KPCRTF State Funded Projects Reporting Template

University of Louisville – Project #2
Anti-GD2 CAR-T cells with intrinsic PD-1 checkpoint blockade, a novel and promising immunotherapy for the treatment of pediatric neuroblastoma and brain tumors
Principal Investigator: William Tsun-Yan Tse

Reporting Period: __ April 2021 - June 2021__

Below please provide a brief summary of the status of the Project listed as well as for each Aim listed below. Include any barriers, how and if they were overcome, and successes achieved.

Summary of Status of Project:

In this project, we have continued to work on developing CAR-T therapies that will be effective against pediatric solid tumors, including neuroblastoma and brain tumors. We have made a series of anti-GD2 CAR-T constructs that co-express a dominant-negative truncated PD-1 receptor or a chimeric PD-1 receptor with a positive signal-transmitting domain. We are testing whether these constructs will have abrogated PD-1 inhibition and thus enhanced anti-solid tumor capabilities. We applied single-cell transcriptomic methodologies to study how different CAR-T cell culture conditions influence the resulting cellular phenotypes and made important findings that can guide optimization of CAR-T cell production. We showed that CAR-T cells are heterogeneous in phenotype and can be grouped into cell clusters that correspond to T cell subsets at different stages of development, such as central memory, effector memory and terminal effector T cells. Different culture conditions contribute to distinctive cluster composition. Basing on these findings, we calculated for each cluster its signaling entropy rate (SR), a quantitative measure of differentiation potency or lineage promiscuity. We showed that differentiated clusters have a lower SR and primitive clusters have a higher SR. We are now correlating gene expression pattern, cell surface epitope distribution, and SR values of each cluster to construct gene signatures that can be used to define the CAR-T cell clusters. We plan to use the multimodal single-cell transcriptomics technology to study how functional capabilities of CAR-T cells might be affected by the solid tumor microenvironment and whether blocking inhibitory signals from the microenvironment could enhance the tumor-killing capability of the CAR-T cells. These studies provide biological insights that will enhance our understanding of the interaction of CAR-T cells with solid tumors and will help the development of effective CAR-T cells against pediatric solid tumors such as neuroblastoma and brain tumors.

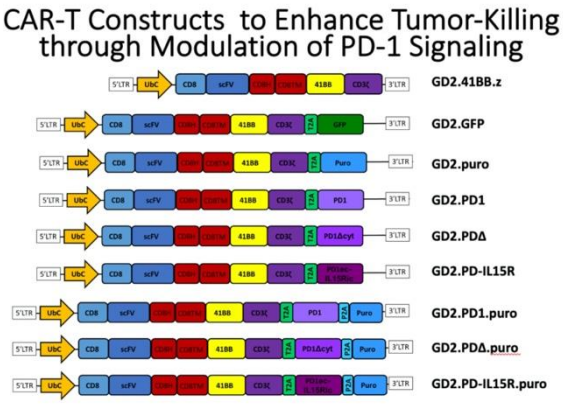
Project #2: Anti-GD2 CAR-T cells with intrinsic PD-1 checkpoint blockade, a novel and promising immunotherapy for the treatment of pediatric neuroblastoma and brain tumors

Aim 1: Improve the cytotoxicity profile of anti-GD2 CAR-T cells by incorporating intrinsic PD-1 blockade

To study the ability of CAR-T cells to kill and eliminate solid tumors, we made a lentiviral construct that expresses on T cells a humanized form of CAR targeting GD2, an antigen present on several pediatric solid tumors, including neuroblastoma, osteosarcoma and malignant brain glioma. We demonstrated that T cells transduced with lentivirus derived from this construct can kill GD2-expressing

tumor cells efficiently and specifically. We used the xCELLigence RTCA System to measure the cytotoxic capacity of the CAR construct and were able to show a linear relationship between cytotoxicity and the level of CAR expression.

