Application of Logic Gate Regulated Constitutively Activated STAT Molecule for CAR T Cell Efficacy Improvement

Taylor Kimiko-Ha Ishida

A thesis submitted in partial fulfillment of the requirements for the degree of

Masters of Science in Bioengineering

University of Washington

2021

Committee:

Michael Jensen

Hao Yuan Kueh

Program Authorized to Offer Degree:

Bioengineering

University of Washington

Abstract

Application of Logic Gate Regulated Constitutively Activated STAT Molecule for CAR T Cell Efficacy Improvement

Taylor Kimiko-Ha Ishida

Chair of the Supervisory Committee:

Michael Jensen, MD

Cancer is a complex disease, therefore the treatment methods used to address it should be robust enough to effectively combat it. Despite their general success, standard methods of treatment such as chemotherapy or radiation are not suitable for many patients, children in particular, as the associated side effects can be extremely detrimental. Chimeric antigen receptor (CAR) T cell therapy is a solution, as it has shown great promise in combating cancer while overcoming the non-specific nature of traditional therapy methods and minimizing side effects. CAR T cell therapy involves isolating T-cells from a patient's blood and introducing recombinant DNA into them, which encodes for the CAR and other various transgenes that provide functional advantages. Despite the success that has been seen with CAR T cell therapy, there still however remains room for improvement. Studies have shown evidence of low T cell persistence, represented by poor cell expansion and tumor infiltration, thus resulting in the therapeutic effect declining over time. This project works to overcome these issues by implementing logic gate regulation of transgenes in a T cell-based therapy method. Various combinations of an inducible synthetic promoter and drug regulation will be explored to control expression of constitutively activated signal transducer and activator of transcription (caSTAT) molecules, which are transcription factors associated with cell survival. The synthetic promoter requires CAR activation for expression of the downstream caSTAT molecule. Fusion of estrogen binding domains (EBDs) to the molecules regulates their activity, based on the presence of an estrogen analog. These regulated systems are designed to achieve time and location control of transgene expression to minimize its side effects. Efficacy of these systems will be explored by applying them in *in vitro* models. The results will better inform the development of more regulated, personalized and efficient CAR T cell therapy methods for treating cancer in patients.

Table of Contents

Table of Contents 1

Table of Figures 2

Introduction 4

Specific Aim 1: To explore regulation of caSTAT3 and caSTAT5a activity by estrogen binding domains: EBD(4-OHT) or EBD(CMP8). 11

Methods: 11

Cloning Plasmids of Interest: 11

Establishing STAT Reporter HEK 293t Cell Line: 12

Developing and Testing a Phosphoflow assay for phosphorylated STAT3 and STAT5a: 13

Observing the Growth of CD4+ T cells with Estrogen Binding Domain Regulated caSTAT3 and caSTAT5a: 15

Results: 15

Establishing STAT Reporter HEK 293t Cell Line: 15

Developing and Testing a Phosphoflow assay for phosphorylated STAT3 and STAT5a: 18

Observing the Growth of CD4+ T cells with Estrogen Binding Domain Regulated caSTAT3 and caSTAT5a: 23

Specific Aim 2: To characterize caSTAT3 and caSTAT5a function in primary human T cells when constitutively expressed and under the regulation of a CAR-inducible promoter. 25

Methods: 25

Cloning Plasmids of Interest: 25

Electroporating CD4+ and CD8+ T cells to produce CAR T cells: 26

Utilizing Incucyte analysis to understand cytotoxicity of CAR T cells with caSTAT in Dual Promoter Systems: 27

Results: 28

Cloning Plasmids of Interest: 28

Electroporating CD4+ and CD8+ T cells to produce CAR T cells: 29

Utilizing Incucyte analysis to understand cytotoxicity of CAR T cells with caSTAT in Dual Promoter Systems: 33

Specific Aim 3: To understand effects of caSTAT3 and caSTAT5a on CAR T cell activity in dual promoter systems, when regulated by estrogen receptors and constitutive or CAR inducible promoters. 40

Methods: 40

Cloning Plasmids of Interest: 40

Summary and Future Work: 41

References 43

Table of Figures

Figure 1: endogenous STAT phosphorylation, and therefore activation, occurs via the JAK/STAT pathway, [21] 7
Figure 2: the activation pathway for caSTAT, or constitutively phosphorylated/activated STAT is dependent on the presence of an estrogen analog 8
Figure 3: iSynpro, a CAR-inducible synthetic promoter allows for controlled transgene expression, dependent on the presence of antigen expressing cells 9
Figure 4: Iterations of caSTAT3 or caSTAT5a fused with EBD(4-OHT) or EBD(CMP8), when driven by EF1 11
Figure 5: caSTAT5b-EBD(4-OHT) did not provide any proliferative advantage to CD8+ CAR T cells, when subjected to serial tumor challenges 11
Figure 6: STAT3 or STAT5a responsive elements serve as a binding site for the dimerized STAT3 or STAT5a transcription factors, which reads off GFP or mScarlet 13
Figure 7: new STAT reporter plasmids were engineered to include CD19t, a surface markers that could be used for gating purposes for analyses 13
Figure 8: three experimental Phosphoflow protocols were tested and compared to a standard surface staining method, to establish the process that would minimize cell death due to the harsh permeabilization step 14
Figure 9: Flow results to analyze the transduction efficiency of STAT reporter plasmids into HEK 293t cells, shows that there was successful transduction for both STAT3RE and STAT5RE, but STAT3RE was more efficient. 16
Figure 10: STAT3RE HEK 293t cells treated with estrogen analogs at 12-hours post-EP 16
Figure 11: STAT5RE HEK 293t cells treated with estrogen analogs at 12-hours post-EP 17
Figure 12: STAT3RE HEK 293t cells treated with estrogen analogs at 24-hours post-EP 17
Figure 13: STAT5RE HEK 293t cells treated with estrogen analogs at 24-hours post-EP 18
Figure 14: the results of the Phosphoflow protocol opimization experiment, indicates that Pre-Fixation is the method that will provide the most promising results 19
Figure 15: the efficiency of the CD4+ T cell transduction is indicated by Her2tG+ 20
Figure 16: the efficiency of the CD8+ T cell transduction is indicated by Her2tG+ 20
Figure 17: the Phosphoflow results revealed no expression of pSTAT3 above the unstimulated Mock cells, indicating there is no detectable expression of exogenous pSTAT3 21
Figure 18: the Phosphoflow results revealed expression of pSTAT5a above the unstimulated Mock cells in the Her2tG+ quadrant, indicating there is some detectable expression of exogenous pSTAT5a 21
Figure 19: quantification of the percent of cells with pSTAT3 or pSTAT5 expression greater than the Mock cells reiterates and clarifies the results of these assays, and emphasizes the importance of further investigating the pSTAT3 Phosphoflow experiment 22
Figure 20: the growth curve assay of transduced CD4+ T cells indicates ca-STAT5a-EBD(4-OHT), when treated with 4-OHT, provides the greatest proliferative advantage 23
Figure 21: caSTAT3 CD4+ T cells treated with CMP8 immediately declined in cell count, indicating the drug may be inducing a cytotoxic effect 23
Figure 22: experimental plasmids with caSTAT3 or caSTAT5a driven by constitutive promoters: EF1 and hPGK, or the CAR inducible promoter, iSynPro (S161) 25
Figure 23: experimental plasmids with GFP driven by various constitutive promoters, with a PBPMK backbone 25

Figure 24: experimental plasmids with GFP driven by various constitutive promoters, with a PBNP backbone 25
Figure 25: iSynPro dual promoter plasmid, and CAR alone control plasmid that were used as controls for the electroporation and experimental processes 26
Figure 26: the "TICLE" procedure for CD8+ and CD4+ elecroporations require addbacks of CD19+ PBMCs from the same donor, allowing for CAR expressing T cell limited expansion 26
Figure 27: Incucyte image 8 days after the initial plating of CAR T cell effector cells and mCherry labeled Be2 target cells 27
Figure 28: previous experiments, with the intention of identifying a constitutive promoter to use in a dual promoter system, applied MND and compared it to iSynPro (S161) 28
Figure 29: flow results of MND or iSynPro driving GFP, the marker downstream of caSTAT, indicates that in the EGFRt (CAR) positive populations, MND hinders GFP expression, compared to iSynPro 29
Figure 30: the dual promoter constructs with PBNP backbones showed greater transgene expression compared to those with PBPMK backbone when electroporated into CD4+ and CD8+ T cells 30
Figure 31: transgene positivity for these dual promoter systems with PBPMK backbones was weak, indicating the plasmids required further development 30
Figure 32: flow results for CD4+ T cells from Donor A showed some electroporation efficiency, but cell growth was so low there were few events to record 31
Figure 33: flow results for CD8+ T cells from Donor A showed poor electroporation efficiency. Additionally, cell growth was so low there were few events to record 32
Figure 34: flow results for CD4+ T cells from Donor B showed strong transgene expression in the majority of the conditions, indicating successful electroporations 32
Figure 35: flow results for CD8+ T cells from Donor B showed varying levels of transgene expression, and therefore electroporation efficiency in the majority of the conditions 33
Figure 36: Incucyte image of CD19+/mCherry+ Raji cells (red) clumping in a 96-well plate, when treated with Mock CD4+ T cells 34
Figure 37: Incucyte results of electroporated CD4+ CAR T cells over the course of around 13 days, indicate the cytotoxic advantage of cells with caSTAT3 or caSTAT5a transgenes under various promoters, over the Mock CAR T cells 35
Figure 38: Incucyte results of electroporated CD8+ CAR T cells over the course of around 13 days, indicate the cytotoxic advantage of some conditions with caSTAT3 or caSTAT5a transgenes under various promoters, over the CAR Control or Mock CAR T cells 36
Figure 39: Incucyte results of electroporated CD4+ and CD8+ CAR T cells, with the caSTAT3 transgene being driven by inducible and constitutive promoters 37
Figure 40: Incucyte results of electroporated CD4+ and CD8+ CAR T cells, with the caSTAT3 transgene being driven by inducible and constitutive promoters 38
Figure 41: the cytotoxic potential of caSTAT3 and caSTAT5 in both CD4+ and CD8+ CAR T cells can be compared when driven by iSynPro 39
Figure 42: a total of 12 plasmids, with various combinations of promoters and caSTAT transgenes fused with either EBD(4-OHT) or EBD(CMP8) will be cloned for eventual downstream assays 40

Introduction

Cancer is a collection of diseases that involve the rapid growth and spread of the cells in a patient's body. When healthy, human cells grow and divide to form new cells as they are needed and die off when they are old or damaged. Cancer disrupts this orderly cycle, resulting in cells that become abnormal and may form tumors [1]. Depending on the location and type of cancer, the physiological impacts on the patients differ. These may include decreased functionality, due to the blocking of channels in the body, or tumors pressing against neighboring tissue or organs. Cancer may also diminish a patient's immune system and alter hormone levels [2]. Cancer is a result of changes to genes that control how our cells function, in particular, those affecting how they divide and grow. These mutations may be inherited or may be due to damage to DNA that may be caused by various environmental factors [1].

Acute lymphoblastic leukemia (ALL) is the most common form of cancer in pediatric patients. It is a cancer of the bone marrow and blood. [3]. ALL begins with lymphoma B cells, which derive from pluripotent hematopoietic stem cells in the bone marrow. Mutations that result in the development of cancers are a result of structural chromosomal abnormalities, including hyperdiploidy, hypodiploidy, translocations, and rearrangements. The genes that are often mutated are the ones involved in encoding for proteins with key roles in lymphoid development, cytokine receptors, tumor suppression, epigenetic regulation, etc [4].

Current treatment methods for cancer have been shown to be quite effective, as the 5-year survival rate for pediatric cancer overall is around 85% [5]. Despite this success, standard methods of treatment are antiquated and not suitable for children, as there are detrimental side effects associated with treatment. Chemotherapy is a therapy method that utilizes powerful chemicals to kill rapidly growing cells, thus targeting both cancerous and healthy cells. Childhood survivors of cancer who undergo chemotherapy are at an elevated risk of chronic morbidity and early mortality, often from adverse cardiovascular events that are related to exposure to treatment [6]. They are also more susceptible to obesity, endocrine and metabolic disorders, muscle weakness, neurosensory impairment, decreased resistance to infection and neurocognitive deficits [7]. Stem cell transplantation (SCT) is a procedure in which a patient's stem cells that have been damaged by cancer or cancer treatments, are replaced with healthy stem cells via infusion into the bloodstream. The stem cells travel to the bone marrow and form new healthy blood cells. This therapy method may result in long-term side effects including non-malignant organ or tissue dysfunction, infections due to abnormal tissue reconstitution and development of secondary cancers [8]. In addition to the pediatric patients themselves being subject to these side effects, the parents or caregivers of the child are prone to significant psychological stress [9].

Chimeric Antigen Receptor, or CAR T cell therapy may help overcome these shortcomings. This process involves the isolation of T-cells from a patient's blood and introducing recombinant DNA into them [10, 11]. This DNA has been engineered to encode for the chimeric antigen receptor, which are expressed on the surface of the T cells. These receptors are capable of recognizing specific antigens that are typically expressed on the surface of cancer cells, allowing them to target and kill the cells upon reinfusion into a patient's bloodstream. The side effects associated with CAR T cell therapy do not have the same long term impacts on patients' health as those due to chemotherapy treatment or other traditional therapeutic methods. The most common side effect (affecting around 70-90% of patients) of CAR T cell therapy is cytokine release syndrome (CRS). For around five to seven days, CRS will leave patients with a severe case of the flu, or fatigue, due to the immune response in their body as the T cells are rapidly multiplying and attacking the cancer cells [12].

CARs are fusion proteins of single-chain variable fragments from antibodies and T-cell receptor components, which target particular antigens. In the case of the anti-CD19 CAR, it is targeting the CD19 expressing cancer cells, which are commonly ALL associated cells. Following the molecular workflow, these CARs may be expressed in a patient's T cells, allowing for specific CAR T cell responses. The results of this method for combating cancer have shown promise. According to studies that have already been conducted, mice with acute lymphoblastic leukemia, who are treated with CART T cell therapy, had reported longer survival time compared to the other treatment groups [13]**.** Additionally, clinical trials have shown promising results for end-stage patients, who use CAR T cell therapy. Up to 92% of patients with ALL have achieved full recovery.

The CAR expressed on the surface of the T cell can be broken down into three parts: the ectodomain, transmembrane domain and the endodomain. The ectodomain is outside the cytoplasm and exposed to the extracellular space, thus it is responsible for the signaling nature of the CAR T cell. A primary component of the ectoderm, is the single-chain Fragment variant (scFv) which helps provide the specificity of the targeting. It is formed by fusing the variable portions of the heavy and light chains of an immunoglobulin via a flexible linker. A spacer is utilized to connect this antigen binding domain to the transmembrane domain. The transmembrane domain consists of a hydrophobic alpha helix that spans the membrane and provides stability to the receptor. It has been studied that the CD28 transmembrane domain provides the most stability. The endodomain provides the functional end of the CAR, consisting of costimulatory and stimulatory molecules. Upon antigen recognition, the receptors cluster and the signal is activated, thus transmitting it to the T cell [14].

CAR T cell therapies have shown remarkable progress in treating cancers, in particular ALL and some B cell lymphomas. However, treatment resistance remains a major barrier to future implementation. A meta-

analysis reveals relapse rates as high as 37% [95% CI, 29.4%-45.4%] for CD19 CAR T cell therapy, or 75.6% [95% CI, 56.4%-88.2%] for non-CD19 CAR T cell therapy [15]. Relapse presents in two major patterns: early relapse of antigen-positive cancer or later relapse associated with antigen loss [16]. Loss of CAR T cell persistence following administration is thought to be a major contributor to antigen-positive relapse. Strategies to improve persistence include optimized CAR design, administration of T cell antigen-presenting cells (T-APCs), checkpoint inhibitors, improved gene editing techniques, and reinfusion of CAR T cells. These approaches seek to address persistence by increasing proliferation, though they remain limited in their capacity to allow for tunable proliferation that would be critical for preventing exhaustion.

Implementing constitutively activated signal transducer and activator of transcription (caSTAT) molecules into the CAR T cell system may help address issues of persistence while offering control over proliferation [17]. These molecules can improve CAR T cell function [18]. There are several members of the STAT protein family, including STAT1, STAT3, STAT5a, and STAT5b. caSTAT5a is a molecule that has been studied quite a bit thus far, and is understood to promote the proliferation of T cells. When studying mice who were deficient in STAT5a, it was observed that they had abnormally low levels of white blood cells as well as an extreme deficiency in T cell proliferation [19]. Studies have shown that CAR T Cells with activated STAT5 (one of the several members of the STAT protein family), demonstrated “superior” *in vivo* persistence and antitumor effects in both liquid and solid tumors when compared to CAR T cells with only the CD28 or 4-1BB co-stimulatory domains alone. This implies that antigen responsive effector T cells with the caSTAT5a proteins are “long lived” and will thus provide a therapeutic response over a greater time period.

While STAT5a has been reported to provide advantage to CAR T cell systems via proliferative advantage, STAT3 has been found to do so via increased survival. STAT3 enhances the survival of activated T cells by upregulating OX-40 and Bcl-2, and down-regulating FasL and Bad expression. This suggests that STAT3 and FoxO pathways may converage to ultimately regulate the lifespan of T cells. STAT3 has been considered a transcription factor that regulates the proliferation in some mammalian cells and has been referred to as an oncocgene, due to its constitutive activation in some primary tumors. However, it has been revealed that STAT3 is capable of promoting CD4+ T cell quiescence and inihiting

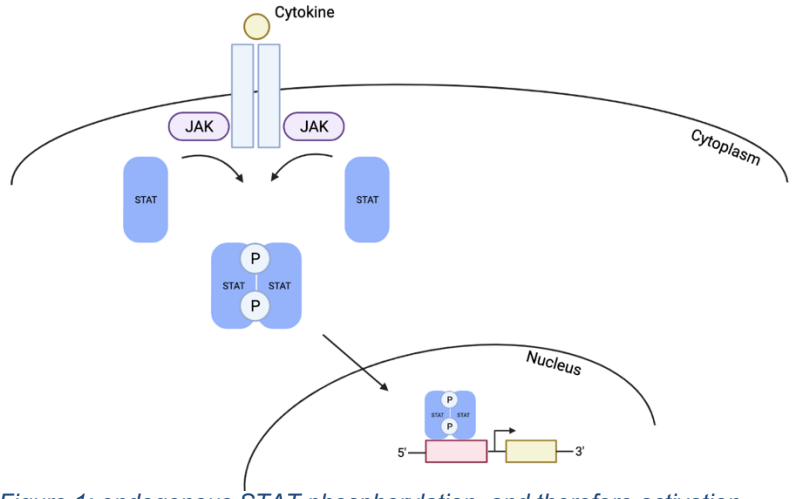


Figure 1: endogenous STAT phosphorylation, and therefore activation, occurs via the JAK/STAT pathway

IL-2 production, thus promoting stability and overall survival of the activated T cells via upregulation of anti-apoptotic genes [20].

These caSTAT molecules may be introduced into the CAR T cell systems, via the engineered plasmid. STAT proteins are found inherently in cells [21]. Via the JAK/STAT signaling pathway, as seen in figure 1, these endogenous proteins are phosphorylated by janus kinases (JAKs) in response to various cytokines, including prolactin, IL-2, IL-3, IL-5, IL-7, granulocyte-macrophage colony-stimulating factor (GM-CSF), G-CSF, M-CSF, erythropoietin (Epo), thrombopoietin, and growth hormone (GH) [22]. These phosphorylated STAT proteins can then dimerize, translocate into the nucleus and bind to the promoters of target genes. These target genes are associated with division, resulting in proliferation of the CAR T cells [13]. Exogenous caSTAT proteins have the same downstream effects, however, do not rely on JAK phosphorylation. To be constitutively phosphorylated on their tyrosine residues caSTAT3, caSTAT5a and caSTAT5b have mutations described in table 1. These mutations allows for the dimerization of the caSTAT proteins without the reliance on cytokine binding, thus inducing constitutive transcriptional activity [22].

