. The disialoganglioside, GD2, was recently identified as an immunotherapeutic target in DMG[4]. Building off the preclinical success of GD2-directed CAR T cells in DMG xenografts [4], a recent clinical trial opened (NCT04196413) in which all three patients experienced evidence of tumor regression[5]. Unfortunately, all three patients subsequently progressed and little is known about the mechanism of tumor escape. One patient exhibited a distant metastatic site in the brain that was revealed to be GD2 positive, but no other available information from the additional relapses[5].
CHOP is currently planning a phase 1 GD2-directed CAR T cell trial for DMG patients. Understanding the mechanisms leading to eventual recurrence is thus of the utmost importance, as combinatorial approaches may be incorporated to enhance this promising treatment.

PRELIMINARY DATA
An mRNA based platform for generation of GD2 specific CAR T cells. The Foster Lab has previously completed work to optimize mRNA for use in CAR T cells (Fig. 1)[6] and applied these methods in GD2-directed CAR T cells against DMG patient-derived models in vitro and in vivo (Fig. 2). This approach is advantageous because mRNA delivery allows rapid manufacture of CAR T cells and the transient nature of mRNA affords the ability to regulate and reduce toxicity which is a major challenge in CAR T treatment of DMGs[4, 5].

An optimized CRISPR based approach for targeting epigenetic dependencies in DMGs. The Phillips Lab has generated a chromatin focused CRISPR library containing sgRNAs targeting ~500 epigenetic regulators i.e. genes encoding chromatin ‘writer’, ‘reader’, ‘eraser’ and ‘remodeler’ proteins which regulate chromatin structure and gene expression. Prior pooled screens in patient derived DMG cell-lines have identified chromatin factors which sensitize cells to specific treatments (Fig. 2). Our experimental and bioinformatic pipeline is thus poised to identify chromatin factors which regulate the response to CAR T cells.

Figure 1. Chromatin focused CRISPR screen pipeline. Left: schematic for pooled sgRNA library loss of function screen to identify modulators of treatment response. Right: sgRNA abundance deconvoluted from prior screens showing top depleted sgRNAs (genes which sensitize effect of treatment).

Figure 1. Mice engrafted with 7316-6349 treated with four intratumoral doses of 4 ×10^6 CAR T cells, tumor burden measured over time with bioluminescent imaging. GD2 CAR n=10, CD19 CAR n=9. **** p<0.0001

APPROACH
Specific Aim 1: Identify functional epigenetic regulators of the CAR T cell response using a high throughput CRISPR screen in vivo. Lentiviral transduction will be used to establish stable Cas9 expression in patient-derived DMG cell line 7316-6349. Lentivirus from our chromatin-focused sgRNA library will be used to transduce Cas9 expressing 7316-6349, followed by antibiotic selection in vitro. Xenografts will then be established by stereotactic injection of 500,000 cells into the brainstem of NSG mice (n=20 mice). Following confirmation of tumor engraftment using bioluminescent imaging, mice will be infused with GD2 directed CAR T cells (N=10) or non-targeting CAR T cells (N=10). Mice will be euthanized at two time points (7 days and 14 days post-treatment) and tumor tissue harvested. Genomic DNA will be extracted from post-treatment xenograft tissue and pre-injection cells and the sgRNA cassette PCR amplified. Amplicons will be sequenced on the Miseq platform and the average log2FC of the sgRNA abundance calculated bioinformatically as previously described[9]. sgRNAs which are depleted (sensitzers) and enriched (resisters) are of interest, though we will initially focus on genes whose loss of function sensitize cells to CAR T killing for functional and mechanistic characterization as these will be most rapidly translated to combinatorial therapy.