To examine whether CAR T cells with intrinsic blockade of the PD-1 inhibitory pathway will have enhanced anti-tumor property, we made a series of anti-GD2 CAR-T constructs that co-express dominant-negatively acting truncated PD- 1 receptor, or a chimeric PD-1 receptor attached to the signal-transmitting domain of the Interleukin-15 receptor. Lentiviruses were produced using these constructs and used to transduce expanded T cells. We are now using the xCELLigence system to quantitatively compare anti-GD2 tumor-killing abilities of CAR-T cells derived from these constructs. We anticipate that we will be able to show that the modified CAR-T cells will have enhanced anti-tumor cytotoxic capability.



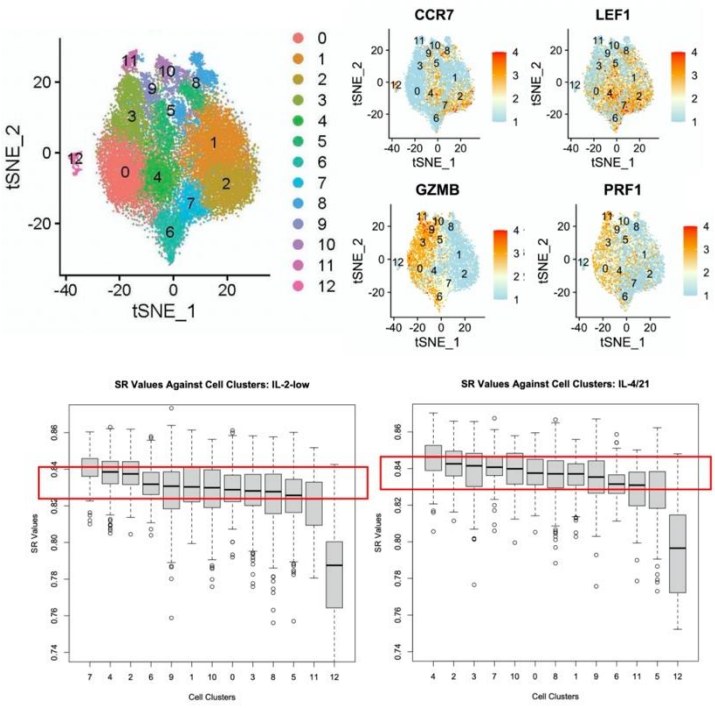
To improve on the cytotoxicity profile of anti-GD2 CAR-T cells, we are designing constructs that have CAR-coding DNA sequences inserted into the endogenous T-cell receptor loci in primary T cells, utilizing the CRISPR-mediated genome editing technique. By placing the CAR-coding DNA sequences under control of the T-cell receptor genes, we can express the CAR protein in a T-cell-specific manner, which has been shown in published data to result in enhanced tumor-killing functionality. In addition, replacing expression of the endogenous T-cell receptors by CAR receptors will abrogate the ability of the engineered T cells to cause graft-versus-host disease in transplanted patients, thus potentially enabling the use of these cells as "off-the-shelf" universal CAR-T cells and decreasing the production cost of these cells. We are setting up a new DNA transfection protocol to mediate CAR DNA transfer by means of a ribonucleoprotein (RNP)-based method, which will eliminate the need for the use of lentivirus as a vector for gene transfer, thus streamlining production of clinical-grade engineered CAR-T cells. Once we have this protocol set up, we will transfer the technology to the GMP laboratory and validate its use in a GMP-compliant manner.

Aim 2: Optimize the manufacturing processes to produce clinical-grade, anti-GD2 CAR-T cells

We upgraded our Good Manufacturing Practice (GMP) Laboratory to improve compliance with strict GMP requirements. WorkingBuildings, a laboratory quality consultant company, performed inspection and auditing of the GMP laboratory. Triple cleaning of the laboratory took place, and test engineering runs of CAR-T cell products were started. Preparation of clinical-grade CAR-T cells will proceed once the laboratory is fully functional. Standard Operating Procedures were revised to ensure the production of high-quality cell products.