Table 1: Mutations and Target Genes associated with caSTAT3, caSTAT5a and caSTAT5b [23, 24, 25]

Molecule	Mutation for Constitutive Activation	Target Genes
caSTAT3	A662C, N664C	c-Fos, HIF-1α, c-Myc, Sox2, Zeb1, Bcl-2, Mcl-1, Bcl-xL
caSTAT5a	S711F, H299R	Bcl2, Junb, Id2, NDRG1, DNAJC6, CBS, PPP2R2B, ST3GAL1, SAMD4A, SSH2, MAP3K5
caSTAT5b	N642H	DOCK8, SNX9, LNPEP, SKAP1, PTGER1, FOXP3

If these caSTAT molecules are not regulated, however, it may result in over proliferation of T cells. This may lead to the CAR T cells reaching the stage of senescence [26], thus leading to cell-cycle arrest of the T cells, rendering the CAR T Cell therapy ineffective, as the cells are no longer capable of responding to the target cells [27]. Additionally, it has been discovered that over proliferation of CAR T cells may result in toxicity in the body. In the past, toxicity was limited and controlled by simply destroying the T cells [28] once the cells have proliferated to toxic levels, rather than regulating the proliferation during production. These effects, which may reduce the length of life of the CAR expression, and render the T cells ineffective, can be avoidable by designing a T cell based therapy method, that is specifically designed to be controlled.

This regulation can be accomplished by introducing an estrogen binding domain (EBD) to the caSTAT molecule [29]. EBDs, when fused to molecules, are capable of controlling their activity. This domain is a heterologous, ligand-inducible dimerization domain and serves as an inducible driver of STAT dimerization in the presence of an estrogen analog. As seen in figure 2, the fusion protein of caSTAT with the EBD is found in the cytoplasm, with heat shock protein 90 (HSP90) surrounding the EBD.

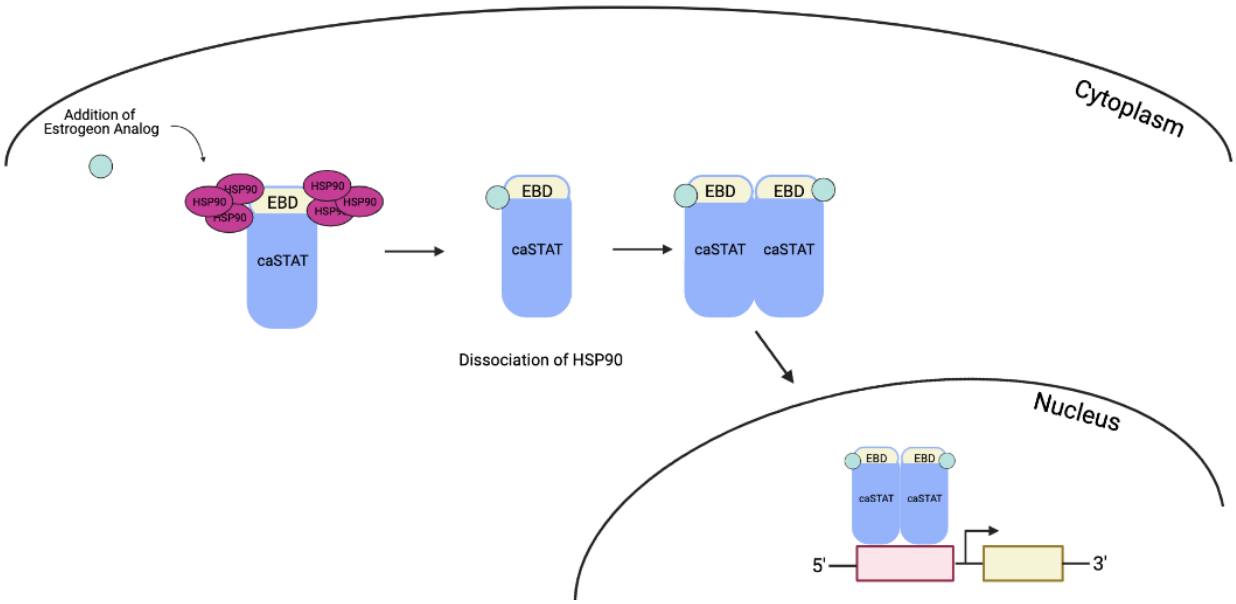


Figure 2: the activation pathway for caSTAT, or constitutively phosphorylated/activated STAT is dependent on the presence of an estrogen analog

Upon addition of the estrogen analog, HSP90 will dissociate, and allow for the molecules to dimerize. The dimerized caSTAT(EBD) protein would then proceed with the same downstream behaviors, by

translocating into the nucleus and inducing transcription activity [30]. This may allow for a more efficient therapy system, as it provides a method of regulating expression of the caSTAT molecules, by administering doses of the estrogen analog drugs at certain variables of time. The EBDs of interest are EBD(4-OHT) and EBD(CMP8). EBD(4-OHT) is a wild type of estrogen receptor alpha ligan binding domain (wt-ERaLBD) with amino acid substitutions at G3400V, M543A and L544A, and has been reported to be responsive to estrogen analogs 4-hydroxy-tamoxifen (4-OHT), and (Z)-Endoxifen. ENEBD(CMP8) is wt-ERaLBD with mutations at L385M, M421G and G521R, and has shown responsiveness to CMP8, 4-OHT and (Z)-Endoxifen [31].

The potential promoters driving regulated caSTAT must also be explored. In the Jensen lab, an Inducible Synthetic Promoter (iSynPro) responsive to CAR activation, was invented by Senior Research Scientist, Dr. Jia Wei. As shown in figure 3, upon CAR binding by the antigen expressing cancer cell, a library of transcription factors will be released, and bind to particular domain of iSynPro, thus allowing for expression of the downstream transgene. This is a tightly regulated system, in that binding of the endogenous T cell receptors (TCRs) or surface receptors, including TNF α, INF α, CD28 or TLR, despite producing transcription factors, will only bind to endogenous promoters, and not to the exogenous promoter (iSynPro) to drive expression of the transgenes. This technology contrasts with constitutive promoters, such as EF1, polyUbc, CMV and hPGK, which will constitutively express the downstream transgenes, regardless of any stimulation [32].

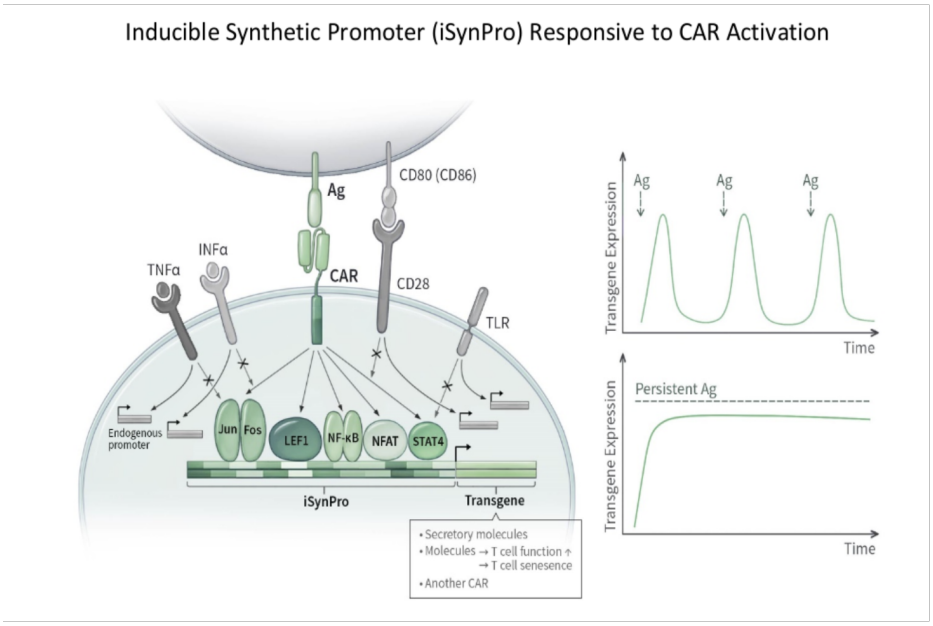


Figure 3: iSynpro, a CAR-inducible synthetic promoter allows for controlled transgene expression, dependent on the presence of antigen expressing cells

Utilizing a dual promoter system, the caSTAT(EBD) gene and the CAR part can be encoded for on the same engineered DNA plasmid but be driven by different promoters. If the CAR is under a constitutive promoter, then it will be expressed on the surface of the T cell, regardless of any environmental stimulus [33]. By introducing caSTAT(EBD) as a transgene regulated by a CAR-inducible promoter, then expression of the pro-proliferative molecule will only occur if the CAR T cell is in the presence of the target cells, and there is antigen-receptor interaction. This provides another level of regulation of the STAT gene expression, and thus CAR T cell functionality, helping to address the concern of T Cell senescence.

Specific Aim 1: To explore regulation of caSTAT3 and caSTAT5a activity by estrogen binding domains: EBD(4-OHT) or EBD(CMP8).

Methods:

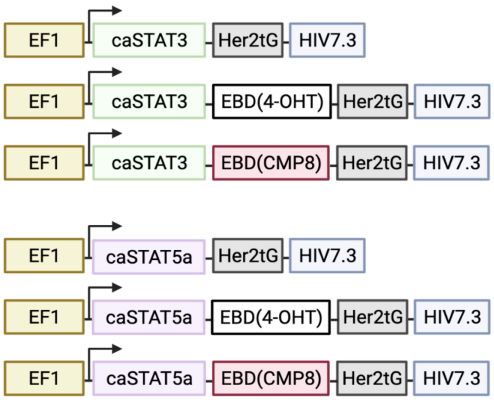


Figure 4: Iterations of caSTAT3 or caSTAT5a fused with EBD(4-OHT) or EBD(CMP8), when driven by EF1

Cloning Plasmids of Interest:

Plasmids containing various combinations of caSTAT3 and caSTAT5a alone or fused with EBD(4-OHT) and EBD(CMP8), seen in figure 4, were produced. caSTAT5b was not cloned into these constructs, or further considered for testing in assays. In an experiment conducted previously observing the growth of CD8+ CAR T cells upon tumor challenges, as shown in figure 5. I tested the functional advantage provided by caSTAT5a-EBD(4-OHT) or caSTAT5b-EBD(4-OHT) when treated with varying concentrations of 4-OHT. As seen in the growth curves for the caSTAT5b-EBD(4-OHT) CAR T cells, highlighted by the yellow box, it is clear the molecule does not provide any proliferative advantage, which was enough evidence to no longer consider it for further functional assays. The molecular cloning process began with synthesizing the fragments necessary for a Gibson Assembly reaction. The vector fragment, containing the EF1 promoter and HIV7.3 backbone (including the Kanamycin resistance gene), was produced utilizing restriction digestion, via the NheI and NotI cutsites. The insert fragments, which consist of the particular caSTAT transgene with or without the EBD, and the Her2tG surface marker, were fabricated utilizing polymerase chain reactions (PCRs). Primers were designed to have overhangs of around 10-15 base pairs, that are complementary to the vector fragment sequence. These DNA fragments were isolated utilizing gel extraction and purification [34]. From there, each of the insert fragments were incubated

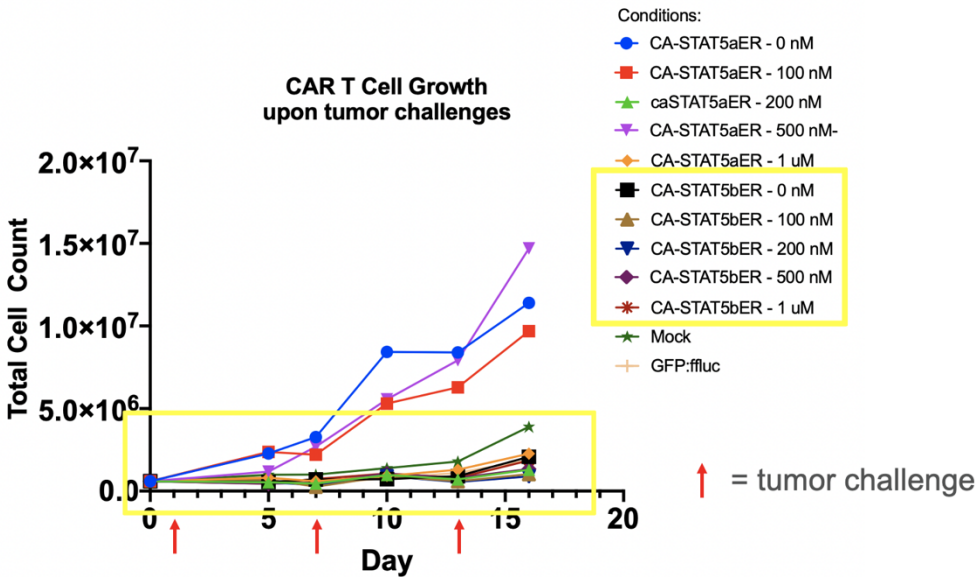


Figure 5: caSTAT5b-EBD(4-OHT) did not provide any proliferative advantage to CD8+ CAR T cells, when subjected to serial tumor challenges

with the vector fragment in individual reactions to undergo the Gibson Assembly procedure. This process employs the enzymatic activity of exonucleases, DNA polymerases and DNA ligases. The exonuclease chews back the DNA at the 5' end, exposing complementary sequences for annealing. The DNA polymerase fills in gaps on the region that anneals with nucleotides, and the DNA ligase removes any nicks by covalently linking the fragments together. The Gibson Assembly products, now circularized DNA, were transformed into NEB Stable Competent *E.coli* (High Efficiency) cells, and plated onto LB (Luria-Bertani) Agar plates with kanamycin. Assuming the kanamycin in the plates will kill any cells that are not expressing the antibiotic resistance gene, all clones that grow out will contain the plasmid of interest. Individual colonies were picked and grown in LB Broth with kanamycin, overnight at 37℃ and 300 rpm. Portions of these cultures were harvested for plasmid preparation, via the Miniprep protocol. This process involves lysing the *e.coli* cells, then purifying the extracted DNA. The Miniprep products were analyzed using Sanger sequencing, to ensure it was the plasmid of interest. Once identifying the colony carrying the correct plasmid, the remainder of the culture was spiked into a large flask with 200 mLs of the LB Broth with kanamycin. This was cultured overnight at 37℃ and 300 rpm. The DNA from this culture was extracted via the Maxiprep procedure, a large-scale plasmid purification process. These products were again analyzed via Sanger sequencing prior to being used in experiments.

Virus Production: In order for cells to express exogenous genes of interest (i.e. CARs, transgenes) the plasmid encoding for these must be delivered into the cells. For the plasmids shown in figure 4, which have HIV7.3 backbones, this transduction will be accomplished via lentiviral delivery. Upon attaching to the cells, the lentiviruses will enter them, and will begin introducing genetic material in the form of RNA. This RNA is then reverse-transcribed into DNA, and integrated into the genome of the cell, allowing the exogenous genetic material to not only be expressed, but also be carried on to daughter cells [35, 35]. Lipofectamine 2000 was used to produce these lentiviruses. On day 1, 3e6 HEK 293t cells were plated. On day two, when the 293ts have reached 70%-90% confluency, the cells were transfected with the plasmids by forming DNA/liposome complexes and applying them to the cells. On day 3, the supernatant media was removed from the plates, and replaced with fresh, complete DMEM and sodium Butyrate. On day four, the lentivirus was harvested from the supernatant media. The titers of the lentiviruses were then determined using Jurkat parental cells.

Establishing STAT Reporter HEK 293t Cell Line:

In order to characterize the activity of the caSTAT dimers, I worked to develop STAT Reporters using HEK 293t Cell Lines. HEK 293ts are an adherent, immortalized human embryonic kidney cell line that expresses a temperature-sensitive mutant of the SV40 large T antigen [37]. The first iteration of the STAT Reporter Lines was created by transducing the 293ts with virus delivering the plasmids shown in figure 6.

Each plasmid has a STAT responsive element (STAT_RE) that is specific to the particular STAT dimer of interest. The STAT_RE essentially serves as a promoter region. When bound, it will activate transcription of the downstream transgene, which in this case is, are various fluorescent markers, and therefore the readout for STAT activity. After one week in culture, these 293ts were transiently transfected with the plasmids shown in figure 4, utilizing Lipofectamine 2000. 24 hours post-transfection, the cells were treated with 4-OHT or CMP8 depending on their conditions, at 250 nM. 24 hours later, the cells were harvested and stained with Live/Dead Near-IR and Herceptin-PE, and analyzed via flow cytometry. The second iteration of the STAT Reporter cell line was established by creating a new set of STAT_RE containing plasmids, following similar molecular cloning techniques as those described under "Cloning Plasmids of Interest." As seen in figure 7, these plasmids include a constitutively expressed CD19t, which is a surface protein that can be utilized for staining purposes. These plasmids were then packaged into viruses and used to transduce the HEK 293t cells. One week post-transduction, cells were harvested to check for successful transduction, indicated by expression of the CD19t marker. The reporter cells were then transiently transfected with the plasmids described in figure 4. In an effort to optimize this protocol, the cells were treated with 4-OHT and CMP8 at concentrations of either 500 nM or 1000 nM at either 12 or 24 hours post transfection. 24 hours later, the cells were harvested and stained with Live/Dead Near-IR, CD19-APC, and Herceptin-PE.

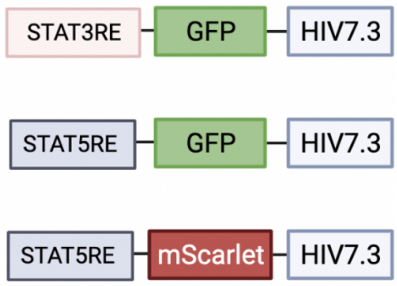


Figure 6: STAT3 or STAT5a responsive elements serve as a binding site for the dimerized STAT3 or STAT5a transcription factors, which reads off GFP or mScarlet

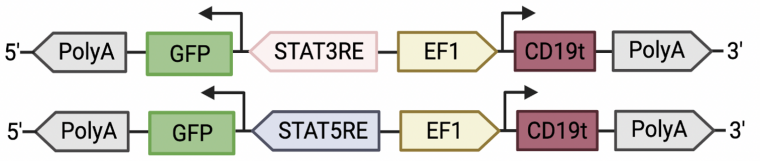


Figure 7: new STAT reporter plasmids were engineered to include CD19t, a surface markers that could be used for gating purposes for analyses

Developing and Testing a Phosphoflow assay for phosphorylated STAT3 and STAT5a:

To understand the state of phosphorylation of caSTAT molecules in T cells when regulated by EBD(4-OHT) or EBD(CMP8), transduced T cells were subjected to analysis via Phosphoflow. Phosphoflow is a method that employs both surface staining and intracellular staining, providing it with the potential for tracking intracellular signaling molecules at a single-cell level [38]. This assay has the potential to be a powerful tool, as it allows for gating on transduced populations based on the expression of surface markers, to help differentiate between endogenous and exogenous gene expression. In particular for this experiment, phosphorylated STAT is found endogenously in T cells, therefore it is important to develop a

method of differentiating this "background" expression from the exogenous phosphorylated STAT molecules that are delivered via the transduced plasmids. Prior to implementing this method of analysis, the protocol had to first be optimized, as it consists of a harsh permeabilization step. This step, although necessary for intracellular staining, may degrade the epitopes of the surface proteins that the staining antibodies bind to, rendering the target population indistinguishable. The iterations of the protocol tested include Pre-Fixation, Post-Fixation and Post-Permeabilization, which pertain to the step during which the cells are stained for the surface marker of interest, as seen in figure 8. The "Standard" condition is a classic surface staining procedure that does not include the permeabilization or intracellular steps and served as the control to ensure Her2 staining is feasible for these cell types. The samples were stained with Herceptin-PE for the Her2 surface marker, and Live/Dead Aqua.

