The Development of a CRISPR-Cas9 Gene Editing Platform to Efficiently Deliver HPV Neoantigen-Specific T Cell Receptors to Cytotoxic CD8+ T Cells

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A thesis

submitted in partial fulfillment of the

requirements for the degree of

Master of Science

University of Washington

2024

Committee:

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Program Authorized to Offer Degree:

Bioengineering

University of Washington

Abstract

The Development of a CRISPR-Cas9 Gene Editing Platform to Efficiently Deliver HPV Neoantigen-Specific T Cell Receptors to Cytotoxic CD8+ T Cells

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HPV-related cancers remain prevalent worldwide despite the development of several efficacious vaccines. Fortunately, the unique expression of E6 and E7 proteins allows for the specific targeting of HPV cancer cells by engineered immune cells with binding specificity to these antigens. The Heath lab is generating a methodology for the treatment of HPV-16 related cancer using TCR-engineered cytotoxic T cell immunotherapy. This lab-wide initiative involves the discovery of TCRs from HPV-16 cancer patient blood samples, TCR assembly, and preclinical functional validations. The non-viral gene editing system CRISPR-Cas9 is used to generate these engineered T cells, whose simplicity and safety allows for the generation of many T cell clones expressing several patient-specific TCR sequences. My role in this project was to first improve the efficiency of CRISPR-Cas9 gene editing of T cells using experimental methods, and to apply this strategy towards the development of HPV-specific CD8+ T cells. Using various methodologies, the CRISPR-Cas9 knock-in efficiency was increased from 0.1% to 10%. This improved method was used to investigate HPV-specific TCR sequences in both Jurkats and primary human T cells, where TCRs 001-006 showed adequate cell surface expression. Unfortunately, these TCRs did not demonstrate functionality in cytokine release assays in primary T cells. However, future TCRs with adequate expression and function will be used in a clinical trial to test the ability of a multiplexed TCR-engineered T cell immunotherapy to have improved clinical outcomes compared to a therapy using a single known T cell clonotype.

Acknowledgements

Thank you to my PI, Jim Heath, and my primary mentor Jongchan Choi for their guidance in this project and in my professional development. Thank you to the following members of the Heath lab at ISB for their help, expertise, and camaraderie at my time at ISB: Sunga Hong, Rongyu Zhang, Vanessa Gutierrez, Conor Brennan, Connor McDonald, Rick Edmark, Kim Murray, Andrew Webster, Yusuf Rasheed, Rachel Ng, Jingyi Xie, Chong Xia, Sarah Li, Jingqi Qi, Shuo Wang, Michaela McKasson, Jimmi Hopkins, Daniel Chen, and Dan Yuan. Thank you to Dr. Connie Trimble for providing the HPV cancer patient samples, and to the U.S. National Institute of Health, National Cancer Institute for the funding that made this project possible. Finally, a sincere thank you to my family, friends, and colleagues for their continued support of me in my academic endeavors.

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Literature Review

HPV-Related Cancers

Human papilloma viruses (HPVs) are epithelial pathogens that are associated with malignant tumors. HPV is the most common sexually transmitted infection in the United States, affecting both men and women[1]. HPV is associated with cervical, oral, penile, vulvovaginal, and anal carcinomas, in which about 20% of oral cancers are associated with HPV positivity[2]. HPV-16, HPV-18, and HPV-31 are the most prevalent high-risk subtypes of HPV associated with the development of such cancers, particularly cervical cancer. HPV types 16 and 18 are responsible for about 70% of cervical cancer cases worldwide[3].

These viruses can cause squamous intraepithelial lesions in several areas of the body including the cervix, penis, and anal cavity[2]. These lesions can ultimately progress to squamous cell carcinoma. The virus first infects dividing cells of the basal epithelium, maintaining a low copy number in these cells before invading terminally differentiated keratinocytes of the inner epithelium where they replicate rapidly and shed progeny virus into the epithelial tissue. HPVs replicate with the host's DNA synthesis machinery assisted by the early-expressed papillomavirus proteins E1 and E2, a DNA helicase and a DNA binding transcription factor, respectively. Mature HPVs express the proteins E6 and E7, which are key players in the development of cancer from HPV infection. When the virus integrates itself into the host genome, viral E6 and E7 genes are preferentially upregulated while other genes like E1, E2, and E5 are often lost. Further, infected cells expressing E6 and E7 genes have a selective growth advantage over episomal HPV genomes[4]. The expression of high-risk HPV E6 and E7 genes in infected keratinocytes has been shown to lead to cell immortalization[5] by inducing and sustaining telomerase activity[6]. This also results in genomic instability, increasing the likelihood of DNA mutations and thus directly increasing the risk of developing a potentially oncogenic mutation[2]. A major factor in the immortalization of infected keratinocytes is the inactivation of the tumor suppressors p53 and pRb by the E6 and E7 proteins, respectively. E6 and E7 also cooperate to create aneuploidy and mitotic defects in infected cells[7]. As highly oncogenic components of the HPV lifecycle, the E6 and E7 proteins are clear targets for HPV cancer treatment.

Fortunately, the development of the first HPV vaccine in 2006[8], and subsequent vaccines in 2009 and 2014, has reduced the incidence of HPV-caused cancers dramatically. HPV vaccines consist of manufactured viral-like proteins from recombinant HPV capsid proteins, specifically the late-expressed HPV protein L1[9]. HPV vaccination has been adopted worldwide, primarily the quadrivalent (qHPV) and bivalent (bHPV) vaccine types, and since 2017 the 9-valent HPV vaccine (9vHPV) has been the only available vaccine in the U.S.[8]. Each of these three vaccines protect against the highly oncogenic subtypes HPV-16 and HPV-18. The qHPV and bHPV vaccines have been shown to remain immunogenic and well tolerated up to 9 years after vaccination[10], and the 9vHPV vaccine demonstrated immunogenicity and tolerance after 10 years post-vaccination[11]. Despite the existence of these vaccines, HPV-related cancer is still prevalent globally. In 2018, it was estimated that HPV led to 620,000 new cancer cases in women and men worldwide[12]. In many low- and middle-income countries the HPV vaccine is not accessible, or

people opt out of the vaccine due to misinformation or superstition despite its proven efficacy[9]. Despite the fact that HPV vaccines are widely accessible in the U.S., in 2018 only 53.6% of women and 27.0% of men reported to have received at least one dose of HPV vaccine in their lifetime[13]. These vaccines were developed relatively recently and have been demonstrated to prevent cancer when administered to women up to 25, 26, and 45 years of age[14,15,16,17], after which it confers significantly less immunity. In addition, men are less likely to take the HPV vaccine despite the fact that approximately 40% of HPV-related cancers occur among men[18]. As a result, sexually active adults, especially older women and men, are still at risk of developing cancer caused by HPV infection, and require adequate treatment.

Cancer Immunotherapy

There have been many cellular technologies that have been developed for the treatment of human cancers. Immunotherapies like immune checkpoint inhibitors, monoclonal antibodies, and cancer vaccines have been shown to be particularly effective in treating certain cancers. A subset of cancer immunotherapy is adoptive cellular therapy (ACT), in which human immune cells are generated and administered to patients to create a targeted immune response against cancer cells. ACT includes four main types: tumor-infiltrating lymphocyte (TIL) therapy, natural killer (NK) cell therapy, chimeric antigen receptor (CAR) T cell therapy, and engineered T cell receptor (TCR) therapy. Key characteristics of these therapies are summarized in Table 1.

Immunotherapy	Immune Cell	FDA-Approved Therapies & Targeted Disease	Toxicity	Specificity
TIL therapy	T cells B cells NK cells	N/A	Low	Moderate
NK cell therapy	NK cells	N/A	Low	Moderate
CAR T cell therapy	T cells (CD8+ and CD4+)	Abecma – multiple myeloma Breyanzi – B-cell NHL Carvykti - multiple myeloma Kymriah – B cell ALL, B-cell NHL Tecartus – MCL, B-cell acute ALL Yescarta - B-cell NHL, follicular lymphoma	High	Low
Engineered TCR therapy	T cells (CD8+ and CD4+)	N/A	Low	High

Table 1: Summary of the four main types of ACTs highlighting the immune cells utilized, which therapies have been approved by the FDA, degree of toxicity, and degree of target specificity. NHL – non-Hodgkin lymphoma, ALL – acute lymphoblastic leukemia, MCL – mantle cell lymphoma.

TILs, including T cells and NK cells, are lymphocytes that naturally recognize and destroy cancer cells[19]. TIL therapy involves isolating TILs from a patient's blood, expanding them *ex vivo*, and reinfusing the amplified cell product back into the patient. Several phase II clinical trials have demonstrated the ability of TIL therapy to treat certain cancers, including melanoma[20] and metastatic cervical cancer[21]. However, no TIL therapies have been approved by

the FDA due to several limitations: TILs rapidly become exhausted when administered to patients after in vitro expansion, it takes two months to expand an adequate amount of TILs for effective therapy, and the quality of expanded TIL products between patients is inconsistent[19].

NK cell therapy utilizes natural killer lymphocytes which can directly kill cancer cells via cytotoxic effects, by recruiting tumor necrosis factor (TNF), or by recruiting IgG antibodies[22]. They also release immunostimulatory molecules that trigger CD8+ T cell differentiation into anti-tumor effector functions. These cells are expanded *ex vivo* before patient infusion, where these NKs can be isolated from the same patient or a donor. An adequate amount of NKs for clinical efficacy can be obtained after a 2-3 week expansion, much quicker compared to TIL therapy, but no NK cell therapy clinical trials have progressed beyond phase II[22].

CAR T cell therapy involves the genetic engineering of T cells to express a synthetic chimeric receptor that recognizes antigens independent of major histocompatibility complex (MHC) expression in the target cell[19]. This allows CAR T cells more flexibility in which molecules they can target than the natural TCR expressed by the T cell, since these molecules do not need to be expressed on an MHC. The CAR single-chain variable antibody domain can be modified to change its binding affinity to target any desired antigen, including cancer cell antigens[23]. The intracellular domain of the CAR protein allows the T cells to proliferate and attack the cancer cell when it binds its target antigen on the cell surface, stimulating an anti-tumor response. Several CAR T cell therapies have been approved as a treatment for blood cancers including large B cell lymphoma[24], multiple myeloma[25], and mantle cell lymphoma[26]. A major disadvantage of CAR T therapies is the lack of specificity to tumor cells, increasing the risk of cytokine release storm (CRS), in which the concentration of cytokines in the blood become high enough to produce side effects like high fever and hypotension[23]. CARs have also been transduced into NK cells to enhance their targeting efficiency and reduce the risk of CRS, but these treatments have not yet been FDA approved[22].

Engineered TCR therapy involves the modification of T cells to express exogenous T cell receptors. TCRs are heterodimeric transmembrane proteins composed of two peptide chains, TCR **α** and TCR **β**, flanked by four CD3 chains[23]. In humans, the **α** and **β** TCR chains are encoded by the TCR alpha constant (TRAC) and TCR beta constant (TRBC) genes, respectively. The variable region of the TCR sequence allows the cell to recognize and bind antigens uniquely expressed on the MHCs of foreign pathogens or cancer cells. High-affinity synthetic TCRs can be engineered to target antigens of interest by changing the variable TCR region. These TCRs can then be manufactured in the laboratory and transfected into a patient's T cells to modulate the cells' antigen specificity, allowing them to mount an immune response against their target when administered to the patient. Commonly, this therapy utilizes the cell-killing behavior of cytotoxic CD8+ T cells, which release cytokines into the cytoplasm of their target cell when both the TCR and CD8 co-receptor proteins bind the MHC-I complex, killing the target cell. In the clinical setting, a patient's T cells are sampled and isolated, then genetically modified, cultured, and infused back into the patient, usually at the site of a solid tumor[19]. The modified T cells can multiply in the body, with the cytotoxic effect lasting several weeks. Despite the potential of this

treatment, no engineered TCR therapies have yet been approved by the FDA. Limitations of clinical translation include non-generalizability due to HLA matching requirements, and the complexity and cost of manufacturing. Active clinical trials of TCR-engineered T cell therapies are attempting to target antigens unique to cancers in solid tumors, including melanoma differentiation antigen MART-1, cancer germline antigen New York esophageal squamous cell carcinoma-1 (NY-ESO-1), and members of the MAGE-A protein family[27].

TCR-engineered T cell therapies offer several advantages over other ACT immunotherapies. They can target antigens with a very high degree of specificity, reducing the risk of CRS responses due to nonspecific or excessive binding. TCRs are also more sensitive to antigens in the body at low concentrations than CARs[28]. This therapy could also lead to a longer period of time between infusions compared to other cancer treatments, which occur every 3-6 weeks in current market immunotherapies[29,30]. Most trials utilize a single engineered T cell product targeting one cancer antigen, but an emerging approach is to use multiplexed therapy with several clonotypes of T cells targeting various cancer antigens. Further, personalized medicine approaches can be used to identify cancer antigens specific to patients using TCR discovery, allowing for the development of a personalized multiplexed TCR-engineered T cell therapy.

Gene Editing Strategies in Immunotherapy

In TCR-engineered T cell therapies, cytotoxic T cells are modified to express an engineered TCR using both viral and nonviral gene editing approaches. Viral gene editing can be used to both knock-out (KO) the endogenous TCR genes TRAC and TRBC, and to knock-in (KI) the engineered exogenous TCR to modify T cells. Viral approaches include retroviral, adenoviral, and lentiviral transduction.

Retroviruses transfect dividing cells by passing through nuclear pores of mitotically dividing cells. Retroviral vectors carry RNA and allow for *in situ* delivery of genetic material by reverse transcription and linear integration into the host cell genome[31]. They have been used to treat X-linked severe combined immune deficiency[32] and used in an FDA-approved treatment for soft tissue sarcoma, osteosarcoma and pancreatic cancer[33]. Retroviruses are still limited clinically by low *in vivo* efficiency, immunogenic effects, and the risk of abnormal integration resulting in oncogenic effects and cancer development[31,34].

Adenoviruses type 2 and 5 carry and deliver DNA particles up to 38 kb into dividing and nondividing cells, but do not integrate into the host genome and are thus short-lived[35]. They are also highly immunogenic[36] and, although rare, gene therapies utilizing adenoviral vectors have led to serious side effects and death in some patients[37]. The transient nature of adenoviruses have given them clinical advantages over retroviruses due to enhanced safety, allowing for their approval by the FDA to treat nasopharyngeal cancer[38], head and neck cancer[39], ebola virus[40], and COVID-19[41,42,43]. Adeno-associated vectors (AAVs) have a similar mechanism to adenoviral vectors but much weaker replication capabilities and pathogenicity effects, leading to low immunogenicity and increased safety. As a consequence they can only carry small transgenes up

to 4.8 kb[44]. AAVs have been used in FDA-approved treatments to treat lipoprotein lipase deficiency[45], Lebers congenital amaurosis[46], and spinal muscular dystrophy[47].

Lentiviruses are a subclass of retrovirus originally derived from HIV-1[48] that deliver transgenes in the form of RNA. In contrast to retroviruses, lentiviruses can transfect both dividing and nondividing cells. Lentiviral vectors also have reduced toxicity and risk for insertional mutagenesis than retroviral vectors[49]. No therapies utilizing lentiviruses have yet been approved by the FDA, but several gene therapies using lentiviral vector delivery are under clinical investigation. The efficiency and ease of use of lentiviral transduction makes it a useful tool for research applications, including single gene KO and KI experiments.

Viral delivery methods are effective and have led to the development of several FDA approved therapeutics. However, viral vectors have a smaller cargo capacity, higher immunogenicity, and higher manufacturing cost than non-viral delivery systems[50]. These limitations have reduced the ability of these systems to be used as therapeutics, and thus recent research has focused on non-viral gene editing methods, including transposons, designer nucleases, and CRISPR-Cas9.

Transposons are units of DNA that encode a gene for a transposase enzyme with inverted terminal repeats (ITRs) on each end[51]. These regions of DNA can change their positions in the genome by the transposase enzyme binding to the ITRs and inducing transposition. The Sleeping Beauty (SB) transposon is able to transpose in human cells and has been used in many non-viral cellular engineering applications. This is done by Nucleofection, an electroporation technique that enables donor DNA direct access to the nucleus[52]. Nucleofection of the naked vectorized SB transposon system reduces cell viability, so other forms of SB delivery have been tested, including minicircle vectors and mRNA[51]. This system cannot be targeted for insertion at a specific location in the genome, so there is a risk of deleterious insertions.

Designer nucleases are proteins that can identify specific locations on genomes for targeted KO, including zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). ZFNs are composed of a series of zinc finger protein domains linked to the cleavage domain of the *FokI* endonuclease that can recognize a specific DNA sequence 6-9 nucleotides long[53]. A pair of ZFNs bind to the target genomic site where the *FokI* domains dimerize to cleave DNA at that region, creating a double-strand break (DSB)[54]. TALENs consist of an engineered designer nuclease made up of a *FokI* endonuclease cleavage domain and sequence-specific DNA recognition domain[55]. The recognition domain is derived from bacterial TALEs, which consist of 12-27 repeating units. The size of TALENs has made them more difficult to deliver than ZFNs[56], but they have been used to develop universal allogenic T cells for B cell acute lymphoblastic lymphoma[57]. ZFNs and TALENs are currently being applied in several ongoing cellular therapy clinical trials, but none have moved beyond phase II[58].

