**High dimensional protein characterization of glial tumor tissues and relevance to outcomes in immunotherapy clinical trials**

**Research Proposal**

**Impact/relevance**

Gliomas make up about 30% of all central nervous system (CNS) tumors and 80% of all malignant brain tumors. Under the World Health Organization (WHO) grading system, gliomas are graded from I (least aggressive) to IV (most aggressive). Due to their infiltrative growth patterns and resistance to chemo- and radiation therapy, WHO grade II-IV gliomas are almost always lethal. WHO grade II low-grade gliomas (LGGs) are slow-growing tumors that occur in mainly young adults, most of whom experience tumor progression to aggressive high-grade glioma (HGG) and eventually succumb to the disease 1,2. WHO Grade IV Glioblastoma (GBM) is the most common and lethal primary malignant central nervous system tumor. Even with an aggressive standard of care regimen of surgery, radiation therapy and chemotherapy, 3 year survival rate is just 10.1%. Furthemore, brain tumors are the leading cause of cancer- related mortality and morbidity in children. Children with DIPG have one-year progression-free survival rates below 25% and median overall survival of 9 to 10 months with current treatments 3 4

New therapies are desperately needed for patients diagnosed with glioma. Early studies with single agent immunotherapies such as PD-1 were disappointing and thought to be hindered by the immune-suppressive tumor microenvironment (TME) in GBM 5, but recent studies in the adjuvant setting of anti-PD1 in combination with surgery show significantly increased overall survival and are being extended to more patients 6*.* Further insights into the tumor immunobiology in GBM are needed to guide rational treatment selection and enhance the efficacy of emerging immunotherapeutics for this disease.

Current prioritization algorithms to drive development of immunotherapeutics in glial tumors depend on genomic analysis taken from tissue biopsies when available. These assays are usually done in bulk cell populations, which precludes resolving tumor heterogeneity and makes it extremely challenging to distinguish the role of different components of the tumor microenvironment, including stroma as well as different immune cell subsets. Single-cell analysis methods, such as single-cell sequencing and mass cytometry, have been instrumental in resolving some of these complexities, however a major limitation of these studies is that they require tissue dissociation for analysis, which loses key attributes of the tumor microenvironment including cell types and spatial relationships. Tumor/immune spatial relationships, particularly as they pertain to immune infiltration and/or exclusion from the tumor bed, provide key insights into disease stage and overall prognosis 7.

Exciting results in a patient with GBM were reported by Brown *et. al.*, demonstrating a complete response following chimeric antigen receptor (CAR) therapy targeting IL-13R 8. Further, CAR T cells targeting EGFRvIII led to antigen remodeling and the induction of adaptive resistance, indicative of effective tumor targeting 9. Pre-clinical data demonstrates impressive anti-tumor effects of GD2-CARs in murine orthotopic models of pediatric high grade glioma (HGG). Together these data provide proof-of-concept that engineered T cells can traffic to the CNS and mediate meaningful anti-tumor effects, but they also reveal the challenge posed by antigenic heterogeneity when targeting a single cell surface molecule in this disease. Effective cell-based therapy for GBM and pediatric HGG will no doubt require the development of multispecific approaches and/or approaches that can induce endogenous antitumor immune responses through epitope spreading.

Further, it is well known that dissociation protocols do not recover every cell type with equal efficiency. For example, most protocols are optimized for the recovery of T cells to the detriment of the dendritic and myeloid compartment. This is particularly relevant in glial malignancies, where myeloid cells are thought to have a major role in promoting tumor growth and suppressing immune responses 10 11 12 13 14 15.

We need to develop novel methodologies to evaluate tumor antigenic expression and the phenotype and functions of associated immune cells, in particular glioma-infiltrating myeloid cells. While flow-cytometry- and single cell-sequencing-based evaluations may provide valuable information on dissociated single cells, these technologies will not provide us with critical information related to their localization and physical interactions with other cell populations in the tumor microenvironment.