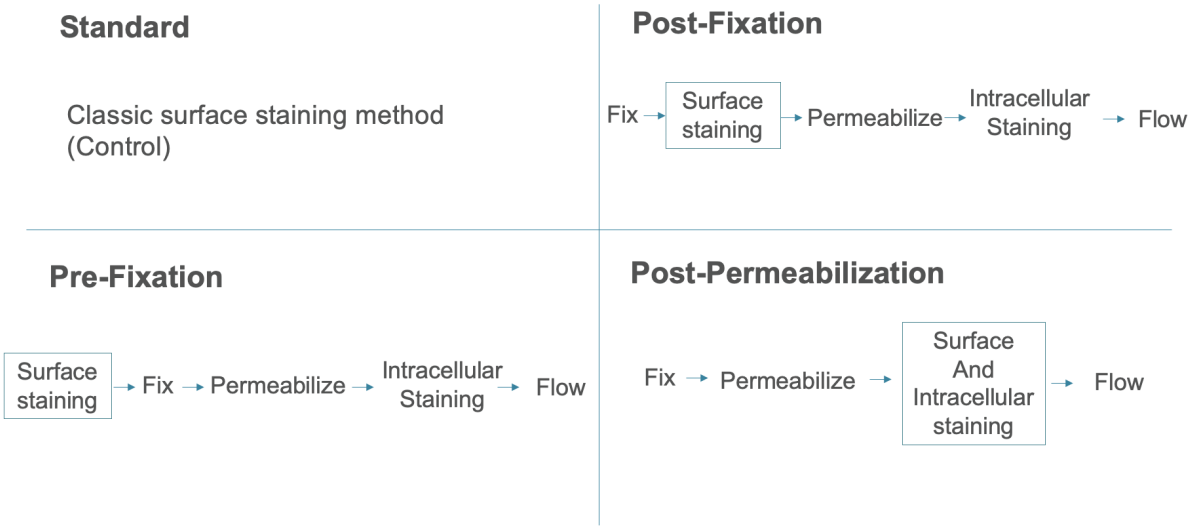


Figure 8: three experimental Phosphoflow protocols were tested and compared to a standard surface staining method, to establish the process that would minimize cell death due to the harsh permeabilization step

The experimental process for optimizing Phosphoflow protocol was performed in parental or Her2+ H9 cells. The parental H9 cells represented a Mock cell population, and for the Her2+ T cell population equivalent, Parental and Her2+ H9 cells were mixed. This is because the Her2+ H9 cell line used was extremely pure in regards to Her2 expression. Meanwhile, in a population transduced with a Her2tG containing plasmid, the Her2 expression would be far less pure. Therefore, mixing the cell types provides a more accurate model. After testing the various protocols in H9 cells, this assay was applied to T cells. CD4+ and CD8+ T cells were transduced with the lentiviruses delivering the plasmids described in figure 4. After 14 days of stimulation via CTS dynabeads, cells were harvested, stained with Herceptin-PE and Live/Dead Aqua, and analyzed via flow cytometry to check for successful transduction. In order for the T cells to eventually be utilized for bulk analysis, the CD4+ T cell populations were enriched for transgene positivity. This process began with staining the cells with the primary antibody, Herceptin-Biotin and the

secondary, Streptavidin-PE. This allowed the Her2+ CD4 T cells to be isolated using the Sony Cell Sorter. These cells were then subjected to a standard Rapid Expansion Protocol (REP), by stimulating them with irradiated peripheral blood mononuclear cells (PBMCs) and lymphoblastoid cell lines (LCLs). Then, the cells were left in culture with penicillin-streptomycin (Pen-Strep), as the Sony Cell Sorter is not a sterile environment, for future assays. The caSTAT5a transduced CD4+ T cell population showed signs of contamination on day five post-transduction, and was therefore bleached. All other conditions reached the end of the 14-day quarantine period without any indication of contamination. The Pre-Fixation Phosphoflow protocol was used to analyze the phosphorylation state of the exogenous caSTAT3 and caSTAT5a expressed in the CD4+ T cell populations. Herceptin-PE and Live/Dead Aqua were used again, and pSTAT3-Alexa Fluor 647 and pSTAT5-Alexa Fluor 647 were used for the intracellular staining. Mock cells treated with either IL-21 or IL-2 served as the positive controls for the phosphorylated STAT3 and phosphorylated STAT5a respectively.

Observing the Growth of CD4+ T cells with Estrogen Binding Domain Regulated caSTAT3 and caSTAT5a:

To understand the influence of estrogen binding domain regulated caSTAT3 or caSTAT5a on the growth of transduced CD4+ T cells, the cells were subjected to analysis via a growth curve assay. On day 8 of the Rapid Expansion Protocol for the cells transduced with plasmids described in figure 4, as explained in "Developing and Testing a Phosphoflow assay for phosphorylated STAT3 and STAT5a," 500,000 cells were pulled from the culture. They were then washed and plated in cytokine-free, compounded RPMI, and grown either in the presence or absence of 4-OHT or CMP8, at 1000 nM. The conditions were performed in duplicates and the cells were counted by hand using the hemocytometer. For analysis purposes, the average cell counts for each condition will be considered. Due to contamination, the caSTAT5a and Mock CD4+ T Cell conditions were not included in this experiment.

Results:

Establishing STAT Reporter HEK 293t Cell Line:

The transduction of HEK 293t cells was shown to be successful via the CD19 positivity. As seen in figure 9, when gated on the unstransduced Mock 293t cells, the STAT3 reporter cell population was around 98% positive, and the STAT5a population was around 32% positive. Although the transduction efficiency of these two reporter cell lines differed significantly, this is acceptable for this experiment, as the samples collected for flow analysis will be gated on the population of interest (CD19+).

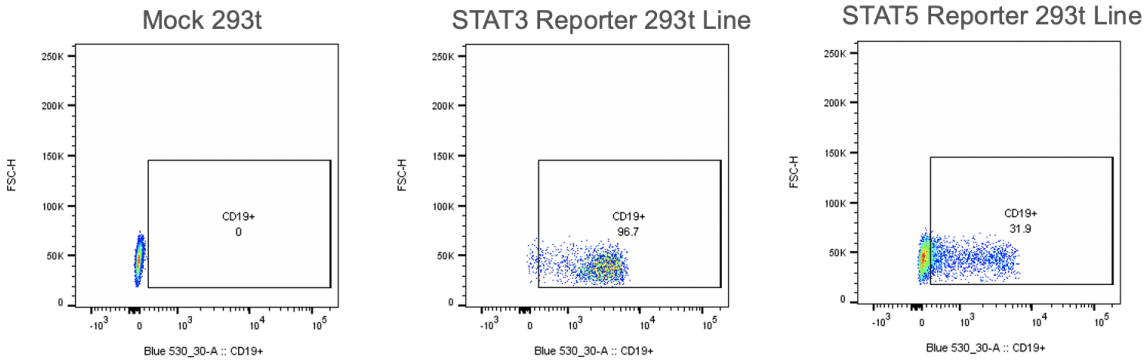


Figure 9: Flow results to analyze the transduction efficiency of STAT reporter plasmids into HEK 293t cells, shows that there was successful transduction for both STAT3RE and STAT5RE, but STAT3RE was more efficient.

The flow plots in figure 10, 11, 12, and 13, show the results of the transfected caSTAT-EBD plasmids in the transduced STAT Reporter HEK 293t cell line. As seen in all four conditions, there is an extremely high GFP background. In particular looking at the STATRE alone samples, in which there is no exogenous STAT present, the GFP signal in the Her2- population is high, indicating this signal is not in the cells carrying the transfected plasmid. It can also be observed in the STAT-EBD(4-OHT) transfected conditions shown in figure 10 and 12, that upon treatment of 4-OHT at both 500 nM and 1000 nM, the dual positive GFP+/Her2+ signal increases, thus indicating a greater amount of STAT binding. Despite these results, the overwhelming GFP background found in these cells makes it difficult to be confident in the results. Therefore this experiment must be repeated to account for this.

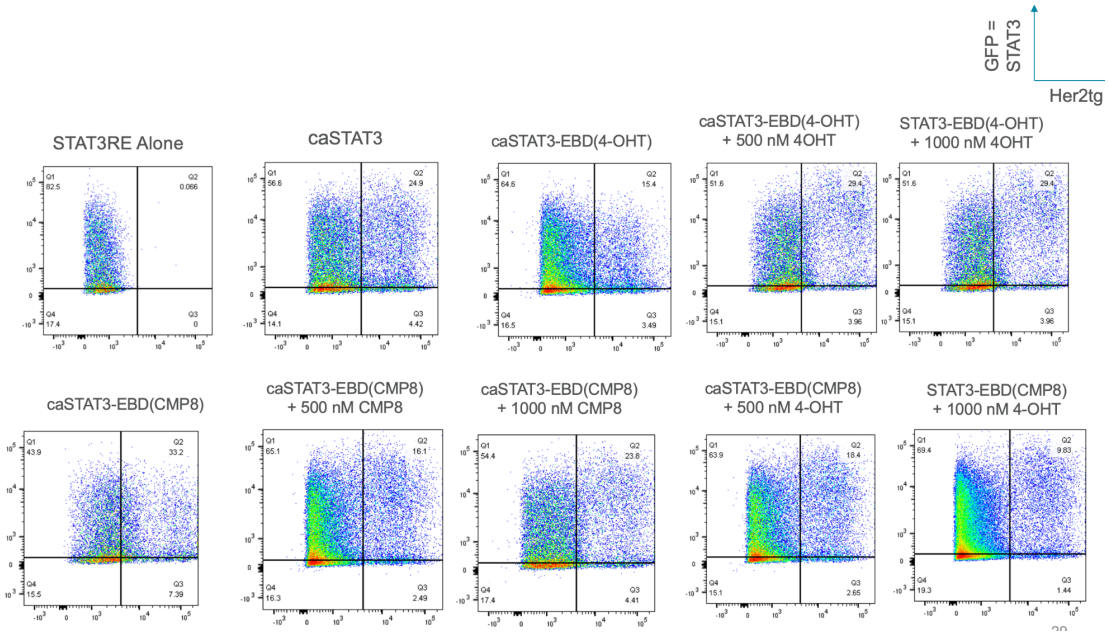


Figure 10: STAT3RE HEK 293t cells treated with estrogen analogs at 12-hours post-EP

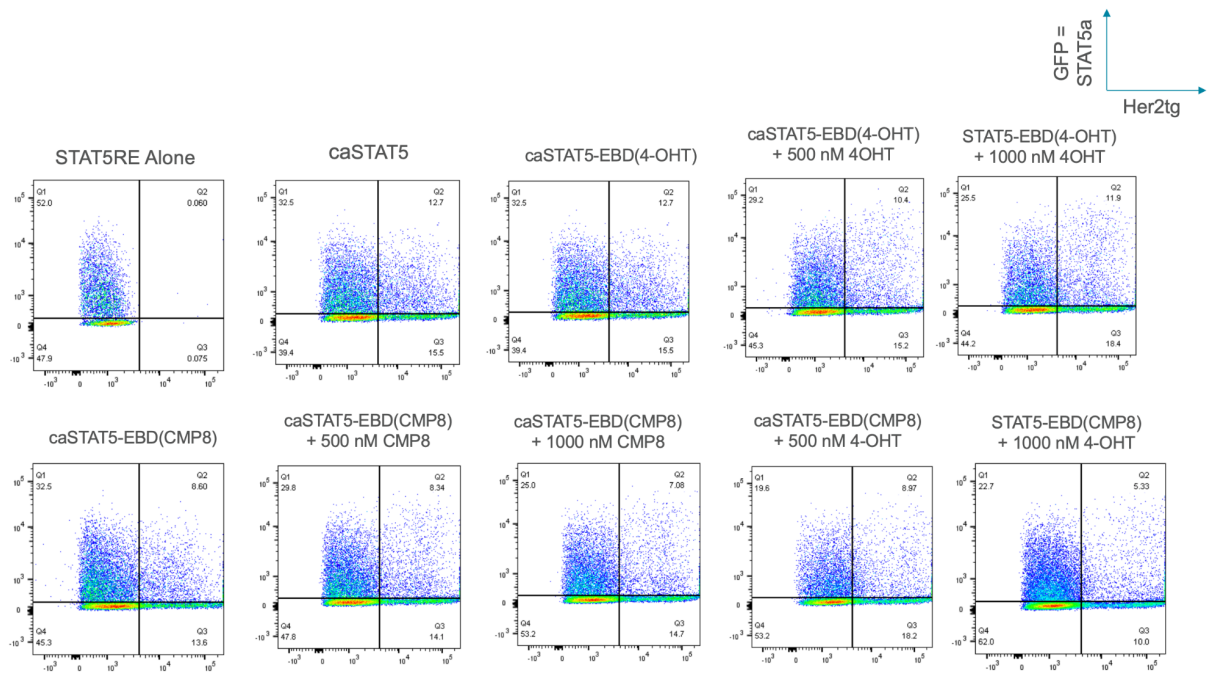


Figure 11: STAT5RE HEK 293t cells treated with estrogen analogs at 12-hours post-EP

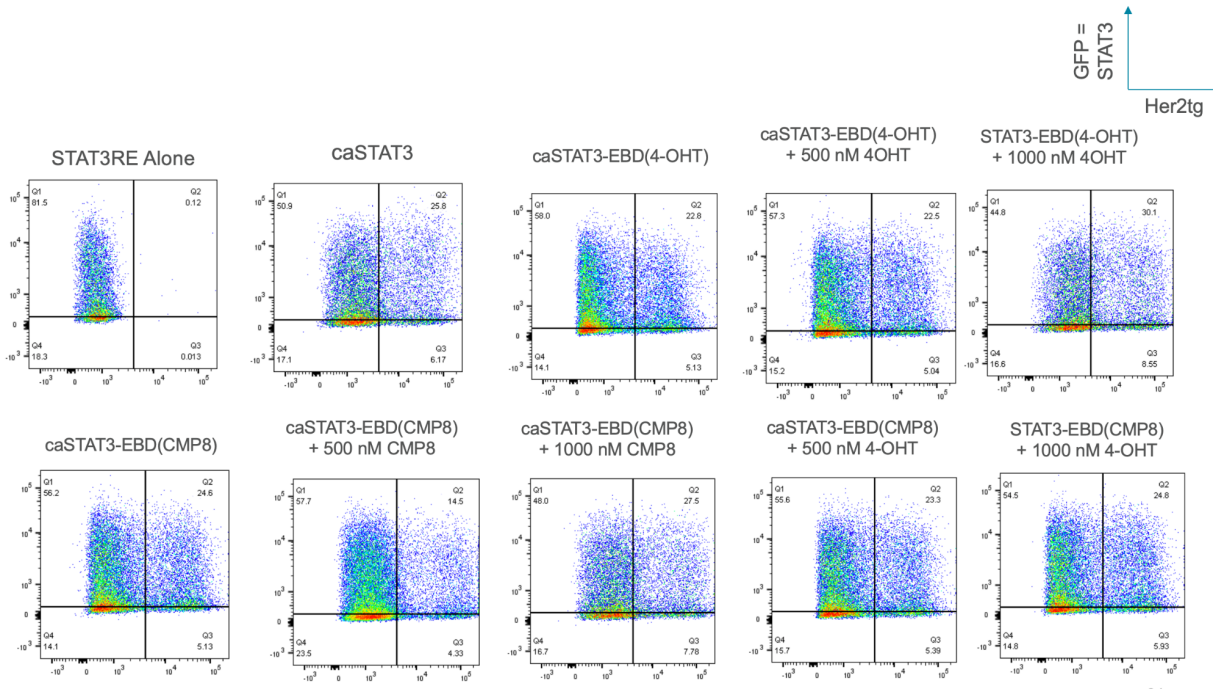


Figure 12: STAT3RE HEK 293t cells treated with estrogen analogs at 24-hours post-EP

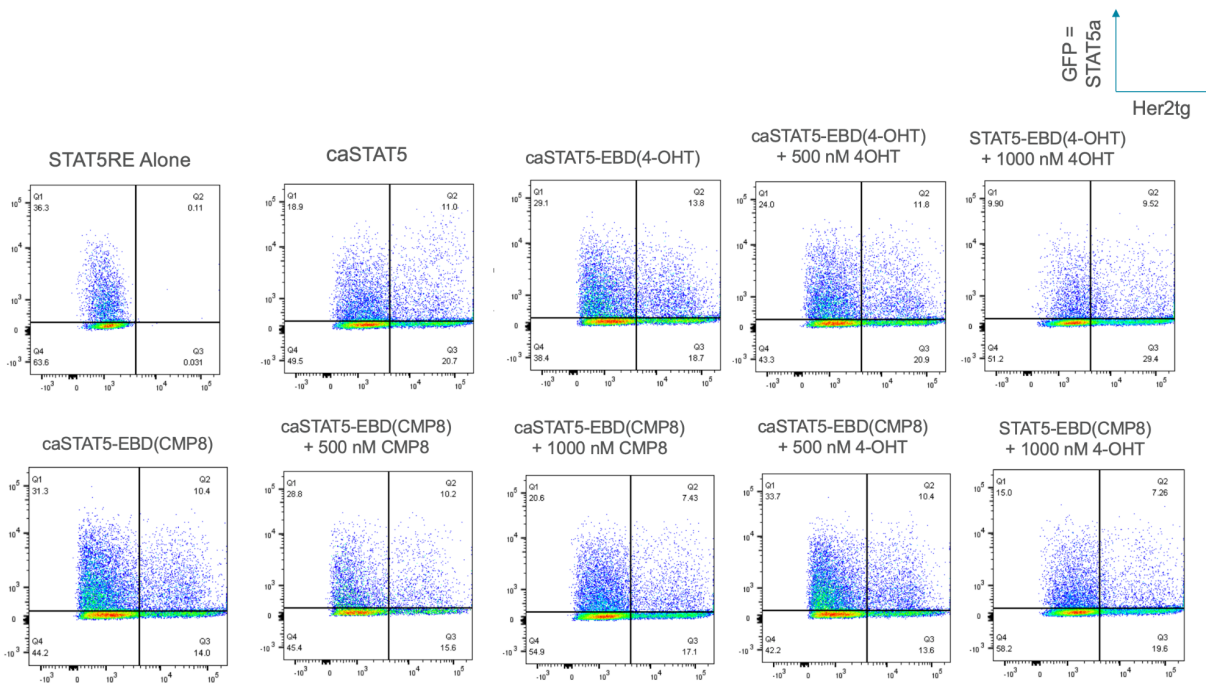


Figure 13: STAT5RE HEK 293t cells treated with estrogen analogs at 24-hours post-EP

Developing and Testing a Phosphoflow assay for phosphorylated STAT3 and STAT5a:

The optimization experiment for the Phosphoflow analysis method revealed that Her2 is detectable in the stained Parental H9/Her2+ H9 cell populations, using any of the staining procedures tested, as seen in figure 14. This indicates that despite the harsh permeabilization step the cells are subjected to, the Her2 epitope can still be bound by the Herceptin-PE antibody, allowing for analysis via Flow Cytometry. Although measurable, the Her2+ population detected using the Post-permeabilization method is sparse, suggesting that the technique leaves fewer viable cells in the sample by the end of the staining procedure. There is a significant Her2+ population presented using the Post-fixation method. However, the Her2+ and Her2- populations are clustered closely and therefore not very distinct. Conversely, the Pre-fixation method provides results showing significant Her2+ and Her2- populations, where there is a distinct shift between them, indicating this is the optimal method to move forward with for further Phosphoflow analyses.