A relatively new nonviral gene editing method, clustered regularly interspaced short palindromic repeats-CRISPR‐associated protein 9 (CRISPR-Cas9), is able to achieve a simultaneous KO of a target gene and KI of endogenous DNA[59]. The CRISPR-Cas9 system utilizes a single guide RNA (sgRNA) strand conjugated to a Cas9 endonuclease, forming a

ribonucleoprotein (RNP) complex. The sgRNA detects complementary sites in the genome including a protospacer-adjacent motif (PAM) sequence, and the Cas9 creates a DSB at the base pair 3 bases downstream from the PAM[60]. Optionally, a donor DNA strand can be inserted into this cut site through homology directed repair (HDR), in which the homologous ends of the engineered donor DNA bind to their complementary sequence on the regions flanking the cut site[61]. In TCR-engineered T cell applications, CRISPR-Cas9 can be used to KO the endogenous TRAC and TRBC genes and KI an exogenous engineered TCR by HDR (see Figure 1).

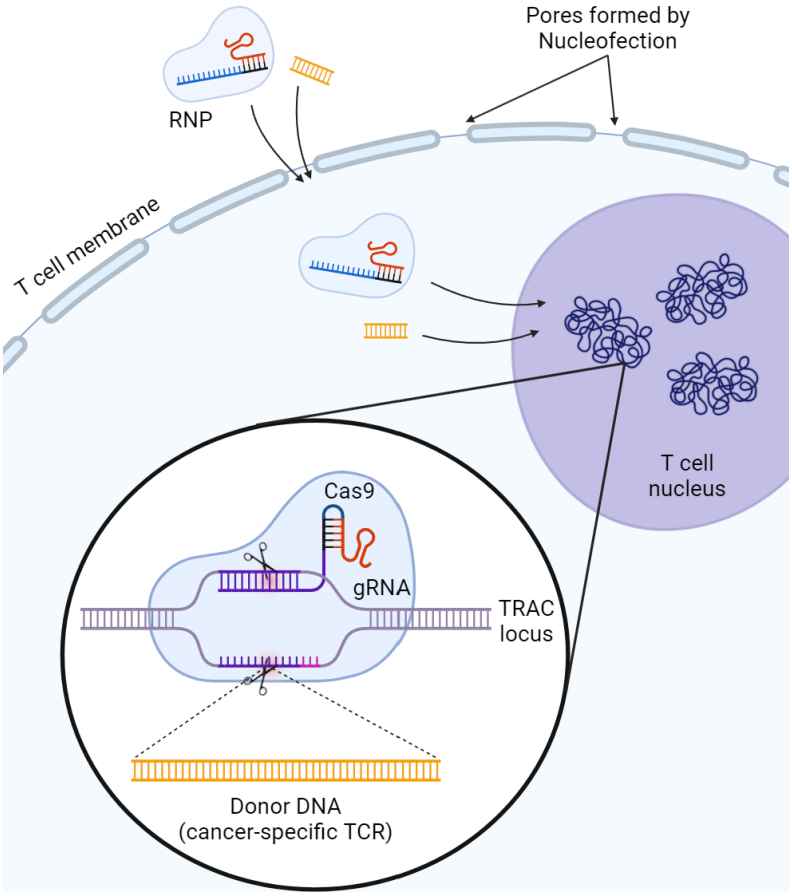


Figure 1: Gene editing T cells for TCR-engineered T cell therapy using CRISPR-Cas9.

A major limitation of CRISPR-Cas9 gene editing is low donor TCR KI efficiency. T cells targeting the NY-ESO-1 antigen have been engineered and tested in clinical trials[60], but most deliver the RNP using lentiviral vectors. This delivery system allows the RNP to exist for longer periods of time in cell culture, whereas transient delivery leads to rapid degradation of the Cas9 and sgRNA[62], leading to a smaller KI efficiency. However, a longer RNP lifetime increases the likelihood of off-target effects[63]. The use of lentiviral vectors also increases cost[62]. To reduce laboratory costs and minimize toxicity, there is a need for a CRISPR-Cas9 gene editing system utilizing an effective transient delivery system of the RNP with high KI efficiencies. There are

several approaches to improving these KI efficiencies that I have applied to this project outlined in the Results and Supplementary Data sections of this report.

TCR-Engineered T Cell Therapy for HPV-Related Cancer

Antigen Selection

To attempt to address current roadblocks in HPV cancer treatment, the Heath lab has launched an initiative to develop a treatment for HPV-related cancers using TCR-engineered T cells developed with CRISPR-Cas9 gene editing technology. The first task was to improve the KI efficiency of the CRISPR technology using the well-validated antigen NY-ESO-1. This antigen has been used as a target of engineered TCRs due to its prevalence on the surface of various tumor cells[64]. It is a popular antigen target in clinical trials due to its high levels of expression in tumors, and low expression in normal tissues, reducing the occurrence of off-target T cell activity[65]. The CRISPR-Cas9 KI efficiencies were successfully increased through my work under the mentorship of Jongchan Choi (see Methods & Results). This improved CRISPR-Cas9 editing methodology was then applied to generate several T cell clones targeting HPV neoantigens.

The HPV cancer antigens of interest are the tumorigenic E6 and E7 proteins, since they are cancer cell-specific and express uniformly in all HPV-related cancer cells and thus the TCR-engineered T cell therapy can be generalized to any variety of HPV-related cancer anywhere in the body. Using these antigens as a target, the Heath lab is working to find appropriate TCR sequences that can bind E6 or E7, generating and validating Jurkat and human T cell lines that express these TCRs, and measuring their ability to create a cytotoxic response when stimulated (see Methods). The ultimate goal is to find several functional T cell clones with specificity and activity to the E6 or E7 antigens that will be used in a phase I clinical trial, in which several of these T cell populations will be administered to HPV cancer patients simultaneously. The simultaneous administration of these T cells, each with specificity to E6 or E7 HPV antigen, act as a multiplexed cell immunotherapy treatment that is hypothesized to bring about better therapeutic outcomes than a treatment consisting of a single TCR-engineered T cell therapy.

TCR Selection and Development

PhD students Jingyi Xie, Rongyu Zhang and Rachel Ng are working to discover TCRs from HPV-16 patient blood samples that can bind E6 or E7 HPV cancer antigens. They, in collaboration with other members of the Heath lab, are working to develop a TCR discovery pipeline. This pipeline would allow for the creation of a large HPV-specific TCR library with specificity to many E6 and E7 HPV-16 cancer antigens. With a large enough library, these TCRs could be used to create multiplexed immunotherapies that target all major HLA alleles, which differ in dominance across race and ethnicity. This TCR discovery pipeline could also enable the creation of personalized TCR-engineered immunotherapies, where a patient's CD8+ T cells are isolated and analyzed, TCRs specific to the cancer of interest are sequenced and manufactured,

and a population of the patient's T cells are genetically modified with CRISPR-Cas9 to express those TCRs, which are administered back to the patient. This approach circumvents a central limitation of generalizable immunotherapies, which is the diversity of HLA class I alleles in the human population. There have been over 24,000 human HLA alleles recorded as of a 2015 paper[66] which vary widely across racial and ethnic groups[67].

Methods

Development of the CRISPR-Cas9 Editing Method

The general method for CRISPR-Cas9 editing of T cells in the Heath lab was initially developed by Jongchan Choi based on the known scientific literature. The overall procedure involves the isolation and activation of CD8+ T cells from peripheral blood mononuclear cells (PBMCs), which are then electroporated with a manufactured TCR and the CRISPR-Cas9 RNP. The manufactured antigen-specific TCR was based on a paper from Roth et al.[68]. The antigen target used for validation was NY-ESO-1, which is a well-characterized antigen with a validated homologous TCR sequence. KI efficiencies were evaluated using flow cytometry. The general method is visualized in Figure 2.

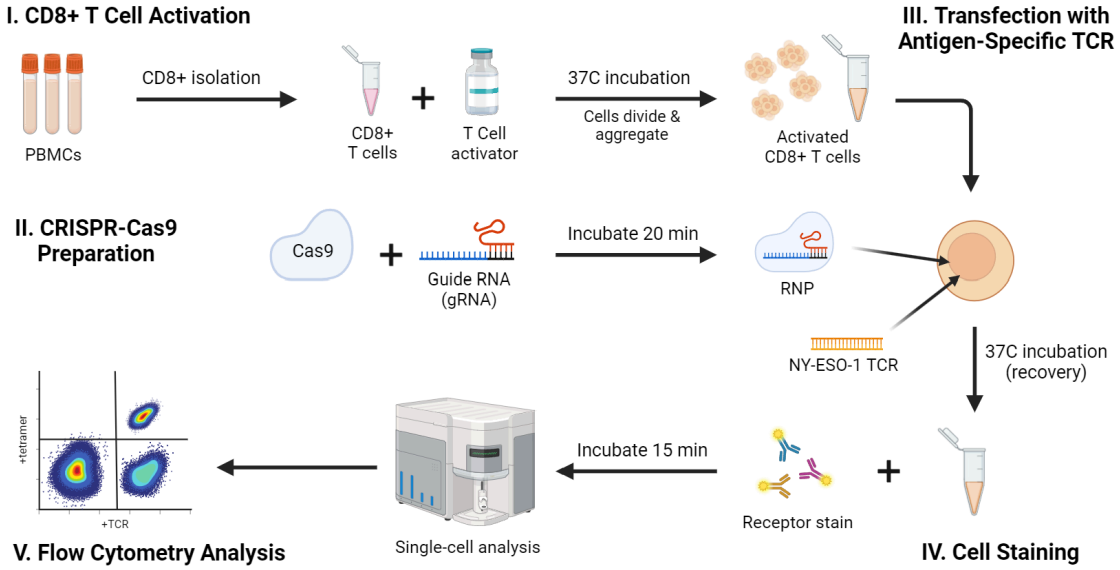


Figure 2: Overall methods for the optimization of the CRISPR-Cas9 T cell editing method using an NY-ESO-1 specific TCR.

Isolation & Activation

CD8+ T cells were isolated from samples of PBMCs isolated from cancer patient blood samples that were donated to the Heath lab. These samples were stored in liquid nitrogen at -80C to preserve cell viability. All samples used were positive for the common A:02+ HLA antigen. These CD8+ T cells were isolated using either negative or positive isolation methods. After isolation, the number of CD8+ T cells was approximated with a hemocytometer. Each

experimental condition to be tested required a minimum of 200,000 cells to generate a meaningful result. The method for T cell activation also varied in certain iterations. The CD8+ T cells were activated over 48 hours of incubation at 37°C, resulting in rapid cell division and clustering. This stimulation induces the transcription of genes in the T cells, preventing them from packaging their DNA into chromatin[69]. This increases the efficiency of Cas9 DNA binding, causing the cells to be more susceptible to genetic editing in the electroporation step

Electroporation

Activated CD8+ T cells were electroporated using two main systems: Neon NxT transfection, and subsequently Lonza 4D Nucleofector transfection. This step involves the creation of the RNP, incubation of the RNP with the donor DNA and CD8+ T cells, and the electroporation of these mixtures to allow the RNP and donor DNA to enter the T cell cytosol and nucleus. The electroporated T cells were allowed to recover before analysis during a 3-5 day incubation at 37°C. Each experiment had a KO control without donor DNA to ensure the Cas9 was functioning properly.

Flow Cytometry

To determine the presence of the donor TCR in the electroporated T cell populations, the cells were tagged with the following fluorophores and analyzed on the Attune NxT flow cytometer: CalceinUV live-dead, Alexa Fluor 488 anti-CD8, APC anti-CD3, and PE NY-ESO-1 tetramer (see Table 2). CD3 expression was used as an indirect measure of TCR expression. The tetramer cognate antigen stain was created in-house using peptide-major histocompatibility complex (pMHC) multimers[70], where antigen pMHC is incubated with PE fluorescent dye and biotin.

Cell Marker	Fluorophore	Attune Channel
Live/Dead	CalceinUV	VL1
CD8	Alexa Fluor 488	BL1
CD3	APC	RL1
Tetramer	PE	YL1

Table 2: Flow panel for NY-ESO-1 TCR CRISPR-Cas9 KO/KI analysis on the Attune NxT.

The anti-CD8 stain indicated the presence of the surface CD8 protein expected from CD8+ T cells, acting as a measurement of cell viability. The anti-CD3 stain indicated the presence of a T cell receptor, measuring the CRISPR-Cas9 KO efficiency. Finally, the NY-ESO-1 stain indicated the presence of the donor TCR, measuring the KI efficiency. Often, cell viability was the limiting factor for obtaining statistically significant data. Both cell viability and

knock-out efficiency were generally high throughout the project and across iterations. However, KI efficiency was initially 0.1% with the original editing method. Using the graphical cytometry software FlowJo, the cell data was gated and graphed. Flow cytometry was the primary method to evaluate each experimental iteration's success, and these results informed the majority of future directions for experimental design.

Finalized Editing Method

After testing several conditions using different TCR development techniques and T cell culture conditions (see Results & Supplementary Data), the finalized protocol is shown below.

1. Obtain A:02+ PBMCs.
2. Isolate CD8+ T cells with positive selection using Miltenyi human CD8 MicroBeads (#130-045-201, Miltenyi).
3. Resuspend CD8+ T cell pellet in 1 mL of TexMACS media with 3% human serum supplemented with 12.5 ng/μL IL-7 (581904, Biolegend) and 12/5 ng/μL IL-15 (570304, Biolegend) per 10^6 cells.
4. Activate 1 M T cells by adding 10 μL of TransAct human CD3/CD28 activator (#130-111-160, Miltenyi) and incubate for 48 hours.
5. Transfect the CD8+ T cells with the Lonza Nucleofector system.
    a. Pre-warm a 16-well Lonza Nucleocuvette strip at 37C.
    b. Incubate Cas9 (Alt-R™ S.p. Cas9 Nuclease V3, IDT) and sgRNA (IDT) together in a 1:3 ratio to create the RNP, RT for 20 min. Prepare the RNP separately for TRAC and TRBC.
    c. Create the Nucleofector buffer by combining P3 Primary Cell Nucleofector solution and Supplement 1 in a 4.5:1 ratio.
    d. Spin down the activated T cells and wash with 1 mL PBS. Resuspend washed cells in 18 μL of Nucleofector buffer.
    e. Combine RNP and 2 μg of PACT TCR DNA, incubate at RT for 1 min. Add electroporation enhancer (Alt-R™ Cas9 Electroporation Enhancer, IDT) and activated T cells.
    f. Transfer samples to a 16-well Lonza Nucleocuvette strip and electroporate T cells with the Lonza Nucleofector on the EO-115 setting.
    g. Allow cells to recover by incubating the Nucleocuvette strip at 37C for 15 min.
    h. Transfer cells to a 48-well plate with 500 μL TexMACS-IL media with 3% human serum, incubate at 37C for 5 days.
6. Stain the cells with Apotracker™ Green (427402, Biolegend), anti-CD8 BV421 (344748, Biolegend), anti-CD3 APC (317318, Biolegend), and tetramer PE, and analyze the cells with flow cytometry to confirm the presence of edited T cells.
7. Isolate the edited T cells with cell sorting.

Engineering HPV-Specific Cytotoxic T Cells with the CRISPR-Cas9 Method

The CRISPR-Cas9 T cell engineering method described previously is currently being applied to generate cytotoxic T cells with binding specificity to HPV-16 E6 and E7 oncogenes. This is one step in a larger HPV-16 clinical trial initiated by the Heath lab in collaboration with Dr. Connie Trimble from Johns Hopkins, Dr. Toni Ribas from UCLA, and Dr. Christian Henrichs. This trial is a lab-wide initiative which requires HPV-specific T cell discovery, TCR sequencing and assembly, T cell editing, and functional validation. Each step is led and contributed to by different members of the Heath lab, which will be noted where applicable.

HPV-Specific TCR Discovery & Sequencing[71]

PBMC samples from 14 HPV-16 patients were provided by Dr. Connie Trimble for analysis, where samples from 11 of these patients were used for CD8+ T cell capturing and analysis, with 2 healthy donors as controls. Each patient had vials donated at 1-3 separate time points. Jingyi Xie and Rongyu Zhang collaborated to apply this protocol. Jingyi Xie first enriched and stained HPV-specific T cells from these PBMCs, then sequenced them. First, each vial was Hashtaged with patient and time point IDs using peptide-encoded DNA primers. CD8+ T cells were then enriched by FACS sorting, and HPV-specific CD8+ T cells were stained with a pool of 82 class I dextramers.

The dextramer pool was prepared based on a high-throughput platform developed by Dr. William Chour to generate pMHC libraries using single chain trimer (SCT) technology, which are utilized to capture antigen-specific CD8+ T cells expressing any class I HLA allele[72]. Peptides with binding affinity to HPV E6 and E7 oncoproteins were selected from public databases and literatures[73], and Class I HLA alleles with high frequencies in the North American population were selected, including HLA-A*01:01, HLA-A*02:01, and HLA-C*07:01[74]. From the population of HPV-specific T cells, the high throughput single cell sequencing platform 10x Genomics was combined with gene expression analysis to generate V(D)J gene expression of HPV-specific TCRs. These TCRs were then selected by dextramer binding.