Specific Aim 2: Characterize the epigenomic adaptation of DMG xenografts to CAR T cell treatment. DMG xenograft models 7316-6349 and SUDIPG-XIIIIP* have been created as previously described[7], and treated with GD2-directed mRNA CAR T cells (Fig. 3). Brains have been harvested from the mice of these experiments and flash frozen. Five brains each from GD2-treated and control (CD19-treated) mice will be used to perform ATAC-seq and RNA-seq as previously described[8]. Briefly, tumor tissue from each brain will be dissected and nuclei isolated and subjected to a transposition reaction (n=5 per group). Indexed libraries will be generated and subjected to paired end sequencing on the NextSeq platform. Following alignment, peaks will be called with MACS. Log2 fold change of accessibility will be calculated as log2 averaged FPKM on replicates of GD2 CAR T treated versus non-targeting CAR T treated tumor using DESeq2. Differentially accessible regions will be subjected to unbiased pathway and motif analysis to mechanistically characterize changes in chromatin state observed. Candidate trans acting factors will be functionally assessed using knockdown experiments in DMG xenografts followed by assessment of growth kinetics in the presence of GD2 directed CAR T cells.
REFERENCES


BUDGET

**Personnel**

**Jessica Foster** (co-PI, 5% effort, no salary requested) is an Assistant Professor in the Division of Oncology at CHOP and the Perelman School of Medicine at the University of Pennsylvania. She leads a research program focused on immunotherapeutic approaches for pediatric central nervous system tumors. She will co-direct this project, and her laboratory will be responsible for generation of CAR T cells, providing murine xenografts that have been treated with CAR T cells, and flow cytometry.

**Richard Phillips** (co-PI, 5% effort, no salary requested) is a Presidential Assistant Professor in Neurology and Genetics at the University of Pennsylvania. Dr. Phillips directs a research program studying mechanisms of epigenetic regulation in brain cancer and the development of novel therapeutics. Dr. Phillips will co-direct this project and his lab will be responsible for epigenomic analysis of tissue (ATAC-seq, bioinformatics analysis), CRISPR library lentivirus production and cell transduction, and bioinformatic deconvolution of CRISPR screens.

**Ezra Beaubien** (research technician – Foster lab, 50% effort) is a technician in the Foster lab who will provide CAR T cell generation, cell culture, flow cytometry and additional hands-on support for this project.

**James, Innes** (postdoctoral fellow - Phillips Lab, 30% effort) is a postdoctoral fellow in the Phillips lab who will generate lentivirus from the sgRNA library, transduce cell-lines, conduct epigenomic analyses (such as ATAC-seq) and perform bioinformatics analyses.

*Total personnel costs: $53287* ($29,687 from Foster Lab, $23,600 from Phillips Lab)

**Reagents**

**Foster Lab:**
- T cells ($3,000): T cells will be purchased from the Penn Immunology Core.
- mRNA ($8,000): mRNA will be in vitro transcribed through the Penn RNA Core.
- Mouse in vivo work ($9,313): Animal husbandry, surgical supplies, tumor imaging.

*Foster lab reagent total: $20,313*

**Phillips Lab:**
- Molecular biology reagents ($6000): custom oligos and cloning reagents will be purchased from Genewiz and IDT
- Library Preparation ($6000): Library preparation kits will be purchase from NEB
- Sequencing ($14,400): Sequencing will be performed at the Penn Sequencing Core

*Phillips Lab reagent total: $26,400*

*Total reagent costs: $46713*

**Timeline**

Aim 1 and 2 will be pursued in parallel over the course of 1 year. The work will be divided between the Foster and Phillips lab, and thus the total award amount will be split evenly between both labs.
BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors. Follow this format for each person. DO NOT EXCEED FIVE PAGES.

NAME: Jessica Foster

eRA COMMONS USER NAME (credential, e.g., agency login): fosterjb

POSITION TITLE: Assistant Professor

EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)

<table>
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<th>INSTITUTION AND LOCATION</th>
<th>DEGREE (if applicable)</th>
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<th>FIELD OF STUDY</th>
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<td>Columbia University, New York, NY</td>
<td>B.A.</td>
<td>05/2006</td>
<td>Neuroscience and Behavior</td>
</tr>
<tr>
<td>University of Virginia, Charlottesville, VA</td>
<td>M.D.</td>
<td>05/2010</td>
<td>Medicine</td>
</tr>
<tr>
<td>Cincinnati Children’s Hospital, Cincinnati, OH</td>
<td>na</td>
<td>06/2014</td>
<td>Pediatrics residency and Chief residency</td>
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<tr>
<td>The Children’s Hospital of Philadelphia, Philadelphia, PA</td>
<td>na</td>
<td>06/2017</td>
<td>Hematology/ Oncology Fellowship</td>
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A. Personal Statement