**As a first step toward generating a comprehensive understanding of both the TME and the antigenic heterogeneity of GBM and high grade gliomas, here we propose to use Multiplexed Ion Beam Imaging (MIBI) to perform multiplexed imaging of intact, well annotated low grade glioma (LGG) and GBM tissue from pediatric and adult patients in response to vaccine, checkpoint inhibitor and cellular therapies** (Table 1).MIBI enables quantitative, high-resolution (500 nm), simultaneous imaging of ~40 protein markers in FFPE tissue that is readily available for these cohorts. This dataset will inform tumor target expression profile, immune inhibitory protein expression, and the type and functional status of T cells and myeloid cells within the context of an intact tumor micro-environment. The tumor and immune characteristics will be analyzed and interpreted in the context of patient outcome, molecular or other clinical characteristics. As many protein and cellular targets currently under clinical development will be analyzed, **we envisage** **these data would provide unique and broad rationale for therapeutic selection for CART, vaccine and immunotherapy combination trials for glial tumor patients.**

**Table 1. Cohorts of tumor samples for analysis.**

|  |  |  |
| --- | --- | --- |
| **Sample Set** | **Number of cases** | **Associated Datasets** |
| Pediatric HGG  Primary & recurrent | 70 TMAs, 30 with molecular datasets | WGS, RNA, clinical. |
| Paired primary and recurrent gliomas & site documented biopsies | 20 – 25 pairs (50)  50 | WES, RNA, TCRseq, clinical. |
| WHO G2 glioma receiving randomized neoadjuvant vaccines | 30 | RNA-seq, CyTOF. TCRb seq, clinical |
| GBM off label neoadjuvant Niovlumab and pembrolizumab | 30 | WES, RNA, TIL / PBMC cyTOF |
| GBM CART (IL-13R2, Her2) | 90 | CSF & blood cytokines and FC. |

Abbreviations: high grade glioma (HGG), glioblastoma multiforme (GBM), chimeric antigen receptor T cell (CART), tissue micro-array (TMA), whole genome sequencing (WGS), whole exome sequencing (WES),ribonucleic acid (RNA), T cell receptor sequencing (TCRseq), tumor infiltrating lymphocyte (TIL), peripheral blood mononuclear cell (PBMC), cytometry by the time of flight (CyTOF), cerebral spinal fluid (CSF), flow cytometry (FC).

The **specific aims** of this research proposal are to:

1. Optimize tumor and immune analysis panels and generate tissue microarrays for analysis.
2. Define the expression of protein targets of immunotherapy on tumor cells and other cells in the tumor microenvironment. Define functional phenotypes of tumor-associated myeloid cells and lymphocytes.
3. Integrate tumor-immune protein expression data with clinical and other molecular datasets.

**Aim 1. Optimization of tumor and immune panels and assay setup**

Rationale: Aim 1 will lead out the project with the development and optimization of two customized antibody analysis panels. Both panels contain common immune lineage and cell subset markers allowing cross-comparison (Table 2). The ‘immuno’ panel will have a deeper dive into myeloid functional subsets and immunotherapy targets (Table 3).The ‘tumor’ panel will focus on tumor-associated therapeutic targets (Table 4).

Limitations in MIBI data acquisition (image acquisition and analysis of relatively large regions of interest (ROI)) make TMAs particularly useful for this project. In generating TMAs, multiple cores, or ROI from a patient block will be selected by a pathologist to represent different histological areas including tumor, stroma, immune infiltrate and TME heterogeneity. TMAs reduce the number of slides that need to be analyzed, increasing the throughput of data acquisition, and enabling the analysis of the total cohort size of 300 samples. Moreover, the fact that multiple samples are stained simultaneously on the same slide minimizes experimental variability. Finally, serial sections of the same cohorts can easily be analyzed with multiple panels, such as the immune and tumor panel described here, plus any other validation stains that may arise as follow ups from this work.