Each TCR sequence was analyzed using an in-house algorithm developed by Rachel Ng, where the TCR **α** and **β** chains were combined to generate paired TCR sequences. Using the antigen barcode alignment, a selection of the top peptide antigen sequences were identified for each TCR.

TCR Binding Validation in Jurkats

After selection of the top TCRs, the full TCR sequence is processed by a simple Python script written by William Chour. This script changes nucleotides without changing the amino acid sequence to stabilize the protein structure, and outputs 4-5 fragments with 30 bp overlap that can be ordered from a DNA manufacturer. The output of the script contains 4 fragments for CRISPR assembly and 5 fragments for lentiviral assembly.

These fragments are ordered from a DNA manufacturer and assembled by Gibson into a vector backbone for sequencing validation. Before moving to the functional analysis in primary

T cells, since the discovered TCRs are not previously validated in the literature, the ability of the selected TCRs to bind their target antigen must first be determined. This is done in Jurkat cells, which are more robust than primary human T cells and are easier to work with. Specifically, TCR binding validation was done using a Jurkat cell line developed in-house by Yusuf Rasheed and Andrew Webster, which express CD8 surface protein and NFAT-GFP fluorescent protein. Critically, this cell line was developed to not express TCR, so a new TCR can be introduced by gene editing as the primary functional TCR. These Jurkats were transduced to express the HPV-specific TCR using lentiviral transduction. Subsequent flow cytometry analysis was used to determine the ability of the TCR to bind its cognate antigen, again using fluorescent tetramer technology as described previously. Since the identity of cognate antigen is not known with certainty, the top likely antigen sequences are tested simultaneously, followed by a separate antigen stain. For the TCRs with positive tetramer signal, they are incubated with peptide-presenting artificial antigen presenting cells (aAPCs) and the GFP activation signal is measured to determine the TCR-antigen binding efficiency.

Functional Analysis in Primary Human T Cells

HPV-specific TCRs that demonstrated strong binding to their cognate antigen in Jurkats were then used to engineer primary human CD8+ T cells. This editing was done using the CRISPR-Cas9 method described previously. Expression of the TCR was evaluated with flow cytometry. If a significant proportion of the T cell population expressed HPV-specific TCR, the functionality of the cells was measured by Sunga Hong and Jongchan Choi. This was done using the following assays: Interferon gamma (IFN-γ) expression, cell-killing, alloreactivity, alanine scan, and cross reactivity[75]. The IFN-γ and cell-killing assays were done by stimulating the cells with their TCR's cognate antigen, allowing them to incubate overnight, and measuring the concentration of secreted IFN-γ as well as the ability of the cells to lyse cells expressing the cognate antigen. The stimulation step is done using lymphoblastoid cell lines (LCLs) ordered from the Coriell Cell Repository, which are induced to express particular peptides on their surface. The alloreactivity and cross reactivity assays were used to determine the potential toxicity of the TCRs when applied in a future clinical trial.

Results

Generation of the CRISPR-Cas9 Editing System

To increase the KI efficiencies of the CRISPR-Cas9 editing method, a series of approaches were tested. The experiments that led to the final selection of the method compared three different strategies to deliver the exogenous TCR. The first was a linear, double stranded DNA (dsDNA). The second were purchased Nanoplasmids that express the TCR, inspired by a protocol from PACT Pharma[76]. The third used TCR-expressing minicircles (MCs) which were developed in-house with a production kit from Miltenyi, offering a cheaper alternative to the proprietary Nanoplasmid technology. For each of these three TCR delivery methods, two TCR

sequences were compared, one based on the PACT Pharma protocol, and the other based on a paper from Oh et al.[77]. For each TCR sequence, two forms of MC were developed, one with a promoter (MN100B1) and one without a promoter (MN100A1).

dsDNA vs MC Donors

All four MCs, Oh-MN100B1, Oh-MN100A1, PACT-MN100B1, and PACT-MN100A1, were tested against their respective dsDNA TCRs, along with the original double-stranded TCR based on the paper by Roth et al.[68]. All were tested at 3 μg/μL concentrations. These samples were incubated for an additional 5 days post-electroporation to see if the edited cell populations would preferentially expand. This flow data is shown in Figure 3.

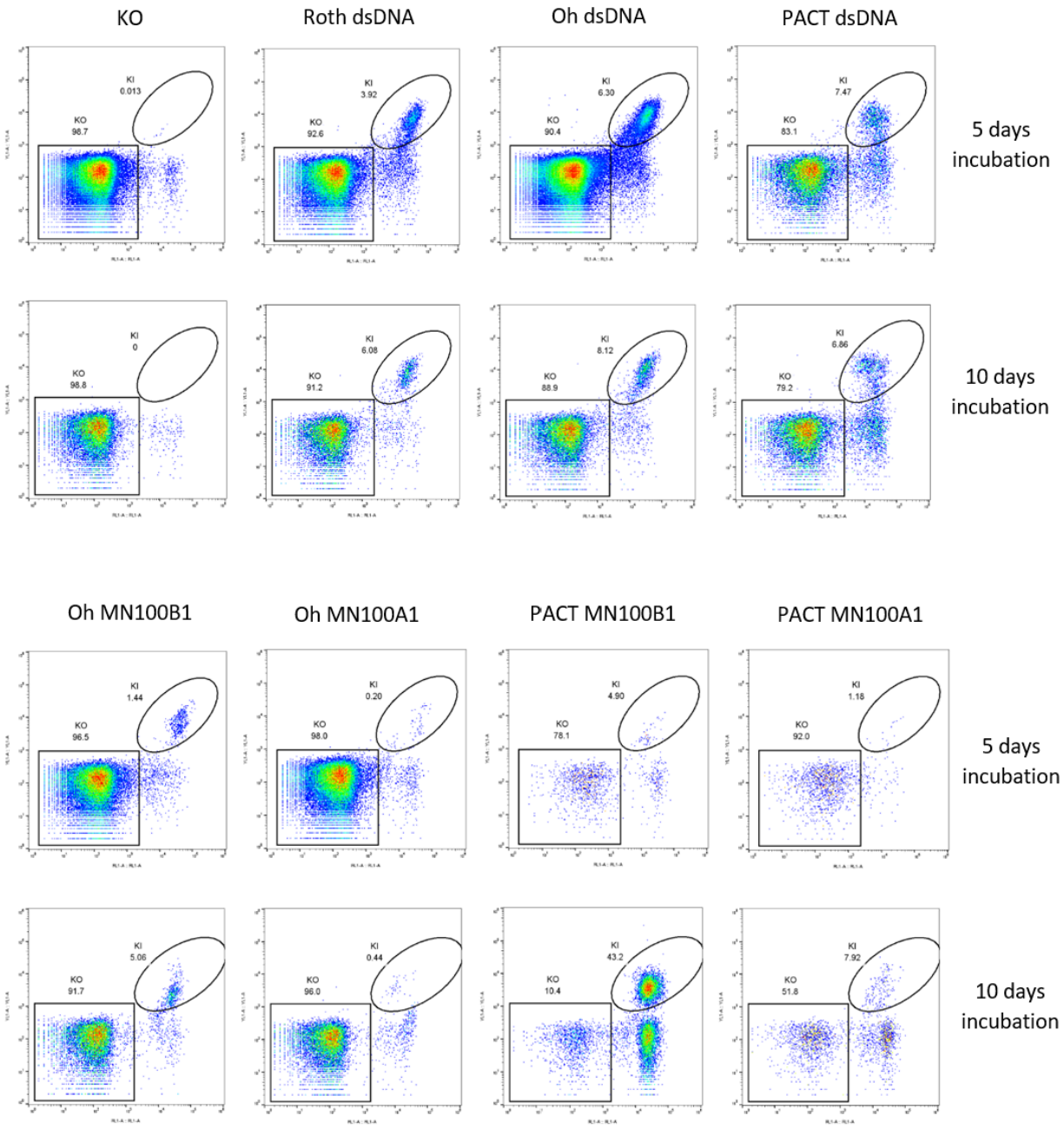


Figure 3: Flow cytometry data comparing 3 dsDNA TCR sequences and 4 MC TCR donors after 5 and 10 days of incubation after electroporation.

For most samples containing a donor TCR, the KI efficiency increased after 5 additional days of incubation. For these samples, the initial number of edited cells was sufficient to expand into the larger populations shown. Among the dsDNA donors, the TCR from Oh et al.[77] produced the largest KI efficiency after 10 days of incubation. This sample also had the highest cell viability after 5 days compared to Roth and PACT dsDNA. Among the MC donors, the MCs

containing a promoter had generally higher KI efficiencies than those without. The MC that produced the highest KI efficiency was PACT-MN100B1 after both 5 and 10 days of incubation. The efficiency after 10 days was about 40%. This value is 10-fold higher than the second highest efficiency of Oh-MN100B1 after 10 days, and 1000-fold higher than previous efficiency results on the order of 0.1% (see Supplementary Data). This MC condition and the PACT dsDNA condition were investigated in subsequent experiments.

The best condition, PACT-MN100B1, as well as the PACT dsDNA sample were analyzed with an ELISA cytokine secretion assay. This assay measured the concentration of tumor necrosis factor alpha (TNF-**α**) and IFN-**γ** cytokines, and granzyme B (GZMB) protease which stimulates cytokine release. These concentrations were measured with and without NY-ESO-1 peptide in the sample. This data is shown in Figure 4.

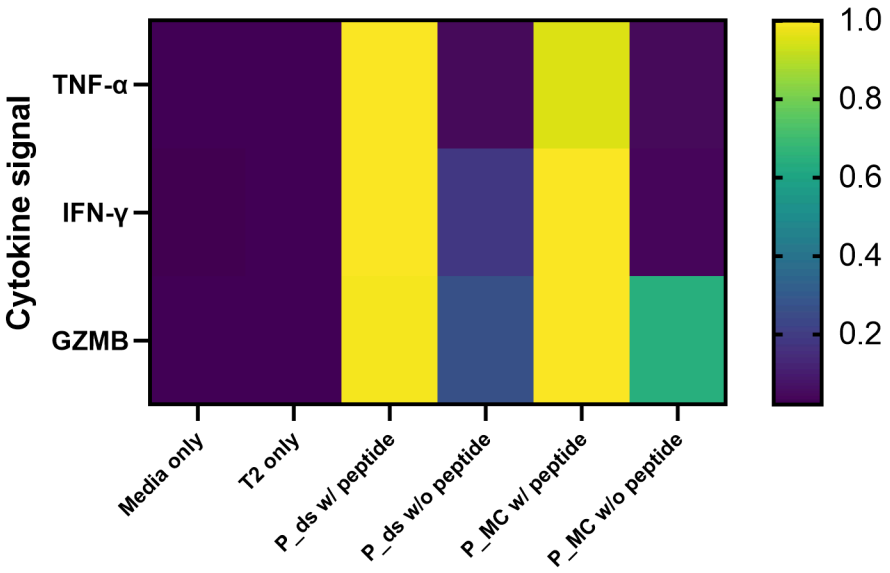


Figure 4: ELISA secretion assay heat map showing the concentration of three molecules involved in CD8+ T cell killing activity, TNF-**α**, IFN- **γ**, and GZMB, normalized to the highest concentration value.

The samples containing the target NY-ESO-1 peptide had the highest cytokine expression, indicating that the cells generated with this method have cytotoxic functionality and can attack and kill cancer cells if used as an immunotherapy treatment.

Cas9:sgRNA Ratio

Both Oh et al.[77] and the PACT Pharma patent[76] described different ratios of Cas9 and sgRNA that assemble into the RNP, 1:3 and 1:6, respectively. To avoid using large quantities of sgRNA, only the 1:3 condition was tested compared to the current 1:1 condition used in all previous experiments. These ratios were tested with PACT dsDNA, PACT-MN100B1 and the

manufactured Nanoplasmid containing the PACT TCR. The flow cytometry data for this experiment is shown in Figure 5.

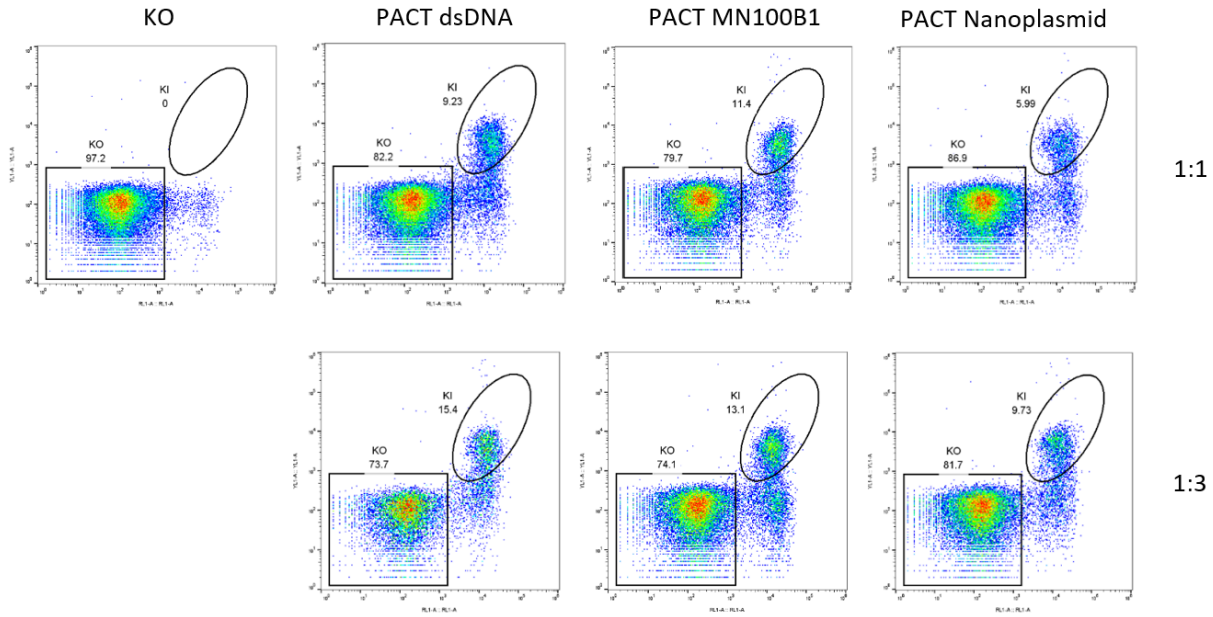


Figure 5: Flow cytometry data comparing all three PACT TCR vehicles, PACT dsDNA, PACT-MN100B1, and PACT Nanoplasmid, at two different Cas9:sgRNA ratios.

PACT dsDNA and MN100B1 MC had higher cell viabilities compared to the previous experiment, and the KI efficiency after 5 days of incubation post-electroporation was higher in this experiment as well. The Nanoplasmid had smaller efficiencies than both dsDNA and house-made MC donors. The house-made MCs have comparable KI efficiencies to the Nanoplasmids. This is a promising result, since making the MCs in-house is significantly less expensive than purchasing the manufactured Nanoplasmids. For all samples, the 1:3 Cas9:sgRNA ratio produced higher KI efficiencies than the 1:1 condition. The 1:3 ratio was used in future experiments.

CRISPR-Cas9 T cell editing with an MC donor produced the highest KI efficiency out of all other conditions tested, reaching about 40% for the PACT-MN100B1 MC sample after 10 days post-electroporation (see Figure 3). However, this result should be considered an outlier, since this efficiency was not reproducible in future experiments. The PACT dsDNA produced a similar KI efficiency to the PACT-MN100B1 MC at both 1:1 and 1:3 Cas9:sgRNA ratios (see Figure 5) and led to a similar cytotoxic response in the edited cells after NY-ESO-1 stimulation (see Figure 4). Since the in-house MC production was a long, time-consuming process with many areas for potential error, the PACT dsDNA method was selected to use in future experiments. Further experiments compare these two methods to verify the reproducibility of the PACT dsDNA KI efficiency results.

TRAC/TRBC KO

In the Roth et al.[68] paper, from which the original TCR was based, only the TRAC region of the TCR genome was targeted by the CRISPR-Cas9 RNP for a DSB. Single TRBC KO has been shown to result in comparable KI efficiencies[68]. This experiment tested the effect of using two different RNPs, one with a sgRNA strand targeting the TCR **α** chain and the other targeting the TCR **β** chain, both separately and combined. The combined **α**/**β** condition was also used to test the effect of using half the normal concentration of RNP. These four conditions were tested with the PACT dsDNA and MN100B1 TCRs. The flow cytometry data for this experiment is shown in Figure 6.

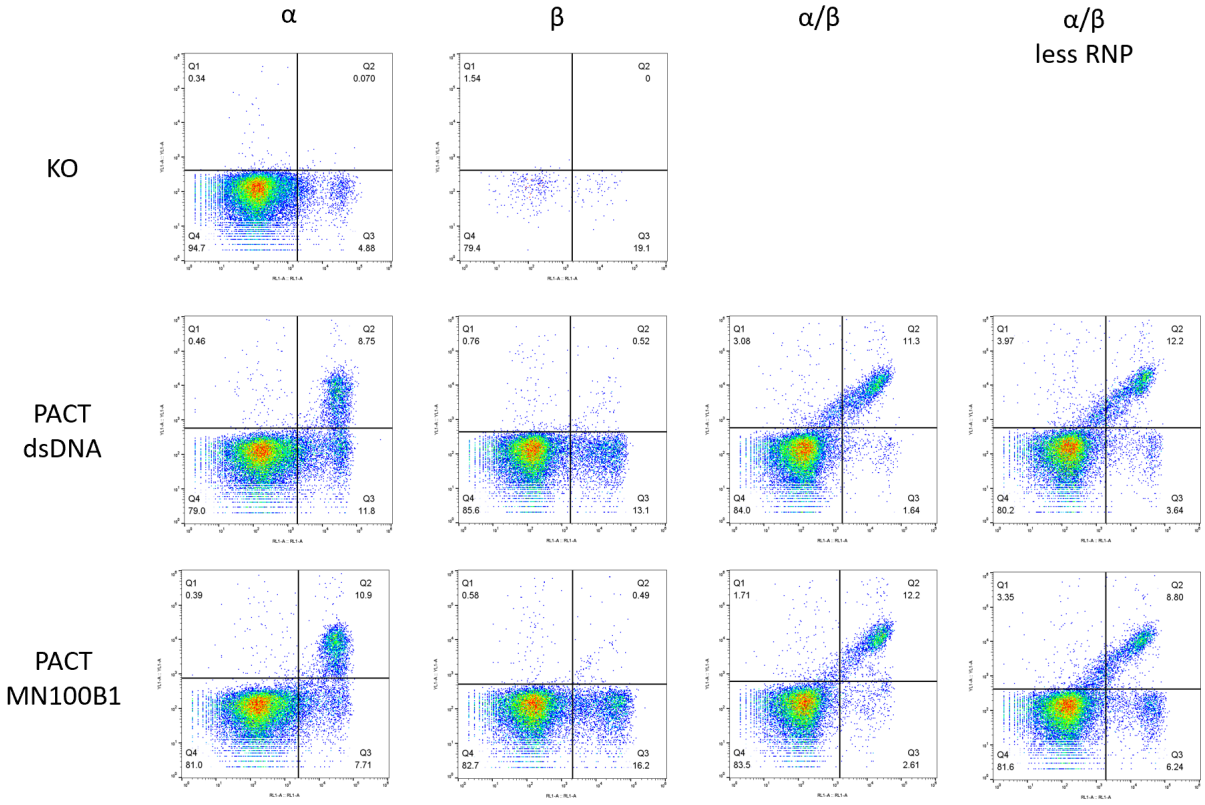


Figure 6: Flow cytometry data comparing PACT linear dsDNA and MN100B1 plasmid with four different KO conditions: TRAC only, TRBC only, combined TRAC/TRBC, and combined TRAC/TRBC with half the normal concentration of RNP.

For both dsDNA and MC TCRs, the combined **α**/**β** KO produced higher KI efficiencies than **β** KO alone. However, the single **α** KO/KI produced comparable KI efficiencies to the dual KO for both TCRs. For robustness, the dual **α**/**β** KO condition was used in future experiments. Notably, the beta KO condition resulted in the death of most of the cells. Some experimental error may have occurred with this sample, since the single KO of TRBC has been shown to produce similar cell viabilities and KI efficiencies as single TRAC KO[78,79]. In addition, the dual **α**/**β** KO conditions with halved concentrations of **α** and **β** RNPs also produced comparable KI

efficiencies. For conservation of RNPs in the future, this concentration of RNP was used in future experiments to improve the CRISPR-Cas9 method.

HA Length

The original TCR used previously[68] had homology arms (HA) 300 bp in length. These arms refer to the regions of DNA in the engineered TCR before the start of the TCR gene and after the stop codon of the gene, which have homology to the TCR genome flanking the cut site and are essential for integration during KI. The PACT MC produced better KI efficiencies than the PACT dsDNA, and we hypothesized that this may be due to the 800 bp length of the HAs in the PACT MC TCR. To test this hypothesis, TCRs from PACT and Oh et al.[77] with 800 bp HAs were manufactured and compared against the standard 300 bp HA version. The flow cytometry data is shown in Figure 7.

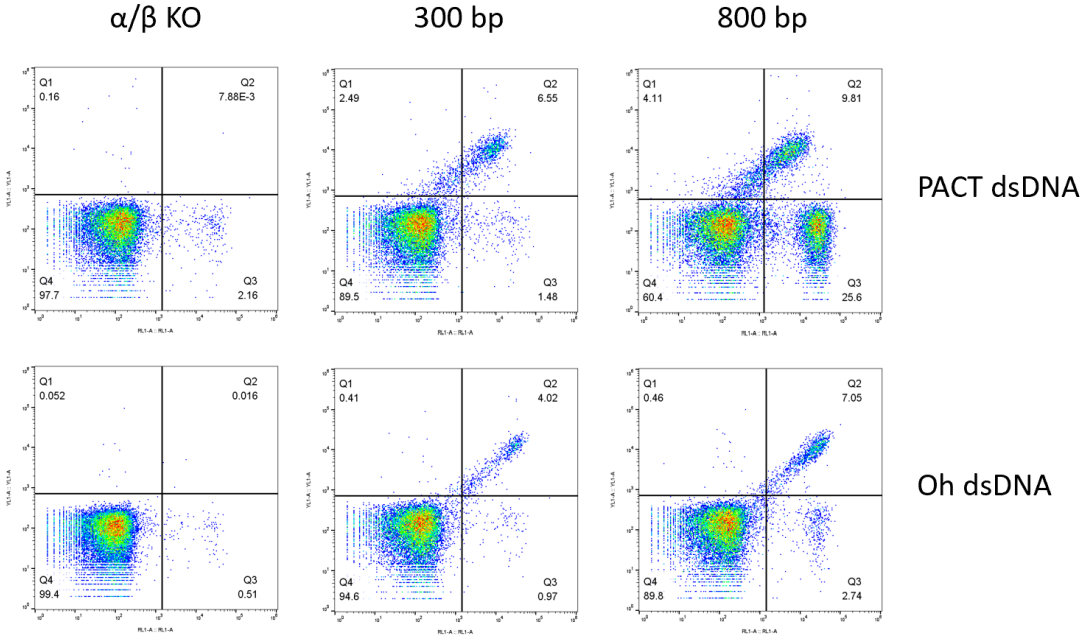


Figure 7: Flow cytometry data comparing linear dsDNA TCRs derived from PACT and Oh et al.[77] at 300 bp and 800 bp HA lengths.

For both PACT and Oh et al.[77] dsDNA TCRs, those with 800 bp HAs had higher KI efficiencies than the TCRs with 300 bp HAs. This length of HA was used in future TCR manufacturing processes.

800 bp HA TCR Concentrations

For further optimization, three different concentrations of 800 bp HA PACT dsDNA were tested, 1, 3, and 5 ug. Also, the condition with half the concentration of RNP was added to see if the earlier results were reproducible. The flow cytometry data is shown in Figure 8.

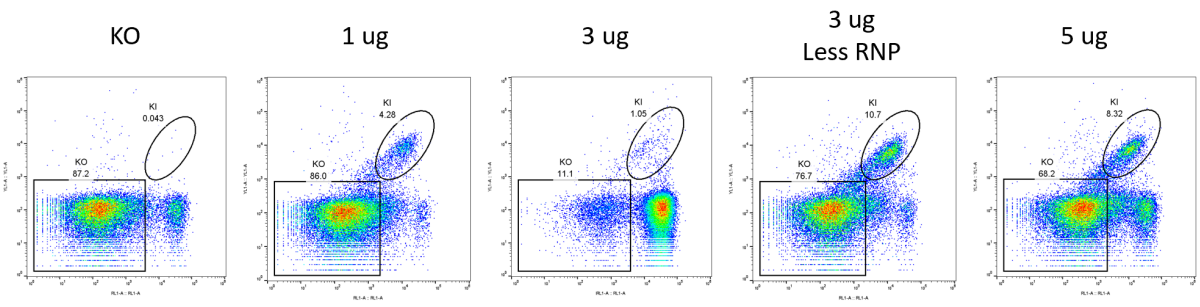


Figure 8: Flow cytometry data comparing 3 different concentrations of PACT dsDNA, including one condition with half the normal concentration of RNP in the CRISPR-Cas9 reaction.

The concentrations producing the best KI efficiencies were the 3 μg less RNP and 5 μg conditions. It is likely that some error occurred for the standard 3 μg condition, since the KI was incomplete and this result isn't consistent with previous results for PACT dsDNA. Generally, the more TCR DNA added, the higher the KI efficiency will be. To conserve manufactured DNA while maximizing KI efficiency, the 3 μg condition was used for future experiments.

Application of CRISPR-Cas9 to Create HPV-Specific CD8+ T Cells

After the optimization of the CRISPR-Cas9 editing protocol, this system was applied to create HPV-specific T cells to assist in the HPV-16 clinical trial project. Almost 100 TCRs have been identified and assembled, and about half of these sequences have been validated in Jurkats. The following are flow cytometry analyses of the first 11 TCRs, with some analyses done by myself and some by other members of the Heath lab.

Pooled Peptide Binding Analysis

Using a selection of the top potential binding peptides for the first 11 HPV-specific TCRs with human growth hormone (HGH) signal peptide, fluorescent tetramers were assembled and a pooled binding experiment with subsequent flow analysis was conducted to see if any of the selected peptides were potential cognate antigens. Each TCR was also stained with a pool of the top 3 HLA-unmatched peptides for each. This was used as a control to confirm if the tetramer PE signal may be from the binding of HLA-unmatched SCTs rather than true binding. This data is shown in Figure 9.

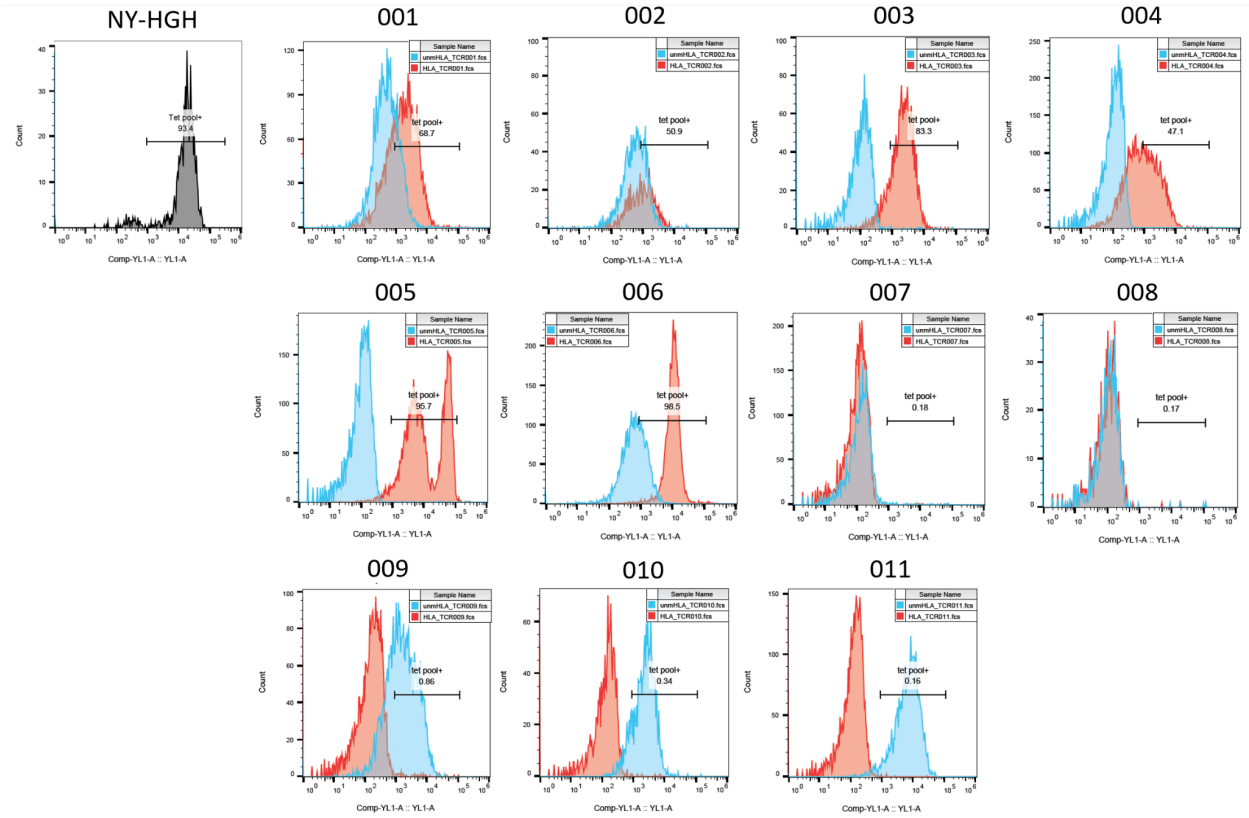


Figure 9: Flow cytometry data of the pooled tetramer experiment in REP-expanding primary T cells expressing a control NY-ESO-1 specific TCR and the first 11 HPV-specific TCRs, showing the frequency of tetramer positive cells. The red peaks are signals from the HLA-matched SCT pool stain, and the blue peaks are signals from the HLA-unmatched SCT pool stain. This data was processed by Rongyu Zhang.

For each sample, a positive tetramer signal was gated at above 10^3 YLA laser fluorescence as a conservative measure to ignore low-intensity PE fluorescence that is likely due to errant binding of the tetramer molecule to the cell surface. As expected, the NY-ESO-1 positive control with HGH signal peptide showed positive tetramer signal. TCRs 001-006 had positive signals from the HLA-matched tetramer pool, while TCRs 007-011 did not express a positive tetramer signal. TCRs 001 and 002 expressed signal from the HLA-unmatched pool, suggesting that the positive HLA-matched tetramer binding signal was not entirely due to true tetramer binding. TCR 005 showed two distinct tetramer positive peaks. This may be due to differential binding affinity to the KFY-group of HPV TCR-specific peptides which vary by only one amino acid. TCRs 009-011 showed that their high tetramer signals were from HLA-unmatched tetramer binding, likely because the patient TCRs are never trained to tolerate non-self HLAs, so they are able to recognize the unmatched HLA tetramers[71].

TCRs 001-006 showed positive tetramer binding signal, so these TCRs and tetramer pools were tested in a subsequent flow experiment with separate tetramer staining. Despite the HLA-unmatched pool signal, TCRs 001 and 002 were included in the analysis. The separate

stain of each peptide will determine if TCR 005 showed differential binding to the two KFY peptides.

Unpooled HPV TCR Binding

Using the first 6 HPV-specific TCRs with HGH signal, each tetramer was tested separately to identify a potential cognate antigen. TCR 003 did not express TCR, so it was omitted from this analysis to conserve tetramer. The flow cytometry data is shown in Figure 10.

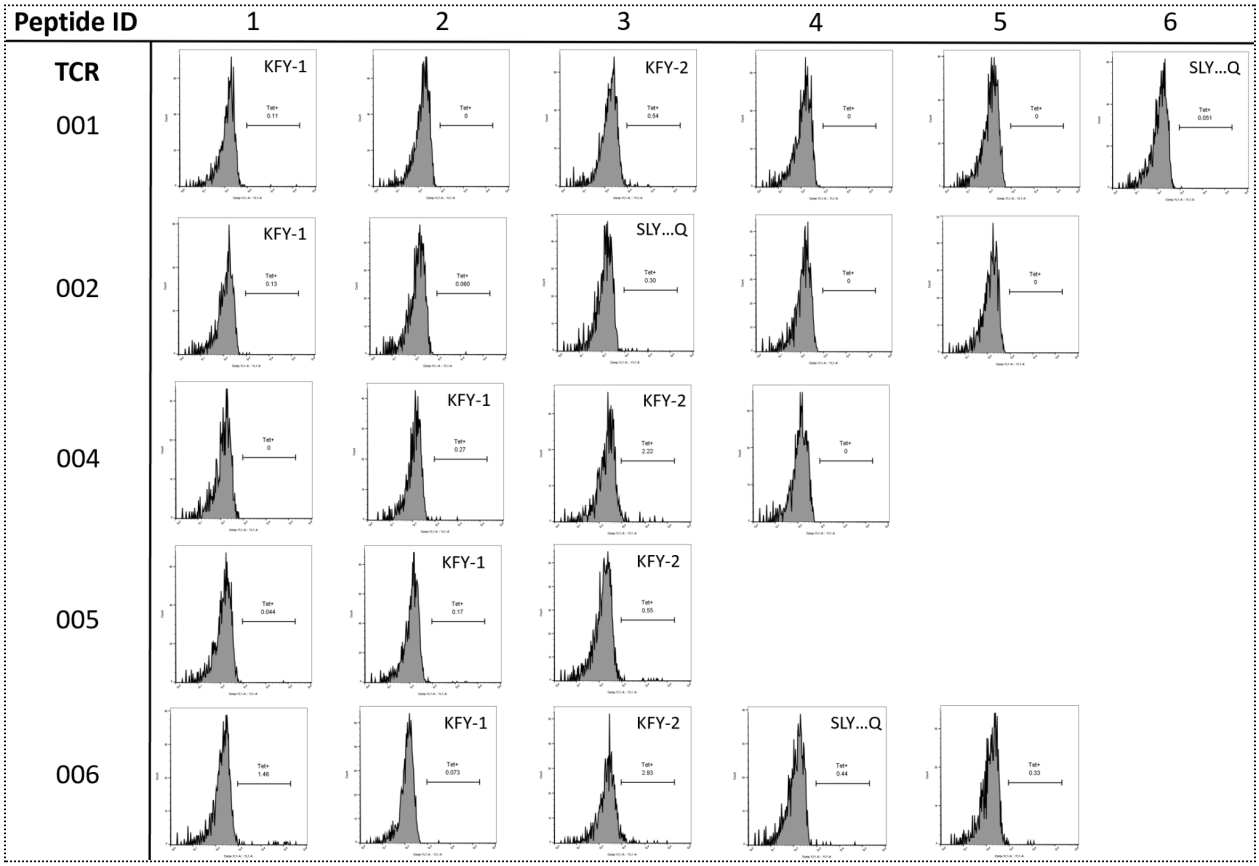


Figure 10: Tetramer binding results for HPV-specific TCRs 001, 002, and 004-006, with the top peptides ordered by rank for each TCR. The two KFY peptides differing by one amino acid are indicated where applicable. Another peptide family of future interest, SLY, is also indicated.

All TCRs showed the highest population of tetramer positive cells when bound to their third-ranked potential cognate peptide. The highest among these proportions was about 2% for both TCR 004 and 006. TCR 006 is the only TCR which showed binding for their top-ranked cognate peptide, which was at 1.5% population proportion. This observation may indicate a flaw in the ranking system for the top peptide TCR binders. Notably, most of the third-ranked peptides were one version of the KFY peptide, which may suggest some nonspecific binding occurred. TCR 005 showed a low tetramer binding signal to both versions of the KFY peptide, which may account for the two peaks shown in the pooled peptide binding experiment.

Based on these results, it is evident that TCRs 001-006 expressed effective tetramer binding to at least one of their top ranked cognate peptide antigens. Further analysis was done on these TCRs in an attempt to replicate these results.

The primary human T cell cultures prepared for this experiment were expanded by REP, including T cells edited with HPV-specific TCR 003. REP expansion has been observed to initially downregulate TCR expression before recovering after about a week[80]. The REP expanding samples were stained with anti-TCR and tetramer to see if this recovery of TCR expression was achieved after 8 days. The peptides chosen to stain with are from the SLY family, which were high ranking cognate peptide antigens for TCRs 001-006. These peptides did not produce a positive signal in an initial TCR binding experiment, but were revisited to determine if TCR 005 was showing differential binding to these antigens instead of the KFY peptides. This data is shown in Figure 11.

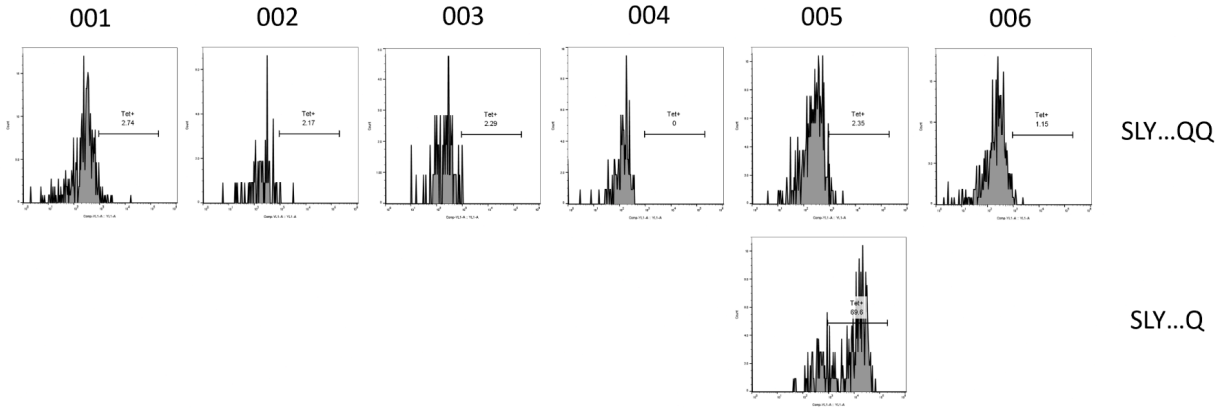
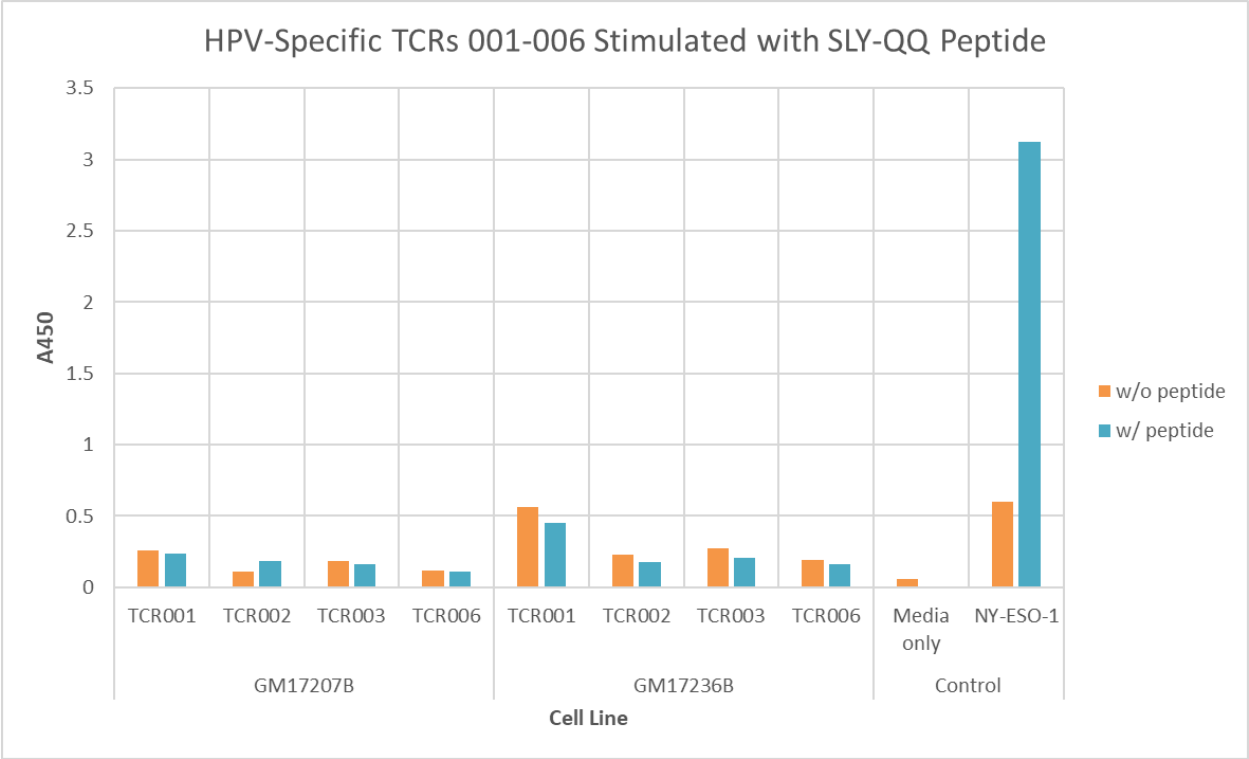


Figure 11: Tetramer binding results for HPV-specific TCRs 001-006 in REP expanded primary human T cells, stained with two different SLY tetramers differing only by the number of terminal glutamine residues.

TCR 005 bound to the SLY peptide ending with a single glutamine showed the highest tetramer binding signal of all the samples. These peptides and all 6 TCRs were used in a functional analysis with ELISA in the following experiment.

Functional Validation of TCRs 001-006[75]

These TCRs were tested in a cytokine release assay to measure concentrations of cytokine secreted after stimulation with aAPCs expressing each SLY cognate antigen. Two different LCLs were used, GM17207B and GM17236B. Each LCL was induced to express SLY-QQ and SLY-Q peptides. TCRs 001-004 and 006 were stimulated with SLY-QQ peptide and TCR 005 was stimulated with SLY-Q peptide only, and the level of IFN-γ was measured for each condition. This data is shown in Figure 12.



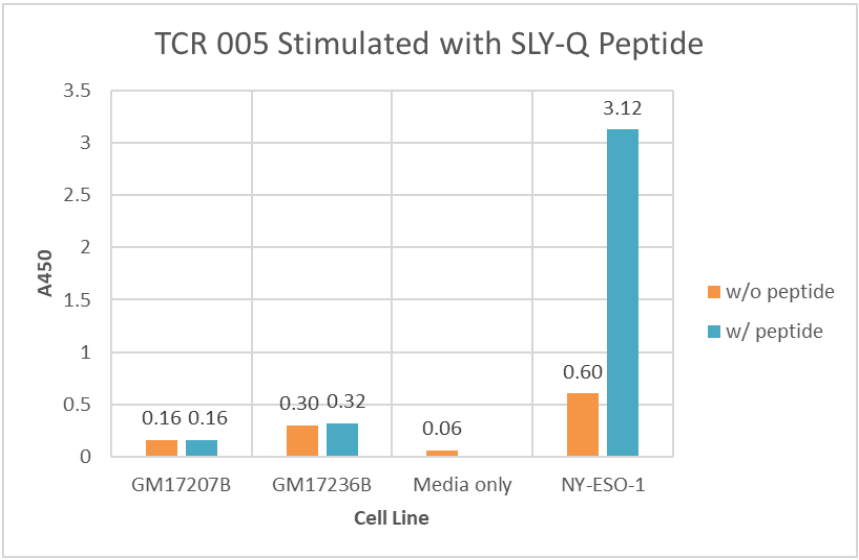


Figure 12: IFN-γ secretion of primary human T cells expressing TCRs 001-006 after stimulation with LCL cell lines expressing SLY-Q and SLY-QQ cognate peptide antigen. The optical density A_{450} is used as an approximation of IFN-γ concentration levels. This data was collected by Sunga Hong.

None of the 6 HPV-specific TCRs showed higher cytokine release when stimulated with their cognate peptide antigen compared to unstimulated cells. This indicates that these T cell clones are non-functional. Unfortunately, none of these TCRs can be used for the future HPV clinical trial due to their lack of TCR functionality in the presence of their cognate antigen.

Discussion

CRISPR-Cas9 T Cell Editing System

By identifying and applying strategies from the current scientific literature, the CRISPR-Cas9 T cell editing protocol was improved. The use of the linear dsDNA TCR based on the PACT Pharma[76] design, and the optimization of genome KO location, reagent ratios, HA length, and TCR concentrations, the KI efficiency of this method was increased hundredfold from 0.1% to 10%. These results demonstrate that CRISPR-Cas9 editing technology can be utilized to efficiently engineer tumor-specific T cells, which is an idea well documented in the literature. Although this KI efficiency isn't as high as some papers have reported, this was still an important improvement for the Heath lab to continue research in the area of CRISPR-Cas9 engineered TCR immunotherapy.

CRISPR-Cas9 precision gene editing technology has opened the door for improved safety and feasibility of TCR-engineered therapies in the clinical setting. CRISPR-Cas9 is more specific, simpler, and safer than alternative viral and non-viral gene editing methods. The mechanism of CRISPR-Cas9 allows it to have very high specificity for where exogenous DNA is inserted into the cell genome. Viral vector delivery of the engineered TCR is not guaranteed to disrupt the cell's original TCR, and the expression of both endogenous and exogenous TCRs can increase the frequency of mispairing by binding to one another, reducing the availability of TCRs to bind antigen and achieve therapeutic functionality[27]. The specificity of CRISPR-Cas9 avoids this limitation, potentially leading to a more functional therapeutic T cell product. Its simplicity compared to ZFNs and TALENs has led to its dominant use in recent T cell engineering clinical trials[81]. CRISPR-Cas9 gene editing is also less toxic to patient cells than viral vectors[61]. Therefore, this genetic engineering strategy is the optimal choice for the HPV clinical trial.

CRISPR-Cas9 technology is significantly easier and faster than other gene editing methods, giving it a unique advantage in clinical trials. The use of CRISPR-Cas9 gene editing technology could lead to combination therapies wherein tens of distinct engineered T cell clones could be developed in the laboratory and administered to patients as a personalized multiplexed cancer treatment. It isn't feasible to generate several personalized clinical-grade viral vectors per patient, but a CRISPR-Cas9 editing workflow with high editing efficiency and consistent KI results could realize this potential. Foy et al.[82] have demonstrated the feasibility of this concept, applying CRISPR-Cas9 to make personalized multiplexed TCR-engineered T cell therapies using TCR discovery in a phase I clinical trial.

Despite these benefits, CRISPR-Cas9 still has limitations in clinical applications. Although this report demonstrates a 10-fold increase in KI efficiency to the order of 10%, that efficiency is still very low. This poses a problem when scaling up the cell product to generate a high quantity and quality immunotherapy treatment. Cell sorting and expansion is more difficult when the initial edited cell populations are small, increasing production time and complexity. In addition, there is a risk for off-target gRNA binding, leading to KO mutations in other portions of the T cell genome that may be oncogenic. Off-target DSBs are rare, reported at a frequency of

about 6% of all DSBs in the Stadtmauer et al.[60] clinical trial. This risk can be mitigated by increasing the length of TCR HAs to reduce the probability of mispairing. For effective use in clinical trials, this off-target effect should be quantified, but online gRNA tools are limited by lack of training data and limited algorithms[83] and thus may not provide an accurate risk assessment. Another limitation is that DSBs have been shown to induce cell apoptosis[84]. Cells with functioning p53 protein are more likely to undergo apoptosis, so there is a selection bias toward cells with abnormal p53 expression which are more likely to develop into cancer[85].

There are also limitations of the methods of this study. First, since CRISPR-Cas9 editing efficiencies were at or below 10% for the duration of this research, the edited cell populations were usually very small. Flow cytometry may have poor accuracy when analyzing such small cell populations in a larger cell sample, due to a higher signal-to-noise ratio[86]. This may have led to the dismissal of some experimental condition that could have produced higher KI efficiencies. Another limitation was low overall cell counts after primary T cell isolation. To compensate and make the best out of the experiment, the KO control was omitted several times. This reduced the certainty of the results, but allowed the project to progress. Ultimately, sacrificing the KO control did not hinder the discovery of an effective CRISPR-Cas9 method with higher KI efficiencies. This practice was not continued when applied to work on the HPV clinical trial.

In the Heath lab, the CRISPR-Cas9 protocol will continue to be improved as more information is discovered about its mechanism to edit primary human T cells. Recently, the addition of the non-homologous end joining inhibitor AZD7648[87] was added to the protocol by Jongchan Choi and has shown moderate improvements in KI efficiency[80]. Novel transient approaches to RNP and donor DNA delivery to cells are continuing to be developed in the literature to further improve the performance of CRISPR-Cas9 in the clinical trial space.

Generation of HPV-Specific CD8+ T Cells

The CRISPR-Cas9 T cell engineering protocol was applied to validate the sequences of the first batch of HPV-specific TCRs in primary human T cells. TCRs 001-006 were the first sequences to show effective expression in primary T cells and were used to create the first batch of HPV-specific CD8+ T cell clones for functional validation. Unfortunately, the TCRs were not functional when expressed in primary T cells, and were not able to progress to the clinical trial stage. This lack of functional activity isn't surprising, as Foy et al.[82] reported only 73/127 TCRs, or 57%, discovered from patients secreted significant amounts of IFN-γ cytokine after cognate antigen stimulation in preclinical validations. This lack of TCR function may be due to protein misfolding, or indicate that the tetramer positive signals in the initial binding assays may be capturing nonspecific tetramer binding. Members of the Heath lab are continuing their search for additional HPV-specific TCRs that demonstrate both cell expression and cytotoxic function.

The use of this method to treat HPV-related cancers could fill a need where the HPV vaccines have failed, allowing patients with severe cases of such cancers a chance for recovery and providing treatment for unvaccinated individuals. The discovery of HPV-specific TCRs from these patients could lead to the development of an HPV-specific TCR library with specificity to

every possible E6 and E7 HPV-16 cancer antigen. Theoretically, for each TCR with specificity to a particular E6 or E7 antigen, there would be several variants with different HLA expression. These could be used for multiplexed TCR-engineered immunotherapy with matched HLA identity to cover as many target HPV antigens as possible. The HLA coverage of the TCR library would also allow for the treatment of a much broader population of patients, since HLAs have differential dominance across race and ethnicity. The relative affordability of CRISPR-Cas9 gene editing compared to viral vectors could allow this treatment to reach disadvantaged populations with less access to HPV vaccines. Further, a more ambitious idea is to apply the TCR discovery pipeline to each individual patient from scratch to develop a highly personalized HPV immunotherapy treatment. Demonstrating the efficacy of such a method could revolutionize the T cell immunotherapy field and make possible the elimination of several types of antigen-presenting cancers simultaneously[82].

Although it poses a smaller risk compared to CAR-T cell therapies, TCR-engineered T cell immunotherapy may still result in CRS after cytotoxic T cell transfusion. Therefore, it is important to fully understand the cytotoxic activity of the edited T cells to assess and minimize CRS risk in patients. This becomes especially important when discovering novel patient-specific TCRs, which may have unpredictable cytotoxicity profiles.

Despite the fact that the personalized TCR discovery and immunotherapy approach of the clinical trial could have a great positive impact on patient lives, it is by nature not generalizable and therefore would be difficult to commercialize. The TCR discovery and T cell engineering pipeline must be highly efficient to make this method feasible. In particular, the TCR and cognate antigen discovery pipeline is slow, with very few discovered TCRs able to bind antigens in Jurkats, and none yet able to demonstrate functionality in primary human T cells. Accelerating the process of TCR validation in Jurkats is necessary to make adequate progress in the phase I HPV trial, which requires a more multiplexed and high-throughput approach. A high-throughput validation pipeline is much easier with CRISPR-Cas9 in the primary T cells, but more difficult to accomplish with lentiviral transduction in Jurkats for initial binding validation. It may be beneficial to generate a method for editing the Jurkat cells with CRISPR-Cas9 to accelerate this process. As of now, the process is time-consuming and expensive, but the Heath lab is working to accelerate and validate these pipelines.

Conclusion

Using linear dsDNA donors from the PACT method, an effective CRISPR-Cas9 gene editing system was developed. The new design with improved KI efficiency made possible the editing of tens of CD8+ T cells to validate TCRs specific to HPV cancer E6 and E7 neoantigens. This method of gene editing is simpler, cheaper, and safer than viral methods, making this solution useful to the Heath lab and appropriate for use in the high-throughput development of HPV-specific T cells in the HPV cancer clinical trial. As relevant literature is published, new strategies will be tested by the Heath lab to improve the editing method over time.

The HPV trial is ongoing in the Heath lab, and the data presented in this thesis is not complete. The Heath lab has worked to discover approximately 100 TCRs from patient blood samples, and nearly half of them have been tested in Jurkats and primary T cells. Moving forward, more TCRs will be discovered, manufactured, and utilized to generate HPV-specific CD8+ T cell clones. Primary T cells with adequate expression of significantly functional HPV-specific TCRs will be used in a phase I clinical trial comparing the effectiveness of a multiplexed regimen of TCR-engineered CD8+ T cells and well-validated T2 cell treatment expressing NY-ESO-1 TCR. This trial will hopefully fill in the treatment gaps that are not adequately met by the HPV vaccine and other cancer treatments.

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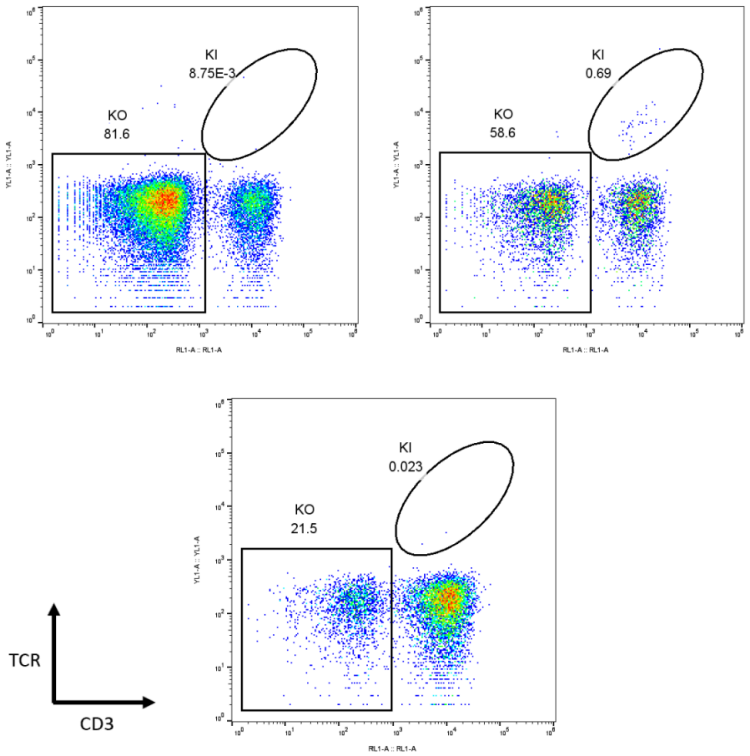
Supplementary Data

Iteration of Design Components

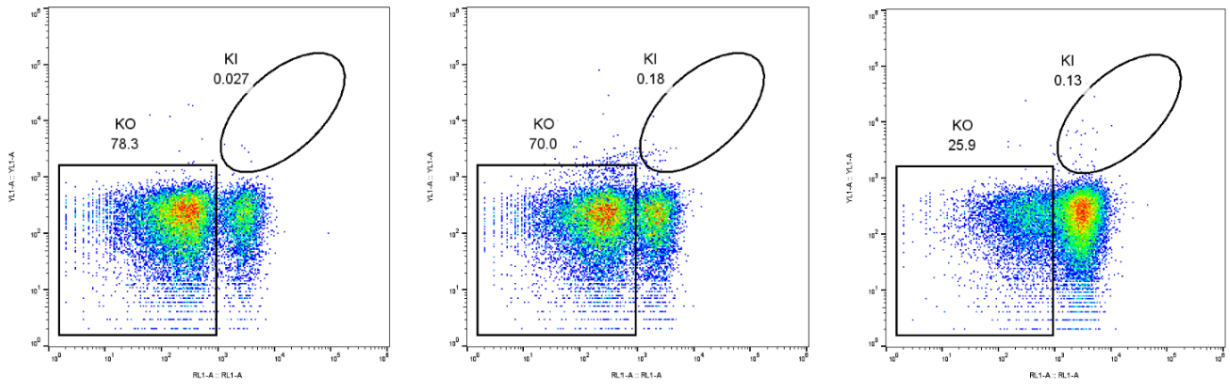
dsDNA vs ssDNA

In the first iteration of the base experiment, the ability of single stranded DNA (ssDNA) donors to better integrate into the T cell genome compared to dsDNA was tested. This idea was inspired by a paper by Roth et al.[68] where ssDNA was shown to effectively correct a IL-2**α** receptor mutation in T cells after CRISPR knock in. Single stranded forms of donor DNA were shown to reduce off-target integration. This ssDNA was produced through exonuclease digestion of the original dsDNA donor TCR sequence. The flow cytometry data is shown in Supplementary Figure 1.



Supplementary Figure 1: Flow cytometry data for an experiment comparing dsDNA and ssDNA forms of the TCR donor. Samples are KO control, dsDNA, and ssDNA.

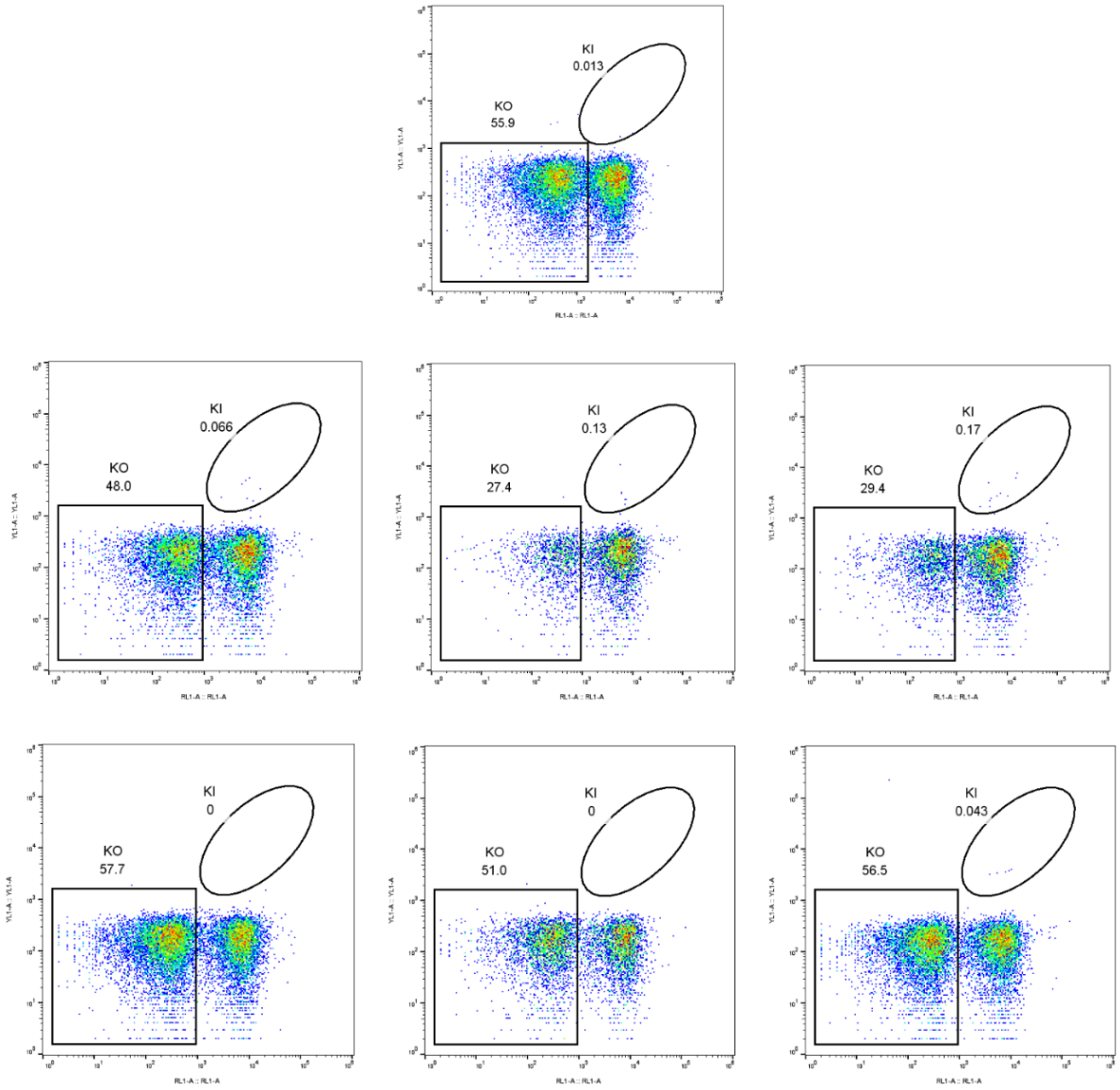
The knock-in efficiency for the dsDNA condition was about 10-fold higher than for the ssDNA condition. A repeat of this experiment showed similar results (see Supplementary Figure 2), so future iterations used dsDNA donors.



Supplementary Figure 2: Flow cytometry data for a repeat experiment comparing dsDNA and ssDNA forms of the TCR donor. Samples are KO control, dsDNA, and ssDNA.

DNA Cas9 Target Sequences (CTSs)

Inspired by a paper by Nguyen et al.[88], the dsDNA and ssDNA donors were modified to have an additional 20 base pair Cas9 target sequence (CTS) at each end. This CTS sequence is designed to bind to the RNP complex, allowing the donor DNA to enter the cell nucleus and increasing knock in efficiency. In addition, two types of RNP complexes were added to one sample of CTS-dsDNA. Each complex had a different sgRNA strand, one targeting the TRAC region, and the other targeting the TRBC region. TRBC is another candidate region for a Cas9 double strand break that has been shown to result in comparable KI efficiencies[68]. The rest of the samples used only the TRAC-targeting sgRNA in the RNP complex. The flow cytometry data is shown in Supplementary Figure 3.

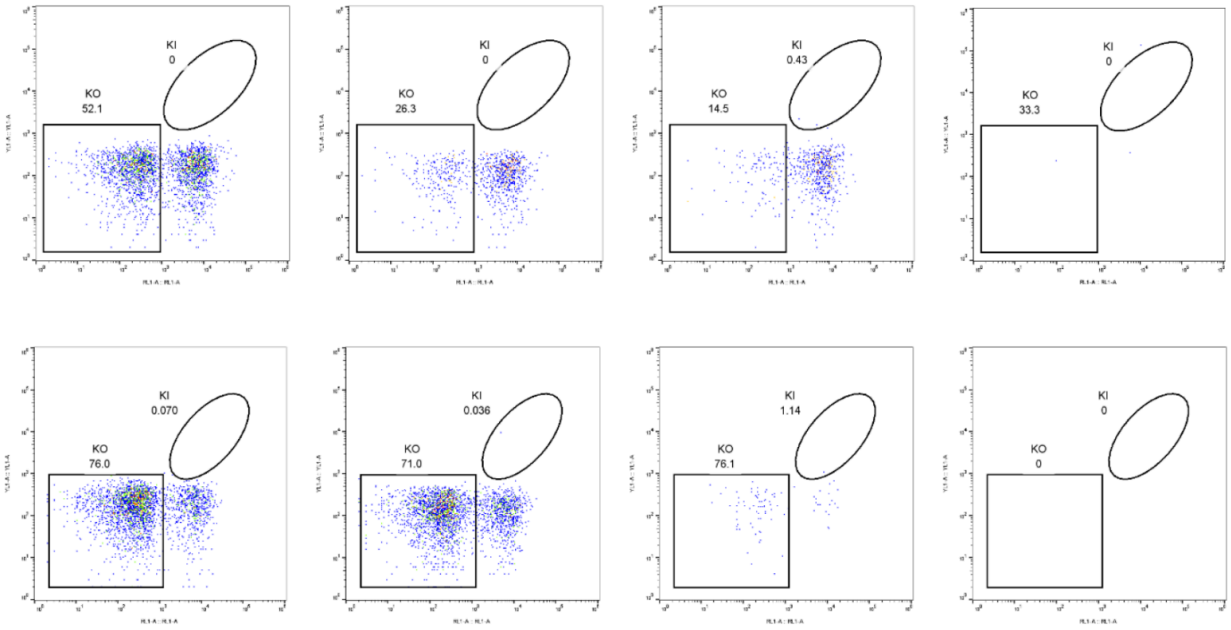


Supplementary Figure 3: Flow cytometry data for an experiment testing the effectiveness of binding the donor TCR to the RNP by adding a CTS sequence at each end of dsDNA and ssDNA donor TCRs. This experiment also compared the single KO of TRAC to the dual KO of TRAC and TRBC regions of the TCR gene. Samples are KO control, dsDNA, ds-CTS-**α**, ds-CTS-**α**/**β**, ssDNA, ss-CTS-**α**, and ss-CTS-**α**/**β**.

The DNA tagged with CTS did not produce a meaningfully higher KI efficiency compared to DNA without CTS. Again, dsDNA samples appeared to produce higher efficiencies than ssDNA samples. Each AB sample seemed to have higher efficiency than their respective 1x condition, which is probably because there are more cut sites for the DNA to integrate, increasing the KI probability. This increase wasn't large enough to justify continuing with this condition.

Anti-Cas9 and Anti-His

The high affinity reaction between biotin and streptavidin was utilized to attempt to noncovalently bind the TCR donor to Cas9. The TCR donor was biotinylated, and an antibody conjugated to streptavidin was designed to have specific affinity to Cas9. When incubated together, the resulting RNP should have the biotinylated donor bound to the Cas9-specific antibody. A second condition used an antibody specific to a His tag on the N-terminal end of the Cas9. This interaction could help shuttle the donor TCR into the nucleus, increasing KI efficiency. The flow cytometry data is shown in Supplementary Figure 4, including data from a repeat of this experiment.

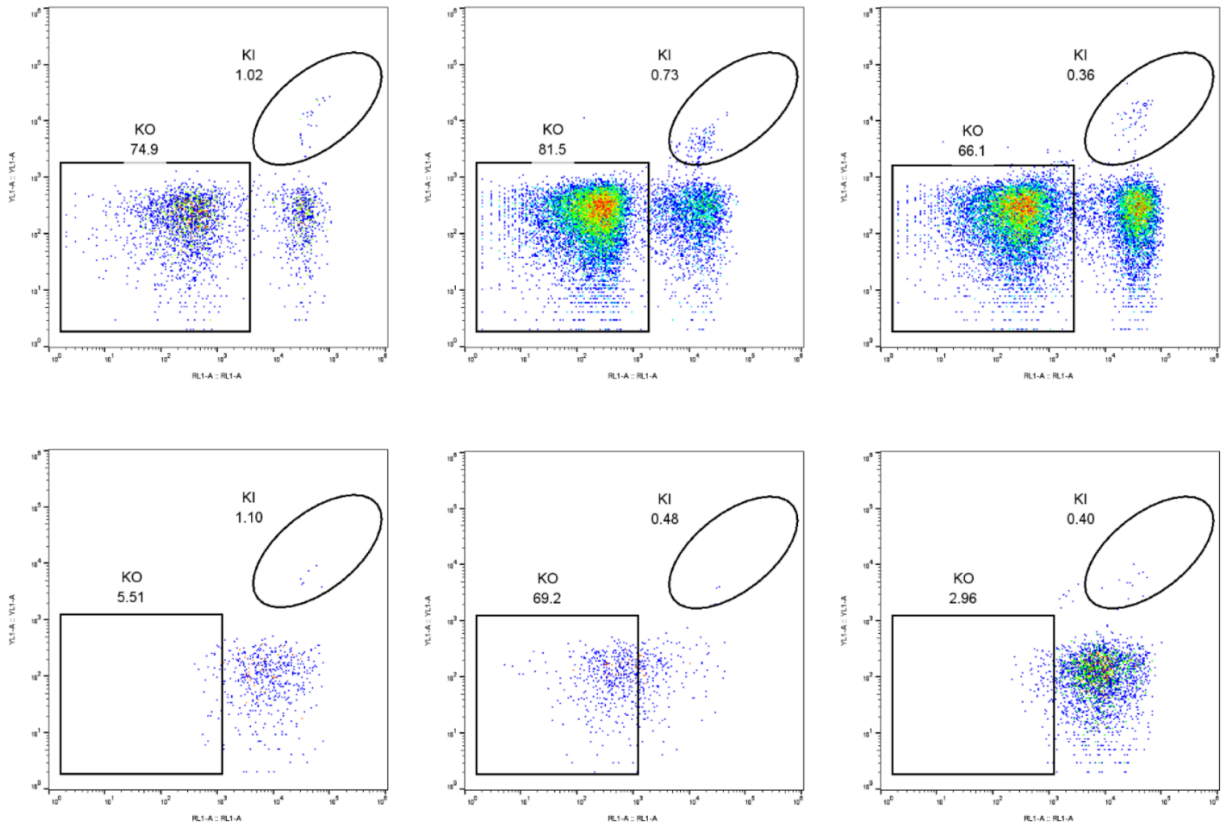


Supplementary Figure 4: Flow cytometry data for an experiment testing the effectiveness of binding the TCR to the RNP through a biotin-streptavidin interaction using two antibodies, anti-Cas9 and anti-His. Data from the original experiment are in the top row and data from a repeat experiment are on the bottom row. Sample columns are KO control, dsDNA, ds-anti-Cas9, and ds-anti-His.