My research and clinical interests lie in bringing immunotherapy to use in pediatric brain tumors. I have been particularly moved during my clinical experience by the significant need to improve outcomes for pediatric brain tumors. I have also been able to witness firsthand the success of immunotherapy in curing what were previously considered incurable leukemias, sparking a scientific curiosity to bring that same success to central nervous system tumors. During my pediatric oncology fellowship, I joined the laboratory of John Maris, MD internationally renowned expert in neuroblastoma, with co-mentorship from Adam Resnick, PhD a leading expert in pediatric brain tumors. In collaboration with David Barrett, MD, PhD and Stephen Grupp MD, PhD who have successfully translated chimeric antigen receptors (CARs) from the bench to the bedside for the treatment of relapsed acute lymphoblastic leukemia, my work focused on developing CAR T cells for pediatric brain tumors and improving CAR T cell efficacy and safety using RNA-based technologies.

My research began during oncology fellowship, where using a CD19 CAR model, we were able to improve existing mRNA CAR technology through the use of a novel purification method, paving the way for mRNA CAR T cell therapy in solid and brain tumors. My goal moving forward has been to apply these same mRNA synthesis techniques toward CAR T cells targeting pediatric solid and brain tumors. During my time in the lab I have published on this work and now taken on multiple projects developing CAR therapy for neuroblastoma and high-grade pediatric brain tumors. These projects include targeting GD2 with mRNA CAR T cells for diffuse midline glioma (DMG) and development of new CAR structures such as GPC2 for neuroblastoma, medulloblastoma and high grade glioma (HGG). I am also investigating new immunotherapeutic targets for DMGs and HGGs through molecular analysis, RNA sequencing, and proteomic data from large cohorts of DMG and HGG patients. This Synergy grant will investigate mechanisms of CAR T cell resistance and escape for DMG to improve therapeutic targeting and ultimately lead to a clinical trial. Our labs and institutional success with chimeric antigen receptors combined with the Phillips’ lab expertise in molecular and epigenetic drivers of brain tumors makes us well poised to successfully bring CAR T cell therapy to these devastating childhood cancers.
Ongoing and recently completed projects that I would like to highlight include:

Mentored Clinical Scientist K08 NIH/NCI
Foster (PI)
09/09/2021-08/31/2026
Defining a therapeutic strategy for DIPG with mRNA CAR T cells and microglia inhibition

PRCRP Career Development Award, Department of Defense
Foster (PI)
04/15/2021-04/14/2024
Effective locoregional delivery of mRNA CAR T cells for diffuse intrinsic pontine glioma

St. Baldrick's-Stand Up to Cancer Pediatric Cancer Dream Team
Maris and Mackall (MPI), Role: Young Investigator
07/01/2015-05/31/2022
Immunogenomics to Create New Therapies for Pediatric Cancers

U54 CA232568, NIH/NCI
Maris and Mackall (MPI), Role: Young Investigator
09/01/2018-08/31/2023
Discovery and Development of Optimal Immunotherapeutic Strategies for Childhood Cancers

Hyundai Hope On Wheels Young Investigator
Foster (PI)
09/21/2018- 12/31/2020
Development of GPC2-directed RNA chimeric antigen receptor therapy for neuroblastoma

Matthew Larson Foundation
Foster (PI)
05/01/2019 –08/31/2020
Development of an mRNA GD2-directed CAR T cell platform for the treatment of DIPG

5K12CA076931-19
Hunger (PI), Role: Appointee
07/01/2017 –06/30/2019
Cancer Center research program, University of Pennsylvania

Citations:


B. Positions, Scientific Appropriations, and Honors

Positions and Scientific Appropriations
2022-present Assistant Professor, Division of Oncology, Children's Hospital of Philadelphia
2017-2022 Instructor in Pediatrics, Division of Oncology, Children’s Hospital of Philadelphia
2014-2017 Fellow in Pediatric Hematology-Oncology, Children's Hospital of Philadelphia
2013-2014 Chief resident in Pediatrics, Cincinnati Children’s Hospital
2010-2013 Resident in Pediatrics, Cincinnati Children’s Hospital