**Table 2. Common lineage panel.** Pan markers across the two antibody panels for the project.

|  |  |  |
| --- | --- | --- |
| **Marker** | **Cell type & function** | **MIBI validated** |
| CD45 | All differentiated hematopoietic cells. Protein tyrosine phosphatase, regulates immune receptor signaling. | ✓ |
| dsDNA | Nucleated cells. | ✓ |
| CD31 (PECAM1) | Endothelia, angiogenic tumors, monocyte, neutrophil, T cell | ✓ |
| Ki-67 | Nuclear marker. Proliferating cells. | ✓ |
| Cyclin D1 | Nuclear marker. Proliferating cells. | 🗶 |
| Neu-N | Neurons. Nuclear protein. | ✓ |
| CD45 (PTPRC) | Differentiated hematopoietic cells. Protein tyrosine phosphatase. | ✓ |
| TMEM119 | Microglia. Trans-membrane. Unknown function. | ✓ |
| Glial fibrillary acidic protein (GFAP) | Astrocyte. Cytoskeletal protein. | ✓ |
| CD105 | Tumor associated angiogenic blood vessels. Receptor for TGFb. | ✓ |
| CD133 | Cancer stem cell marker | ✓ |
| CD163 | Macrophage. Hemoglobin scavenger R. | ✓ |
| CD3 | T cell, NK T cell. TCR signaling. | ✓ |
| CD8 | CD8+ T cell, DC, NK. Binds HLA class 1. | ✓ |
| CD4 | CD4+ T cell, DC, monocyte, macrophage. Binds HLA class 2. | ✓ |
| FoxP3 | Early activ. T cell. CD4+ T regulatory cell. Transcription factor. | ✓ |
| HLA Class 1 | All cells. Presentation of endogenous antigenic peptides to CD8+ T cells. | ✓ |
| HLA-DR | DC. Macrophage. Activ. B & T cell. Tumor cell. Presentation of exogenous antigenic peptides to CD4+ T cells. | ✓ |
| CD11b | Pan myeloid. Leukocyte adhesion. | ✓ |
| CD20 | B cell. Calcium channel. | ✓ |

**Table 3. Markers specific to the immuno panel**

|  |  |  |
| --- | --- | --- |
| **Markers** | **Cell type / functional relevance** | **MIBI validated** |
| CD14 | Monocyte. Co-receptor for LPS. | ✓ |
| CD15 | Neutrophil. Adhesion, phagocytosis and chemotaxis | ✓ |
| CD68 | M1-like macrophage. Phagocytic, homing | ✓ |
| iNOS | M1-like macrophage. Synthase of nitric oxide. Tx in clinical dev. | ✓ |
| IDO-1 | M1-like macrophage, DC, tumor cell. Enzyme in the kynurenine pathways. Immunosuppressive. Tx in clinical dev. | ✓ |
| Arginase 1 | M2-like macrophage. Enzyme in the urea cycle. Tx in clinical dev. | ✓ |
| CD33 | AML, CML, Monocytes, pDC, Myeloid “suppressor” cell. Sialic acid binding Ig-like lectin (3) | 🗶 |
| CD141 | Endothelia, monocytes, Cross presenting DC1 (CD103 equiv). Thombomodulin – anti-coagulant by thrombin conversion | ✓ |
| CD209 (DC-SIGN) | DC2 (mDC), macrophages. Adhesion molecule | ✓ |
| CD208 (Lamp3) | Mature dendritic cell. Antigen presentation on HLA class 1. | 🗶 |
| CD123 | AML, pDC, DC, basophil. IL-3 R. | 🗶 |
| Mast cell chymase | Mast cell. Peptidolysis. Pro-inflammatory. | ✓ |
| PD-1 | Activated CD8+, CD4+ Teff, Treg. Tex. Co-inhibitory molecule. Anti-apoptotic. Tx in clinical dev. | ✓ |
| PD-L1 | Tumor cell, activated CD4+ CD8+ T cell, NK cells, monocyte, TAM, MDSC. Co-inhibitory molecule / ligand. Tx in clinical dev. | ✓ |
| CD47 | Monocyte, TAM, DC, NK, Bcell. Phagocytosis. Tx in clinical dev. | ✓ |
| CD38 | Activated B cell, Breg, activ. CD4+ T cell, CD8+ T cell. TermEx. MDSC. TAM. Activ. NK. ADP ribosyl cyclase. Immunosuppressive. Tx in clinical dev. | 🗶 |
| CD39 | Activ. CD4+ CD8+ T cells. CD4+ Treg. MDSC. NK cells. Ectonucleotidase. Immunosuppressive. Tx in clinical dev. | 🗶 |
| CD86 | Dendritic cells, macrophages. Co-stimulatory ligand. | 🗶 |
| CD40 | Dendritic cells, macrophages, B cells. Co-stimulatory ligand. Tx in clinical dev. | 🗶 |
| Eomes | Activated NK, CD8+ Teff, Tex. CD4+ Th1. Transcription factor for IFN | ✓ |
| TOX1 | Ubiquitous expression. Tex. | 🗶 |
| GLUT1 | All cells. Glucose transporter. | ✓ |