Using single-cell RNA sequencing (scRNA-seq) technology, we showed that CAR-T cells are highly heterogeneous in nature and can be clustered into distinct subsets that reflect different stages of T cell development. The clusters span the developmental continuum from primitive central memory T cells

(e.g., positive for CCR7 and LEF1) to terminally differentiated effector T cells (e.g., positive for GZMB and PRF1). To evaluate the developmental potential associated with each cluster, we calculated the signaling entropy rate (SR) of each cluster cultured under different condition. SR measures the differentiation potency of cells by correlating gene expression patterns with connectivity of protein interacting networks. As expected from the definition of SR, clusters that contain differentiated effector cells have a low SR whereas clusters that contain primitive cells with high differentiation potency have a high SR. Samples that were cultured in IL-4/IL-21 have higher SRs across the board, suggesting that this culture condition promote production of CAR-T cells what have a high differentiation potency. We are currently correlating gene expression patterns of the clusters with their culture conditions to derive gene signatures that define different subsets of CAR-T cells. Such analyses will facilitate characterization of culture conditions that can promote production of CAR-T cells with high functionality and persistence.



Aim 3: Characterize quantitatively the cytotoxic activity of anti-GD2 CAR-T cells directed against human neuroblastoma and brain tumor cell lines and primary isolates

To enable quantitative measurement of the cytotoxicity function of CAR-T cells, we set up an xCELLigence Real-Time Cell Analysis System, which works by providing continuous monitoring of target cell viability through measurement of electrical impedance. This system was used to measure the rate of tumor cell killing by CAR-T cells by culturing the 2 cell types together in a chamber slide. Using specific CAR-T cells and target tumor cells, we calibrated the xCELLigence system and validated its software programs. We showed that the tumor cell-killing data acquired using the xCELLigence assay provide a reliable quantification of the functionality of CAR-T cells and that this new assay is an improvement over the conventional chromium-release cytotoxicity assay.

To create an *in vitro* model of the tumor microenvironment, we generated human myeloid-derived suppressor cells (MDSC) by culturing peripheral blood mononuclear cells in the presence of IL-6 and GM-CSF. We added MDSC to mixed cultures of CAR-T and tumor cells and tried to use the xCELLigence system to evaluate the ability of MDSC to suppress the ability of CAR-T cells to kill tumor cells. Unexpectedly, the MDSC produced a high background in the assay, thus presenting a technical roadblock that we are currently trying to resolve. Once we can overcome this roadblock, we will continue to develop this system to screen for pharmacological agents that can be used to block the negative effect of MDSC.

Aim 4: Generate data on the viability, purity, stability, activity, and safety of anti-GD2 CAR-T cells produced in compliance with GMP requirements

As discussed above, we have upgraded our GMP laboratory to enhance its capacity to produce clinical-grade cellular products necessary for clinical trials. Once the laboratory preparation work and process optimization studies have been completed, large-scale engineering runs will be performed to produce CAR T cells for testing of viability, purity, stability, activity and safety.

Timeline:

Aim (check when completed)	**Month 1-6**	**Month 7-12**	**Month 13-18**	**Month 19-24**	√
Improve CAR construct with PD-1 blockade	√	√			√
Optimize production process for CAR-T cells	√	√	√		√
Analyze cytotoxicity profile of CAR-T cells		√	√	√	√
Obtain safety data for CAR-T cells		√	√	√	√

Deliverables:

Check when deliverable is completed:	√
Completed validation of the effectiveness of an anti-GD2 CAR-T cell design, with incorporation of intrinsic PD-1 checkpoint blockade	√
Completed optimization of the process for the production of the anti-GD2 CAR-T cells	√
Completed quantitative analysis of the cytotoxicity profile of the anti-GD2 CAR-T cells	√
Completed characterization of the safety profile of the anti-GD2 CAR-T cells produced in a cGMP-compliant environment	√
Completed collection of pre-clinical data for an IND application and initiation of the IND submission process	√

Reports should be returned to:
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