KPCRTF State Funded Projects Reporting Template

University of Louisville Project
Bringing Next-Generation CAR-T Therapies to Children with High-Risk Malignancies
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 towards developing effective CAR-T therapies to fight high-risk malignancies in children. We are refining a draft protocol for a clinical trial in children to test the safety and effectiveness of a new generation of anti-ALL CAR-T cells that co-express an immune cell enhancing modulator. Our collaborator, the UofL Cellular Therapy Laboratory, is completing upgrading of its GMP facility and is conducting test engineering runs to produce clinical-grade CAR-T cells. We applied cutting-edge single-cell transcriptomic methodologies to study how different CAR-T cell culture conditions influence the resulting cellular phenotypes. We 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 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 contributed 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 characterize the CAR-T cell clusters. We made use of the highly sensitive nature of the xCELLigence cytotoxicity assay and worked to create an *in vitro* model of tumor microenvironment that will allow quantitative analysis of inhibition of CAR-T cells by myeloid-derived suppressor cells and screening of pharmacological agents that can block this inhibition. These studies generated useful biological data and established technical capabilities that are moving us towards the goal of bringing novel and effective CAR-T therapies to children with high-risk malignancies.

Aim 1. Optimizing the manufacturing process and enhanced anti-leukemia functionality of a novel class of anti-CD33/anti-CD123 compound CAR-T cells that target acute myeloid leukemia (AML) in children.

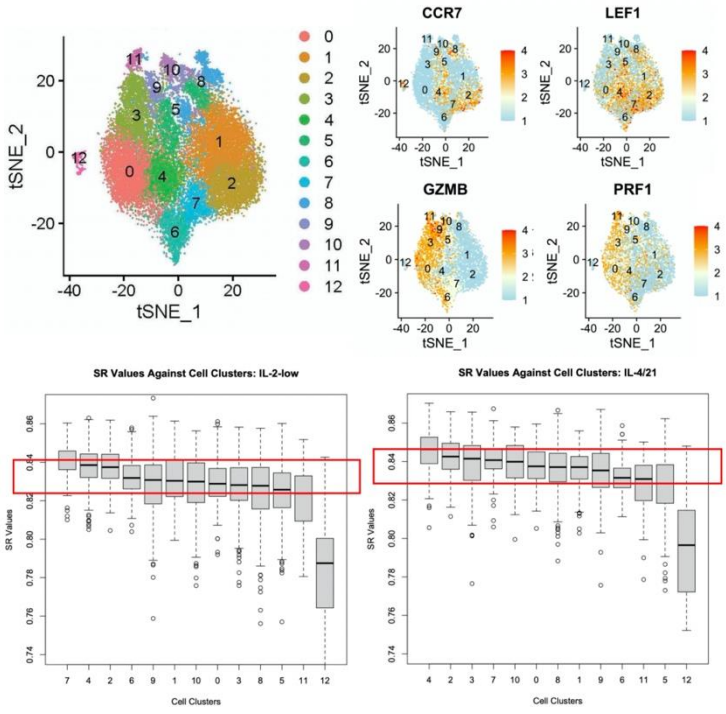
Working with our biotech industry collaborator, iCell Gene Therapeutics, we have made DNA constructs expressing on human T cells compound CAR that target both CD33 and CD123 antigens on AML cells. To allow manufacturing of clinical grade CAR-T cells for clinical trials, our collaborator, the UofL cellular therapy laboratory, have upgraded its Good Manufacturing Practice (GMP) facility and improved compliance of the facility with strict GMP requirements. WorkingBuildings, a laboratory quality consultant company, is performing auditing of the GMP facility and revision of its operating protocols. Engineering runs of CAR-T cell production are being conducted.

To lay the groundwork for a clinical trial to evaluate the use of compound anti-CD33/CD123 CAR-T cells to treat pediatric AML, we are refining the draft if a clinical protocol designed to evaluate the safety and feasibility of the use of a novel anti-CD19 CAR-T product to treat pediatric ALL. This CAR-T product co-expresses both the leukemia-targeting CAR and an Immune Cell Enhancing Modulator (see below), which is expected to enhance the functionality and persistency of the CAR-T cells. Once the anti-ALL protocol has been submitted for IRB review, a similar protocol will be drafted to evaluate the use of the compound anti-CD33/CD123 CAR-T cells to treat pediatric AML.

Aim 2. Optimizing the manufacturing process and enhanced anti-leukemia functionality of a novel class of anti-CD19 CAR-T cells co-expressing an immune modulator that positively affect T and NK cells and targeting acute lymphoid leukemia (ALL) in children.

Working with our biotech collaborator, iCell Gene Therapeutics, we have made DNA constructs designed to co-express on human T cells an anti-CD19 chimeric antigen receptor and an Immune Cell Enhancing Modulator (ICEM). This CAR-T product, designated CD19-ICEM CAR-T cells, has been shown in pre-clinical studies to exhibit increased persistence in vivo and enhanced antileukemia functionality. We are refining a draft clinical protocol to use the CD19-ICEM CAR-T cells to treat pediatric patients with refractory and relapsed ALL. We are currently preparing the protocol for submission to IRB for review and approval. With the upgrade of the UofL GMP facility for CAR-T cell production close to completion, engineering runs are being planned to produce the CD19-ICEM CAR-T cells in preparation for the clinical trial.

Using single-cell RNA sequencing (scRNA-seq) technology, we shown 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 ranging 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 CAR-T cell clusters cultured under different conditions. SR measures the differentiation potency of cells by correlating gene expression pattern with connectivity of protein interacting networks. As expected from the definition of SR, clusters that contain differentiated effector cells are shown to have a low SR whereas clusters that contains primitive cells with high differentiation potency are shown to 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 that have a high differentiation potency. We are currently correlating the gene

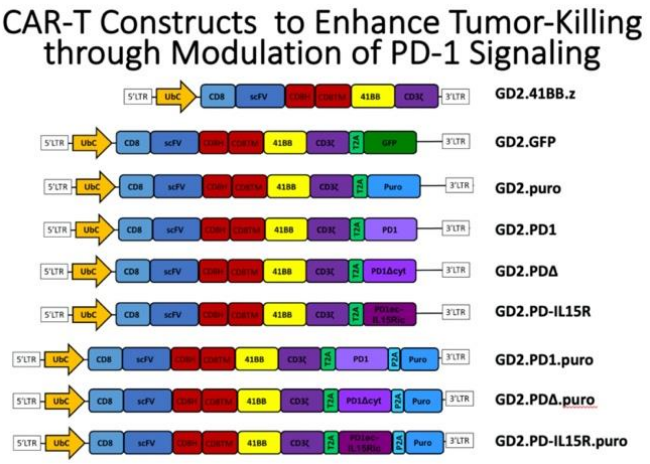


expression patterns of the clusters with their culture conditions to derive gene signatures that can characterize the different subsets of CAR-T cells. Such analyses will facilitate definition of specific culture conditions that can promote the production of CAR-T cells with high functionality and persistence.

Aim 3. Characterizing the anti-tumor functionality of a novel class of anti-GD2 CAR-T with intrinsic PD-1 blockade cells against neuroblastoma and other solid tumors of childhood.

To enable quantitative measurement of the cytotoxicity function of CAR-T cells, we have 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 have continued to calibrate the xCELLigence system and use to system to compare the relative abilities of different types of CAR-T cells to kill tumor cells. 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 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.



Aim 4. Developing a quantitative platform to study the inhibition of anti-GD2 CAR-T cells by myeloid-derived suppressor cells (MDSC) in the microenvironment surrounding neuroblastoma and other solid tumors of childhood and use this platform to screen pharmacological agents that will block the inhibition.

To establish a system that will allow the study of interaction between CAR-T cells, tumor cells, and MDSC in the microenvironment of solid tumors, we make use of the xCELLigence system, which comprises a microchamber where CAR-T cells, tumor cells and MDSC can be placed, and an electrode circuitry that allows reading out of killing of tumor cells by CAR-T cells. To set up this real-time cytotoxicity assay, we first validate the system by examining the relative ability of CAR-T cells to kill

target cells, after cultured in different conditions: 1) low-dose IL-2 alone; 2) high-dose IL2 alone; 3) IL-4, IL-7 and IL-21; or 4) IL-7 and IL-15. The resulting CAR-T cells were co-cultured with target tumor cells and the killing of target cells monitored using the xCELLigence system.

To create an *in vitro* model of the tumor microenvironment, we generated human 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.

Timeline:

Aim (check when completed)	**Month 1-6**	**Month 7-12**	**Month 13-18**	**Month 19-24**	**√**
Study compound CAR-T cells targeting AML	√	√			
Study modulator CAR-T cells targeting ALL	√	√			
Characterize CAR-T cells with PD-1 blockade	√	√			
Characterize CAR-T cells with MDSC depletion	√	√			

Deliverables:

Check when deliverable is completed:	**√**
Completed collection of IND-enabling pre-clinical data for cGMP-compliant manufacturing of anti-CD33/anti-CD123 compound CAR-T cells targeting pediatric AML.	
Completed collection of IND-enabling pre-clinical data for cGMP-compliant manufacturing of anti-CD19 CAR-T cells co-expressing an immune modulator and targeting pediatric ALL.	
Completed characterization of anti-GD2 CAR-T with intrinsic PD-1 block targeting solid tumors in childhood.	
Completed characterization of anti-GD2 CAR-T with extrinsic MDSC depletion targeting solid tumors in childhood.	

Reports should be returned to:
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