**Table 4. Markers specific to the tumor panel**

|  |  |  |
| --- | --- | --- |
| **Marker** | **Therapy / functional relevance** | **MIBI validated** |
| IL13R2 | Tumor growth. Tx in clinical dev. | 🗶 |
| EGFRvIII | Tumor growth. Tx in clinical dev. | 🗶 |
| Her2 | Tumor growth. Tx in clinical dev. | ✓ |
| Epha2 | Tumor growth. Tx in clinical dev. | 🗶 |
| GD2 | Signaling. Tx in clinical dev. | 🗶 |
| B7H3 | Immune inhibitory. Tx in clinical dev. | 🗶 |
| EGFR | Tumor growth. Tx in clinical dev. (806 mab) | ✓ |
| CD70 | Tumor growth, T cell co-stim. Tx in clinical dev. | 🗶 |
| CSPG4 | Tumor-stroma adhesion. Tx in clinical dev. | 🗶 |
| MMP-2 (Chlorotoxin receptor) | Malignant glial cell marker. Imaging in clin. Dev. | 🗶 |
| CD133 | GBM, Ped. HGG membrane protein. | ✓ |
| CMV ISH? |  | 🗶 |
| ApoE | Tumor, glia, macrophages. Lipogenesis / metabolism. Tx in clinical dev. | ✓ |
| Histone-3 | Nucleated cells. Chromatin structure. | ✓ |
| H3K27me3 | H3 methylation status. DNA repair. LGG clinical subtype marker. | ✓ |

Aim 1.1 Optimization of tumor and immune panels

**The panels will be comprised primarily of antibodies currently undergoing validation in the Angelo and Bendall labs.** The immune common lineage (Table 2) and immuno (Table 3) panels are already established, so panel standardization and validation can begin immediately on whole slides of 5 patients per tumor type (HGG, GBM, LGG). This analysis will establish patterns of protein expression and the utility of individual markers in interpreting data.

In order to construct the proposed panels, the following workflow will be carried out at Stanford.

* *Initial quality control (QC) procedures for reagents.*Reagents previously tested and validated by single-plex chromogenic IHC and MIBI are reported in Table 2-4. Analytic validation was carried out on human FFPE tissue microarray conceived by **the Angelo and Bendall labs**. Antibody purity was evaluated before conjugation by SDS-PAGE in reducing conditions. Conjugates were tested and titrated by single-plex chromogenic IHC and subsequently by MIBI on the same tissues. For each metal-conjugated antibody, the working titer was determined as the titer that yielded maximal separation between signal and noise across tissues. Tissue processing, staining and quantitative imaging were carried out as described 7. Unvalidated reagents listed in this proposal will be screened and tested before conjugation by single-plex IHC. Antibodies showing valid staining pattern across different tissues and providing probative information about the cohort will be incorporated in the pipeline for MIBI reagents validation. Attempted validation of these targets will be carried out although without ensuring a successful outcome.
* *Constructing metal labeled antibody panels.* After the initial QC procedure, we will assign the appropriate metal conjugates to the antibodies such that the proposed markers will accurately define each population of interest. The standardized workflow for each panel will be evaluated in a TMA for validation. The proposed TMA will include normal and neoplastic and tissue types as appropriate. An initial hematoxylin and eosin (H&E) section will be created, followed by six serial section on MIBI slides (gold slides) in order to generate six titers. This approach will help to assess the specificity and sensitivity for each target with regard to its cellular localization, subcellular localization (nuclear, membranous, cytoplasmic), and co-expression of proteins across different tissue types. The expected staining pattern should be noted for reaction in comprising of neoplastic and non-neoplastic tissue controls. When a negative tissue control is unavailable, appropriate ROIs with both positive and negative areas will be evaluated. Once the appropriate concentration for each antibody is established on the TMA for validation, a master stock of the antibody cocktail will be prepared and used for the staining of the pilot cohort.
* *Evaluation criteria for the antibody panels.*Results will be evaluated by comparing the MIBIscope expression pattern to the corresponding immunohistochemistry in combination with visual comparison to those markers expected to show either co-expression based on known biologic properties of the marker or no significant co-expression due to expression in a different compartment of the cell or on a different cell type.