Honors
2021 American Association for Cancer Research Team Science Award (Team member)
2021 American Society of Pediatric Hematology Oncology Young Investigator Award
2020, 2021 Early Career Faculty Teaching Honor Roll, Children’s Hospital of Philadelphia
2019 Forbeck Scholar Award – DIPG consensus meeting
2013 Diamond-Blackfan Award for Research in Hematology-Oncology, Cincinnati Children’s Hospital
2010 Leonard Tow Humanism in Medicine Award
2010 Glasgow-Rubin Citation for Academic Achievement, American Medical Women’s Association
2010 Robert M. Blizzard Pediatric Scholar Award, University of Virginia Medical School
2009 C. Richard Bowman Scholar, University of Virginia Medical School
2009 Gold Humanism Honor Society
2008 Alpha Omega Alpha
2008 American Society of Clinical Pathologists Award of Excellence and Achievement
2006-2010 Pratt Scholarship, University of Virginia Medical School
2006 Phi Beta Kappa, Columbia University

C. Contributions to Science

I. Undergraduate – early career laboratory research
During my time as an undergraduate student in the neuroscience laboratory of Rae Silver PhD, I investigated circadian rhythms as governed by the suprachiasmatic nucleus (SCN) in the brain. Using a hamster model subjected to constant light, we were able to identify two separate compartments within the SCN corresponding to the retinorecipient neurons and the adjacent non-retinorecipient neurons, with novel protein production rhythms identified in each compartment. These two compartments within the SCN showed neural connections to the adjacent hypothalamus, suggesting a neural basis for the internal clock of the brain. In addition, we studied the role of dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32) in the circadian system, as DARPP-32 is an important target in the dopamine signaling pathway. Through behavioral experiments of DARPP-32 knock out mice in constant darkness and constant light, as well as examination of DARPP-32 expression in the retina and mouse brain of normal controls, we showed that DARPP-32 is involved in the retinal pathway transmitting photic information that resets the circadian clock. I was primarily responsible for data collection, as well as editing and reviewing manuscripts.

Peer-reviewed publications:


II. Residency – clinical research
During my residency, I worked with Denise Adams, MD, to conduct a systematic review of a rare vascular tumor, Kaposiform hemangioendothelioma. We investigated the literature to seek out all reports on this rare tumor and gather information regarding characteristics, treatment, and outcomes. Our data revealed the vast differences...
in treatment of these tumors amongst different physicians, as well as poorer outcomes associated with visceral involvement of the tumor and Kasabach-Merritt phenomenon, a bleeding coagulopathy that can often occur with these tumors. I was primarily responsible for data collection, as well as writing and reviewing results, which were presented as a poster at the American Society of Pediatric Hematology Oncology national conference in April 2013 and The International Society for the Study of Vascular Anomalies 20th International Workshop, international conference, April 2014.

Meeting abstracts:


III. Pediatric Oncology – immunotherapy laboratory research

During my oncology fellowship, I began to investigate optimizing mRNA for use in CAR T cells. By utilizing recent mRNA *in vitro* production techniques such as modification of uracil and purification to remove double strands, I have shown improved efficacy for mRNA CAR T cells in a leukemia model. I am now applying these new techniques to solid and brain tumor targets. My most recent work has been developing glypican 2 (GPC2)-directed CAR sequences, used for mRNA CAR T cell therapy against neuroblastoma, medulloblastoma and high grade glioma models. I am also studying GD2-directed CAR T cell therapy for diffuse midline gliomas, as described in this proposal. I also have investigated the thoracic duct as a source of naïve T cells for use in cellular therapy. Finally, I am exploring novel immunotherapeutic targets for DMG and high grade pediatric brain tumors through evaluation of large datasets including whole genome sequencing, RNA sequencing, and proteomic data. As the PI, I am leading all of these investigations and am responsible for conceptualization of the projects, data collection and writing results.