In the first experiment, the anti-Cas9 condition produced a slightly higher KI efficiency than the lone dsDNA condition. Unfortunately, in both experiments the cell viability was lower than expected, so it is difficult to elucidate an accurate conclusion from the data. However, the cell viability for the anti-Cas and anti-His conditions were very low compared to the KO and dsDNA samples. It's possible that the Cas9 bound to the TCR donor produced a large complex that induced cell apoptosis. This method is more complex, expensive, and not consistently effective, so this interaction was not repeated in future experiments.

T Cell Activators

The Rezalofti et al.[69] review paper inspired the following experiments. Three T cell activators were compared: CD3/CD5 Dynabeads, CD3/CD28/CD2 tetramer, and TM-LCL activator. Also, the order of electroporation materials during RNP, donor DNA, cell, and electroporation enhancer combination was changed slightly to allow the RNP and DNA to associate by incubating for 1 minute at room temperature first, then adding enhancer followed by CD8+ T cells. In addition, electroporation and flow cytometry was conducted twice on these samples to compare a 2-day activation and a 5-day activation period. It is possible that a longer activation produces larger cell clusters and a more actively dividing culture. Notably, 5 days of incubation is slightly longer than the optimal range of activation time reported in the literature, 2 to 4 days[69]. The flow cytometry data is presented in Supplementary Figure 5.

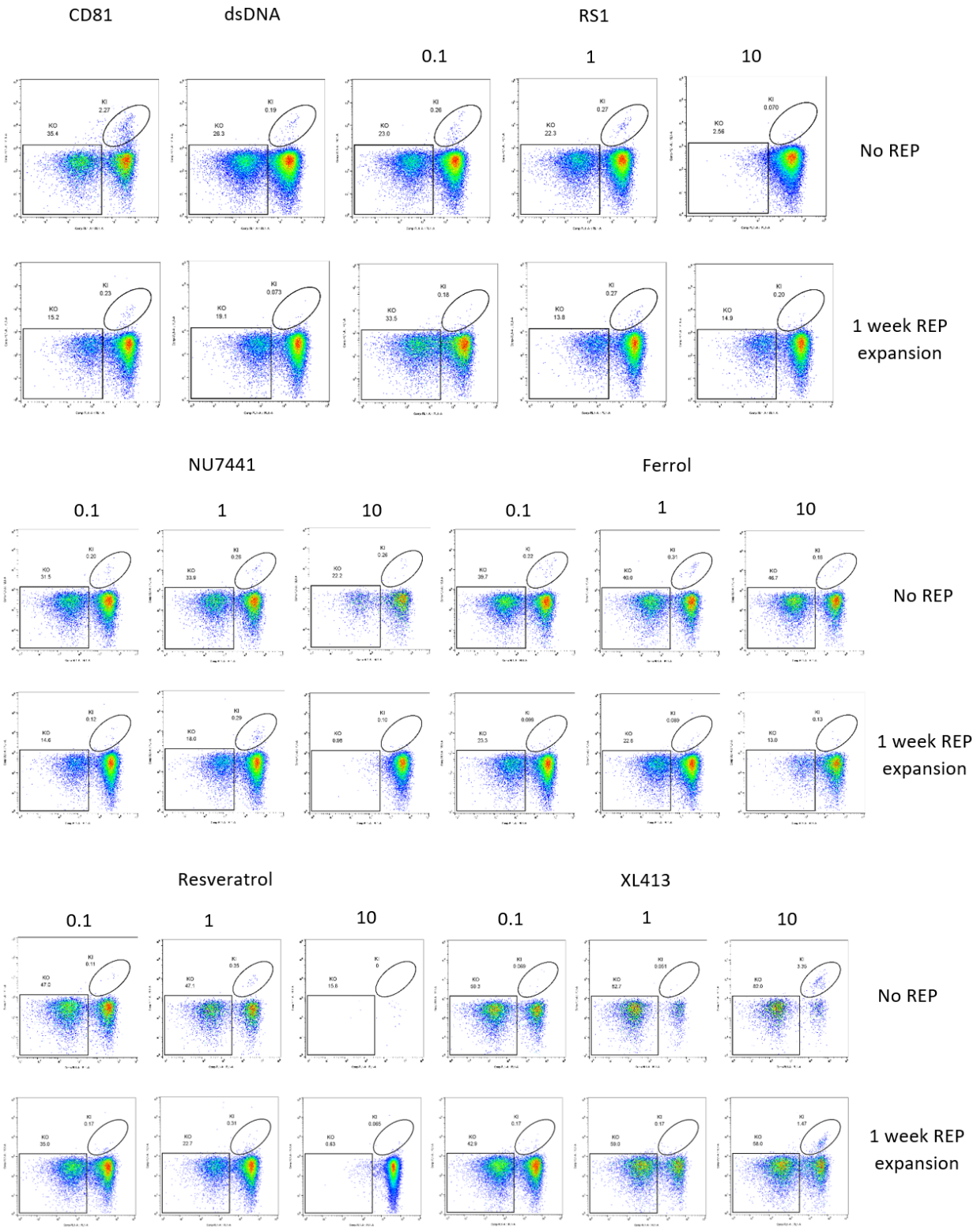


Supplementary Figure 5: Flow cytometry data for an experiment comparing CD3/CD5 Dynabeads, CD3/CD28/CD2 tetramer, and TM-LCL activators, after 2 days (top row) and 5 days (bottom row) of T cell activation. Sample columns are Dynabeads, tetramer, and TM-LCL.

The CD3/CD28/CD2 tetramer struck the best balance between cell viability and KI efficiency. Cell viabilities were much lower for the samples after 5 days of incubation, indicating that longer activation periods do not support more active cell cultures. The cells seem to have died after this length of time. The tetramer activator and 2-day activation time were used in future experiments.

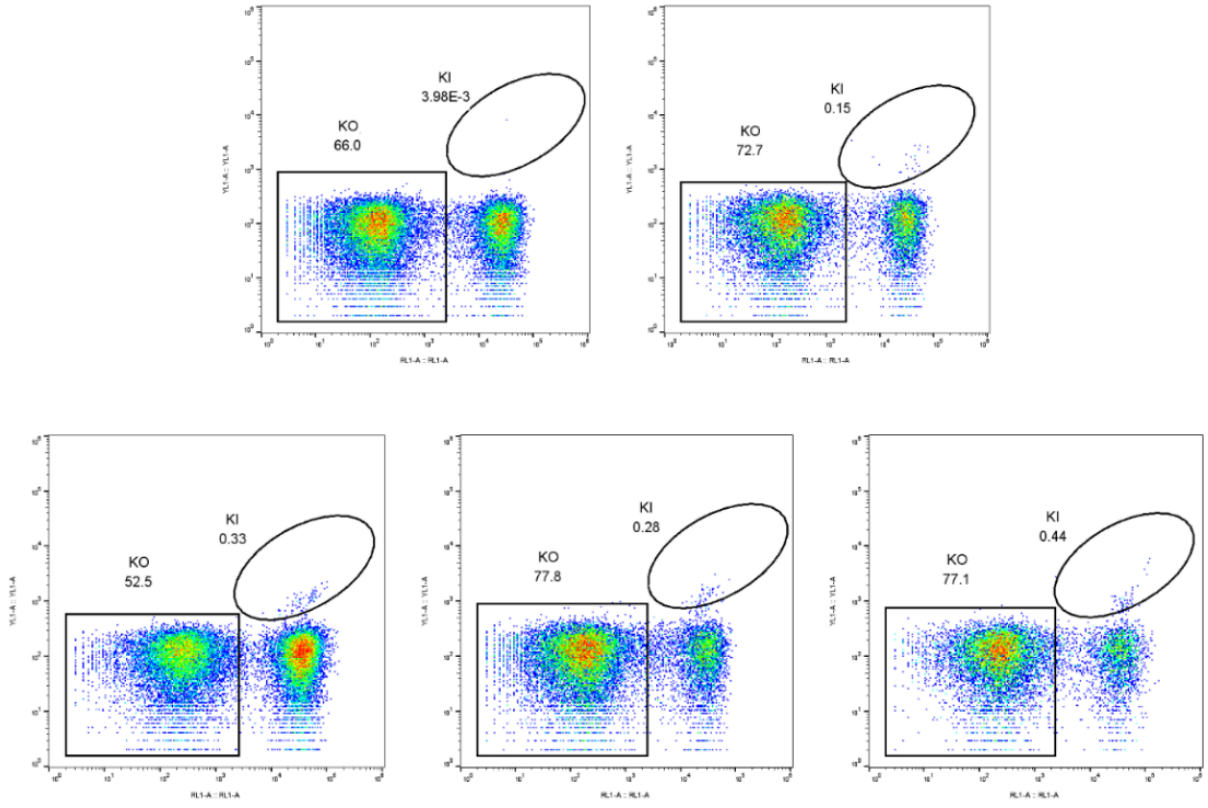
Small Molecule Inhibitors

Small molecules have been shown to upregulate and downregulate certain cellular processes. Certain molecules have been shown to enhance the activity of proteins involved in the HDR pathway of cells, and others inhibit proteins involved in the non-homologous end joining (NHEJ) pathway[90]. HDR and NHEJ work in opposition to repair a DNA double strand break, and less NHEJ and more HDR theoretically causes increased donor DNA insertion. From a selection of papers, six small molecules were incubated with cells after electroporation to see if the donor DNA could integrate better into the T cell genome, increasing KI efficiency. The small molecule HDR activators were Farrerol[91], RS-1[90,] and XL413[92], and the small molecule NHEJ inhibitors were Resveratrol[93] and NU7441[90]. Each molecule was tested at 0.1, 1, and 10 μg/μL concentrations. In addition, the co-activator CD81 was incubated with one T cell sample after activation. Finally, these samples were analyzed using flow cytometry after the standard 5 days of incubation and after an additional week of REP expansion, which utilizes specialized REP media that quickly grows T cell populations. The flow cytometry data is shown in Supplementary Figure 6.



Supplementary Figure 6: Flow cytometry data comparing the effectiveness of 5 small molecules to increase KI efficiency by promoting HDR or inhibiting NHEJ. Samples were tested after the standard 5 days post-electroporation and after an additional week of REP expansion.

Most small molecule conditions produced a higher KI efficiency than dsDNA alone. The efficiency by concentration varies for each small molecule, but for all molecules besides XL413 the 1 μg concentration produced the highest efficiency for samples without REP expansion. This was the case for RS1 and NU7441. This is probably the concentration that induces effective activator or suppressor activity while not disrupting other cellular functions. REP expansion had a mixed result on KI efficiencies for each sample. Since this expansion protocol takes a lot of time and resources, this condition will not be used in future experiments. The highest KI efficiency with and without REP expansion was produced by adding XL413 at 10 ng/uL. The next best condition without REP expansion was the addition of CD81. These two conditions resulted in relatively lower cell viability, so these samples were tested again before moving forward with these conditions. XL413 at 10 ng/μL and CD81 co-activator conditions were tested individually and together, and the flow data from this experiment is shown in Supplementary Figure 7. It was hypothesized that the effects from each molecule would be additive in the combined condition.

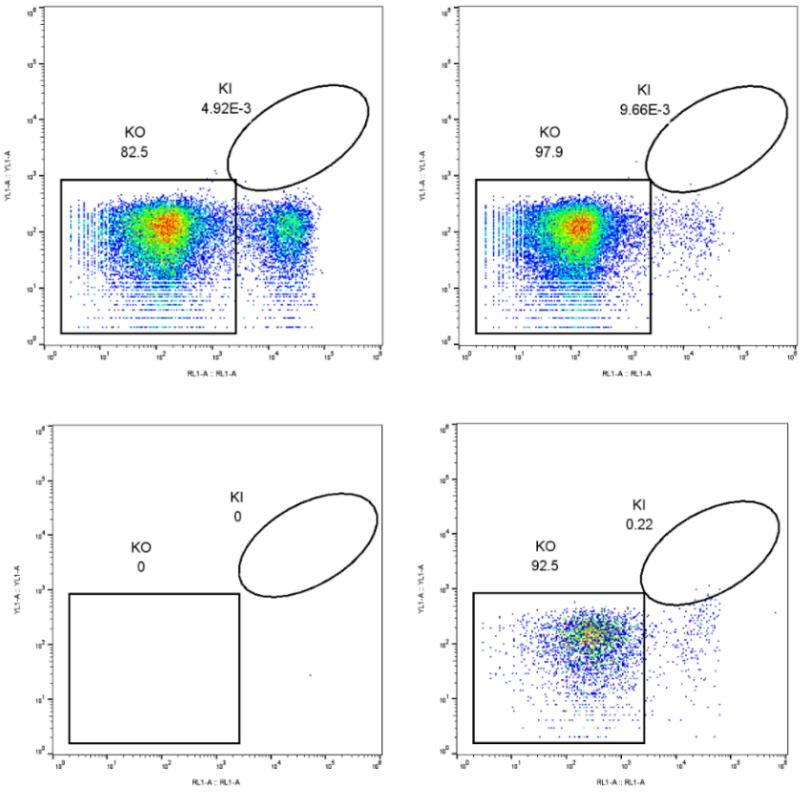


Supplementary Figure 7: Flow cytometry data for an experiment testing the two molecules that produced the highest KI efficiency from the previous experiment, XL413 and CD81. Samples are KO control, dsDNA, XL413 at 10 ng/μL, CD81, and XL413 at 10 ng/μL + CD81.

Once again, both individual conditions produced a higher KI efficiency compared to only dsDNA. However, these effects were not additive as predicted, even with a relatively high KO efficiency for the combined condition. These molecules could have been interacting with or inhibiting one another to negate any potential additive effects. Since XL413 seemed to produce a higher efficiency with moderate consistency, this molecule will be tested in some future experiments.

Neon vs Nucleofector

The Heath lab had purchased a Lonza 4D Nucleofector which was reported to consistently produce transfection efficiencies of about 90%[94]. This experiment compared the electroporation efficiencies of the Neon and Nucleofector transfection systems. The flow cytometry data is shown in Supplementary Figure 8.

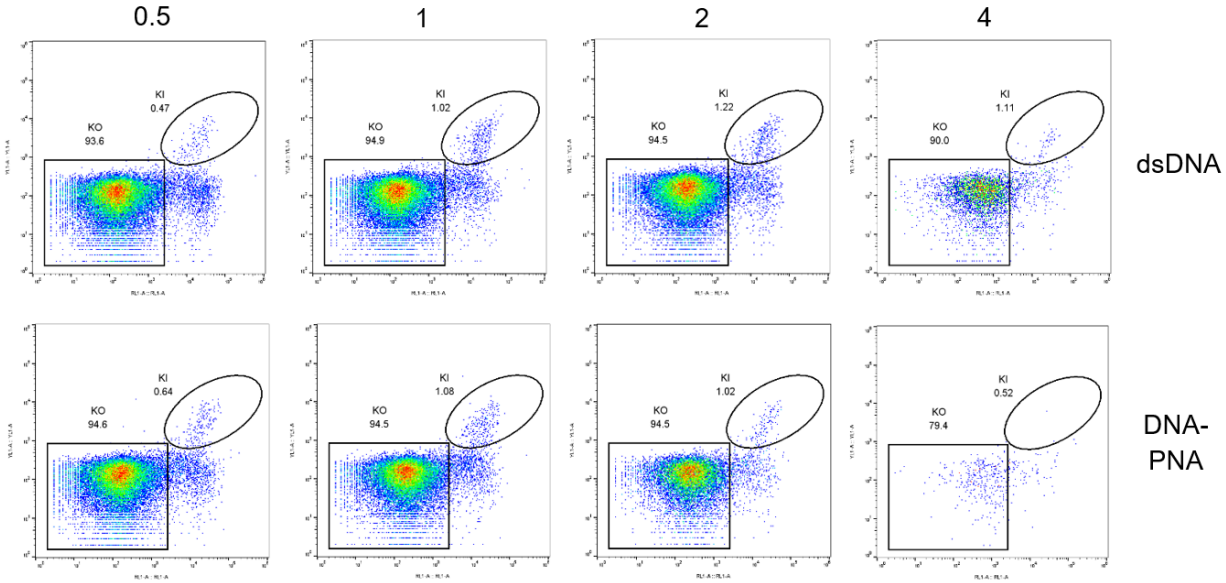


Supplementary Figure 8: Flow cytometry data comparing the Neon NxT and Lonza 4D Nucleofector transfection systems. Samples are Neon KO control, Nucleofector KO control, Neon dsDNA, and Nucleofector dsDNA.

The Nucleofector system produced greater KO efficiencies than the Neon system, at >90%. Although KI efficiencies didn't significantly increase, the Nucleofector was used in all future experiments due to the relative ease of the Nucleofector procedure.

DNA-PNA

Peptide nucleic acids (PNAs) are synthetic DNA analogs that can bind DNA. PNA molecules with specificity to one end of the donor TCR were generated to create molecular interactions between the TCR and the Cas9. dsDNA and DNA-PNA were tested at 0.5, 1, 2, and 4 μg/μL concentrations. Flow cytometry data for these conditions is shown in Supplementary Figure 9.

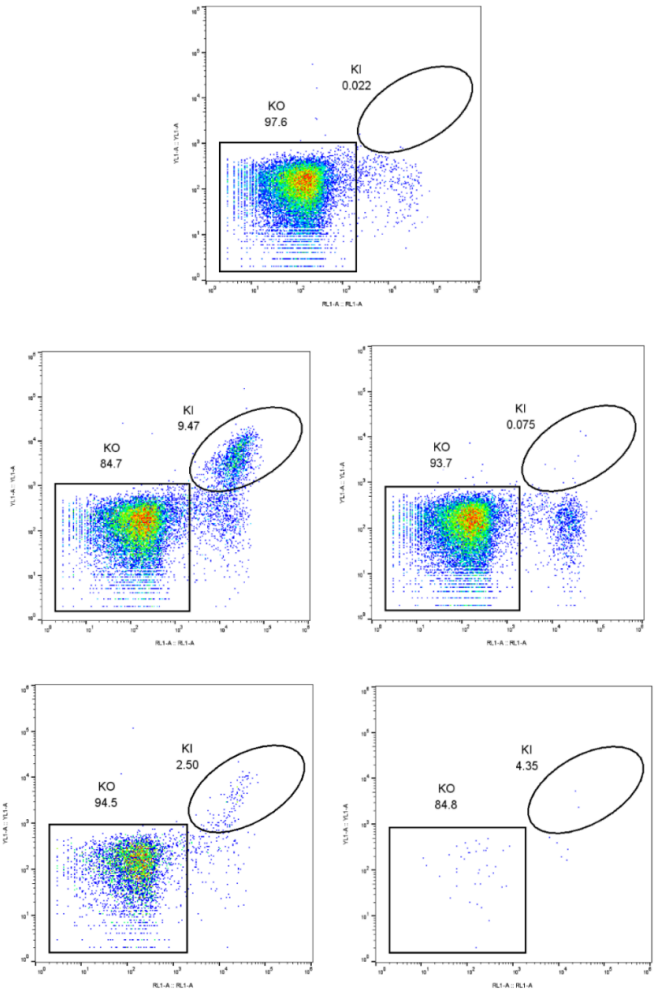


Supplementary Figure 9: Flow cytometry data for an experiment testing the effectiveness of binding the donor TCR to the RNP using DNA-PNA interactions. Both dsDNA and DNA-PNA samples were tested at 0.5, 1, 2, and 4 μg/μL concentrations.

DNA-PNA did not increase KI efficiencies compared to dsDNA for any concentration besides 0.5 μg/μL. The increase in efficiency at this concentration was too minor to justify making more PNA, a time-consuming and expensive process. Among the dsDNA samples, the concentrations that produced the highest KO and KI efficiencies were 1 and 2 μg/μL. These concentrations were used in future experiments.

TCR α/β KO and small molecules

The dual KO of TRAC and TRBC was tested with and without the addition of XL413 at 10 ng/μL concentration. The flow cytometry data for this experiment is shown in Supplementary Figure 10.

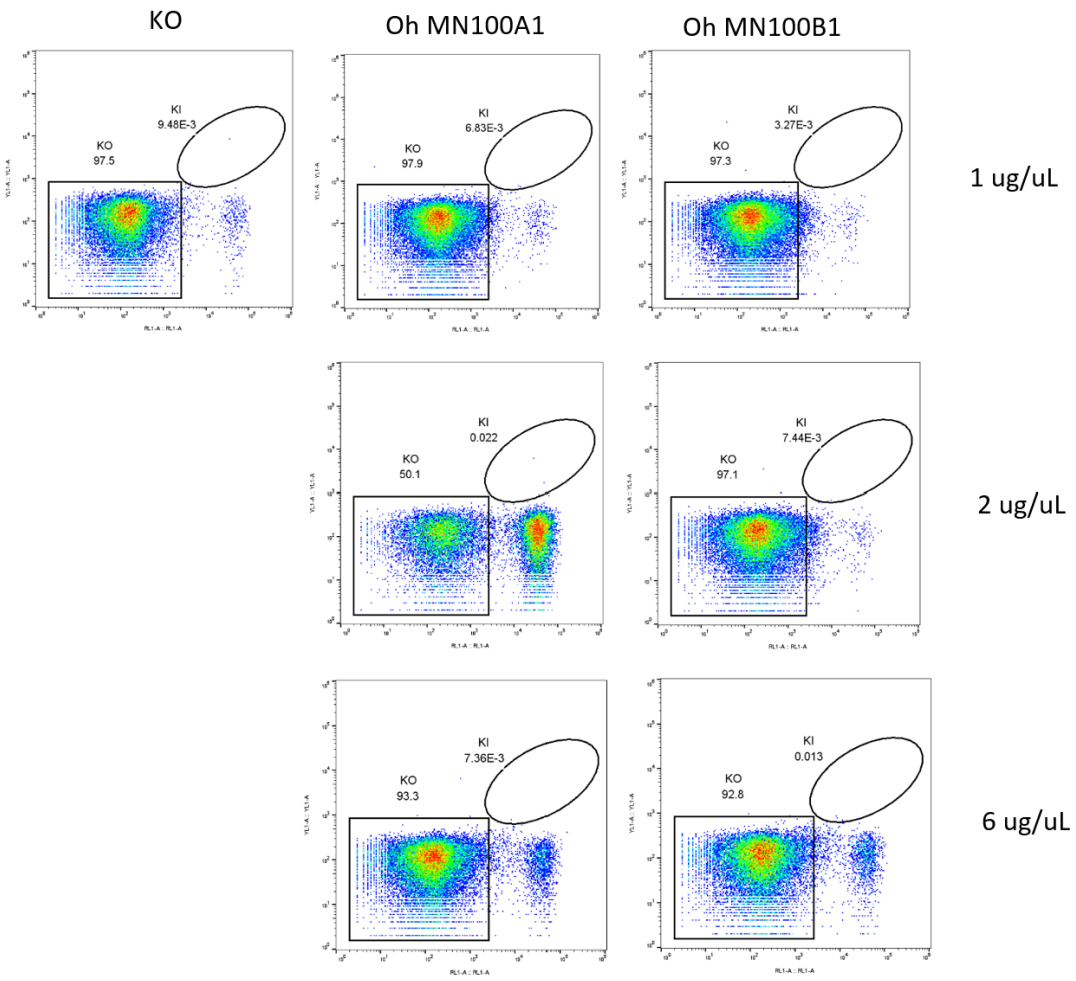


Supplementary Figure 10: Flow cytometry data comparing the effectiveness of single TRAC KO and dual TRAC/TRBC KO with and without the small molecule XL413 at 10 ng/μL concentration. Samples are KO control, **α**, **α-β**, **α**+XL413, and **α-β**+XL413.

In contrast to previous data, the **α** condition had a 100-fold higher KI efficiency than the **α-β** condition without inhibitors, and the XL413 inhibitor did not increase efficiency. Since the potential benefit of dual KO and adding inhibitors is unclear, these conditions were not used in future experiments.

DNA Concentration of dsDNA & MC TCRs

Optimal donor TCR concentrations from the Oh et al. paper[77] and PACT Pharma protocol[76] were identified as 2-4 μg/μL and 6 μg/μL, respectively, and tested. The first two MCs manufactured, Oh-MN100B1 and Oh-MN100A1 were tested at 1, 2, and 6 μg/μL concentrations. The flow data is shown in Supplementary Figure 11.



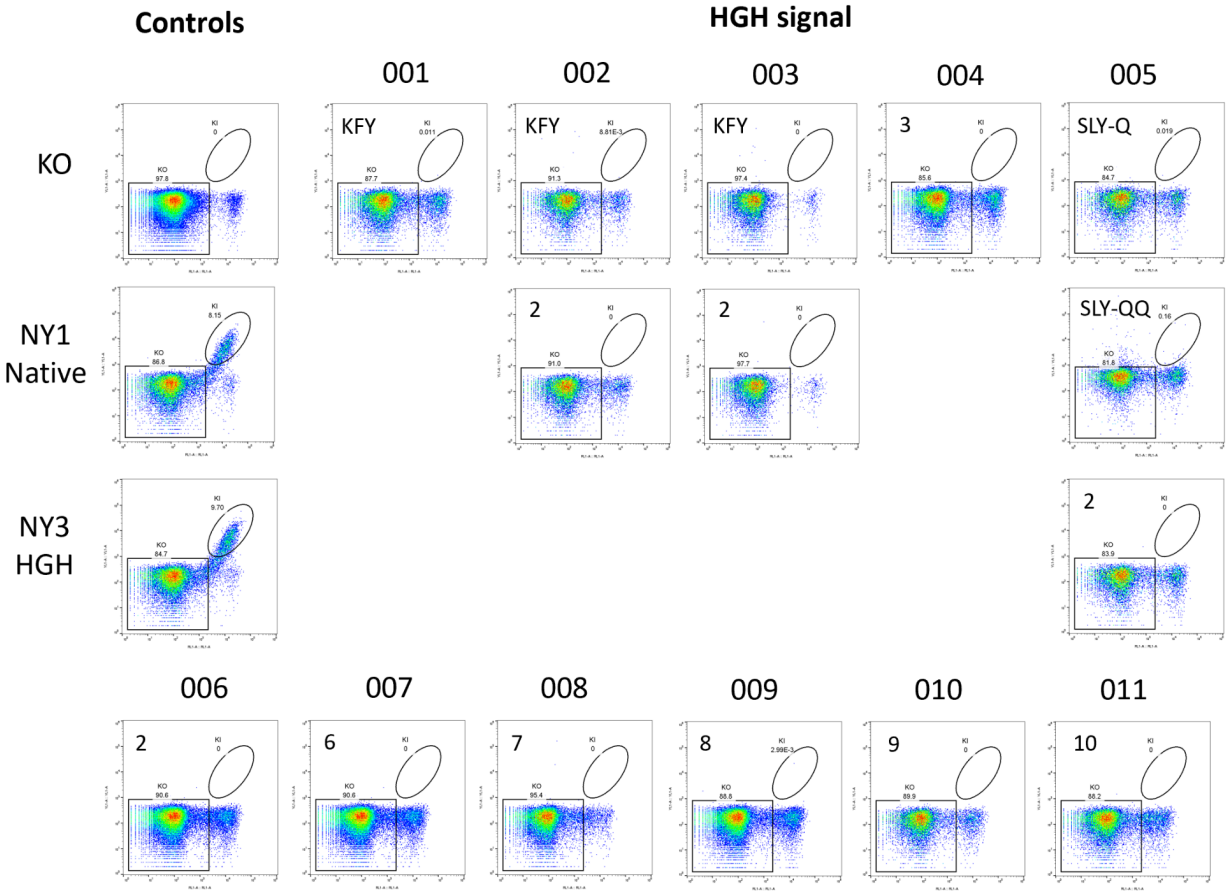
Supplementary Figure 11: Flow cytometry data comparing 1, 2 and 6 μg/μL concentrations of the MC TCR donors Oh-MN100A1 and Oh-MN100B1.

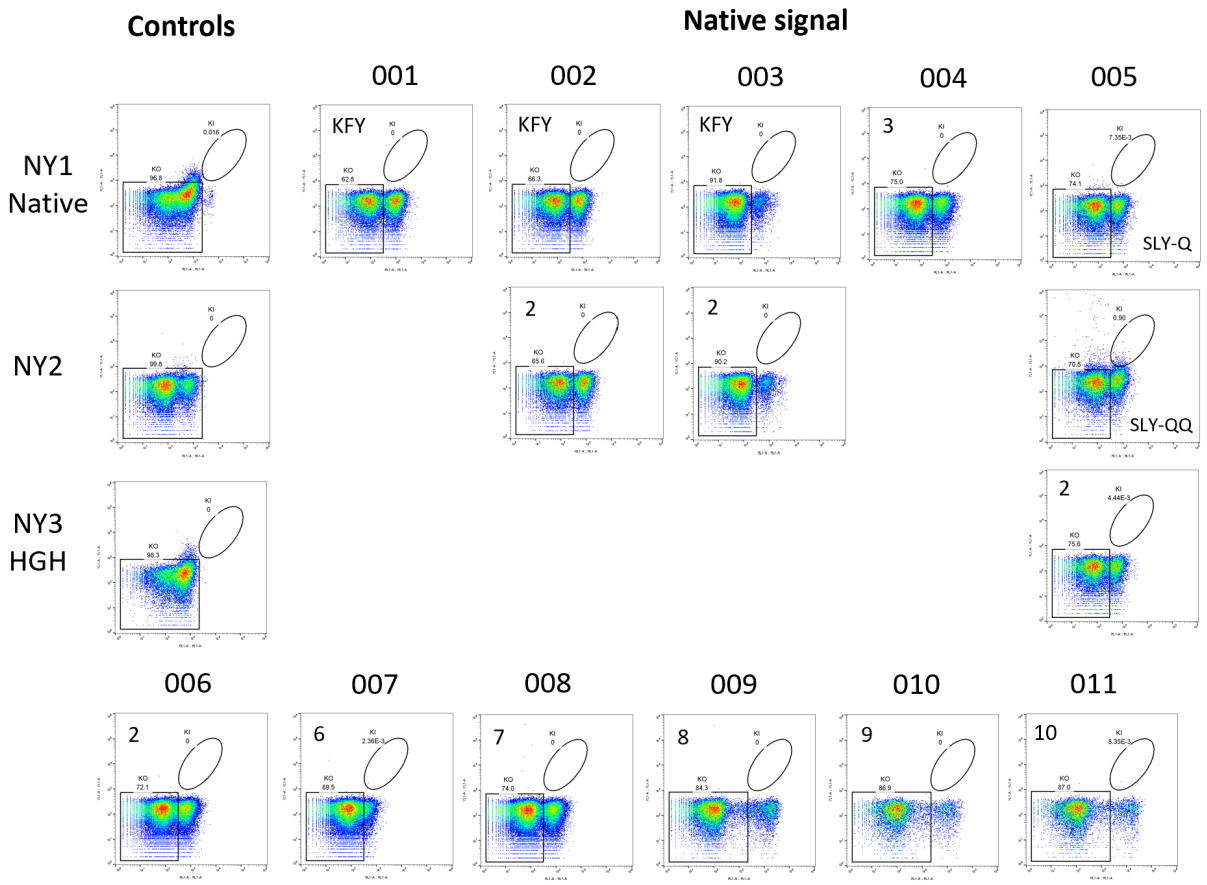
KI efficiencies were very low in this experiment, so it was difficult to elucidate an accurate conclusion from the data. There was also no consistent pattern for which concentration produced the highest KI efficiency between the MCs. Therefore, the best concentration defined in previous experiments was used.

TCR Signal Peptide

The first batch of 11 HPV-specific TCR sequences were assembled using the Python script written by William Chour (see Methods) for CRISPR-Cas9 editing applications only. Two versions of each TCR were assembled, one with the native signal peptide and another with the HGH signal peptide. These signal peptides are responsible for secreting recombinant proteins, and were initially tested to compare their functionality in primary T cells. The HGH signal peptide was chosen based on the PACT Pharma TCR design, which was hypothesized to increase

functional activity of the T cells after stimulation compared to the native TCR signal peptide. The flow cytometry data for two combined experiments is shown in Supplementary Figure 12.





Supplementary Figure 12: All 11 HPV-specific TCRs with native signal peptide and HGH signal peptide, including NY-ESO-1 controls. Numbers on each flow cytometry graph indicate the peptide ID used for tetramer staining. Relevant peptides from the families KFY and SLY were indicated where appropriate.

This experiment was unsuccessful, as none of the HPV-specific TCRs were able to bind anything to indicate their expression in the T cells. It operated on the assumption that these TCRs would be able to bind their top ranked cognate antigen, which was not the case as shown in future validation experiments (see Results). Moving forward, the cognate antigen pooling method was used to stain the cells (see Methods). This experiment showed that both NY-ESO-1 control TCRs had effective expression with both native and HGH signal peptides.