This data should be available in the first 6 months and will inform whether further refinement to the panels is necessary, before they can be deployed on the larger cohort. Further panel development for subsequent analysis in Aims 2 and 3 will be continued if additional biology and priorities by the team arise. The tumor panel (Table 4) will be developed at project initiation. Antibodies compatible for FFPE tissues will be triaged for incorporation **into the tumor panel. The samples selected for tumor panel validation analysis will be pre-screened by molecular assays for expression of the targets of interest. All antibodies will be validated based on positive and negative staining in relevant FFPE tissues. Tissues will be identified to use as controls throughout the project.**

Aim 1.2 Assay setup and generation of TMAs

Each cohort included in the study will be in a TMA format, as whole sections would impose significant logistical issues such that the scope of work would need to be revised. The initial identification and collection of patients samples represents the greatest portion of the work associated with the TMA construction. The cohort TMAs will be constructed on site by investigators supplying each sample cohort. Areas of interest will be marked on an H&E stained section and representative cores from the corresponding paraffin block will be compiled into a tissue microarray for the study. These steps will be repeated for each donor block to be incorporated into the TMA. The overall tissue quality will be evaluated on a serial H&E section under light microscopy. Tissue cores with poor preservation will be excluded from the MIBI analyses.

A total of four serial TMA sections will be generated per cohort, such that the first and last section will be H&E stains, while the intermediate ones will be used for the proposed panels. Adjacent H&E sections will serve as a reference standard for histological examination.

**Aim 2. Define the expression of protein targets of immunotherapy on tumor cells and other cells in the tumor microenvironment. Define functional phenotypes of tumor associated myeloid cells and lymphocytes.**

Rationale: MIBI provides unprecedented high dimensional protein analysis (40 – 50 markers) of FFPE tissues at a single cell resolution. The two panels will contain overlapping markers (Table 2), that will allow interpretation of markers in Table 3 and 4 across all samples analyzed. This will provide insights on myeloid and T cell subsets and tumor protein expression patterns and heterogeneity to a level not currently appreciated, and will inform the whole field of neural oncology and immunotherapy, highlighting correlates of biology that could aid patient stratification and biomarkers of therapeutic intervention with high confidence.

Aim 2.1. Define functional phenotypes of tumor associated myeloid cells and lymphocytes.

The immuno panel (Table 2 and 3) will be used to interrogate the following cell types:

* brain-resident microglia and astrocytes.
* infiltrating monocytes, macrophage and neutrophils defined as inflammatory “M1-like” and anti-inflammatory/tissue healing “M2-like”, which have been implicated as suppressors of T cell activation and are associated with tumor progression. Also, recent studies 10 indicated that glioma-infiltrating myeloid cells can express both M1 and M2 markers as single cells, indicating that we have to re-visit the classical dichotomy of M1 vs. M2 classification of these cells in the glioma microenvironment.
* dendritic cells associated with antigen-specific activation of CD8+ and CD4+ T cells, as well as antiviral plasmacytoid dendritic cells (pDC).
* CD8+ and CD4+ T cell ‘effector’ and ‘exhausted’ subsets
* CD4+ Foxp3+ regulatory T cells, which are associated with tumor progression and resistance to immunotherapy.