Peer reviewed publications:


Complete List of Published Work in MyBibliography:
A. Personal Statement

Malignant gliomas remain amongst the most difficult-to-treat malignancies with poor clinical outcomes. A major insight gained from recent tumor exome-sequencing efforts has been the identification of frequent mutations in epigenetic regulators in cancer. Indeed, specific glioma subtypes are now defined by mutations in histone H3 and isocitrate dehydrogenase (IDH) which reprogram the epigenome, highlighting transcriptional dysregulation as a major event in the development of cancer. Interestingly, these mutations are enriched in children and young adults with malignant gliomas, suggesting the developing/young brain may be particular susceptible to these defects.

The goal of my research program is to: 1) define how fundamental epigenetic mechanisms drive the formation of gliomas and identify specific pathways that can be targeted therapeutically, 2) understand how epigenetic mechanisms drive immune evasion in glioma, and 3) leverage high-resolution epigenomic data in malignant gliomas to engineer novel research tools (cell state specific reporters) and therapeutic agents (gene therapy vectors). The long-term goal is to use our understanding of epigenetic regulation in malignant gliomas to develop novel therapies for this incurable group of tumors.

B. Positions and Honors

Positions and Employment
2020- Presidential Assistant Professor in Neurology and Genetics, University of Pennsylvania, PA
2015-2020 Instructor in Neurology, Memorial Sloan Kettering Cancer Center, New York, NY

Other Experience and Professional Memberships
2014- Member, Society of Neuro-Oncology
2014- Member, American Society of Clinical Oncology
2014- Member, American Association for Cancer Research
2011- Member, American Academy of Neurology

Honors/Awards
2022 V Scholar Award
2019 Team Jack Brain Tumor Foundation Award
2018 MSKCC Center for Experimental Therapeutics Award
2017 DIPG Collaborative Grant
2017 MSKCC IDEA Award
2015 CURE Childhood Cancer Foundation Award
2015 American Brain Tumor Association Basic Research Fellowship
2007 FD Amenities Award
2007 Thomas Arno Award
2006 Morris Fellowship
2003 British Transplantation Society PhD Award
2001 Goldberg Schachmann Scholarship, University of London, London, United Kingdom

C. Contributions to Science

1. Identification of oncogenic pathways as therapeutic targets in epigenetically driven gliomas
As a post-doctoral researcher I investigated the mechanism of action of a menin-inhibitor, MI-2, in DIPG following up on studies which identified a menin-inhibitor (MI-2) as the top 'hit' in a chemical screen in a new model of (Funato et. al, 2014, Science). Menin is an epigenetic regulator which we hypothesized may be specifically required in the setting of H3K27M induced epigenetic dysregulation. I demonstrated that MI-2 exhibits anti-glioma activity in H3K27M mutant and H3 wild-type glioma subtypes and showed menin is not the relevant molecular target for this drug in gliomas. Instead, using an integrated approach employing genetic, biochemical and metabolomic methods, we characterized the direct molecular target of MI-2 in glioma as lanosterol synthase, a cholesterol biosynthesis enzyme; revealing a novel metabolic vulnerability in glioma and more broadly implicating cholesterol homeostasis as an attractive pathway to target in this malignancy.

2. Development of novel therapeutics in glioma targeting epigenetic mechanisms
We identified the chromatin regulator EZH2 as a context-specific dependency in H3K27M gliomas and through computational modeling of existing, non-brain penetrant EZH2 inhibitor scaffolds, we devised a chemical strategy which led to discovery of the first brain-penetrant small molecule targeting EZH2 for brain tumors.

3. Treatment approaches for management of brain tumors
Medulloblastoma is the most common primary brain tumor in children and management of extra-neural recurrence is a controversial and difficult-to-treat clinical scenario in Neuro-Oncology. We demonstrated efficacy of a combination standard-dose chemotherapy regimen which obviated the need for high-dose
chemotherapy and stem cell transplantation (which both have significant potential side-effects) to induce long-term remission in this clinical entity.


4. Mechanisms of immunological tolerance in transplantation
As a doctoral student in the laboratory of Wilson Wong Ph.D., I investigated mechanisms of immune tolerance to transplant allografts. I focused on interactions between the innate immune system and the T cell response. I developed a novel model of donor-lymphocyte induced tolerance to transplant allografts and demonstrated the production of complement from the donor-graft is critical for induction of tolerance, establishing a link between innate immunity and T-cell regulation.


Complete List of Published Work: