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Development of conditionally reprogrammed cells (CRCs) as an *in vitro* model for T-cell based cancer immunotherapy

A Thesis submitted in partial satisfaction of the requirements for the degree
Master of Science

in

Biology

by

Claire Chen

Committee in charge:

Professor Stephen P. Schoenberger, Chair
Professor Ananda Goldrath, Co-Chair
Professor Cornelius Murre

2019

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The Thesis of Claire Chen is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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University of California San Diego

2019

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ACKNOWLEDGEMENTS

First and Foremost, I would like express genuine gratitude to my advisor, Dr. Stephen P. Schoenberger, for his continuous support and guidance. His enthusiasm and passion for his work inspire me every day. Furthermore, being able to witness his generosity, patience and kindness has taught me life skills that I will carry throughout my life.

I would like to thank Dr. Ananda Goldrath and Dr. Cornelius Murre for being members of my committee.

I would like to thank Dr. Martin Naradikian, Dr. Leslie Montero, Jessica Xiao, Spencer Brightman, and Dr. Joseph Dolina for their valuable advice and support in making this thesis possible.

I'd like to thank the rest of the members of the Schoenberger lab for all of their guidance and support over the years.

And I would like to especially thank Dr. Aaron Miller for being my mentor and guiding me throughout this journey. Those of us fortunate enough work with him know of his dedication for his research and people. This thesis would not have been possible without him.

Figure 7 is co-authored by Dr. Martin Naradikian.

ABSTRACT OF THE THESIS

Development of conditionally reprogrammed cells (CRCs) as an *in vitro* model for T-cell based cancer immunotherapy

by

Claire Chen

Master of Science in Biology

University of California San Diego, 2019

Professor Stephen P. Schoenberger, Chair
Professor Ananda Goldrath, Co-Chair

Adoptive T-Cell Therapy (ACT) is a novel cancer therapy in which tumor-specific T-cells are obtained from patients, expanded in the laboratory, and then infused back into the patient to eradicate the cancerous cells. While conventional treatments for cancer such as surgery, radiation, and chemotherapy have been used to successfully eradicate tumors, they work by using non-specific methods to eliminate cancer cells, which in turn can also affect normal, healthy cells. Through Adoptive T-cell Therapy, we are able to take advantage of the natural ability of T-cells to recognize cancer cells and infuse them back into the patient to specifically target malignant

tumors and avoid off-tumor toxicities. ACT has shown a lot of promise as a personal cancer treatment not only due to its ability to specifically target cancer, but also because ACT has been seen to produce deep, enduring responses especially in melanoma patients. Some current ways to increase efficacy in ACT treatment including lymphodepleting the patient prior by irradiation and/or using a chemotherapy drugs. While these methods have shown to enhance ACT outcomes, they can also result in serious side effects like fevers or infections that can be life-threatening for the patient. As a result, there is still a need to further study effective strategies that can improve the efficiency of ACT, and thus decrease complications for the patient.

To develop an *in vitro* model that that would allow researchers to better investigate ACT, we looked into the conditionally reprogrammed cell (CRC) methodology, which is an innovative protocol that allows for the generation of primary epithelial cancer cells in 7 days at a high success rate. To explore whether CRCs can be an immunological model for ACT, we generated conditionally reprogrammed cells from Head and Neck Squamous Cell Carcinoma tumors that express the same cancer mutations as the patient's and presumably allow natural processing and presentation of antigen by MHC-I. Through this model, we were able to show CRCs can be recognized and killed by tumor-specific cytotoxic T-cells. In addition, when compared with cisplatin, a chemotherapy drug, cytotoxic T-cells were able to induce faster eradication of the cancer cells. We herein demonstrate that CRC models retain their original tumor phenotype by staining of cancer stem cell markers, CD44, EGFR, EpCam, BMI1, ALDH1 and have the ability to be used as an immunological surrogate for primary tumor in T-cell based cancer immunotherapies.

INTRODUCTION

Cancer is the second leading cause of death in the United States with a projected estimate of 600,000 cancer deaths and 1.7 million new cases in 2018 and 2019 (Siegel, Miller, & Jemal, 2018, 2019). Cancer arises when the normal cells in the body acquire changes in their genes that cause those cells to divide uncontrollably (Hassanpour & Dehghani, 2017). These genetic changes can be instigated through many ways including exposure to carcinogens, infection with oncogenic viruses, or even by genetic or random circumstances. Consequentially, those transformed cancer cells can develop into malignant tumors which invade essential organs and can often metastasize or spread, causing further damage in other tissues.

Current common treatments for cancer include surgery, chemotherapy, or radiation therapy, which work by physically removing the primary tumor and then delivering cytotoxic drugs and/or radiation to kill the disseminated cancer cells (Works, 2018). One major drawback of current treatments is that although these biologically hazardous substances kill cancer cells, they can also kill the healthy cells in the body. As a result, some carry long-term side effects including heart damage, immune suppression, opportunistic infections, neuropathies, infertility, memory deficits, and distress (Shamim et al., 2008).

$CD8^+$ cytotoxic T-cells normally patrol the body to recognize for any infected or cancerous cells (Corthay, 2014). When the T-cell receptor (TCR) recognizes any foreign peptide presented by the MHC-I on a cancerous cell, the T-cell engages the infected or cancerous cell via its surface T cell receptor (TCR) leading to the secretion of cytokines and cytotoxic molecules such as Granzyme B and Perforin to induce apoptosis of the target cell (Kerkvliet & Lawrence, 2010). However, cancer cells that avoid detection from the immune system can then freely divide and eventually develop into malignant tumors that interfere with normal bodily functions.

One common method of immune escape is when cancerous cells express negative or inhibitory regulators such as PD-L1 (Programmed death-ligand 1), a transmembrane protein that turns-off cytotoxic T-cells by interacting with the PD-1 exhaustion marker on T-cells (Ohaegbulam, Assal, Lazar-Molnar, Yao, & Zang, 2015). Another mechanism of immune escape is establishing an inhibitory tumor microenvironment in which cancer cells can secrete chemokines such as CCL22 to recruit T regulatory cells to further suppress T-cells (Curiel et al., 2004). Through these and many more mechanisms of immune escape, cancer cells are able to evade the adaptive immune system and continue to grow into malignant carcinomas, which can lead to metastasis in other organs.

A novel approach that is quickly emerging to combat cancer and avoid some of the toxicities of current treatments is Adoptive T-Cell Therapy (ACT) (Rosenberg & Restifo, 2015). ACT focuses on enhancing and employing the body's own immune system to target cancerous cells. This is done by acquiring tumor infiltrating lymphocytes (TIL) from the patient tumors, expanding them in large quantities, and then infusing them back into the patient to eradicate the tumor (Grupp & June, 2011). TIL are autologous lymphocytes that have the capacity to recognize, attack and infiltrate into the tumor (Prieto, Durflinger, Wunderlich, Rosenberg, & Dudley, 2010). The reason why ACT is such a powerful tool is because it utilizes T-cells, which have the ability to discriminate between cancerous and healthy cells to allow for specific targeting. In melanoma cancer patients, Adoptive T-cell Therapy using TIL has shown response rates of over 50%, which is considerably higher compared to standard chemotherapy, which had an objective response rate of less than 20% (Lee & Margolin, 2012).

Since the onset of ACT, researchers have developed variations on ACT such as using transgenic T-cells, to create more effective strategies at targeting cancer cells. Instead of using TIL,

TCR-engineered T-cells are used to be infused back into the patient. To do this, T-cells that are specific to particular neoantigens - antigens located on cancerous cells yet not on healthy cells – are identified; neo-antigen specific T-cells have been found to important in the regression of tumors in patients (Robbins et al., 2013). By using next generation sequencing, a specific TCR with a high avidity or reaction to the tumor neoantigen can be identified and transduced on lymphocytes. Using transgenic T-cells that are specific for neoantigens has shown a lot of potential because this method only requires the sequence of one strong TCR as it can then be transduced on multiple lymphocytes to hone in on the specific cancer target in the body.

While Adoptive T-cell Therapy has shown strong promise as a cancer treatment, there is still an urgent need to improve and develop more effective strategies with it. There are still risks to ACT which include pre-conditional chemotherapy or irradiation for lymphodepletion. Lymphodepletion is the destruction of lymphocytes and is used because it has shown to drastically improve the effects of ACT by reducing the number of suppressing factors in the tumor microenvironment and increasing access to homeostatic cytokines (Gattinoni et al., 2005). However, both these methods can result in serious side effects including higher risk to infections, fevers, infertility, or fatigue which can threaten the health of the patient (Wrzesinski et al., 2010). These side effects only underscore the necessity to study and research more ways to lessen the complications and increase the success rate of Adoptive T-cell Therapy.

In order to promote further advancement in ACT, we sought to develop a personalized *in vitro* model that would represents the patient's specific cancer profile. Establishment of a cancer cell line that retains the genotype of tumor in the body would allow researchers to test the efficacy of ACT treatments directed against cancer cell presented neoantigens or viral oncoproteins. To this end, Richard Schlegel's team in Georgetown University recently established a "conditionally

reprogramming" method, which allows for the propagation of epithelial cells derived from healthy human tissue or cancer biopsies without genetic manipulation (Liu et al., 2012). Historically, many primary models of patient-derived cancer cell generation have a lot of limitations such as low success rates or large time-consumption, especially for certain cancer types (Miki & Rhim, 2008). With the CRCs, however, Schlegel's group has not only has it been able to demonstrate that these conditionally reprogrammed cells (CRCs) can be grown indefinitely, but also that the CRCs can be generated rapidly with high success in 7 days. By culturing primary tumor cells with Rho Kinase Inhibitor (Y-27632) and irradiated fibroblast feeder cells, they have found that in 2 days, epithelial cell colonies are readily visible (Liu et al., 2017). Because of the high success rate and conservation of phenotype from the CRC protocol, this method can be used as an essential *in vitro* preclinical model for drug discoveries and translation cancer research.

Since then, there has been some research on conditionally reprogramming cells for drug testing, and the ability to retain somatic mutations from the original tumor (Saeed et al., 2018). However, it's been unknown whether these CRCs can serve as immunogenic models to predict efficacy in cancer immunotherapies. We are particularly interested in exploring whether these conditionally reprogrammed cells can be used as an *in vitro* preclinical model for Adoptive Cell Therapy (ACT). Using TIL and TCR-engineered lymphocytes, we hope to demonstrate that conditionally reprogrammed cells retain the original tumor antigens and can thereby be recognized by T cells in these various ACT approaches. By doing so, we believe that conditionally reprogrammed cells can be a powerful tool in the exploration and improvement of cancer immunotherapies.

MATERIALS AND METHODS

Establishment of Hu_056 CRCs

The human tumor tongue biopsy was obtained from the Moores Cancer Center in La Jolla, California with informed consent of the patients. The tumor biopsy was chopped into $1mm^3$ pieces and digested using the Tumor Dissociation Kit, human (Miltenyl Biotec) using the 'tough tumor' protocol. The cell suspension was then filtered through a cell strainer (70um; Fisher Scientific) and plated on irradiated Swiss 3T3 fibroblasts (J2 strain) feeder cells with conditional media. The conditionally reprogrammed cells (CRCs) established from the human tumor tongue biopsy were maintained following the Conditional Reprogramming protocol (Liu et al., 2017).

Generating Hu_056 TIL Fragments

TIL were obtained from tumor biopsy in the UC San Diego Moores Cancer Center in La Jolla, California with informed consent of the patients. The tumor biopsy was cut into around $1mm^3$ sized fragments and cultured with TIL media and 5,000 IU/uL IL-2. The TIL was rested in TIL media and Recombinant Human IL-2 (PeproTech) for 4-5 days until lymphocytes started to proliferate.

The TIL fragments designed with 'REP' underwent a rapid expansion protocol established by Aaron Miller MD PhD at the Schoenberger Lab of the La Jolla Institute of Allergy and Immunology.

Flow Cytometry

For phenotyping the conditionally reprogrammed cells, the human antibodies used were CD44 BV421 (BJ18) , EGFR Pe-Cy7 (AY13) , EpCAM PE (9C4), MHC-I PB (W6/32), ALDH1

PE (#03), BMI1 Af647 (P51-311), PD-L1 V500 (29E.2A3). For TIL and CRC co-culture, extracellular human antibodies used were CD3 PE-CF594 (UCHT1), CD4 FITC (OKT4), CD8 PB (SK1). The intracellular human antibodies used were IFN-γ APC (4S.B3) and TNFα BV510 (MAb11). After Live-Dead exclusion, cells were gated for $CD3^+$ and then analyzed for cytokine secretion levels. For the transduced J76 and T2 cells co-culture, human antibodies used were CD3 PeCy7 (UCHT1), CD8 APC-Cy-7 (SK1), PD-1 BV711 (EH12.2H7).

For the transduced J76 and CRC co-culture experiment, the human antibodies used were CD3 FITC (17A2), CD69 PerCP-Cy5-5 (FN50), CD34 Af647 (581), PD-1 PeCy7 (EH12.2H7). Cells were gated for $CD3^+$ $CD34^+$ for transduced J76 cells. When looking TIL CD4 and CD8 populations, human antibodies used were CD3 PE-CF594 (UCHT1), CD4 FITC (OKT4), and CD8 PB (SK1). For the TIL Phenotyping, human antibodies used were CD3 PE-CF594 (UCHT1), CD4 V500 (RPA-T4), CD8 FITC (SK1), CD56 PE (HCD56), FoxP3 PB (259D), CD11b APC (ICRF44), CD19 PE-Cy7 (HIB19), CD25 VB650 (BC96), CD45RO PerCP-Cy5-5 (HI100), CD45RA AF700 (UCHL1), PD-1 APC (MIH4). To look at the effects of IFN-γ stimulation with CRCs, the human antibodies used were MHC-II Af647 (Tu39), MHC-I PB (W6/32), PD-L1 V500 (29E.2A3).

All human antibodies were from Biolegend except for ALDH1 PE (#03) (Sino Biological Inc), BMI1 Af647 (P51-311) (BD Biosciences), CD3 PE-CF594 (UCHT1) (BD Biosciences).

Live/Dead antibodies used were APC-eF780 (ThermoFisher) and DAPI (ThermoFischer). The L/D APC-eF780 was diluted for staining. DAPI was diluted 1:4000 for staining. Each experiment was gated for Live-Dead exclusion.

The extracellular antibodies were diluted to 1:200 and incubated with the cells for 12 minutes at 4°C. The Live/Dead antibody was diluted and incubated with the cells for 30 minutes at 4°C. Then, the cells were fixed with Cytofix/Cytoperm (BD Biosciences) for 20 minutes at 4°C.

The intracellular stains were diluted to 1:150 and incubated with the cells for 12 minutes at 4°C. The cells were then run on the BD LSRFortessa™ and analyzed with FlowJo V10. Single stains were done on compensation beads (BD Biosciences) to compensate for each experiment.

To stimulate the lymphocytes, Purified Anti-Human CD3 (OKT3) (Tonbo Biosciences) at 1:200, PHA (Sigma Aldrich) at 10 µg/mL or PMA (Sigma Aldrich) at 40ng/mL with Ionomycin (Sigma Aldrich) at 2 µg/mL were used.

PCR and Gel Electrophoresis

Forward and reverse primers were designed to amplify HPV16 regions of E2, E4, E5, E6/E7 and L1:

The primers to amplify the E2 region are: 5'ATGGAGACTCTTTGCCAAC3' and 5' TCATATAGACATAAATCCAGTAGACA 3'.

The primers to amplify the E4 region are: 5' TGATCCTGCAGGTACCAAT 3' and 5' TGGGTGTAGTGTTACTATTACAG 3'.

The primers to amplify the E5 region are: 5' ATGACAAATCTTGATACTGCATC 3' and 5' TTATGTAATTAAAAAGCGTGCAT 3'.

The primers to amplify the E6/E7 region are: 5' CCCAGCTGTAATCATGCATGGAGA 3' and 5' GTGTGCCCATTAACAGGTCTTCCA 3'.

The primers to amplify the L1 regions: 5' GCMCAGGGWCATAAYAATGG 3' and 5' CGTCCMARRGGAWACTGATC 3'.

Each reaction was carried out in Thermal Cycler (Bio-rad T100™) with the first denaturation step at 94°C for 30 seconds, then the annealing step at 48°C for 30 seconds, and the elongation step at 72°C for 30 seconds. This was repeated for 34 cycles.

The PCR products were then run on a 1% agarose gel with Ethidium Bromide at a final concentration of 0.5 µg/ml for 50 minutes at 150 Voltages. Images were taken using Gel Doc™ XR+ Gel Documentation System (Bio-rad).

CaSki and Hu_056 CRC DNA was acquired using the AllPrep DNA/RNA Micro Kit (Qiagen).

ELISpot Assay

MultiScreen-IP Filter Plate, 0.45 µm 96-well (Millipore Sigma, Burlington, MA) was activated with 70% Methanol. Then, the plates were washed 3 times in 100 µL of distilled water per well prior to coating plate with anti-human IFN-γ mAb 1-D1K (Mabtech) and Anti-human IL-5 mAb TRFK5 (Mabtech), which were diluted 1:200 in PBS. The plate was then placed in 4 °C for 8 hours – 7 days. On the day of the assay, plates were blocked with 100 µL of TIL media for 1 hour. Then, 50 µL of E6 and E7 Pepmix (JPT Peptide Technologies), PHA (Sigma Aldrich) at 10 µg/mL or media were added per well. TIL fragments were plated at 1 x 10^5 cells per well in 50 µL of TIL media. Plates were incubated for 20-22 hours in 37°C with 5% CO_2 in air. Plates were subsequently washed using an automated plate washer (Biotek TS 405) 6 times with 200 µL 0.05% PBS/tween. Then, anti-human IFN-γ mAb 7-B6-1, HRP-conjugated (Mabtech) was diluted 1:200 and anti-human IL-5 mAb 5A10, biotinylated (Mabtech) was diluted 1:1000. After, the developing antibodies were added to each well and incubated for 2 hours at 37°C. Plates were then washed using the automated plate washer 6 times with 200 µL 0.05% PBS/tween. Then, 1 drop from each tube of the Avidin-Biotin Complex Kit (Vector Labs) was added into 10mL of PBS. After, 100 µL was added into each well and the plate was incubated for an hour at 37°C. After incubating, plates were washed with plate washer 6 times with 200 µL of Distilled Water. Vector Blue (Vector Labs) was prepared by adding 4 drops of each tube into 10mL of PBS. This diluted solution was then

added to the wells and incubated for 5 minutes at room temperature. Then after washing with distilled water, the red colorimetric substrate was prepared by combining 2.5mL of N,N, Dimethylformaldehyde, 1 AEC tablet, 25uL of H2O2 and then filtered with a 0.22μm filter. This solution was added onto the wells for 10 minutes. The plate was then washed with distilled water and read on a plate reader.

Transduction of lymphocytes with Hu_056 $E6_{29-38}$ TCR

From the EliSpot Assay, TIL Fragment 12, due to significant secretion of IFN-γ against $E6_{29-38,}$ was selected for cytokine secretion assay. Using the cytokine secretion assay, we identified a population of cells from TIL Fragment 12 that showed reactivity by secretion of IFN-γ against $E6_{29-38.}$ The reactive population of cells underwent single cell sorting and then TCR sequencing. From this, we identified a predominant population of one $CD8^+$ TCR that was specific for the $E6_{29-38}$-peptide.

Using the $E6_{29-38}$-specific $CD8^+$ TCR sequencing data, Aaron Miller, MD, PhD at the Schoenberger Lab of the La Jolla Institute of Allergy and Immunology designed a pHAGE-E6-tCD34 plasmid. This plasmid, pHAGE-E6-tCD34, encodes for the $E6_{29-38}$-specific $CD8^+$ TCR from FR12REP along with a truncated CD34 marker to be packaged by a pHAGE lentivirus.

In order to develop the pHAGE lentivirus encoding the pHAGE-E6-tCD34 plasmid, lentivirus helper plasmids (tat, rev, gag/pol, vsv-g) were obtained from the Shane Crotty lab at La Jolla Institute of Allergy and Immunology. The lentiviral helper plasmids and pHAGE-E6-tCD34 plasmid were then added to 293Ts and transfected following the Lipofectamin 3000 Reagent (ThermoFisher) protocol. To harvest the lentivirus, viral supernatants were collected and filtered through a 0.22μm and 30mL syringe. Then, they were spun and stored in -80°C.

In order to transduce lymphocytes, cells were plated and lentivirus with Polybrene (10mg/mL) was added to it. Then, the concoction was spun for 2 hours at 1000xg at 32°C.

Impedance-based Cytotoxicity Assay

Human primary PBMCs were obtained from an HLA A2.1 donor at the La Jolla Institute of Allergy and Immunology. $CD8^+$ T Cell Isolation Kit (Miltenyl Biotec) were used to isolate $CD8^+$ T-cells through positive selection. Then, these isolated cells were transduced with a pHAGE lentivirus encoding a HLA*02:01-restricted HPV16 $E6_{29-38}$-specific TCR plus a tCD34 marker. Then, the transduced PBMCs were co-cultured with either CaSki (ATCC® CRL-1550™) or Hu_056 CRCs using the ACEA XCelligence system. This protocol was obtained from Martin Naradikian, Phd of the Schoenberger Lab at La Jolla Institute of Allergy and Immunology.

Cell Maintenance

TIL fragments were maintained TIL media which composed of RPMI 1640 supplemented with 10% Human Serum, 2.5% HEPES 1M, 1% Peniciilin-Streptomycin, 0.1% Gentamicin Reagent. Then, the media was filtered in 0.22 µm 500 mL Millipore filter. 10,000IU/µL of IL-2 was also supplemented. The human PBMCs were maintained in the same condition.

CRC media was used to maintain the conditionally reprogrammed cells. This consisted of a 3:1 ratio of Conditional media to F media and 0.1% of Rock Inhibitor (Y-27632). F Media is composed of 75% Complete Media, 25% Ham's F12 Nutrient Mixture (Gibco), 0.1% hydrocortisone/EGF, 0.1% Insulin, 0.1% Fungizone, 0.1% Gentamicin, 0.00086% cholera toxin. Complete media composed of DMEM, 10% Fetal Calf Serum (FCS), 1% Peniciilin-Streptomycin, 1% L-Glutamine. Media was then filtered in 0.22 µm 500 mL Millipore filter.

The conditional media was made by plating irradiated 3T3-Swiss Albino (ATCC® CCL-92™) cells at 1.1 x 10^7 cells per T175 standard Sarstedt flask in 3T3 Complete Media at 37°C with 5% CO_2 in air. After 72 hours, the media was collected, spun down to get rid of any excess dead cells and stored in -80°C. This cell maintenance protocol was obtained from Conditionally reprogramming protocol by the Richard Schlegel group (Liu et al., 2017).

To maintain the 3T3 mouse fibroblast cells, the cells were cultured in 3T3 complete media which composed of DMEM, 10% Fetal Calf Serum (FCS), 1% PenStrep, 1% Glutamax, 1% BME.

To maintain the J76 and CaSki cell line, the media used was in RPMI 1640 with 10% Fetal Calf Serum, and 1% Peniciilin-Streptomycin.

RESULTS

Establishment of HPV16+ HNSCC Hu_056 cell line

A HPV16+ head and neck squamous cell carcinoma (HNSCC) tumor biopsy was acquired from a HLA*02:01 patient (Hu_056) performed for medical need under informed consent. Following the CRC protocol, a cell line was successfully established, which eventually propagated indefinitely without the need of feeder cells and showed an epithelial-like cell morphology **(Fig. 1a)**. In addition, the Hu_056 CRC cells were positive for EGFR and EpCAM, which are often upregulated in epithelial carcinomas (Schartinger et al., 2009). Further phenotypic analysis also demonstrated that the Hu_056 cell line was positive for human cancer stem cell markers, CD44, ALDH1, BMI1 and MHC-I. Furthermore, 32% of the CRCs were found to be positive for PD-L1, a marker important for the suppression of T-cells **(Fig. 1b).** The morphology as well as the phenotyping of the Hu_056 CRCs confirmed that the CRC protocol was able to establish cancer stem cells from the tumor biopsy.

In order to confirm the presence of the HPV16 gene in the CRCs, primers were designed to amplify various regions (E6, E2, E4, E5, and L1) of the HPV16 gene. Then, DNA isolated from Hu_056 CRC and CaSki—a cervical cancer cell line known to be positive for HPV16—was incubated with the primers and underwent PCR. After running the PCR sample on gel electrophoresis, we found that the HPV16 gene was conserved through the conditionally reprogramming process as the E6, E2, E4, E5, L1 bands for Hu_056 CRCs were present. **(Fig. 2b).** Interestingly, the E2 and E4 bands were less intense in the CRCs compared to the bands from the CaSki cell line.

Selection of Hu_056 TIL fragments against E6 pepmix

We first established several different TIL fragments from the Hu_056 tumor biopsy. The Hu_056 TIL morphologically were lymphocyte-like suspension cells **(Supp. Fig. 1a)**. Interestingly, this process of TIL expansion produced almost exclusively CD4 T-cells with very few CD8 T-cells **(Supp. Fig. 1b)**. Furthermore, a majority of these CD4 T-cells were effector cells that expressed CD45RO, a memory T-lymphocyte marker **(Supp. Fig. 2).** There was also a small population of the TIL expressing CD11b showing the presence of some macrophages among the TIL population.

Because the Hu_056 CRC was HPV16 positive, we wanted to isolate TIL fragments that would be reactive towards the E6 protein in HPV16. To do this, we performed an EliSpot Assay, which is a technique that quantifies the frequency of cytokine-secreting cells upon stimulation with cognate peptide. Nineteen of these TIL fragments were incubated with E6 pepmix to identify reactive Hu_056 TIL fragments. E6 pepmix is a pool of 37 peptides that was derived from a peptide scan through protein E6 of HPV16 (Kaneko, Emoto, & Emoto, 2016). Then afterwards, a detection antibody specific to IFN-γ and IL-5 were added, which was followed by a conjugate and precipitating substrate that then resulted in visible spots corresponding to each individual cytokine-secreting cell. Fragments 11, 12, 31, 32, 19, 22, 27, 31REP, 32REP and 33 showed significant secretions of IFN-γ compared to the unstimulated controls, which were not treated with any peptides **(Fig. 3.)**. The fragments that were significant for IL-5 were found to be fragments 27 and 33. Fragment 12 REP showed a negative reading because it was oversaturated with IFN-γ red spots that prevented the plate reader program from distinguishing between individual spots.

Hu_056 TIL do not recognize Hu_056 CRCs treated with IFN-γ

Based on sample availability and reactivity, Fragments 12REP, 19, 27, 31REP and 32REP were chosen to be co-cultured with Hu_056 CRCs. Because the TIL fragments contained predominantly $CD4^+$ T-cells, the Hu_056 CRCs were stimulated with IFN-γ to upregulate MHC-I and MHC-II, molecules important for antigen presentation (**Supp. Fig. 3.)** None of the TIL fragments co-cultured with Hu_056 CRCs showed any induction of IFN-γ or TNF-α compared to the unstimulated controls **(Fig. 4)**. TIL fragments were treated with polyclonal stimulators PMA/Iono to confirm the fragments were capable of activation.

Lack of recognition of Hu_056 CRCs by transduced $E6_{29-38}$-specific J76s

Because of the many variables of TIL fragments, we decided to explore another T-cell candidate using a genetically modified TCR to see if it could recognize the Hu_056 CRCs. To do this, we identified a $CD8^+$ HPV16 $E6_{29-38}$-specific TCR from TIL Fragment 12. Then, we acquired a J76 cell line absent of any TCR and transduced it with a pHAGE lentivirus encoding a HLA*02:01-restricted HPV16 $E6_{29-38}$-specific TCR plus a truncated CD34 marker **(Supp. Fig. 4)**. Through flow cytometry, we saw that 83.5% of the J76 cells were successfully transduced, which was identified through $CD3^+CD34^+$ cells **(Fig. 5a).** We then were able to confirm the functionality of the TCR as the transduced $E6_{29-38}$-specific J76s were able to recognize $E6_{29-38}$ peptide when presented on a HLA*A2 restricted T2 cell line **(Fig. 5b)**. The addition of the irrelevant Flu peptide showed no increase in PD-1 expression demonstrating the transduced TCRs on the J76s are specific for $E6_{29-38}$ peptide. As a positive control, a HPV16 $E6_{29-38}$-specific TCR was obtained from the National Cancer Institute (NCI) and transduced on a J76 cell line as well. Then, the NCI TCR on the J76s was co-cultured with $E6_{29-38}$ peptide presented on a HLA*A2 restricted T2 cell

line. The NCI TCR showed a 65% increase in PD-1 expression, while the transduced J76s with Hu_056 Colony2 TCR originating from FR12 had a 35% increase in PD-1 expression.

Then, these transduced $E6_{29-38}$-specific J76s were co-cultured with Hu_056 CRCs to examine whether these CRCs have the ability to present to the transduced J76s. The transduced J76s co-cultured with Hu_056 CRC and $E6_{29-38}$ peptide showed no significant upregulation of PD-1 or CD69 compared to the J76 only control **(Fig. 6)**. Furthermore, the addition of αMHC-I and αPD-1 also yielded no significant increase compared to the unstimulated J76s. To increase the MHC-I and MHC-II presentation on the Hu_056 CRCs, IFN-γ was added, however, the flow cytometry showed no significant difference with it either **(Fig. 6)**. OKT3 was used as a positive control to stimulate the transduced E6 J76 and was the only condition to show any significant upregulation of PD-1 and CD69.

Transduced $E6_{29-38}$-specific human primary PBMCs can kill Hu_056 CRCs and CaSki

Human primary PBMCs from a HLA *02:01 donor were transduced with the same pHAGE lentivirus encoding a HLA*02:01-restricted HPV16 $E6_{29-38}$-specific TCR plus a tCD34 marker (or control LV alone) as used prior with the J76s. The transduced PBMCs were then sorted for $CD8^+$ cells through positive selection. The resulting $CD8^+$ T-cells were then co-cultured with either Hu_056 CRCs or CaSki cells. We found that the non-transduced CD8+ T-cells temporarily slowed the growth of the Hu_056 and CaSki cells, however after around 50 hours the cells began to steadily increase. The $E6_{29-38}$ TCR $CD8^+$ T-cells drastically decreased the cell number of both the CaSki and Hu_056 CRC cell line demonstrating cytotoxic capability with these transduced T-cells **(Fig. 7).** Furthermore, the $E6_{29-38}$ TCR $CD8^+$ T-cells showed faster killing of both cancer cell lines at 24 hours compared to the chemotherapy drug, Cisplatin.

DISCUSSION

Our results have reaffirmed that the conditionally reprogramming protocol can be used to produce epithelial cancer cells from a head and neck squamous cell carcinoma biopsy. Furthermore, these Hu_056 CRCs upregulate markers such as EGFR and EpCAM, which are important for tumor progression and cell adhesion. Interestingly, the Hu_056 CRCs population were all uniformly expressing CD44, ALDH1 and BMI1, surface markers associated with cancer stem cells. This suggests that this protocol was able to completely reprogram all of the cancer cells to this state. Furthermore, the Hu_056 CRCs maintained a homogenous cancer stem cell phenotype throughout numerous passages.

Often in many head and neck cancers, viral oncogenes serve as attractive targets because they are present on cancerous cells and not on healthy cells. The conditional reprogramming method allows for the conservation of those viral oncogenes especially because it does not require any type of genetic modification through its process. The original Hu_056 tumor biopsy was HPV16+ and this gene was retained in the Hu_056 CRCs. Amplification of various regions of the HPV16 gene demonstrated that the Hu_056 CRCs were positive for HPV16.