**During data analysis, single-cell features extracted using image segmentation will be used for phenotypic clustering and to define neighborhoods of specific composition.** Such a global immune ecosystem characterization will provide insights into mechanisms of immune-cell exclusion and immune cell modulation within the glial tumor microenvironment. We will also interrogate relationships between tumor cell characteristics and immune populations, phenotype and compartmentalization, which will be used to determine the influence of tumor on the immune-microenvironment.

Aim 2.2. Define the expression of protein targets of immunotherapy on tumor cells and other cells in the tumor microenvironment.

We will assess the levels and pattern of expression of immune and tumor targets that are under clinical development that should have application for HGG GBM and LGG (Table 2, 3 and 4). The compartment of expression, the pattern and relative amount of staining, will be determined with respect to the tumor-stromal and immune landscape, help determine relationships between therapeutic targets, and provide insights for combination immunotherapies. This analysis will elucidate both the amount of variation present in individual tumors, as well as the combinatorial pattern of expression of multiple tumor-associated antigens, which is particularly relevant for the development of mono- and bi-specific CAR-T therapies.

Aim 2.3 Comparative analysis between pediatric high grade glioma and adult HGG / GBM with respect to immunotherapy target expression, cell phenotypes and spatial relationships within the tumor-immune microenvironment.

Our cohort (Table 1) includes TMAs that have already been constructed from 70 pediatric HGG cases and are ready for immediate analysis. These samples will be compared with adult HGG/GBM that have associated brain-location specific information. To the best of our knowledge, this is the first time such a comparison is performed. The analysis will elucidate similarities and differences in the tumor-immune protein expression landscape between the pediatric and adult setting, providing key insights into patient subtyping and therapeutic options for both populations that have such high unmet need.

**Aim 3. Integration with clinical data and other molecular datasets**

Rationale: Prior studies led by co-investigators of this project have provided key insights into glial tumor biology and response to immunotherapy that will be incorporated in this study. Multiple studies have highlighted the role of specific immune cell subsets in predicting response to immunotherapy. More specifically, immune-related genes expressed by infiltrating myeloid populations significantly correlate with a poorer prognosis for grade II and III LGG patients 10. Analysis of a ‘super-responder’ GBM patient to IL-13R2-CART therapy revealed that delivery of intra-tumoral CART resulted in an influx into the CSF of peripheral immune cells including monocytes, neutrophils and T cells 8. Finally, in a neoadjuvant / adjuvant anti PD1 (nivolumab) study in surgically resected glioblastoma, the neoadjuvant therapy significantly extended overall survival compared to adjuvant therapy alone 6. This highlighted that patient benefit is experienced when immunotherapy occurs concurrently with the tumor being present, and changes in immune-related protein expression were observed within the tumor microenvironment.

The dataset generated during this project provides a unique opportunity to build on these observations to further characterize the relationship between the composition and structure of the TME and the response to immunotherapy. These analyses will provide a rich protein expression dataset across glial malignancies to characterize the biology of the tumor-immune microenvironment and its interconnection with other molecular and immune-phenome attributes and clinical response to therapies under clinical development. This will markedly advance our understanding of glial tumors and provide insights into tumor and immune targeted therapeutic strategies.

Aim 3.1 Identification of features associated with response

The single-cell features calculated from the imaging data as part of Aim 2.2 will be correlated with response data to identify whether any of them can be used to distinguish responders from non-responders. Such features include the abundance of different cluster of cells, defined by phenotypic marker expression, as well as the expression levels of functional markers in individual clusters. Additionally we will define cell neighborhoods by performing a second clustering run which uses as input the identity (i.e. the phenotypic cluster) of the neighbors of each cell. Both ‘phenotypic’ and ‘spatial’ features will be used as input to multi-variate penalized linear-regression models such as lasso and elastic-net, using clinical response as the response variable.

