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Computational and CRISPR-based screening approaches enhance solid-tumor targeting CAR T cells

By

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Chapter 2: Chapter for the public

2.1 Introduction

As a scientist, I love sharing what I do with others in my life. Unfortunately, scientific papers are often dense, full of jargon, and unreadable to the general public. This chapter strives to present my doctoral research in an accessible manner, targeting a broader audience. I believe that scientific research is for everyone, and it is our responsibility as scientists to convey our findings to the taxpayers who support our work. I would like to thank the Wisconsin Initiative for Science Literacy (WISL) for supporting the creation of this thesis chapter. I would specifically like to thank Professor Bassam Shakhashiri, Elizabeth Reynolds, and Cayce Osborne for their comments, feedback, and support throughout writing this chapter.

2.2 The promise of a living drug

In 2010, a five-year-old girl named Emily Whitehead was diagnosed with Acute Lymphoblastic Leukemia (ALL), a rapidly progressive form of blood cancer. The standard course of treatment for ALL is a concoction of multiple chemotherapeutics; introducing non-specific cell killing agents into the body aimed at poisoning any dividing cell. Since cancer cells typically divide much faster than normal, healthy cells, cancer cells should be killed at a faster rate. However, if there are too many cancer cells to clear, effects on normal, healthy tissue can be extreme. Chemotherapies vary in efficacy against different cancer types and cause a wide range of debilitating side effects. The need for targeted therapeutic agents, that specifically target cancerous cells, is clear; such agents would limit the need to introduce non-specific poisons into a patient's body.

In Emily's case, the bet did not pay off, and her cancer returned following chemotherapy. As a last-ditch effort, Emily enrolled in a clinical trial for an experimental new treatment called **CAR T cell therapy**: a treatment that used a *living drug* consisting of her own immune cells, redirected to fight her cancer. This therapy uses **T cells**, a type of immune cell that normally aids in fighting infections, such as a seasonal cold or flu. However, in the context of cancer, T cells are unable to detect cancer cells as foreign, since cancer cells appear so similar to normal, healthy cells. In CAR T cell therapy, scientists introduce a synthetic protein on the cell surface called a **CAR,** or Chimeric Antigen Receptor. This receptor sits on the surface of the T cell and allows it to recognize and attach to cancer cells, like fitting two pieces of a puzzle together. The CAR on the surface of the T cell binds a unique target on the surface of the cancer cell, called an **antigen** (as seen in the schematic in Figure 2.1). The antigens chosen should have no expression on healthy cells, allowing these weaponized T cells to target only cancer cells.

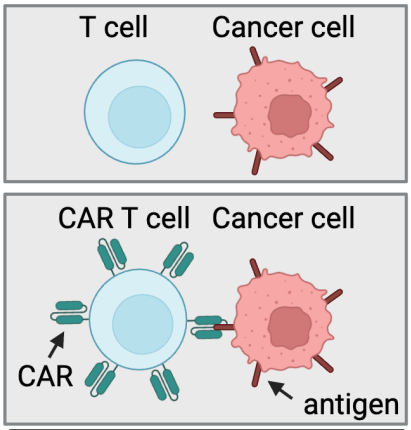

Figure 2.1 Schematic of T cell and CAR-T cell interacting with a cancer cell.

A few weeks after receiving CAR T cell therapy, Emily went into remission. She was the first child in the world to receive CAR T therapy, and she remains in remission to this day, with no detectable cancer in her body. Since Emily's recovery, 7 CAR T cell products have been approved by the Food and Drug Administration, all targeting cancers of the blood and bone marrow. These therapies are used as a last line of defense, when chemotherapy and other treatment options fail, and remain costly, with price tags of over $400,000 per injection.[1]

2.3 Burnout on the battlefield

Although CAR T cell therapies have had success in treating cancers of the blood, such as leukemias and lymphomas, they have not had the same success in treating solid tumors. There are several reasons for this discrepancy. Firstly, solid tumors form masses consisting of large clumps of thousands of tumor cells, and it can be difficult for CAR T cells to effectively penetrate those large clumps of cells. Think of how difficult it can be to make your way through a dense crowd – CAR T cells face this same problem. Secondly, these large clumps of tumor cells can secrete components that dampen the immune response, leading T cells to become unable to kill cancer cells, unable to effectively communicate with other immune cells, and dampening their ability to grow and divide. Imagine navigating a dense crowd where everyone is actively trying to get in your way and halt your progress. This phenomenon is called **T cell exhaustion**. Scientists strive to minimize exhaustion in CAR T cell products, and efforts to mitigate T cell exhaustion are growing in the field of CAR T cell research.

Although researchers have identified many genes associated with T cell exhaustion, the mechanism by which these genes work together to drive T cells into this exhausted state is not fully understood. In this context, genes act as the internal instruction manuals for the cell. Genes are first transcribed into mRNA (think of copying a recipe down from a cookbook) and then from the mRNA, translated into a protein (the final dish made from the recipe). Proteins perform various functions inside and outside the cell, dictating how cells react to their environment and whether they remain active fighters or succumb to exhaustion.

Normally, once a T cell encounters an antigen, it becomes activated. This activation causes the T cell to rapidly divide, creating an army of hundreds of thousands of T cells to respond to the antigen. Some of those T cells become **effector T cells,** which are highly adept at

killing target cells, and communicate with other immune cells to stimulate a larger immune response. Others become **memory T cells,** which are highly proliferative and can mediate a faster, more potent response upon repeat encounter with an antigen. Scientists use these labels of memory, effector, and exhaustion, to group T cells into categories referred to as **phenotypes**. These categories are determined by markers on the cell surface and can change over time: a memory T cell can become an effector T cell, which can in turn become an exhausted T cell. It is a matter of debate in the field whether exhausted T cells can reverse course and become effector T cells again; some scientists believe that exhausted T cells eventually become irreversibly exhausted. In clinical settings, CAR T cell products with a higher proportion of memory T cells are correlated with improved patient responses, underscoring the importance of this T cell subtype.[2] To shift a CAR T cell product to have more memory T cells, we need to understand how T cells become memory or exhausted T cells in the first place.

I was personally curious: how do T cells, which all begin their lives as naïve, untrained cells, ultimately determine their fates? How do some become long-lived, powerful cell killers, fighting foreign invaders for years to come, while others become exhausted, unable to appropriately respond to danger? This is one of the central questions of my research: *What genes are associated with memory and exhaustion in our solid tumor targeting CAR T cell product?* To address this question, we must first understand how CAR T cells are manufactured.

2.4 Teaching the T cells

To manufacture CAR T cells, scientists must introduce a synthetic receptor (the CAR) on the surface of the T cell. In current FDA-approved CAR T cell products, a **viral vector** delivers the CAR to the T cell. This viral vector uses viral machinery to attach to the surface of the T cell and deliver the gene encoding the CAR, which randomly integrates somewhere in the T cell genome. Think of surreptitiously slotting a new recipe page into a cookbook. This gene is then transcribed and translated, becoming the CAR protein which sits on the surface of the T cell, allowing the T cells to recognize and attach to cancer cells. Essentially, the recipe page that the virus added to the cookbook has provided the cell with the necessary instructions to prepare the ingredients and cook the recipe (in this case, our CAR).

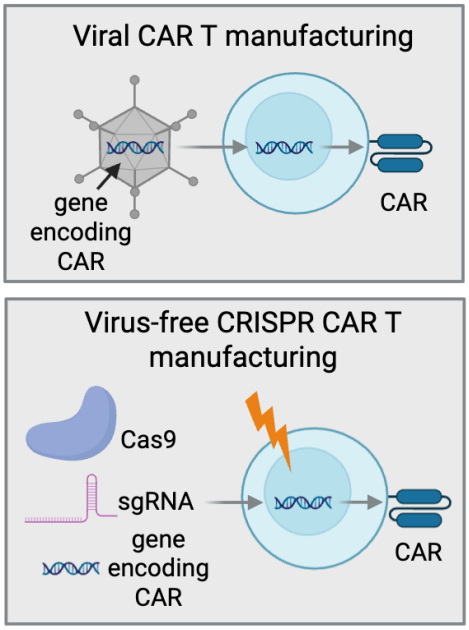

Figure 2.2 Schematic of viral and virus-free CRISPR CAR T cell manufacturing methods.

Although this method of CAR T cell manufacturing is highly efficient, there are some potential issues with viral manufacturing. Since the gene encoding the CAR is inserted at a random position in the genome, there is a possibility that the insertion of the CAR could disrupt the function of an existing gene in the T cell's genome. For a virus, disrupting an existing gene in its host cell is trivial, since viruses only use host cells as viral replication factories, a process that can occur in only a few days. After this process, the host cell usually bursts, releasing hundreds of viruses. However, in the context of precisely delivering a therapeutic product into a host cell, scientists

must ensure that the host cell is not disrupted in any way. These edited T cells are fighting back against cancer cells, so minimizing unwanted disruption of their genome is critical.

The recent discovery of **CRISPR-Cas9** gene editing technology has presented scientists with a new possible method for CAR T cell manufacturing. This method employs **Cas9,** a protein that acts as a pair of molecular scissors, and a **guide RNA** (or **sgRNA**) which directs the Cas9 to cut at a specific site in the genome. Delivery of the Cas9, sgRNA, and the gene encoding the CAR requires an electroporation, an electrical shock delivered to the surface of the cell, which allows these gene editing components to enter the cell, as shown in Figure 2.2. This strategy allows scientists to target a single place in the genome to integrate the CAR, resulting in finer control of CAR expression. Specifically, previous work has shown that targeting the *TRAC* locus, a gene which encodes the T cell receptor (TCR) in T cells, enhances antitumor potency of CAR T cells and creates more memory T cells.[3] Essentially, the *TRAC* locus is like the cell's front-row seat, and by placing the CAR there, it ensures the cell can operate consistently and effectively. Similarly, work from our lab has shown that solid-tumor targeting CAR T cells manufactured with the virus-free CRISPR manufacturing method are more memory-like and less exhausted than CAR T cells manufactured using viral vectors.[4] However, it is not clear why this is the case.

2.5 Delving into the data

To identify genes associated with memory and exhaustion in our CAR T cell products, I started with a huge dataset of the RNA from nearly 80,000 solid tumor-targeting CAR T cells before and after culture in the same dish as cancer cells. A previous graduate student in our lab generated this data using RNA sequencing, a technique that sequences a cell's RNA, providing a snapshot into all the genes expressed at that timepoint in the T cell. Think back to the cell's

genome as a cookbook: a cell's RNA is a representation of which recipes are currently in production in the cell. However, each T cell can express thousands of genes at any given time, resulting in quite a large dataset to process and analyze.

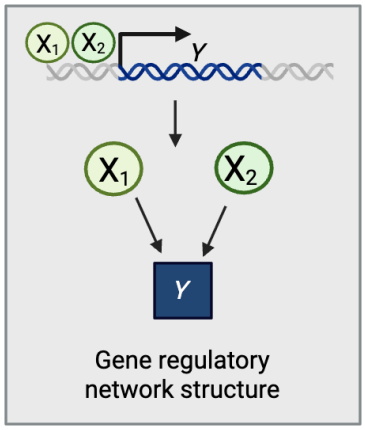

Figure 2.3 An example of a gene regulatory network

One method of analyzing this dataset involves creating networks of genes. For example, in Figure 2.3, proteins X_1 and X_2 are *regulating* the expression of gene Y. Thus, we can create a **gene regulatory network** describing this relationship by drawing arrows from X_1 and X_2 to gene Y. Similarly, these gene regulatory networks can be generated on a much larger scale, using genes with different levels of expression across memory, effector, and exhausted T cells. Using this method, we can identify genes predicted to be regulators of memory and exhaustion in our CAR T cell data. All the computational analyses I will describe in this section were performed by myself and Jeremy Parker Yang in collaboration with Dr. Sushmita Roy's lab, and both algorithms I reference were designed by her lab.

One limitation of gene regulatory network analysis is that each line between a regulator and a target (called an **edge**) is generally considered quite weak. However, when multiple analyses identify the same edges between targets and regulators, or identify the same regulators as associated with T cell phenotypes, confidence in these analyses increases. To increase confidence in the regulators flagged through these gene regulatory network analyses, I identified the regulators that appeared across multiple analyses and selected those regulators for further validation. Specifically, regulators of interest had to appear in at least four computational analyses performed on gene regulatory networks from two different algorithms (MERLIN[5] and

scMTNI[6]) to be considered strong enough hits to pursue. Computational analyses can only predict genes involved in memory and exhaustion; follow-up experiments in human cells are critical to confirm these predictions.

An example of one of these analyses appears below in Figure 2.4. In this gene regulatory network predicted by the algorithm MERLIN, the gene in the center above the black square, *PDCD1*, is a gene known to be associated with T cell exhaustion. *PDCD1* encodes the protein PD1 which appears on the surface of exhausted T cells. The genes represented by diamonds are potential regulators of *PDCD1*. The arrows between the genes represent predictions of gene expression; a red arrow from one gene to another, such as the arrow between *ZFP36L1* and *PDCD1* indicates that as *ZFP36L1* expression increases, *PDCD1* expression increases as well. Conversely, a blue arrow, such as the arrow between *LRRFIP1* and *PDCD1* suggests that as *LRRFIP1* expression increases, *PDCD1* expression decreases.

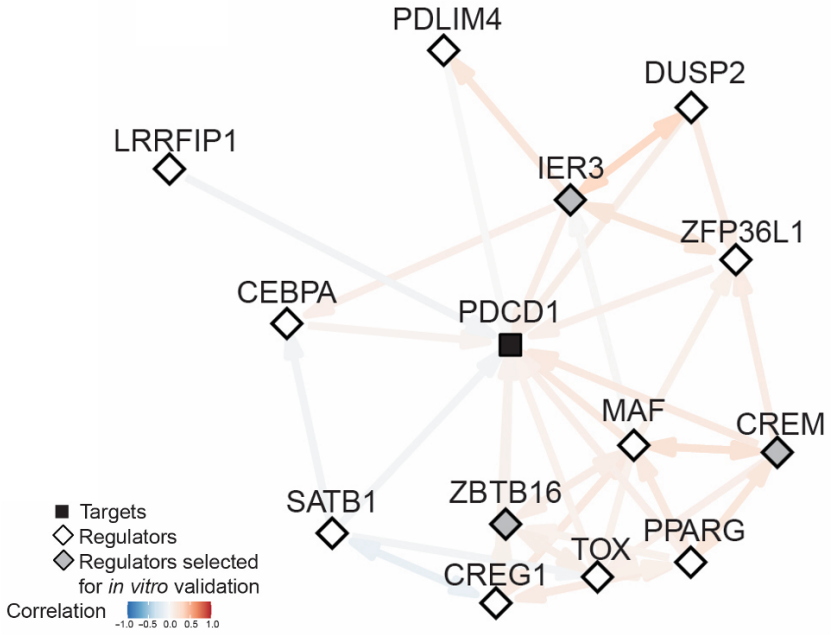

Figure 2.4 Network generated by MERLIN showing regulators (diamonds) interacting with the target gene (square) *PDCD1*.