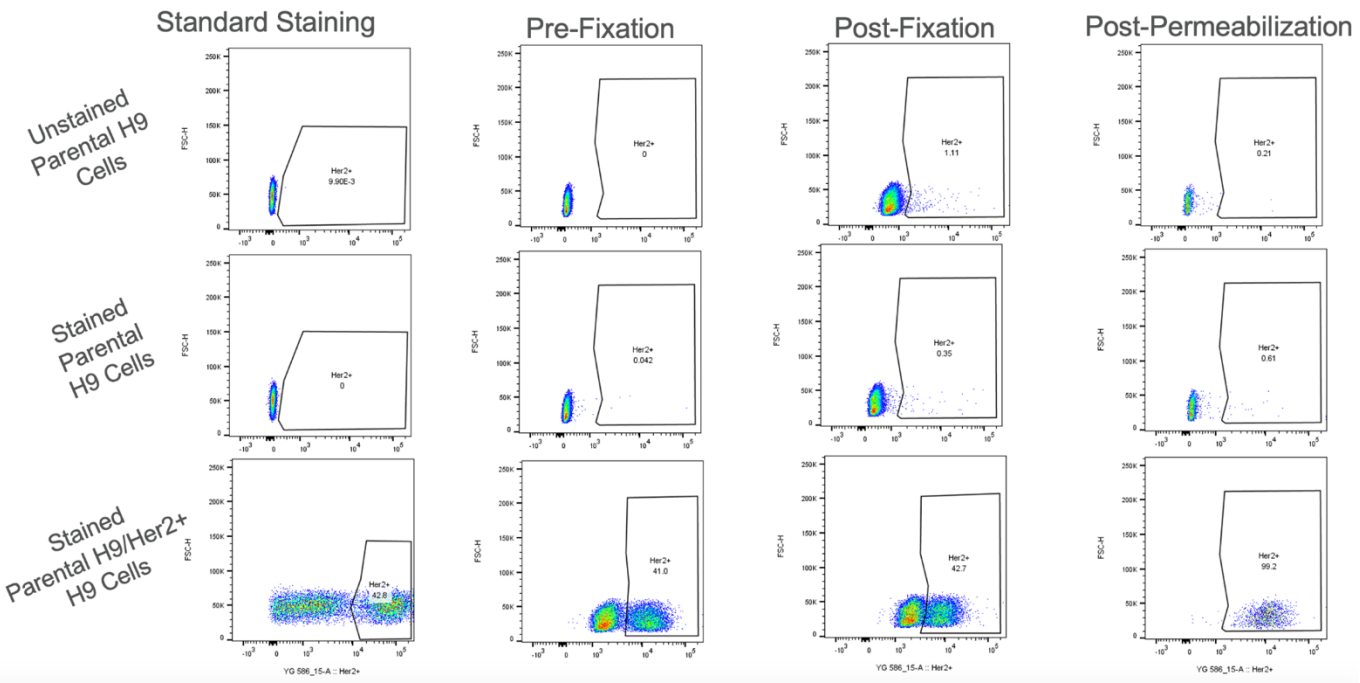


Figure 14: the results of the Phosphoflow protocol opimization experiment, indicates that Pre-Fixation is the method that will provide the most promising results

The transduction efficiency of the plasmids described in figure 4, into CD4+ and CD8+ T cells is shown in figure 15 and 16 respectively. Indicated by Her2 positivity, the transduction efficiency of these T cells varied significantly. In CD4+ T cell transduction, Her2+ ranged from 11-92%, and resulted in a substantial increase in cell count across all of the conditions, after the CTS dynabead stimulation. The CD8+ T cell transduction, alternatively, showed greater and more consistent transduction efficiency, with the Her2 positivity ranging from 68-92%, but resulted in an insignificant increase in cell count following the bead stimulation. Therefore, the transduced CD4+ T Cells were further considered for bulk analyses, but were first sorted and REP'd to ensure more pure and robust populations were available for application in future assays.

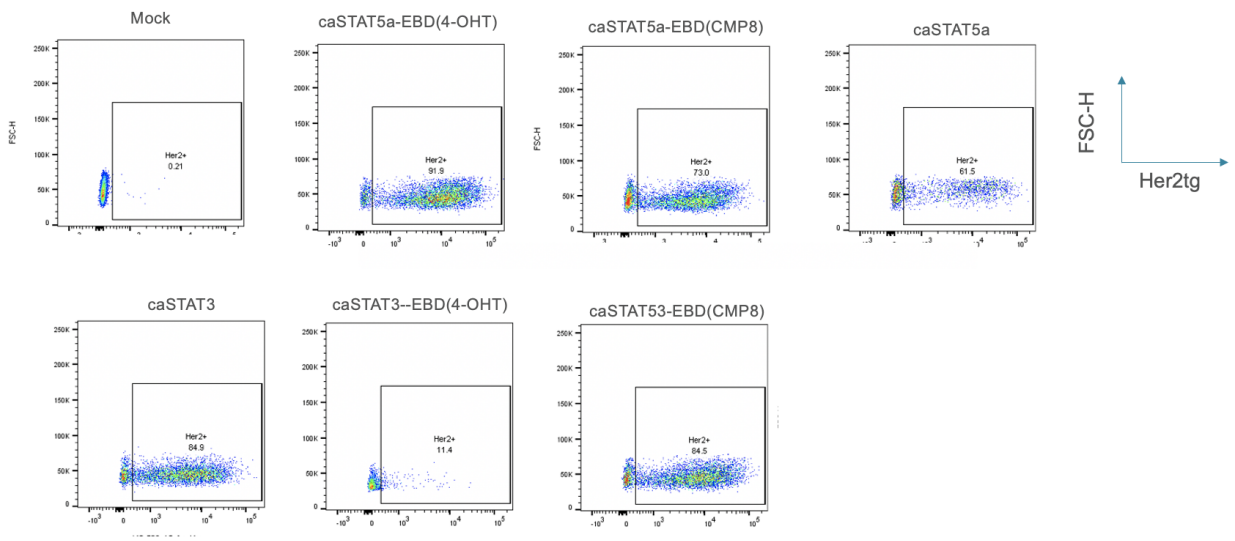


Figure 15: the efficiency of the CD4+ T cell transduction is indicated by Her2tG+

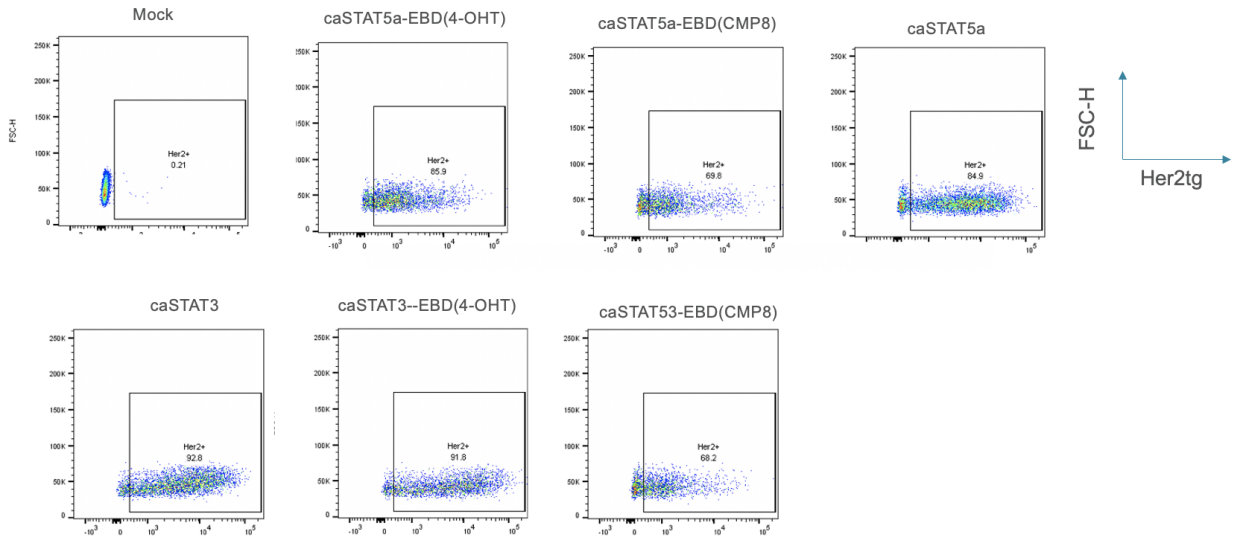


Figure 16: the efficiency of the CD8+ T cell transduction is indicated by Her2tG+

Figures 17 and18 show the results of the Phosphoflow experiment for the caSTAT3 and the caSTAT5a expressing CD4+ T cells respectively. As there is endogenously expressed phosphorylated STAT3 AND STAT5a in T cells, the Mock populations subjected to the entire Pre-Fixation preparation and staining method were used from gating, as the population of interest is the exogenous phosphorylated STAT. With phosphorylated STAT3 or STAT5a (pSTAT3, pSTAT5a) on the y-axis, and Her2tG on the x-axis, the population of interest is the dual positive (STAT+/Her2tG+) population. This quadrant represents the population of transduced cells, indicated by Her2tG+, that are expressing pSTAT+ that is greater than that

of the unstimulated Mock cells. The pSTAT3 positive control of Mock cells treated with IL-21, in figure 17, shows a significant shift in pSTAT3+, indicating that the assay and staining procedure are effective. However, the lack of a dual positive expressing cell population in the pSTAT3 experimental conditions suggests there is no exogenous phosphorylated STAT3 being detected. The pSTAT5a positive control of Mock cells treated with IL-2, in figure 18, also shows that this protocol was successful, as indicated by the shift in the pSTAT5a+. All of the pSTAT5a experimental conditions, whether treated with drug or not, had Her2tG+/pSTAT5a+ populations. These populations represent the Her2tG+ cells that have higher expression of pSTAT5a+ than the Mock cells, meaning they are expressing exogenous pSTAT5a from the transduced plasmid.

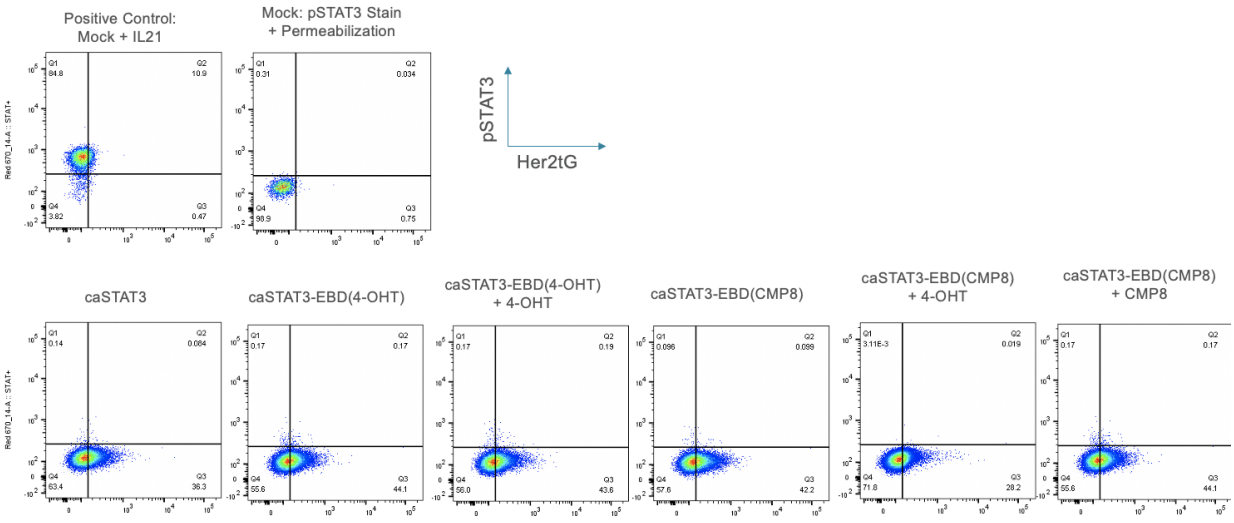


Figure 17: the Phosphoflow results revealed no expression of pSTAT3 above the unstimulated Mock cells, indicating there is no detectable expression of exogenous pSTAT3

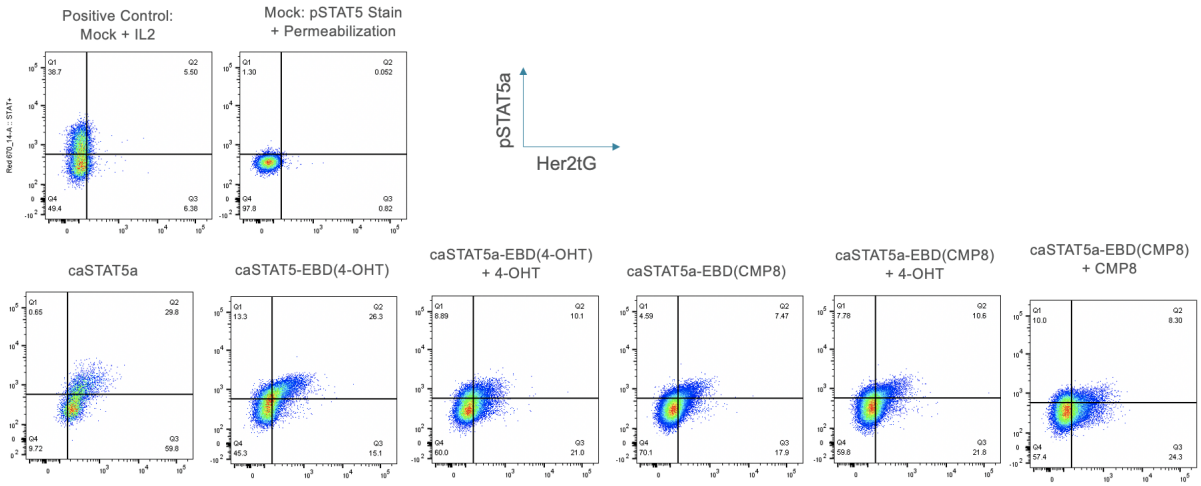


Figure 18: the Phosphoflow results revealed expression of pSTAT5a above the unstimulated Mock cells in the Her2tG+ quadrant, indicating there is some detectable expression of exogenous pSTAT5a

Figure 19 shows the quantification of the percentage of cells in the sampled population that have stronger signals for pSTAT3 or pSTAT5a than that of the baseline signal of the untreated Mock cells. The Mock and positive control conditions are the live, untransduced cell populations, while the experimental conditions are the live, transduced (Her2tG+) populations. The percent of phosphorylated STAT3, shown in the left chart, indicates again that there is virtually no exogenous pSTAT3 in the experimental conditions. As the positive control for pSTAT3 shows that nearly 100% of the cells in the population of interest have pSTAT3+ signal higher than the Mock alone cells, it is clear that this assay worked, ruling that out as the explanation for why there was no pSTAT3+detected in the experimental conditions. Further investigations and experiments will need to be conducted to understand where the issue of a lack of detectable pSTAT3+ expression is occurring. The percent of phosphorylated STAT5a, seen in the right chart, shows that in all of the experimental conditions, there is a significant population of cells that express exogenous pSTAT5a. This suggests that phosphorylation of STAT5a is not hindered by fusion of either EBD(4-OHT) or EBD(CMP8), and therefore will not disturb the molecules' functionality. For the majority of the conditions tested, the percentage of cells expressing higher pSTAT5a+ signal were relatively consistent, and were lower than the percentage shown in the positive control. However, the condition of CD4+ T cells transduced with caSTAT5a-EBD(4-OHT) in the absence of drug, was reported to have a percentage of exogenous pSTAT5a+ cells significantly greater than the experimental and positive control conditions. Further testing will need to be conducted to understand this behavior.

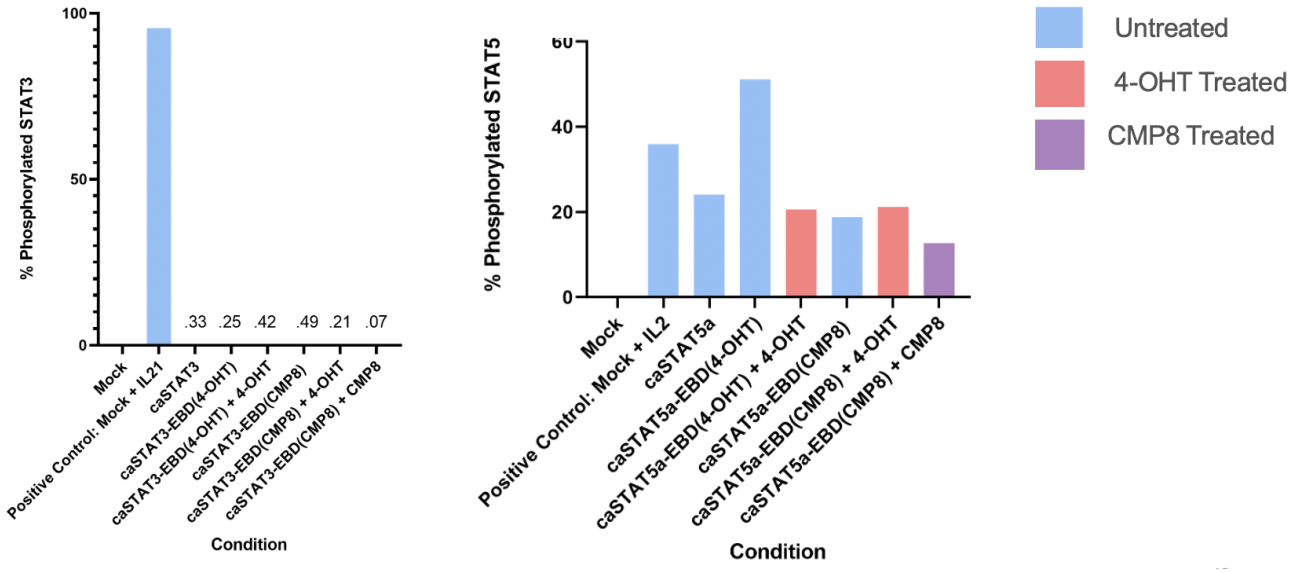


Figure 19: quantification of the percent of cells with pSTAT3 or pSTAT5 expression greater than the Mock cells reiterates and clarifies the results of these assays, and emphasizes the importance of further investigating the pSTAT3 Phosphoflow experiment

Observing the Growth of CD4+ T cells with Estrogen Binding Domain Regulated caSTAT3 and caSTAT5a:

Figure 20 shows the plotted results of the growth curve assay of CD4+ T cells that have been transduced with the plasmids described in figure 4, to understand the effects of caSTAT3 or caSTAT5a with or without the regulation of EBD(4-OHT) or EBD(CMP8).

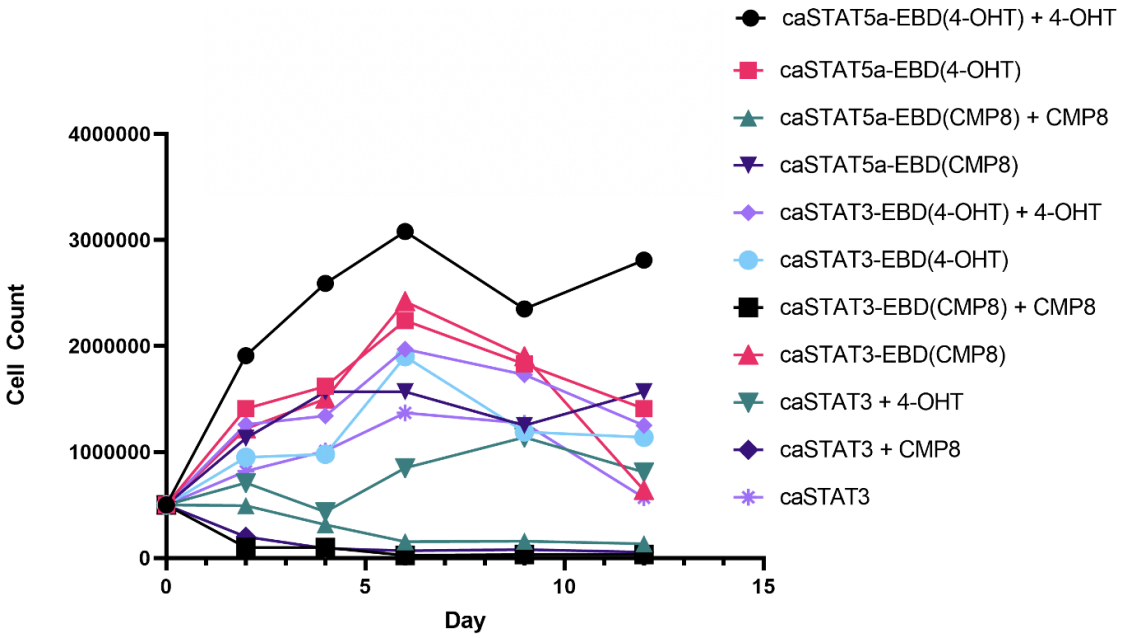


Figure 20: the growth curve assay of transduced CD4+ T cells indicates ca-STAT5a-EBD(4-OHT), when treated with 4-OHT, provides the greatest proliferative advantage

When treated with 4-OHT, the caSTAT5a-EBD(4OHT) CD4+ T cells appear to have the greatest proliferative advantage. This population increased the most in cell count throughout the entirety of the experiment, and by the end of day 12, had the most live cells in culture compared to the other conditions. All the other conditions that were not treated with CMP8 generally had similar proliferative behaviors, where there was a slight increase in cell growth initially, then either a plateau or decline. The counts were never greater than the caSTAT5a-EBD(4-OHT) treated with 4-OHT condition at each time point. All the conditions where the cells were treated with CMP8 (caSTAT5a-EBD(CMP8),

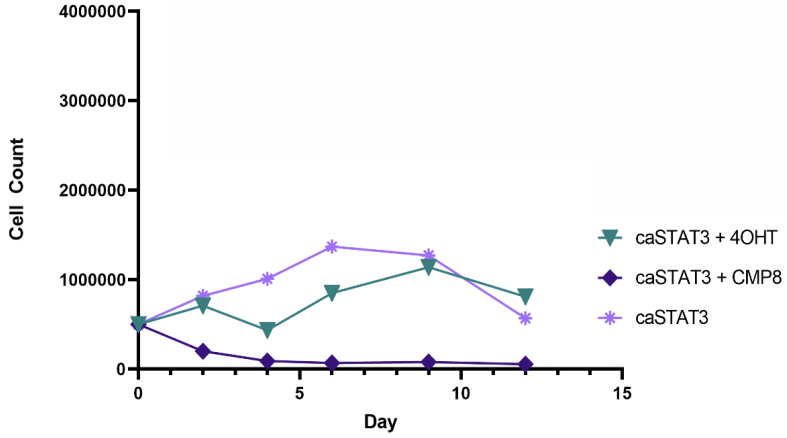


Figure 21: caSTAT3 CD4+ T cells treated with CMP8 immediately declined in cell count, indicating the drug may be inducing a cytotoxic effect

caSTAT3-EBD(CPM8), caSTAT3) showed immediate and continuous decline in cell count throughout this experiment. This suggests that CMP8 may be inducing a toxic effect on these transduced CD4+ T cells. This is further supported by looking at figure 21, which highlights caSTAT3 transduced T cells either in the absence of drug, or treated with 4-OHT or CMP8. As these cells have caSTAT3 without the EBD fused to it, it is expected that they will all behave similarly as none rely on binding of an estrogen analog to activate function. The caSTAT3 and caSTAT3 + 4-OHT conditions show relatively comparable cell counts, where there is an initial increase, until reaching a plateau in counts, indicating that 4-OHT itself is not likely to be causing cell death. However, caSTAT3 + CMP8 shows an overall decline in cell count, that is lower than those of the compared conditions, emphasizing the cytotoxic effect the drug has on the treated cells. As there was no Mock or caSTAT5a alone conditions, it can not be concluded if caSTAT5a-EBD(4-OHT) has a proliferative behavior that is equivalent or greater than that of caSTAT5a, or if the conditions tested provided proliferative advantage over Mock cells, thus this experiment will need to be repeated.

<u>Specific Aim 2:</u> To characterize caSTAT3 and caSTAT5a function in primary human T cells when constitutively expressed and under the regulation of a CAR-inducible promoter.

Methods:

<u>Cloning Plasmids of Interest:</u>

Following similar molecular cloning procedures as those described in "Cloning Plasmids of Interest" under Specific Aim 1, the constructs of interest for this aim, shown in figure 22, 23, and 24 were produced. These plasmids are dual promoter systems, where the first promoter is either an experimental constitutive promoter (hPGK, polyUbc, CMV, or EF1) or the CAR-inducible promoter, iSynPro, driving transgenes. The transgene in figures 22 and 23 is GFP, the green fluorescent protein, and in figure 24, is either caSTAT3 or caSTAT5a with Her2tG, which is surface marker. The second promoter is EF1, driving the anti-CD19 CAR, DHFRDm, which is the Methotrexate resistance gene, and EGFRt, which is a surface marker. The constructs shown in figure 22 have a piggybac PMK backbone, which has a kanamycin resistance gene, therefore the *e.coli* cells transformed with these constructs were grown on LB Agar plates with kanamycin, and cultured in LB Broth with kanamycin. The constructs shown in figures 23 and 24 have the piggybac nanoplasmid (PBNP) backbone, thus the transformed *e.coli* cells were grown on LB Broth Agar Plates with 6% Sucrose and no salt, and in LB Broth with no salt and 6% sucrose. iSynPro, in the context of a dual promoter system, has been implemented frequently in our lab, and thus, is well characterized. However, no constitutive dual promoter system capable of expressing similar dual-positivity to CAR-stimulated iSynPro dual promoter systems, has been established. It is important to identify a constitutive promoter that can be implemented in this

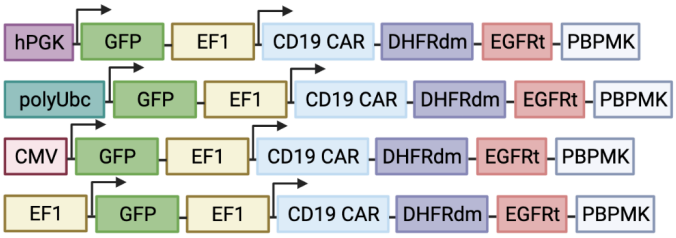


Figure 23: experimental plasmids with GFP driven by various constitutive promoters, with a PBNP backbone

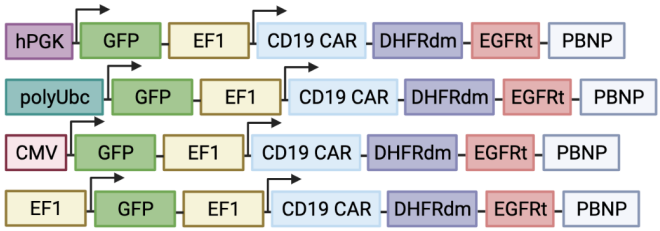


Figure 22: experimental plasmids with GFP driven by various constitutive promoters, with a PBNP backbone

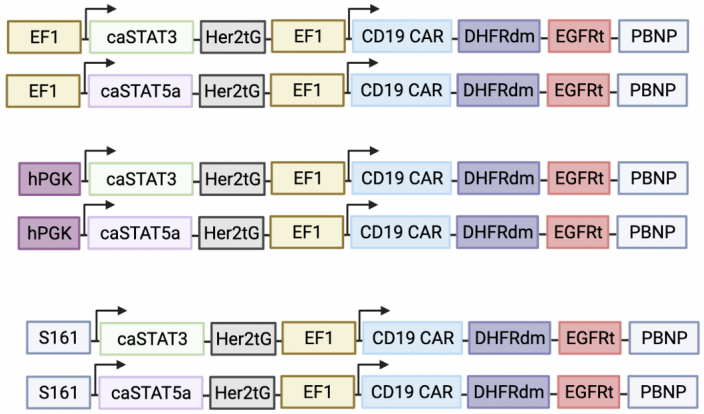


Figure 24: experimental plasmids with caSTAT3 or caSTAT5a driven by constitutive promoters: EF1 and hPGK, or the CAR inducible promoter, iSynPro (S161)

dual promoter system, so that it can serve as a comparison to iSynPro for expressional and functional assays. This comparison is particularly relevant to this project, as it pertains to understanding the level of regulation of caSTAT3 or caSTAT5a that will provide CAR T cells with the greatest functional advantage.

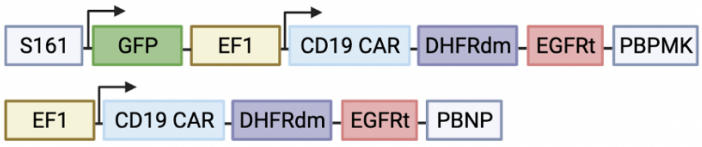


Figure 25: iSynPro dual promoter plasmid, and CAR alone control plasmid that were used as controls for the electroporation and experimental processes

Electroporating CD4+ and CD8+ T cells to produce CAR T cells:

As the plasmids described in figure 21, 22 and 23 have piggbac backbones, they were transfected into cells utilizing non-viral delivery methods. The plasmids described in figure 25 were also transfected to serve as controls. Electroporation is a process in which genetic material, carried via plasmids, can be transiently or stably integrated into the cell genome. The procedure involves resuspending cells in a conductive solution, called electroporation buffer, with the plasmid of interest. This solution and its contents will then be subjected to a quick, electrical pulse that disturbs the phospholipid bilayer of the membrane, causing the formation of pores temporarily. At this point the electric potential across the cell membrane will also be rising, allowing for charged molecules, such as DNA, to drive across the membrane through the pores [39].

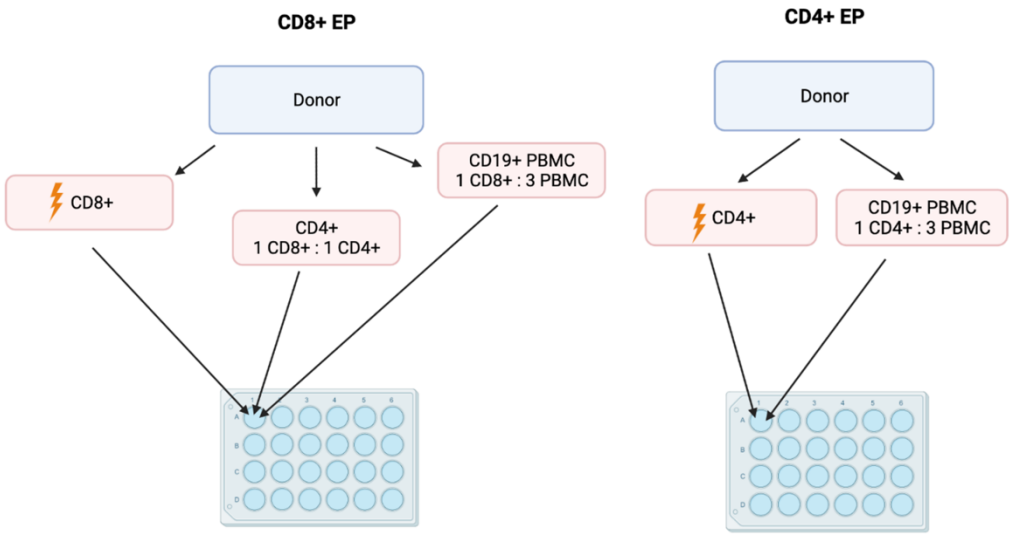


Figure 26: the "TICLE" procedure for CD8+ and CD4+ elecroporations require addbacks of CD19+ PBMCs from the same donor, allowing for CAR expressing T cell limited expansion

As these dual promoter constructs are quite large (nearing 10 kilobases), previous attempts to electroporate them into T cells has yielded low efficiency, therefore the "TICLE" method was employed. "TICLE" which stand for: T cell Interface for CAR Limited Expansion, is a CAR T Cell electroporation and expansion method developed in the Jensen Lab, that has shown the potential of producing a robust CAR T cell population even following an electroporation with an extremely low efficiency. As displayed in figure 26, the procedure for a CD8+ or CD4+ CAR T cell "TICLE" varies. In the case of CD8+ CAR T cells, the CD8+ T cells are electroporated with the plasmids

of interest, and immediately added to a 24-well Grex. In these wells, there is an addback composed of CD4+ T cells at a one-to-one ratio to the electroporated CD8's, and CD19+ PBMCs at a three-to-one ratio, from the same donor. As the PBMCs express CD19, the electroporated CD8+ CAR T cells are stimulated through their anti-CD19 CAR. Similarly for the electroporated CD4+ CAR T Cells, these cells are plated with an addback of CD19+ PBMCs only, and are stimulated through their anti-CD19 CAR. In addition to the anti-CD19 CAR specific expansion, these cells were also treated with Methotrexate (MTX) at a concentration of 50 nM, with the intention of killing any cells that do not express the DHFRdm gene, or essentially any cells that were not successfully transfected. This multi-layered selection method is intended to churn out extremely pure CAR T cell populations by the end of the 21-day cell production period.

Utilizing Incucyte analysis to understand cytotoxicity of CAR T cells with caSTAT in Dual Promoter Systems:

In order to compare the cytotoxic effect of the various CAR T cells described in "Electroporating CD4+ and CD8+ T cells to produce CAR T cells," and further establish a constitutive promoter that is most effective in the context of the Constitutive Dual Promoter system, Incucyte analysis will be employed. The Incucyte is a live cell imaging and analysis platform that tracks the fluorescent signal of cells over time. For the assays described in this section, the Incucyte will be tracking the mCherry expressing target cells, as shown in figure 27. The first target line considered were Raji cells, which are a human B lymphoblastoid cell line that are naturally CD19 expressing and transduced with an mCherry containing plasmid. The other, were Be2s, an adherent neuroblastoma cell line, that were transduced with an mCherry and CD19 containing plasmid. 10,000 target cells were plated in each well of flat-bottom, 96-well Nunc-edge plates. The Nunc-edge plates have reservoirs along the outer perimeter of the plate, which when filled with PBS, can help minimize evaporation in the wells that contain cells. The effector CD4+ and CD8+ CAR T cells were

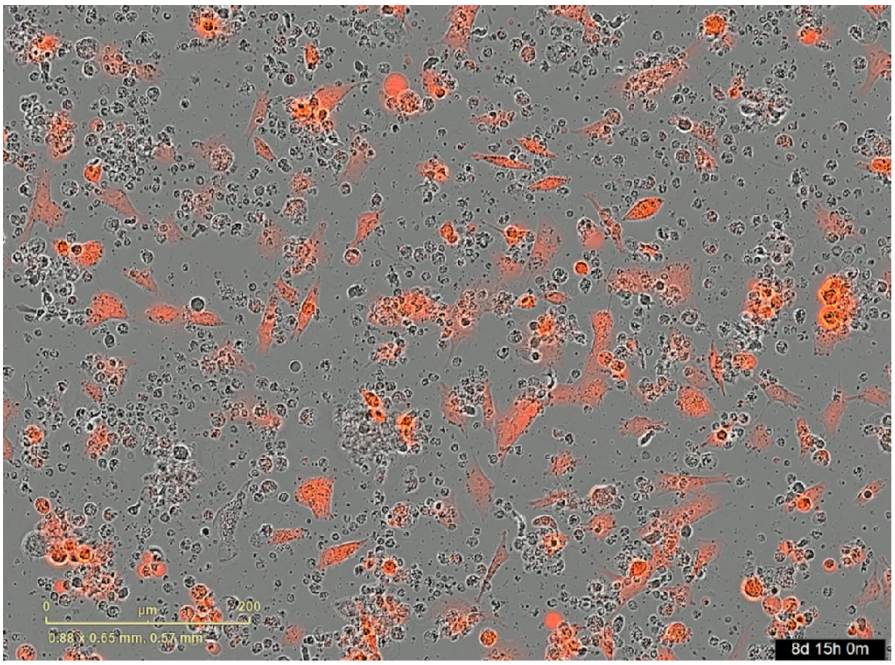


Figure 27: Incucyte image 8 days after the initial plating of CAR T cell effector cells and mCherry labeled Be2 target cells

plated at 4:1 (40,000:10,000) or 1:1 (10,000:10,000) effector to target ratios, based on the CAR positivity of each population on day 22 post electroporation. Each well contained 200 uLs of cytokine-free, compounded RPMI. Tumor challenges and half media changes occurred every three to four days with 10,000 target cells and fresh media. All the produced CAR T cells were subjected to multiple Incucyte *in vitro* analysis, with the exception of the hPGK-caSTAT5a CD8+ CAR cells. The cell expansion of this population was minimal, producing only enough for one assay. Each condition was performed in duplicates on the same plate, where the Incucyte software acquired the average of the two wells and applied those results when conducting any analyses. In addition to duplicates on the same plate, duplicates of the entire 96-well plates were run with identical layouts to confirm results.

Results:

Cloning Plasmids of Interest:

A previous experiment to identify a constitutive dual promoter system that has comparable expressional results as iSynPro, considered MND as a potential candidate. The eight plasmids of interest are shown in figure 28. As shown in figure 29, this assay looked at GFP expression, which is the marker downstream of either MND or iSynPro on the x-axis, and EGFRt expression, which represents CAR positivity on the y-axis, when unstimulated, or stimulated using Raji Cells or PMA/Ionomycin. In the GFP alone conditions, shown on the first row of figure 29, the MND cells show some GFP+/EGFRt+ dual positivity. However, the signals are substantially lower than those of the stimulated iSynPro population. Even looking at the unstimulated conditions, the dual positivity of the MND cells is only slightly higher than in iSynPro cells, which should have virtually no GFP positivity, as iSynPro is not active, and therefore any signal is due to leaky expression. Upon introducing a transgene (caSTATa-EBD(4-OHT), caSTAT5a, or caSTAT5b-EBD(4-OHT)) in front of GFP, there is essentially no dual positivity in the MND cells, while the iSynPro cells continued to have strong dual positivity. These results motivated the need to investigate other potential candidates for the constitutive dual promoter system.

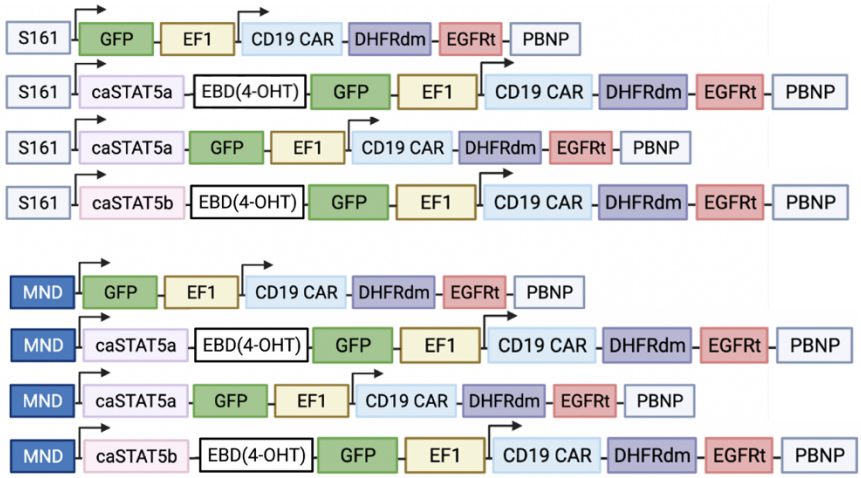


Figure 28: previous experiments, with the intention of identifying a constitutive promoter to use in a dual promoter system, applied MND and compared it to iSynPro (S161)

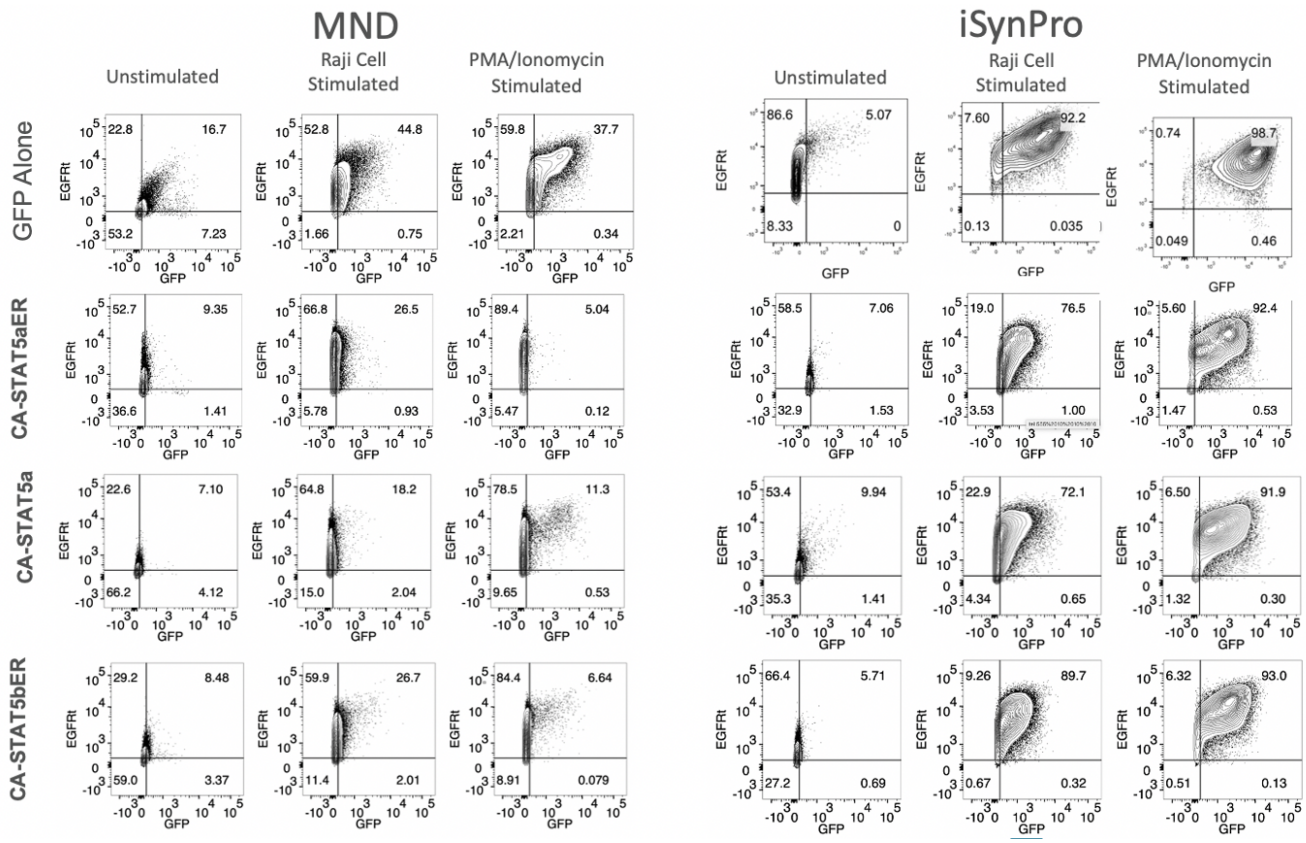


Figure 29: flow results of MND or iSynPro driving GFP, the marker downstream of caSTAT, indicates that in the EGFRt (CAR) positive populations, MND hinders GFP expression, compared to iSynPro

Electroporating CD4+ and CD8+ T cells to produce CAR T cells:

The plasmids shown in figure 22 were electroporated into CD8+ T cells to serve as an initial characterization of the constitutive promoters, EF1, CMV, polyUbc, and hPGK, being considered. While in culture, these experimental CAR T cells did not grow out much. When analyzed via flow cytometry on day 14 and day 21 post-electroporation, as seen in figure 30, there was very little expression of either GFP or EGFRt, which would indicate successful transfection. Other members of the Jensen lab have reported similar challenges with electroporation efficiency when using plasmid with the PBPMK backbone. Therefore, identical constructs were cloned, but with the PBNP backbone instead, as shown in figure 23.

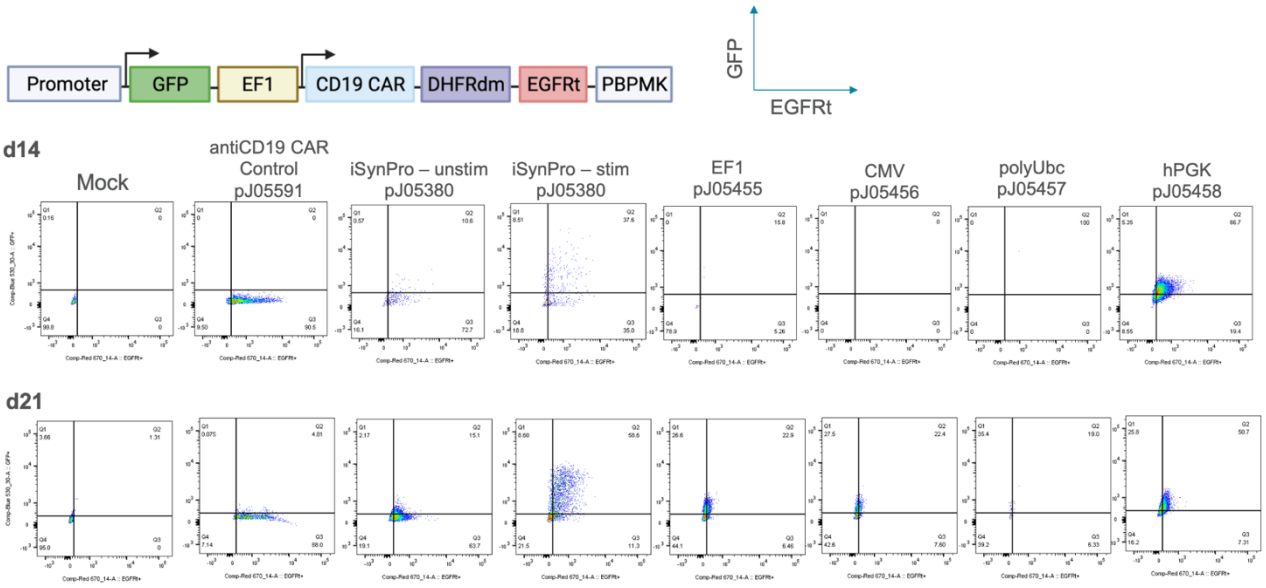


Figure 31: transgene positivity for these dual promoter systems with PBPMK backbones was weak, indicating the plasmids required further development

These plasmids from figure 22 were tested in both CD4+ and CD8+ T cells. The expression of these cells on day 21 post-electroporation are shown in figure 31. In the experimental constitutive promoters, the GFP positivity differs for either CD4+ or CD8+ T cells. This is most evident in the case of polyUbc, where the GFP signal is extremely high in CD4+ cells, but in CD8+ cells, there was extremely limited cell growth, and therefore the GFP signal was nowhere near comparable. This behavior was also observed in the CMV cells. Although the signal was not as high as in polyUbc, the GFP signal was significantly greater in CD4+ T cells compared to CD8+ T cells. Meanwhile, in both EF1 and hPGK, there was a

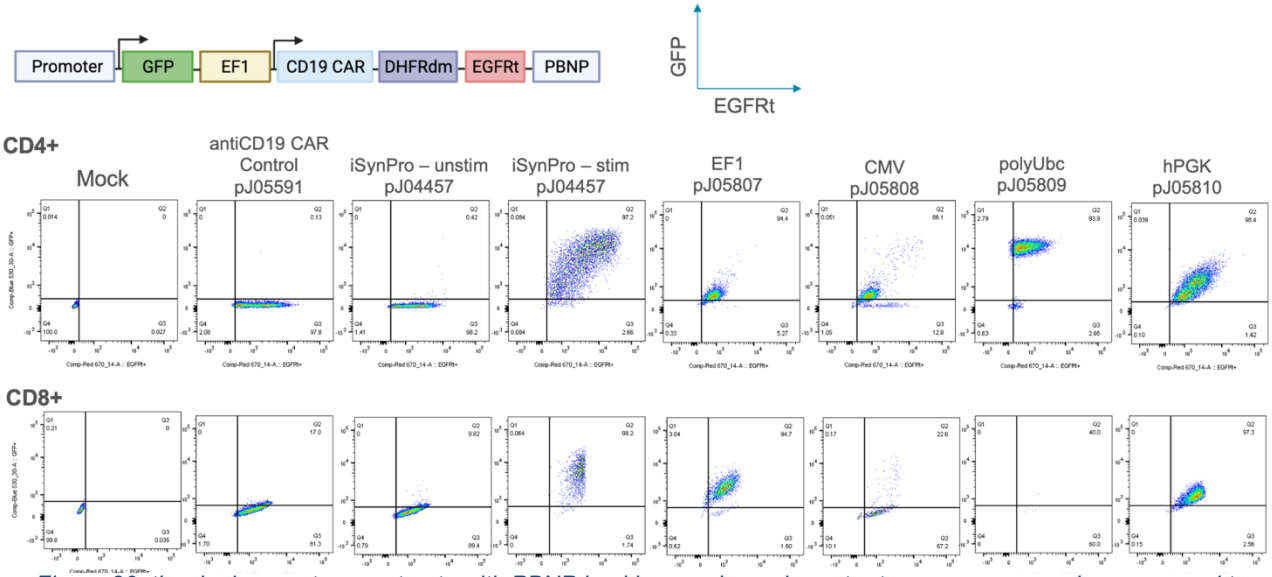


Figure 30: the dual promoter constructs with PBNP backbones showed greater transgene expression compared to those with PBPMK backbone when electroporated into CD4+ and CD8+ T cells

robust dual positive population in both CD4+ and CD8+ cells. Therefore, these promoters were carried forward for further testing, driving caSTAT3 of caSTAT5a.

The electroporation efficiency of the plasmids described in figure 24, in both CD4+ and CD8+ CAR T cells was analyzed on day 22 post-electroporation. The electroporation efficiency of "Donor A' was quite low, in both CD4+ and CD8+ T cells, as seen in figures 32 and 33 respectively. Additionally the expansion of the cell population in this donor was not substantial. For these reasons, these cells were not further considered for testing purposes. Although the results of the constitutive promoter assay were not conclusive, another condition that was tested was the electroporation setting used on the Lonza Nucleofector. The EO-115, or the "high functionality" program is used most frequently in the Jensen lab, however, here it was compared to F1-115, the "high efficiency" program in the CAR control conditions. In the CD4+ T cells, the EGFRt positivity, representing the CAR positivity, is the same for both EO-115 and F1-115 programs. However, by the end of the TICLE procedures, the overall cell count for the cells transfected using the F1-115 program was nearly three times greater than that of the EO-115 cell counts. For CD8+ T cells, there was extremely low CAR positivity expressed in the cell population electroporated using the EO-115 program, indicating the electroporation was inefficient, and resulted in minimal cell expansion. Conversely, the cell population electroporated with the F1-115 program had strong CAR positivity and had robust cell expansion by the end of the TICLE process. In the context of the CD8+ T cell nucleofection, it is not clear if the advantage of the cell growth was attributed to the nucleofection program itself, or just an inefficient electroporation. Meanwhile, in the context of the CD4+ T cell nucleofection, the results are more indicative of the program itself being what provides the F1-115 the cell growth advantage.

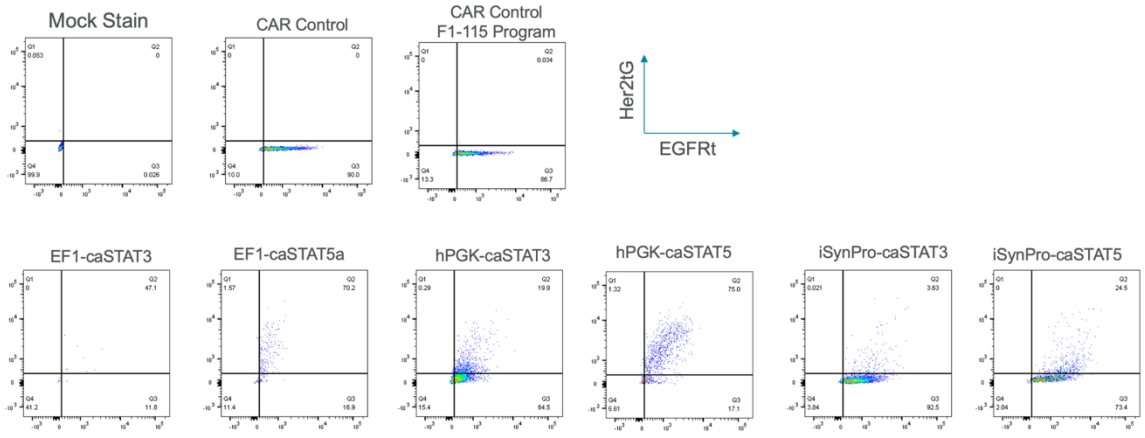


Figure 32: flow results for CD4+ T cells from Donor A showed some electroporation efficiency, but cell growth was so low there were few events to record

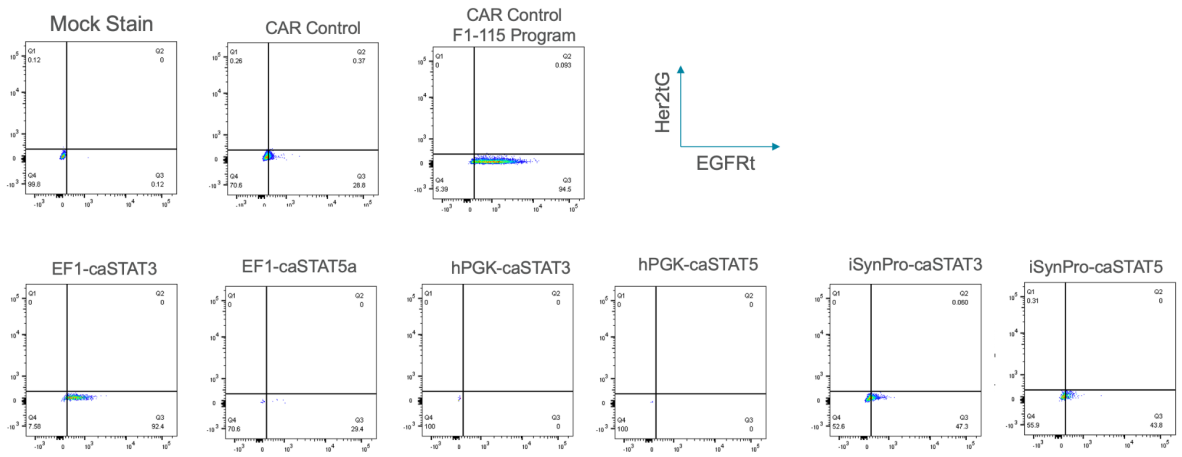


Figure 33: flow results for CD8+ T cells from Donor A showed poor electroporation efficiency. Additionally, cell growth was so low there were few events to record

Donor B on the other hand had substantial expansion in terms of cell growth, with some of the conditions reaching nearly 300 million cells by the end of the 21-day cell production period. On day 22, cells were harvested and analyzed via flow cytometry. As seen in figures 34 and 35, Donor B showed much greater dual positivity in both CD4+ and CD8+ cells compared to Donor A. Dual positivity is much greater in CD4+ CAR T cells than CD8+ CAR T cells. In the CD8+ cells, the EF1-caSTAT3 and hPGK-caSTAT3 expressed little to no dual positivity. hPGK-caSTAT5a had the strongest dual positive signal in CD8s, with EF1-caSTAT5a closely behind. Unfortunately, iSynPro-caSTAT3 and iSynPro-caSTAT5a were only stained when unstimulated, and therefore there is no iSynPro activation, which would drive expression of the exogenous STAT and Her2tG. This flow should be repeated with stimulated iSynPro conditions, to compare the potential dual positivity of the constitutive promoter systems. Despite some issues of dual positivity in the CD8+ CAR T cells, donor B was carried forward for application in functional assays

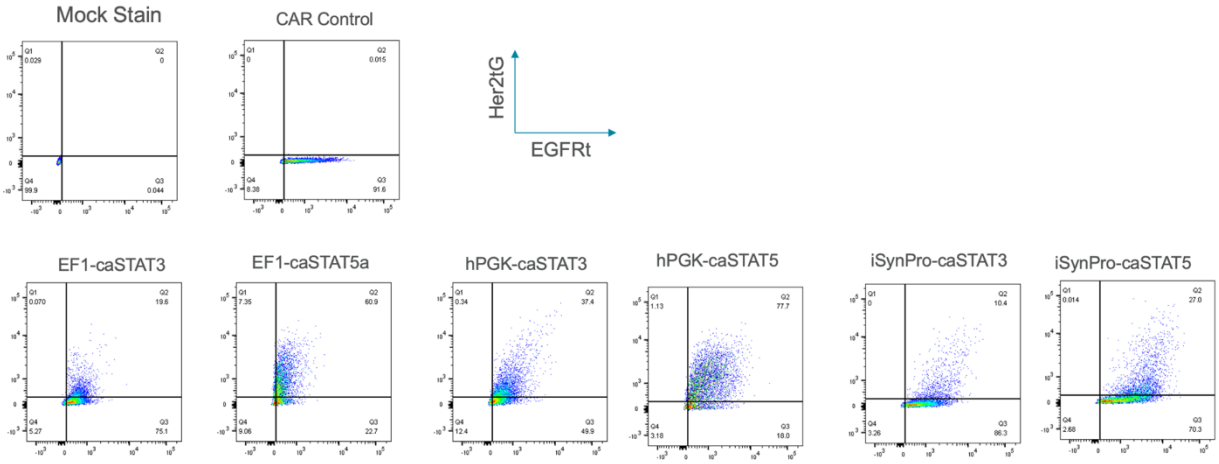


Figure 34: flow results for CD4+ T cells from Donor B showed strong transgene expression in the majority of the conditions, indicating successful electroporations

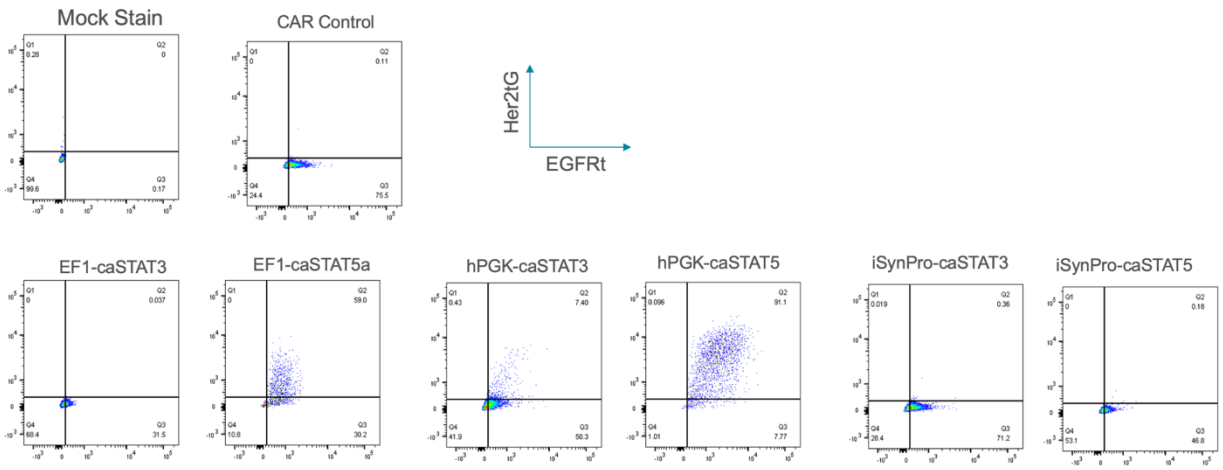


Figure 35: flow results for CD8+ T cells from Donor B showed varying levels of transgene expression, and therefore electroporation efficiency in the majority of the conditions

Utilizing Incucyte analysis to understand cytotoxicity of CAR T cells with caSTAT in Dual Promoter Systems:

The initial Incucyte assay for testing functionality of the CAR T cells, utilized mCherry+/CD19+ Raji cells. As shown in figure 36, which is an image of the well with Raji cells being treated by the Mock CD4+ T cell at five days after the initial plating, clumping of the target cells became a significant problem. These red clumps of cells made interpretation and analysis of the cell behaviors, in both a quantitative and qualitative sense, difficult to interpret. The Incucyte software was not capable of distinguishing cells that were clumped together, skewing the Red Fluorescent readout. Additionally, it was not obvious if the T cells and target Raji cells were mixed uniformly in the culture, which would allow them to interface and interact, and subsequently lead to killing. As the Raji cells began to clump in certain corners of the well, the effector cells often remained in other corners. Although this experiment did not provide reportable results, the observations informed better techniques for subsequent Incucyte assays.

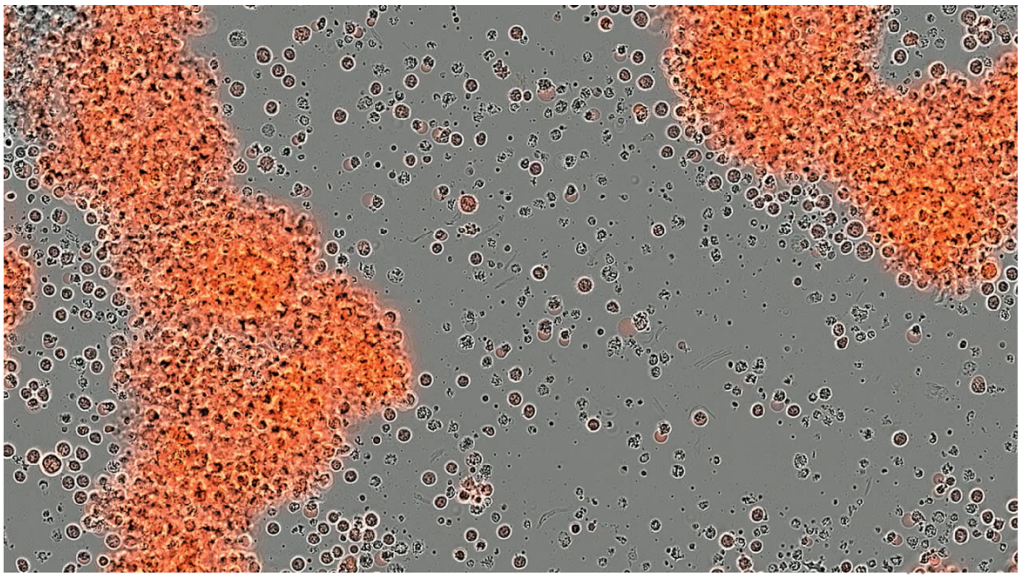


Figure 36: Incucyte image of CD19+/mCherry+ Raji cells (red) clumping in a 96-well plate, when treated with Mock CD4+ T cells

mCherry+, CD19+ Be2 cells were used in place of Raji cells for the following experiments, as this target cell population showed significantly less clumping behaviors. The results of two identical 96-well plate layouts are reported below. Figure 37 is showing the total red object integrated intensity for the various conditions, representing the mCherry signal of the target Be2 cells over the period of 13 days, when subjected to CD4+ CAR T cells with varying conditions. These plots show a broad overview of the behavior of the cells, where the Mock control wells had the overall highest mCherry signal throughout the entirety of the experiment, represented by the highest line of the plots for both Plate 1 and 2. This indicates that the transgenes of interest (caSTAT3 and caSTAT5a) are providing some cytotoxic advantage to the CAR T cells under some of the promoters being considered (EF1, hPGK, iSynPro). Interestingly, EF1-caSTAT3 is also appears to hinder the target cells almost as ineffectively as the Mock cells. Looking back at the expressional results of the EF1-caSTAT3 CD4+ CAR T cells, via the flow plots in figure 34, there was very high CAR positivity, but the Her2tG positivity, which is the marker downstream of caSTAT3, as well as the dual positivity, were not very high. This may suggest that the lack of cytotoxicity for this condition is due to expressional issues of caSTAT3 and will need to be further investigated.

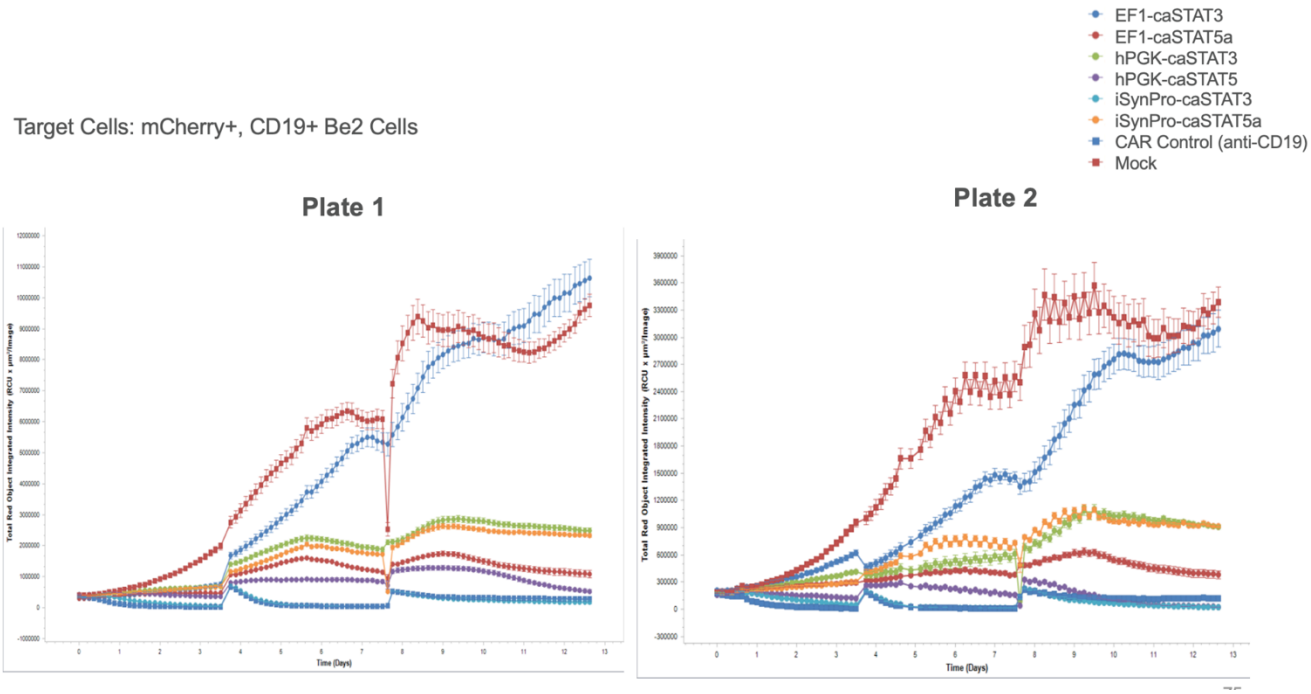


Figure 37: Incucyte results of electroporated CD4+ CAR T cells over the course of around 13 days, indicate the cytotoxic advantage of cells with caSTAT3 or caSTAT5a transgenes under various promoters, over the Mock CAR T cells

Figure 38 displays the same analysis as that shown in figure 37, but with CD8+ CAR T cells rather than CD4+ CAR T cells. In addition to the CAR control and Mock wells, the EF1-caSTAT3 populations again have an upward trend in mCherry signal, indicating that compared to the other conditions, they were less effective at killing, or even hindering the overall growth of the target cells. Looking back at the expressional results of the EF1-caSTAT3 CD8+ CAR T cells, via the flow plots in figure 34, there was very low CAR positivity, and virtually no Hert2G+. Similar to the results of the CD4+ CAR T cells, this may suggest that the lack of cytotoxicity for this condition is due to expressional issues of caSTAT3 and will need to be further investigated. Meanwhile, the hPGK-caSTAT3 population appears to be effectively hindering the growth of the Be2 cells, as well as the iSynPro-caSTAT5a populations doing so even more effectively.

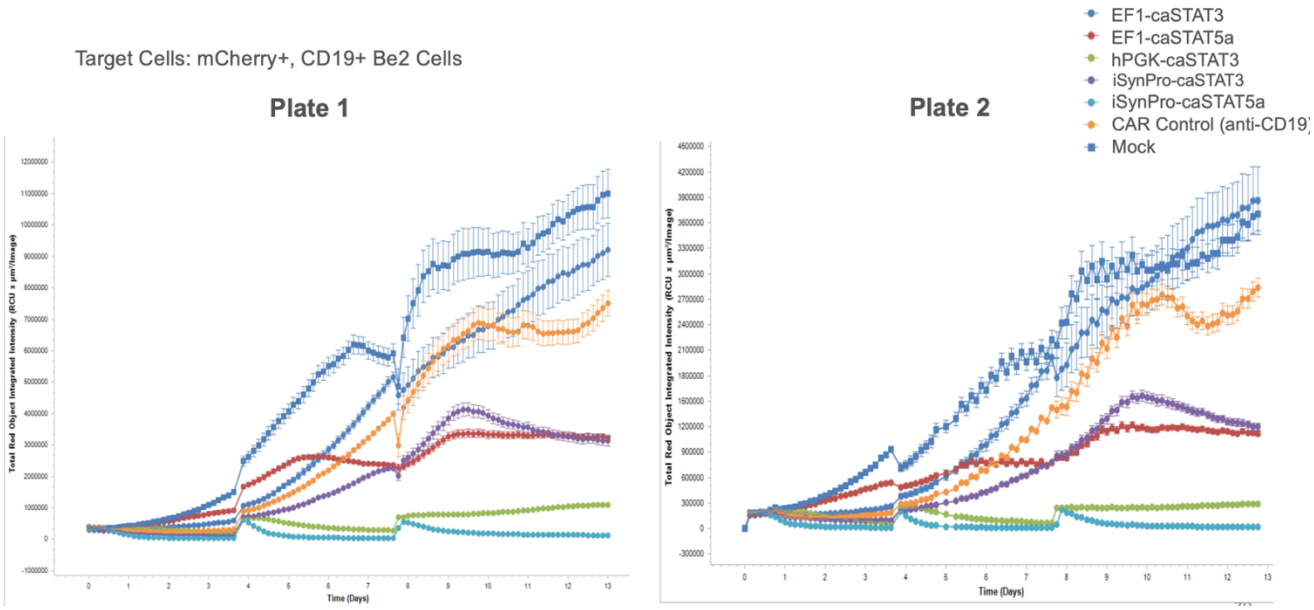


Figure 38: Incucyte results of electroporated CD8+ CAR T cells over the course of around 13 days, indicate the cytotoxic advantage of some conditions with caSTAT3 or caSTAT5a transgenes under various promoters, over the CAR Control or Mock CAR T cells

Figure 39 compares the cytotoxicity of cells where iSynPro, the CAR-inducible promoter, and the experimental constitutive promoters are driving caSTAT3 in either CD4+ or CD8+ CAR T cells. In both CD4+ and CD8+ CAR T cells, the Mock and EF1-STAT3 CAR T cells hinder the target Be2 cells the least. Alternatively, iSynPro-caSTAT3 and hPGK-STAT3 appears to be inhibiting the growth in both CD4+ and CD8+ CAR T cells the most effectively.

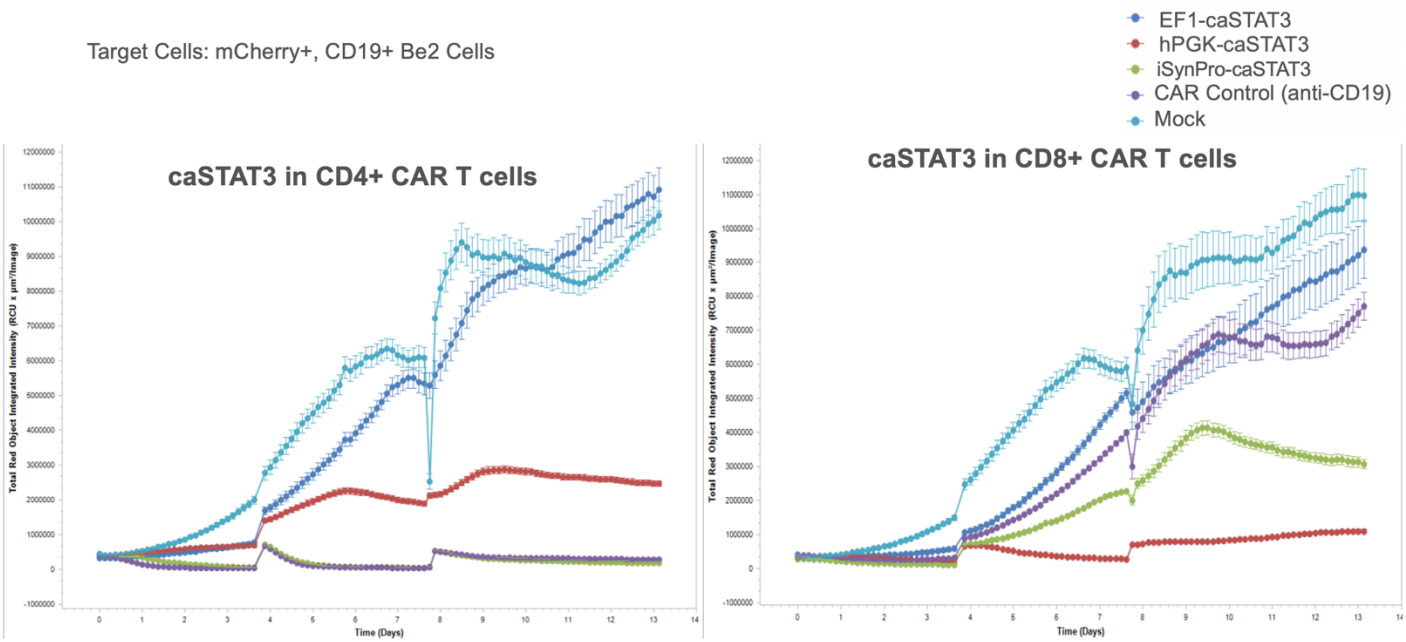


Figure 39: Incucyte results of electroporated CD4+ and CD8+ CAR T cells, with the caSTAT3 transgene being driven by inducible and constitutive promoters

Figure 40 compares the cytotoxicity of caSTAT5a in CAR T cells when driven by either inducible or constitutive promoters. As mentioned in the Methods section for this experiment, the culture for hPGK-caSTAT5a in CD8+ CAR T cells did not grow out well, and thus there were not enough cells to implement in the Incucyte analyses. Therefore, the only constitutive promoter being considered is EF1. Again, Mock shows the least amount of killing among these conditions. Interestingly, however, while iSynPro-caSTAT5a condition in CD4+ CAR T cells showed the least cytotoxic potential among the experimental conditions, it was by far the most effective in the CD8+ CAR T cells. EF1-caSTAT5a appears to have similar effects on hindering the target cells in both CD4+ and CD8+ CAR T cells.

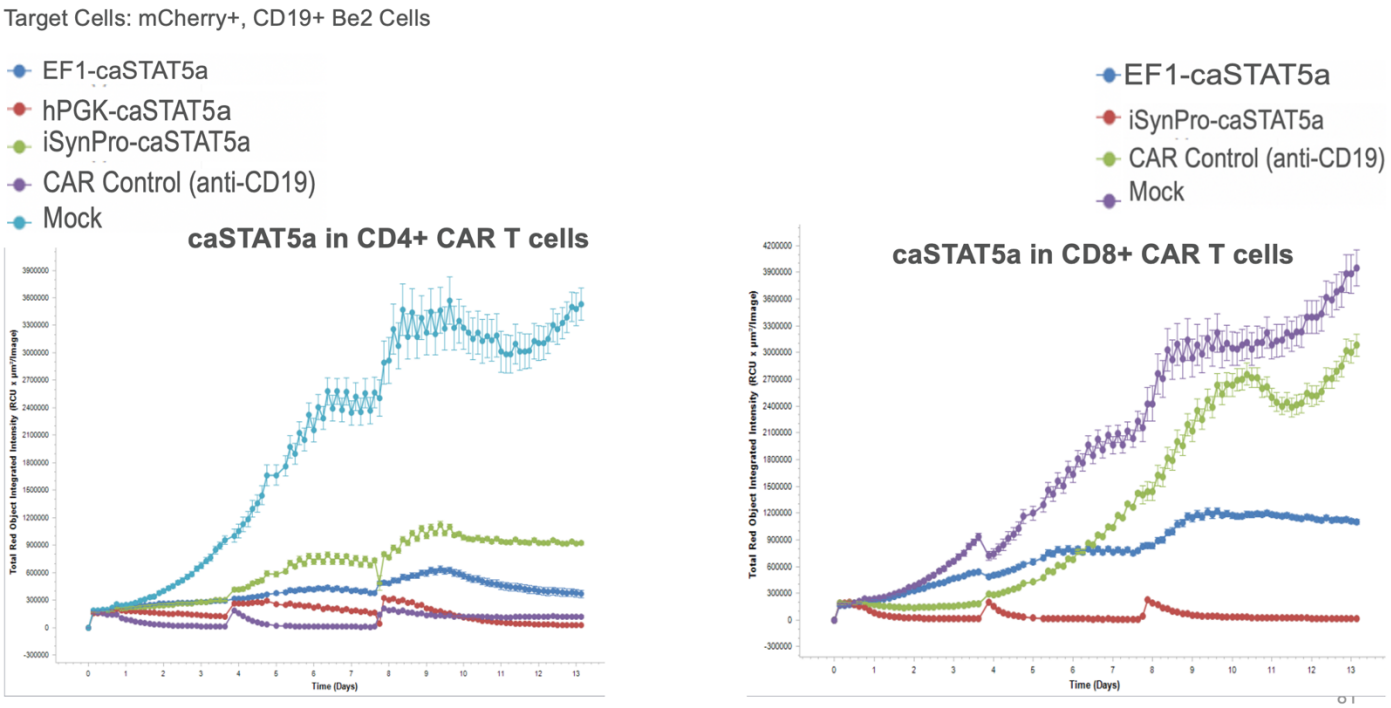
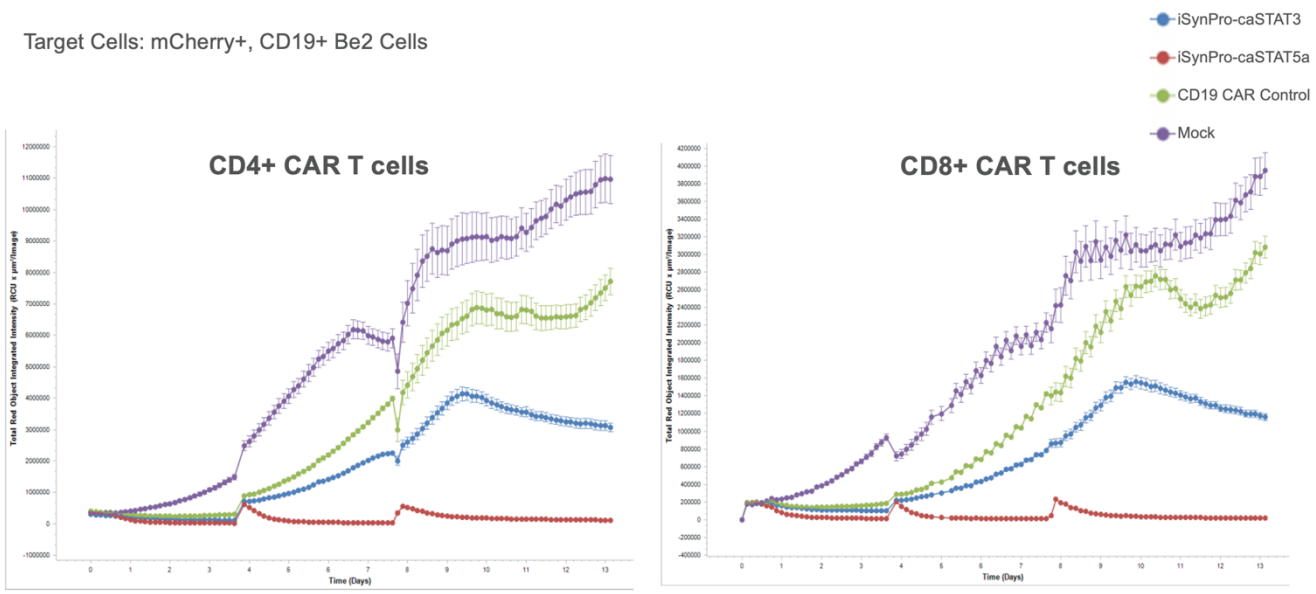


Figure 40: Incucyte results of electroporated CD4+ and CD8+ CAR T cells, with the caSTAT3 transgene being driven by inducible and constitutive promoters

As iSynPro is frequently used in dual promoter systems in the Jensen lab, and therefore is well characterized, the cytotoxic efficiency of caSTAT3 and caSTAT5a in both CD4+ and CD8+ CAR T cells can be compared when driven by iSynPro. Figure 41 shows that compared to the Mock and CAR control conditions, both caSTAT3 and caSTAT5a CAR T cells hinder the growth of the Be2 cells. However, iSynPro-caSTAT5a is far more effective, as the Total Red Object Integrated Intensity only increases at the Be2 rechallenge time points, and decreases in intensity shortly after, indicating the caSTAT5a condition can control the target cell growth efficiently. Meanwhile, for iSynPro-caSTAT3, although it hinders the signal intensity more so than the control conditions, the overall signal does increase throughout the duration of the experiment.



82

Figure 41: the cytotoxic potential of caSTAT3 and caSTAT5 in both CD4+ and CD8+ CAR T cells can be compared when driven by iSynPro

<u>Specific Aim 3:</u> To understand effects of caSTAT3 and caSTAT5a on CAR T cell activity in dual promoter systems, when regulated by estrogen receptors and constitutive or CAR inducible promoters.

Methods:

<u>Cloning Plasmids of Interest:</u>

Following similar molecular cloning procedures as those described in "Cloning Plasmids of Interest" under Specific Aim 1 and 2, the plasmids described in figure 42 are in the process of being cloned. Upon successful production and sequencing, these constructs will be electroporated into CD4+ and CD8+ T cells via the "TICLE" process. These cells will eventually be subjected to *in vitro* assays to further characterize the efficacy of multiple layers of regulation in dual promoter CAR T cell systems.

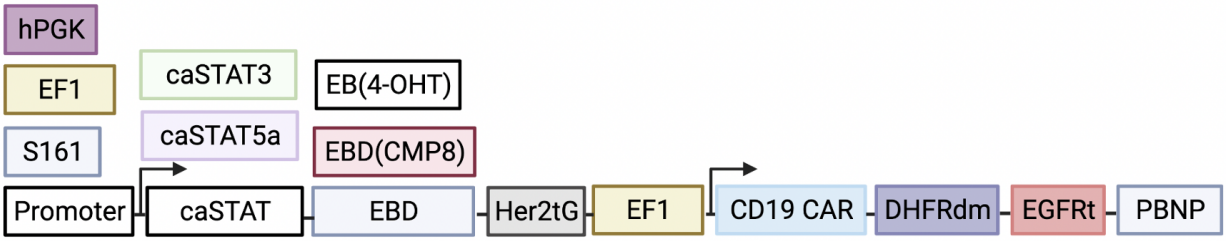


Figure 42: a total of 12 plasmids, with various combinations of promoters and caSTAT transgenes fused with either EBD(4-OHT) or EBD(CMP8) will be cloned for eventual downstream assays

Summary and Future Work:

The findings associated with this project have revealed results that will better inform the further development of a highly regulated and effective CAR T cell system.

Beginning with the results of **Aim 1:** To explore regulation of caSTAT3 and caSTAT5a activity by estrogen receptors, EBD(4-OHT) or EBD(CMP8). The Phosphoflow assays conducted, led to the confirmation that the fusion of estrogen binding domains do not hinder the phosphorylation, and therefore the function, of caSTAT5a molecules. This is an encouraging result, as it means this regulation method can be implemented without fear of disturbing the efficiency of the transcription factor. Additionally, in the presence of 4-OHT, caSTAT5a-EBD(4-OHT) provided greater proliferative advantage to CD4+ T cells, compared to caSTAT3-EBD(4-OHT). This was expected, as studies have found that STAT5 is more commonly associated with increasing proliferation, whereas STAT3 is tied to T cell survival, by hindering proliferation. However, it would be interesting to further explore and understand methods for interpreting "cell survival," if not by cell growth. In addition to the results of Aim 1, that have furthered our understanding of caSTAT in T cells, many processes and components will need to be further developed and investigated. The HEK 293t STAT Reporter Line protocol requires further optimization. The background GFP expression that is detected must be eliminated or minimized, so that the expression that is due to STAT binding is distinguishable. Finally, there were certainly concerns regarding the caSTAT3 transgene expression in T cells. Expressional assays, such as Western or Phospho-Western blots will need to be implemented to deconvolute this expressional issue, and understand where it derives from.

While pursuing **Aim 2:** To characterize caSTAT3 and caSTAT5a function in CAR T cells when constitutively expressed or under regulation of a CAR inducible promoter, methods for optimizing CAR T cell production were further explored. By implementing the "TICLE" protocol, methods and techniques for a more efficient, pure, and robust production were established, including cell culture techniques and nucleofection programs. Additionally, via preliminary expressional assays, candidates for the constitutive dual promoter constructs were narrowed down to EF1 and hPGK, which were then applied to both expressional and functional assays. Via Incucyte analyses, it was revealed that iSynPro driving caSTAT3 and caSTAT5a provides cytotoxic advantage when challenged by mCherry+/CD19+ Be2 target cells. Meanwhile, EF1, one of the constitutive promoters, driving caSTAT3 or caSTAT5a in the dual constitutive promoters does not seem to provide much cytotoxic advantage. hPGK may be a more effective constitutive promoter compared to EF1. The cytotoxic advantage provided by hPGK-caSTAT3 was promising, as well as hPGK-caSTAT5a in CD4+ CAR T cells. However, this experiment would need

to be repeated with a sufficient number of hPGK-caSTAT5a CD8+ CAR T cells, to further characterize the improvement in efficacy it provides.

Ultimately, the goal of this project, would be to integrate the various methods of regulation, by inducible promoter, and by estrogen receptor, into one plasmid, to allow for a highly regulated system. This is reflected in **Aim 3:** To understand effects of caSTAT3 and caSTAT5a on CAR T cell activity in dual promoter systems, when regulated by estrogen receptors and constitutive or CAR inducible promoters. Pursuance of this aim is highly contingent on the findings of Aims 1 and 2, as the various levels of regulation need to be characterized to understand how they can be integrated into one effective, but complex system. It is evident from the findings in the previous aims, that the interactions between the various components of these systems greatly impact the function of the CAR T cells. For example, it cannot be assumed, that iSynPro-caSTAT5a-EBD(4-OHT) will be the most effective in all T cells. Despite iSynPro-caSTAT5a providing the greatest cytotoxic effects in CD8+ T cells, and caSTAT5a-EBD(4-OHT) providing the greatest proliferative advantage in the presence of 4-OHT, when looking at CD4+ T cells, the CAR T cells with iSynPro-caSTAT5a had a lower cytotoxic effect compared to hPGK-caSTAT5a CAR T cells. This further emphasizes the complexity of these biological systems, and the importance of considering all factors when engineering a logic-gate regulated based CAR T cell system.

References

1. What Is Cancer? (n.d.). Retrieved from https://www.cancer.gov/about-cancer/understanding/what-is-cancer
2. Body systems and cancer. (2017, April 07). Retrieved from https://www.cancerresearchuk.org/about-cancer/what-is-cancer/body-systems-and-cancer
3. Childhood Acute Lymphoblastic Leukemia Treatment. (n.d.). Retrieved from https://www.cancer.gov/types/leukemia/patient/child-all-treatment-pdq
4. Zuckerman, T., & Rowe, J. (2014). Pathogenesis and prognostication in acute lymphoblastic leukemia. *F1000Prime Reports*,6. doi:10.12703/p6-59
5. Tidwell, J. (n.d.). Sleep, Fatigue and Caregiver Burden in Parents of Children with Acute Lymphoblastic Leukemia (ALL). doi:10.21007/etd.cghs.2008.0319
6. Weighing the Cost and Value of CAR T-Cell Therapy. (n.d.). Retrieved from https://www.ascopost.com/issues/may-25-2018/weighing-the-cost-and-value-of-car-t-cell-therapy
7. Robison, L. L., & Bhatia, S. (2003). Late-effects among survivors of leukaemia and lymphoma during childhood and adolescence. *British Journal of Haematology*,122(3), 345-359. doi:10.1046/j.1365-2141.2003.04499.
8. Mohty B, Mohty M. Long-term complications and side effects after allogeneic hematopoietic stem cell transplantation: an update. *Blood Cancer Journal.* 2011;1(4). doi:10.1038/bcj.2011.14.
9. Ness, K. K., Armenian, S. H., Kadan-Lottick, N., & Gurney, J. G. (2011). Adverse effects of treatment in childhood acute lymphoblastic leukemia: General overview and implications for long-term cardiac health. *Expert Review of Hematology*,4(2), 185-197. doi:10.1586/ehm.11.8
10. Duong, C., Westwood, J., Yong, C., Murphy, A., Devaud, C., John, L., Darcy, P. and Kershaw, M. (2013). Engineering T Cell Function Using Chimeric Antigen Receptors Identified Using a DNA Library Approach. *PLoS ONE*, 8(5), p.e63037.
11. Hunter, M., Prosser, M., Mahadev, V., Wang, X., Aguilar, B., Brown, C., Forman, S. and Jensen, M. (2013). Chimeric γc cytokine receptors confer cytokine independent engraftment of human T lymphocytes. *Molecular Immunology,* 56(1-2), pp.1-11.
12. DeMarco, C. (n.d.). 9 things to know about CAR T-cell therapy. Retrieved from https://www.mdanderson.org/publications/cancerwise/car-t-cell-therapy--9-things-to-know.h00-159221778.html
13. Grange, M., Buferne, M., Verdeil, G., Leserman, L., Schmitt-Verhulst, A. and Auphan-Anezin, N. (2011). Activated STAT5 Promotes Long-Lived Cytotoxic CD8+ T Cells That Induce Regression of Autochthonous Melanoma. *Cancer Research*, 72(1), pp.76-87.
14. Zhang C, Liu J, Zhong JF, Zhang X. Engineering CAR-T cells. *Biomark Res.* 2017;5:22. Published 2017 Jun 24. doi:10.1186/s40364-017-0102-y
15. Grigor EJM, Fergusson D, Kekre N, et al. Risks and Benefits of Chimeric Antigen Receptor T-Cell (CAR-T) Therapy in Cancer: A Systematic Review and Meta-Analysis. *Transfusion Medicine Reviews.* 2019;33(2):98-110. doi:10.1016/j.tmrv.2019.01.005
16. Shah NN, Fry TJ. Mechanisms of resistance to CAR T cell therapy. *Nat Rev Clin Oncol*. 2019;16(6):372-385. doi:10.1038/s41571-019-0184-6
17. Kagoya Y, Tanaka S, Guo T, et al. A novel chimeric antigen receptor containing a JAK–STAT signaling domain mediates superior antitumor effects. *Nature Medicine*. 2018;24(3):352-359. doi:10.1038/nm.4478.

18. Chang, Z. and Chen, Y. (2017). CARs: Synthetic Immunoreceptors for Cancer Therapy and Beyond. *Trends in Molecular Medicine*, 23(5), pp.430-450.
19. Miliotou, A. and Papadopoulou, L. (2018). CAR T-cell Therapy: A New Era in Cancer Immunotherapy. *Current Pharmaceutical Biotechnology,* 19(1), pp.5-18.
20. Oh, H. M., Yu, C. R., Golestaneh, N., Amadi-Obi, A., Lee, Y. S., Eseonu, A., Mahdi, R. M., & Egwuagu, C. E. (2011). STAT3 protein promotes T-cell survival and inhibits interleukin-2 production through up-regulation of Class O Forkhead transcription factors. *The Journal of biological chemistry*, *286*(35), 30888–30897. https://doi.org/10.1074/jbc.M111.253500
21. Kisseleva T, Bhattacharya S, Braunstein J, Schindler C. Signaling through the JAK/STAT pathway, recent advances and future challenges. *Gene.* 2002;285(1-2):1-24. doi:10.1016/s0378-1119(02)00398-0.
22. Onishi M, Nosaka T, Misawa K, et al. Identification and Characterization of a Constitutively Active STAT5 Mutant That Promotes Cell Proliferation. *Molecular and Cellular Biology.* 1998;18(7):3871-3879. doi:10.1128/mcb.18.7.3871.
23. Carpenter, R. L., & Lo, H. W. (2014). STAT3 Target Genes Relevant to Human Cancers. *Cancers*, *6*(2), 897–925. https://doi.org/10.3390/cancers6020897
24. Kanai T, Seki S, Jenks JA, et al. Identification of STAT5A and STAT5B target genes in human T cells. *PLoS One*. 2014;9(1):e86790. Published 2014 Jan 30. doi:10.1371/journal.pone.0086790
25. Ding, Z. C., Shi, H., Aboelella, N. S., Fesenkova, K., Park, E. J., Liu, Z., Pei, L., Li, J., McIndoe, R. A., Xu, H., Piazza, G. A., Blazar, B. R., Munn, D. H., & Zhou, G. (2020). Persistent STAT5 activation reprograms the epigenetic landscape in CD4+ T cells to drive polyfunctionality and antitumor immunity. *Science immunology*, *5*(52), eaba5962. https://doi-org.offcampus.lib.washington.edu/10.1126/sciimmunol.aba5962
26. Grange M, Giordano M, Mas A, et al. Control of CD8 T cell proliferation and terminal differentiation by active STAT5 and CDKN2A/CDKN2B. *Immunology.* 2015;145(4):543-557. doi:10.1111/imm.12471.
27. Kasakovski D, Xu L, Li Y. T cell senescence and CAR-T cell exhaustion in hematological malignancies. *Journal of Hematology & Oncology.* 2018;11(1). doi:10.1186/s13045-018-0629-x.
28. An, N., Tao, Z., Li, S., Xing, H., Tang, K., Tian, Z., Rao, Q., Wang, M. and Wang, J. (2016). Construction of a new anti-CD19 chimeric antigen receptor and the anti-leukemia function study of the transduced T cells. *Oncotarget,* 7(9).
29. Finney, O., Brakke, H., Rawlings-Rhea, S., Hicks, R., Doolittle, D., Lopez, M., Futrell, B., Orentas, R., Li, D., Gardner, R. and Jensen, M. (2019). CD19 CAR T cell product and disease attributes predict leukemia remission durability. *Journal of Clinical Investigation*.
30. Milocco LH, Haslam JA, Rosen J, Seidel HM. Design of Conditionally Active STATs: Insights into STAT Activation and Gene Regulatory Function. *Molecular and Cellular Biology.* 1999;19(4):2913-2920. doi:10.1128/mcb.19.4.2913.
31. Gallinari, P., Lahm, A., Koch, U., Paolini, C., Nardi, M. C., Roscilli, G., Kinzel, O., Fattori, D., Muraglia, E., Toniatti, C., Cortese, R., De Francesco, R., & Ciliberto, G. (2005). A functionally ORTHOGONAL Estrogen receptor-based TRANSCRIPTION SWITCH specifically induced by a Nonsteroid Synthetic ligand. *Chemistry & Biology*, *12*(8), 883–893. https://doi.org/10.1016/j.chembiol.2005.05.018

32. [Rad S M, A. H., Poudel, A., Tan, G., & McLellan, A. D. (2020). Promoter choice: Who should drive the CAR in T cells?. *PloS one*, *15*(7), e0232915. https://doi.org/10.1371/journal.pone.0232915]
33. Uchibori R, Teruya T, Ido H, et al. Functional Analysis of an Inducible Promoter Driven by Activation Signals from a Chimeric Antigen Receptor. *Molecular Therapy - Oncolytics*. 2019;12:16-25. doi:10.1016/j.omto.2018.11.003.
34. Biolabs, N. E. (n.d.). *Gibson Assembly*®. NEB. https://www.neb.com/applications/cloning-and-synthetic-biology/dna-assembly-and-cloning/gibson-assembly.
35. Milone, M.C., O'Doherty, U. Clinical use of lentiviral vectors. *Leukemia* **32,** 1529–1541 (2018). https://doi.org/10.1038/s41375-018-0106-0
36. Lana, M. G., & Strauss, B. E. (2020). Production of Lentivirus for the Establishment of CAR-T Cells. *Methods in molecular biology (Clifton, N.J.)*, *2086*, 61–67. https://doi.org/10.1007/978-1-0716-0146-4_4
37. Rio, D. C., Clark, S. G., & Tjian, R. (1985). A mammalian host-vector system that regulates expression and amplification of transfected genes by temperature induction. *Science (New York, N.Y.)*, *227*(4682), 23–28. https://doi.org/10.1126/science.2981116
38. Wu, S., Jin, L., Vence, L., & Radvanyi, L. G. (2010). Development and application of 'phosphoflow' as a tool for immunomonitoring. *Expert review of vaccines*, *9*(6), 631–643. https://doi.org/10.1586/erv.10.59
39. Zheng, Y., Li, Z. R., Yue, R., Fu, Y. L., Liu, Z. Y., Feng, H. Y., Li, J. G., & Han, S. Y. (2019). PiggyBac transposon system with polymeric gene carrier transfected into human T cells. *American journal of translational research*, *11*(11), 7126–7136

Acknowledgements

I would like to thank Dr. Jensen for allowing me to pursue this project in his lab. Having joined this group nearly three years ago as an undergraduate research intern, I am incredibly grateful to have had the opportunity to complete both my Senior Research Capstone Project, as well as my Master's Thesis Project, while exploring a field I am so passionate about. I am excited to continue contributing to the flourishing field of immunoengineering!

Thank you to Dr. Kueh for serving on my Thesis Defense committee, and for helping me cultivate my love for immunology and molecular/cellular engineering through his Bioengineering courses.

I would also like to thank Chris Saxby, Jia Wei, and James Rosser for their constant guidance, mentorship and encouragement. I would not have been able to make the progress that I did, without all of your help.

And of course, a huge thank you to the rest of the SCTx Team!