Aim 3.2 Integration of imaging data with sequencing and genomics information

A unique feature of the cohorts that will be analyzed in this study is the fact that extensive orthogonal molecular data, including DNA and RNA sequencing, has already been generated from these samples. As part of this aim, we will correlate these datasets with the imaging data generated during this project, with the aim of resolving some of the complexities in the bulk molecular data. More specifically, similarly to Aim 3.1, we will use imaging features as input to multi-variate models, this time using as response variables molecular features that have been independently identified in the bulk molecular data, e.g. the expression level of genes that have been associated with response, or the presence of specific genetic variants of interest. The outcome of this analysis will be single-cell features (from the imaging data) that correlate with orthogonal features from the bulk datasets (e.g. the expression of specific genes). This type of information can be used to broaden the scope of the image analysis, which is limited to ~40 markers, by including additional markers that can be tentatively assigned to one of the cell populations identified from the imaging. To make a concrete example, if the abundance of cell-type X, as identified in the imaging, is correlated with the bulk expression of gene Y, it can be reasonably assumed that cell type X represents the subset where gene Y is primarily expressed, thus helping deconvolve the bulk data and potentially informing the development of new panels.

**Timelines for project**

**Year 1 – Aim 1**

* Complete antibody panel development.
* Run initial proof of concept on a small set of slides from representative cases.
* Complete TMA construction

**Year 2 – Aim 2**

* Complete data acquisition
* Start data analysis

**Year 3 – Aim 3**

* Complete data analysis
* Integrate data
* Publications

**Personnel**

**Michael Angelo MD PhD. Stanford. Principal investigator.** Mike Angelo is a clinical pathologist with expertise in high dimensional imaging, cancer immunology, and elemental mass spectrometry.  Mike will oversee the development of antibody staining panels outlined in the proposal, MIBI data acquisition and image analysis.  Additionally, mike will coordinate with members of his team and with all co-investigators to ensure timely progression of project goals as stated in the proposal.

**Sean Bendall PhD. Stanford. Co-investigator.** Dr. Bendall is a biochemist and stem cell biologist with deep expertise in mass spectrometry instrumentation for bioanalysis. He is also a world expert in the technologies and analytical approaches in single cell molecular analysis ranging from the discovery of new cell populations and processes, to the derivation of predictive clinical signatures. As a PI Dr. Bendall will coordinate much of the project design, execution, and analysis. Dr. Bendall will oversee the statistical learning and data analysis of single cell signatures derived from the images. He will also be responsible for assembling reports and publications.

**Hideho Okada MD PhD. UCSF. Co-investigator.** Dr. Okada has been dedicated to brain tumor immunology and development of immunotherapy for over 20 years. Dr. Okada has a number of seminal contributions, such as discoveries of glioma-specific/associated antigens and conduct of immunotherapy trials targeting those antigens. Furthermore, most relevant to the current proposal, Dr. Okada has delineated and reported for the first time on local immunosuppression mechanisms in IDH-mutated gliomas. Dr. Okada’s expertise can bridge between the lab and clinical sciences for patients with brain tumors.

**Crystal Mackall MD. Stanford. Co-investigator.** Crystal Mackall is a pediatric oncologist with expertise in cancer immunotherapy.  She has led numerous first-in-human and first-in-child trials of immunotherapies and has generated preclinical data demonstrating activity of several CAR T cells against adult and pediatric brain tumors.  She will oversee the acquisition of pediatric samples on this project and interpret the results as they relate to pediatric gliomas. She will also serve as the conduit to disseminate these results within the larger pediatric immune-oncology community.

**Christine Brown PhD. City of Hope. Co-investigator.** Dr. Christine Brown is the Heritage Provider Network Professor in Immunotherapy in the Department of Hematology/Hematopoietic Cell Transplantation and Deputy Director of the T cell Therapeutics Research laboratory (TCTRL). She provides scientific oversight for the TCTRL translational program, overseeing cGMP cell manufacturing and CAR-T cell regulatory activities. Dr. Brown’s on-going research is focused on developing and clinically translating CAR T cell therapy for the treatment brain tumors, both primary and metastatic. For this study she will oversee the acquisition and evaluation of patient glioblastoma samples from two clinical trials being carried out at City of Hope (NCT02208362 and NCT03389230).  She will also participate in the interpretation of results with respect to patient findings, and work collaboratively with the team to utilize this transformative high-dimensional protein evaluation of patient samples to help inform response and resistance to CAR T cell therapy.

**Robert Prins PhD. UCLA. Co-investigator.** Robert Prins has a broad background in solid tumors and immunotherapy, with over 20 years of specific training and expertise in brain tumor immunotherapy, dendritic cell vaccines, and checkpoint blockade in pre-clinical and clinical studies.  He will be responsible for the acquisition of data from recent neoadjuvant PD-1 blockade samples from recurrent glioblastoma patients, as well as the interpretation of correlative data.

**Pier Federico Gherardini PhD. PICI. Co-investigator.** Pier Federico Gherardini has expertise in computational biology, image analysis and single-cell analysis. He will be responsible for coordinating the analysis of the data, and its dissemination to the research community.

**Samantha Bucktrout PhD. PICI. Co- investigator.** Samantha Bucktrout is an immunologist with expertise in myeloid and T cell biology, cancer immunotherapy and immunotherapy drug development. She will be responsible for the overall design, execution and interpretation of the proposed aims. Dr. Bucktrout will coordinate activities of all collaborating team members to meet the goals outlined in the proposal.

**Bibliography**

1. Sanai, N., Chang, S. & Berger, M. S. Low-grade gliomas in adults. *J Neurosurg* **115**, 948-965 (2011).

2. Westphal, M. & Lamszus, K. The neurobiology of gliomas: from cell biology to the development of therapeutic approaches. *Nat Rev Neurosci* **12**, 495-508 (2011).

3. Kebudi, R. & Cakir, F. B. Management of diffuse pontine gliomas in children: recent developments. *Paediatr Drugs* **15**, 351-362 (2013).

4. Schroeder, K. M., Hoeman, C. M. & Becher, O. J. Children are not just little adults: recent advances in understanding of diffuse intrinsic pontine glioma biology. *Pediatr Res* **75**, 205-209 (2014).

5. Sampson, J. H., Maus, M. V. & June, C. H. Immunotherapy for Brain Tumors. *J Clin Oncol* **35**, 2450-2456 (2017).

6. Cloughesy, T. F. et al. Neoadjuvant anti-PD-1 immunotherapy promotes a survival benefit with intratumoral and systemic immune responses in recurrent glioblastoma. *Nat Med* **25**, 477-486 (2019).

7. Keren, L. et al. A Structured Tumor-Immune Microenvironment in Triple Negative Breast Cancer Revealed by Multiplexed Ion Beam Imaging. *Cell* **174**, 1373-1387.e19 (2018).

8. Brown, C. E. et al. Regression of Glioblastoma after Chimeric Antigen Receptor T-Cell Therapy. *N Engl J Med* **375**, 2561-2569 (2016).

9. O’Rourke, D. M. et al. A single dose of peripherally infused EGFRvIII-directed CAR T cells mediates antigen loss and induces adaptive resistance in patients with recurrent glioblastoma. *Sci Transl Med* **9**, (2017).

10. Müller, S. et al. Single-cell profiling of human gliomas reveals macrophage ontogeny as a basis for regional differences in macrophage activation in the tumor microenvironment. *Genome Biol* **18**, 234 (2017).

11. Antonios, J. P. et al. Immunosuppressive tumor-infiltrating myeloid cells mediate adaptive immune resistance via a PD-1/PD-L1 mechanism in glioblastoma. *Neuro Oncol* **19**, 796-807 (2017).

12. Wu, J. et al. MerTK as a therapeutic target in glioblastoma. *Neuro Oncol* **20**, 92-102 (2018).

13. Achyut, B. R. et al. Canonical NFκB signaling in myeloid cells is required for the glioblastoma growth. *Sci Rep* **7**, 13754 (2017).

14. Hutter, G. et al. Microglia are effector cells of CD47-SIRPα antiphagocytic axis disruption against glioblastoma. *Proc Natl Acad Sci U S A* **116**, 997-1006 (2019).

15. Tomaszewski, W., Sanchez-Perez, L., Gajewski, T. F. & Sampson, J. H. Brain Tumor Microenvironment and Host State: Implications for Immunotherapy. *Clin Cancer Res* **25**, 4202-4210 (2019).