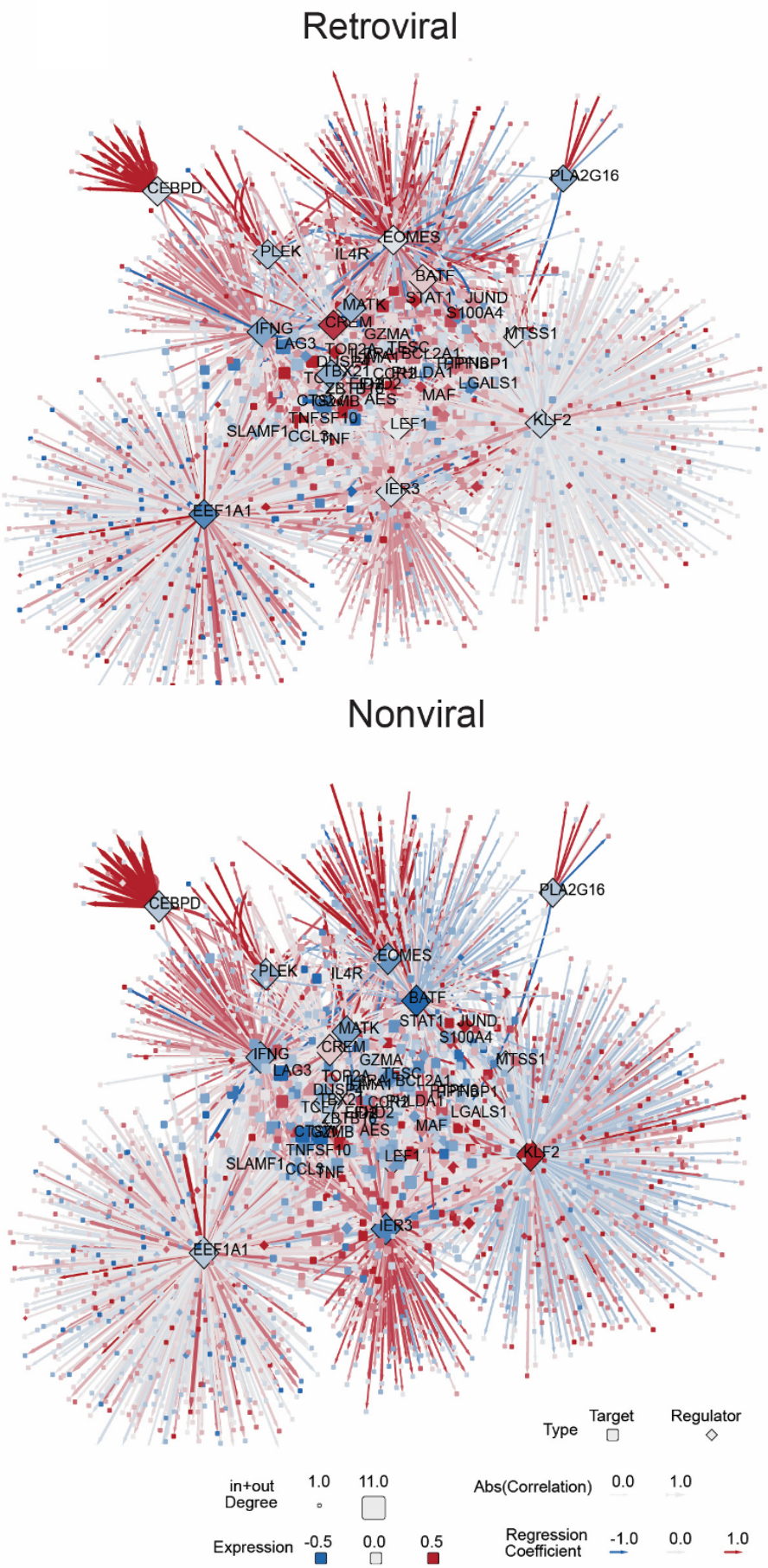
Retroviral
CEBPD
PLA2G16
EOMES
PLEK
IL4R
BATF
STAT1
JUND
S100A4
MATK
IFNG
CREM
GZMA
MTSS1
LAG3
LGALS1
MAF
KLF2
TNFSF10
SLAMF1
CCL3
TNF
LEF1
IER3
EEF1A1
Nonviral
CEBPD
PLA2G16
EOMES
PLEK
IL4R
BATF
STAT1
JUND
S100A4
MATK
IFNG
CREM
GZMA
MTSS1
LAG3
LGALS1
MAF
KLF2
TNFSF10
SLAMF1
CCL3
TNF
LEF1
IER3
EEF1A1
Type
Target
Regulator
in+out Degree
1.0
11.0
Abs(Correlation)
0.0
1.0
Expression
-0.5
0.0
0.5
Regression Coefficient
-1.0
0.0
1.0

Figure 2.5 Network visualization of retroviral (top) and nonviral (bottom) CAR T cells generated by scMTNI. Regulators (diamonds) and targets (squares) are larger in size based on larger outdegree (the number of targets they are predicted to regulate). They are colored based on expression: blue indicates decreased expression, red indicates increased expression.

Several of these genes have been previously described in the scientific literature as regulators of T cell exhaustion, while others have no known role in exhaustion. I selected the three genes in light grey for further validation, because they appeared in multiple other computational analyses. Across the computational analyses I performed in collaboration with Dr. Roy's lab, one common thread was that the gene regulatory networks predicted for exhaustion and memory were quite dense and complex, with dozens or even hundreds of genes predicted to be involved in regulating these phenotypes. These analyses suggest that there is much more work to be done to fully characterize the cell signaling pathways that lead to memory and exhaustion in T cells. However, the density of these networks also suggests many potential target genes that can modulate T cell phenotype to improve CAR T cell immunotherapies.

In addition to creating gene regulatory networks with known exhaustion- and memory-related genes as targets, I was interested in using gene regulatory networks to compare virally manufactured CAR T cells with our lab's virus-free CRISPR CAR T cells, to identify potential differences in gene regulation based on manufacturing method. One of the networks we generated is shown in Figure 2.5: on the top is the network for the virally manufactured CAR T cells, and on the bottom is the network for the virus-free CRISPR CAR T cells. Both networks have the same regulators (diamonds). This network visualization was created by Ryan Moreno, a student in Dr. Roy's lab.

However, the colors of the regulators change and the edges between regulators and targets within the network change significantly. For example, *KLF2* on the lower right has much higher expression in the nonviral network on the righthand side as compared to the network for the virally manufactured CAR T cells. Many of its edges also shift between the two groups. *KLF2* is a gene known to be active in memory T cells, and high levels of this gene correlate with development of memory.[7] Previous work in our lab has shown that nonviral CAR T cells are more memory-like as compared to retroviral CAR T cells. It is possible that increased expression of *KLF2* could be one reason for this difference, since increased expression of this gene is associated with memory. Overall, this computational analysis confirms changes in gene expression across viral and nonviral CAR T cells. Similarly to previous analyses, these gene regulatory networks are extremely dense, showcasing the complexity of the cell signaling pathways regulating these CAR T cells.

From these dense networks of hundreds of genes, I identified fifteen genes that appeared in four or more computational analyses and selected these genes for further validation work. Nine of those fifteen genes had been characterized in previous literature as associated with T cell memory or exhaustion. Three of the genes were associated with other T cell functions, and the remaining three genes had no known role in T cells. The appearance of genes known to be involved in T cell memory and exhaustion further supports the validity of the networks generated through these computational analyses. However, the finding that some of the top genes across these analyses have no known role in T cells indicates that this computational approach can identify novel or poorly characterized genes related to CAR T cell phenotype.

2.6 Validation of top regulators

Since these genes were only identified through computational work, it was critical to follow up this work with validation of these genes *in vitro* (meaning, in the laboratory, using cells in a dish). To perform this validation in the laboratory, I decided to disrupt the function of these genes, one by one, in our solid tumor targeting CAR T cells using CRISPR-Cas9. To manufacture these cells, I first isolated T cells from a healthy donor and proceeded with the CRISPR-Cas9 manufacturing strategy described earlier to manufacture solid tumor targeting CAR T cells. Next, I performed another electroporation to introduce a second sgRNA targeting

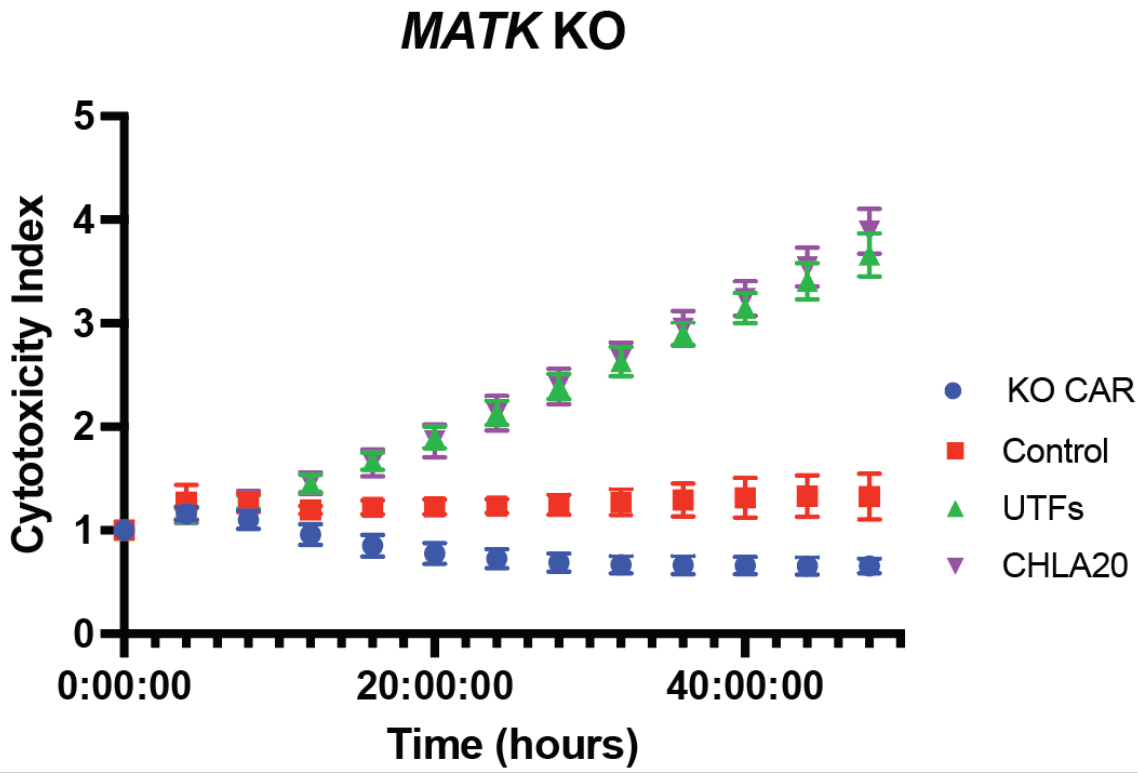

Figure 2.6 Results from 48-hour co-culture with cancer cells. *MATK* knockdown CAR T cells (blue), control CAR T cells (red), untransfected (UTFs, green), and cancer-only (CHLA20s, purple) were monitored over 48 hours. Y-axis represents cytotoxicity index (indicating the proportion of live cells as compared to time 0 – lower numbers indicate more cell death), while the x-axis is time.

one of the genes of interest from my computational analyses along with a Cas9. This second

electroporation disrupted the gene of interest from my computational analyses, and I repeated this electroporation for all fifteen genes I identified.

Following this gene disruption, I co-cultured these modified knockdown CAR T cells with a neuroblastoma cancer cell line for 48 hours. CAR T cells with no additional gene disruption served as a control for this experiment. The results for one gene are shown here in Figure 2.6: on the x-axis is the amount of time the CAR T cells were co-cultured with the cancer cells and on the y-axis is a measure of the number of cancer cells present in culture over time. The lower the number, the more cancer cell death. In green (UTFs) and purple (CHLA20s) are controls with unedited T cells with cancer cells and cancer cells alone, respectively. In red are the control CAR T cells with no additional gene disruption. In blue are CAR T cells with an additional gene disruption in the gene *MATK.* The blue line shows increased killing as compared to the control CAR, indicating that cells with these gene disrupted can kill cancer cells better as compared to the control CAR T cells in red.

To assess whether disrupted CAR T cells had shifts in memory and exhaustion, I performed flow cytometry, a technique used to identify markers on the cell surface. For this experiment, I assessed the expression of markers related to memory and exhaustion before and after co-culture with cancer cells. All in all, I identified three genes: *MATK, PLEK,* and *MTSS1,* that, when disrupted, improved CAR T cell killing in co-culture with cancer cells and shifted T cell phenotype away from exhaustion and towards memory.

2.7 Limitations & future directions

Although the findings of this work are exciting, there are also significant limitations to my chosen experimental approach that are important to consider. For example, I chose only to

disrupt the various genes, instead of choosing other methods such as overexpression of those genes. Overexpression of genes can identify genes that *enhance* a desired phenotype (such as memory); this could be relevant therapeutically, since scientists could then intentionally enhance a desired phenotype. Also, if the genes identified from my computational analyses work together with other genes, disrupting a single gene might be insufficient, due to potential redundancy in function. Additionally, the changes in cell killing demonstrated by *MTSS1, MATK,* and *PLEK,* although significant, still do not eliminate all cancer cells, indicating that there is still room for improvement. The manufacturing process to create these CAR T cells with gene disruptions is quite cumbersome and lengthy, and would not be a clinically viable strategy, so further improvement would need to be made to target these genes and their pathways in a CAR T cell therapy.

In the future, the computational approach I applied to the RNA sequencing data could be applied to other publicly available datasets of RNA sequencing from other CAR T cell studies. Many studies collect similar datasets from other CAR T cell constructs, both *in vitro* and *in vivo.* These datasets contain hundreds of thousands of datapoints on gene expression in CAR T cells. Since the gene regulatory networks behind CAR T cell phenotype are not fully understood, maximizing the information that we can obtain from these already existing datasets could help us construct more robust gene regulatory network predictions that can inform the design of future CAR T cell therapies.

2.8 Exhaustion without antigen

Have you ever woken up after a full night of sleep feeling just as tired as you were when you went to bed? As it turns out, a similar phenomenon can occur in the context of CAR T cells. **Tonic signaling** is a state of chronic activation that occurs in T cells prior to contact with antigen

and can lead to increased expression of exhaustion markers and activation markers such as the cell surface protein CD69.[8] Oddly enough, solid tumor targeting CAR T cells have higher levels of tonic signaling than liquid tumor targeting CAR T cells currently used in the clinic.[8] Furthermore, CAR T cells with high levels of tonic signaling are less effective: they have higher levels of exhaustion and are not able to target and kill cancer cells as effectively. However, it is not clear why solid tumor targeting CAR T cells would exhibit heightened tonic signaling. While some recent research shows that modifications in CAR design can contribute to tonic signaling, the genes regulating tonic signaling in CAR T cells are poorly understood.[9] In my thesis work, I was interested in exploring this topic further: How are CAR T cells becoming exhausted without ever encountering a cancer cell? Are there certain genes that we can identify in our CAR T cells that are involved in tonic signaling, and if so, can we knock out these genes to decrease levels of tonic signaling?

To address these questions, I designed a new set of experiments using our solid tumor targeting CAR T cells. First, I created a list of genes potentially involved in tonic signaling based

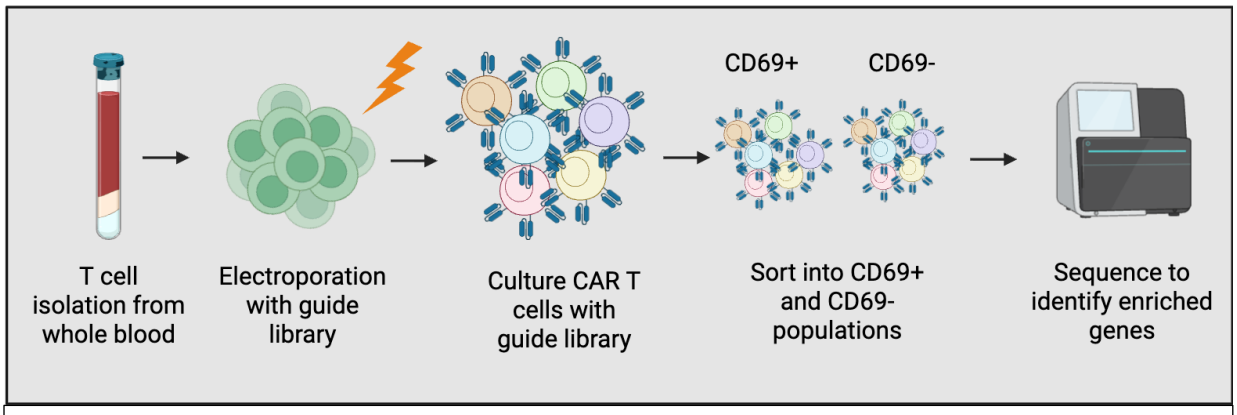

Figure 2.7. Schematic of pooled CRISPR screen experimental design.

on previous literature, my computational work above, and publicly available databases of genes involved in T cell function. All in all, I selected 776 total genes. Knocking out these genes one by one would be incredibly time consuming and inefficient; instead, I employed a technique called a

pooled CRISPR screen. Using this approach, I was able to screen all my genes of interest simultaneously in my solid-tumor targeting CAR T cell model. First, I isolated T cells from whole blood and electroporated them to manufacture CAR T cells, as I described earlier. However, I incorporated an additional element into the gene encoding the CAR: a **guide library**. This library consists of guides targeting each one of my 776 genes of interest (along with some control guides that do not target any human genes).

You can think of a pooled CRISPR screen a bit like a lottery. In this lottery, everyone (in my case, every CAR T cell) receives a single, unique lottery ticket (one guide from my guide library). This lottery ticket might be a losing ticket (a guide targeting a gene that is not important to tonic signaling) or might be a winning ticket (a guide targeting a gene that persists in culture). To identify the winning lottery ticket in my pooled CRISPR screen, I sorted my cells into CD69+ and CD69- populations. The CD69+ population contained cells that were chronically activated and exhibited tonic signaling, while the CD69- population contained cells that were not exhibiting tonic signaling. Then, I sequenced cells from each of those populations to identify the enriched guides, and I repeated this screen three times in different donors. A schematic for the experimental design is shown in Figure 2.7.

2.9 Lottery Winners

From this screen, I identified seven winning lottery tickets - genes strongly associated with tonic signaling across all three donors. In this case, these knockdowns act like removing the brakes: when each of these genes were knocked out, CAR T cells had an increase in tonic signaling. This indicates that normally, these genes function as *repressors* of tonic signaling. Two of the seven genes stood out to me: *MYH9* and *FLNA.* Both genes are associated with the cell cytoskeleton, the internal network of proteins and filaments in the cell that acts like scaffolding,

allowing the cell to be able to move and change shape. *MYH9* encodes a portion of the myosin II motor protein involved in cell adhesion, migration, and division.[10] *FLNA* encodes the protein filamin A, a structural component of the cell cytoskeleton.[11]

I manufactured knockdown CAR T cells with *MYH9* and *FLNA* knocked out using the same methods I described in the previous section about validating regulators. I then co-cultured the knockdown CAR T cells, alongside controls, with cancer cells. My data, as shown in Figure 2.8 below, shows that at CAR T cell to cancer cell ratio of 1-to-1 (meaning that in the co-culture, there is one CAR T cell for every cancer cell), *MYH9* knockdown CAR T cells performed significantly better than controls. *FLNA* knockdown cells performed slightly, but not significantly better. My data indicates that in a short-term co-culture assay, increased tonic

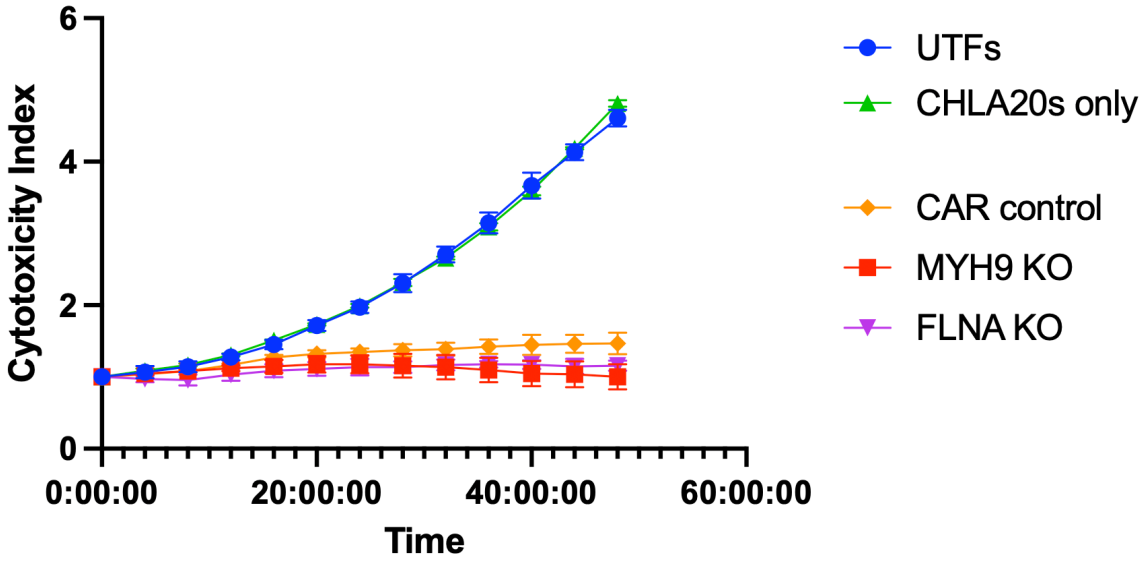

Figure 2.8 Results from 48-hour co-culture of *MYH9* and *FLNA* knockdown CAR T cells with cancer cells. *MYH9* knockdown CAR T cells (red), *FLNA* knockdown CAR T cells (purple), control CAR T cells (orange), untransfected (UTFs, blue), and cancer-only (CHLA20s, green) were monitored over 48 hours. Y-axis represents cytotoxicity index (indicating the proportion of live cells as compared to time 0 – lower numbers indicate more cell death), while the x-axis is time.

signaling helps CAR T cells kill cancer better. This data is only from one donor, and in the future, I plan to repeat this experiment to obtain more robust data. There can be significant variation in CAR T cells manufactured from different healthy donors, so repeating experiments with cells from different donors strengthens confidence in the data.

Previous research has shown that tonic signaling in solid tumor-targeting CAR T cells causes the CAR proteins to clump together on the cell surface.[8] I hypothesized that since my gene knockdowns are associated with an increase in tonic signaling, my knockdown CAR T cells might have more clumps of CAR on the cell surface as compared to controls. I imaged my knockdown CAR T cells in collaboration with Dr. Joshua Brockman's lab at UW-Madison. I prepared the CAR T cells for imaging, and Monica Umesh from the Brockman lab performed the

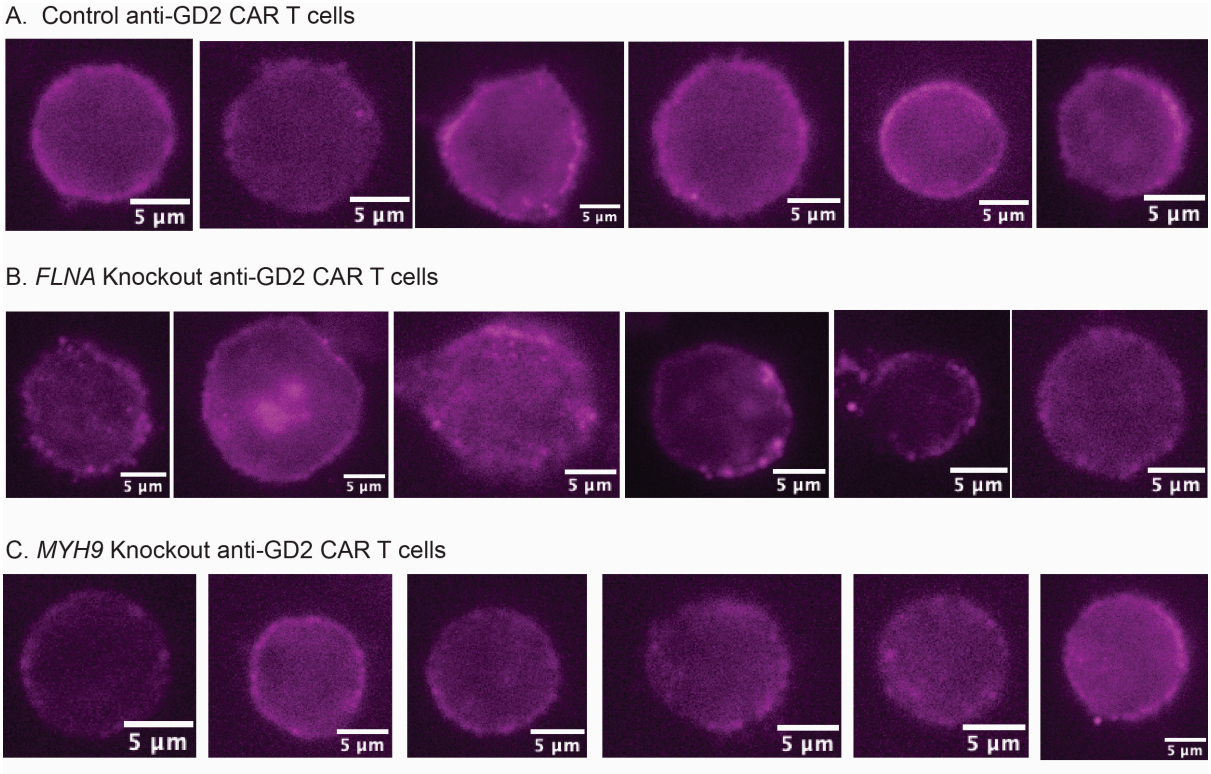

Figure 2.9 Results from imaging of *FLNA* and *MYH9* knockdown CAR T cells as compared to control CAR T cells. Purple indicates anti-GD2 CAR expression on the cell surface.

microscopy, since the Brockman lab frequently images CAR T cells at high resolutions. What we found, as shown in Figure 2.9, is that there are some changes in the clustering of the CAR on the cell surface. When we quantified these changes in CAR clustering using an image processing software, Fiji[12], we found that there are significantly more clumps on the cell surface of *FLNA* knockdown CAR T cells as compared to the controls. This indicates that knockdown of *FLNA* causes CAR proteins to clump together on the cell surface.

2.10 Summary & Future Directions

When cells move through their environments, as T cells do when they circulate through the bloodstream and when they move into tissue to combat infection, they exert force on their surroundings. The Brockman lab also studies cell force in the context of CAR T cells. Since both genes I identified are associated with the cell cytoskeleton, they may be involved in changes in cell force. In the future, it would be interesting to measure whether my knockdown CAR T cells have differences in the amount of force they exert on their surroundings. This could provide insight into changes in cell motility associated with tonic signaling.

My tonic signaling screen was able to identify biologically relevant genes involved in tonic signaling, including *MYH9* and *FLNA*. Additionally, *MYH9* knockdown caused CAR T cells to kill cancer cells better in a short-term co-culture. In the future, I think it would be interesting to delve deeper into the other five genes identified by this screen to better understand how they are involved in tonic signaling. However, my findings that cytoskeletal components contribute to tonic signaling are novel. Previous literature focused on how changing aspects of the design of the CAR itself can modulate tonic signaling, my work instead focused on how genes within the cell contribute to tonic signaling.

In the future, understanding the networks of genes governing shifts in T cell state such as tonic signaling, memory, and exhaustion will be key to developing better CAR T cell therapies to target solid tumors. My work demonstrates how dense and complex these networks are: clearly, there is much more to learn. However, my research also shows that there are a large number of potential genes to target to create better CAR T cell therapies in the future.

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