We found that the TIL generation from the Hu_056 tumor biopsy through the enrichment of IL-2 was successful. However, we noticed the longer the TIL remained in culture, the more certain populations of T-cells started to become more prevalent. This could be due to the fact that the different T-cells compete within the limited resources, so some populations will clonally expand while others will die out. We found that a majority of the T-cells that predominated the TIL population were effector CD4 T-cells. This also suggests that continuous expansion of the TIL can result in the loss of potential tumor-specific T-cells.

Using the generated TIL, we observed that tumor-specific T-cells from the TIL population were able to be isolated. Because the Hu_056 CRCs were HPV16+, we surmised that there would be T-cells obtained that were reactive to the E6 protein. Thus, we identified the TIL fragments that were reactive against E6 pepmix, a collection of multiple peptides from E6 protein. Significant responses in IFN-γ suggested that several TIL fragments were capable of recognizing and responding to the HPV16+ head and neck tumor. Based on growth and reactivity, we focused on five TIL fragments to see whether Hu_056 CRCs could present and stimulate them.

We found that the Hu_056 CRCs treated with IFN-γ were unable to activate any of the E6 reactive TIL fragments. The Hu_056 CRCs were treated with IFN-γ prior to promote antigen processing and presentation on MHC-I and MHC-II, especially since a majority of the TIL contained CD4 T-cells. The inability for TIL to recognize the CRCs could be because the Hu_056 CRCs were not able to properly present, or that the CRC is not presenting the specific E6 peptide that the TIL are reactive to. It could also be due to the fact that these TIL are exhausted from frequent IL-2 stimulation, which is why for Fragments 12REP and FR19, the polyclonal stimulators weren't able to induce a higher response. Furthermore, the Hu_056 CRC could also be expressing enough PD-L1, to completely turn off T-cells and evade detection.

Due to the multiple variables, we then shifted our focus to using a HLA*02:01-restricted HPV16 $E6_{29-38}$-specific CD8 TCR plus a tCD34 marker which was transduced using a pHAGE lentivirus on a J76 cell line. With the lentivirus system, we were able to achieve a high transduction efficiency with J76 cell lines, but wanted to confirm that the TCR was capable of recognition. To do so we co-cultured the transduced J76 with T2 cells (HLA*A2) and $E6_{29-38}$ peptide and found that our Hu_056 TCR could successfully increase PD1 expression in response to antigen. However,

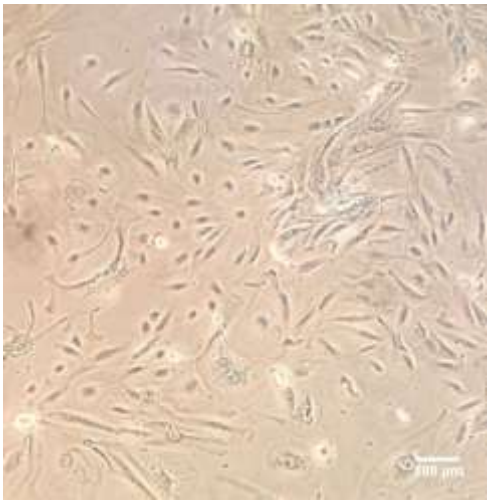
compared to the NCI TCR, the Hu_056 TCR showed a lower response most likely due to the different avidity of the TCRs.

With the Hu_056 TCR transduced on J76s, we found that the Hu_056 CRCs were not able to present and be recognized by the lymphocytes through flow cytometry analysis. There was no increase in PD-1 or CD69 expression in the transduced J76s with CRCs and E6 peptide. However, when the Hu_056 TCR was transduced on human $CD8^+$ TCRs, the Hu_065 TCR T-cells showed killing on the Hu_056 CRCs. This could be due to the fact that J76s are a hybridoma cell line and thus may not have the necessary signaling or may require more presentation of the E6 peptide on the CRCs to be activated. Another observation we noticed is that as transduced J76 remained longer in culture, they had a lower response to cognate antigen. Perhaps, these transduced J76 cells were cultured for too long, which resulted in failure of recognition. To investigate whether the J76 lines were the issues, we moved on to looking at $CD8^+$ T-cells derived from a human primary donor. Then, through an impedance-based cytotoxicity assay, we were able to see that transduced $E6_{29-38}$-specific- $CD8^+$ T-cells were able to engage in a cytotoxic response and decrease the growth of Hu_056 CRCs.

The conditionally reprogrammed cells show potential as an immunological model for T-cell based immunotherapies. We have shown that in this head and neck cancer case, transgenic T-cells can recognize and even kill CRCs. However, because of the variability of the TIL or the exhausted state we found that it was not able to recognize CRCs. Future experiments will be explored to see if recognition is repeatable in other patient cases. We also eventually aim to see if these conditionally reprogrammed cells can be applied as an *in vivo* mouse model for T-cell based immunotherapies.

FIGURES

A



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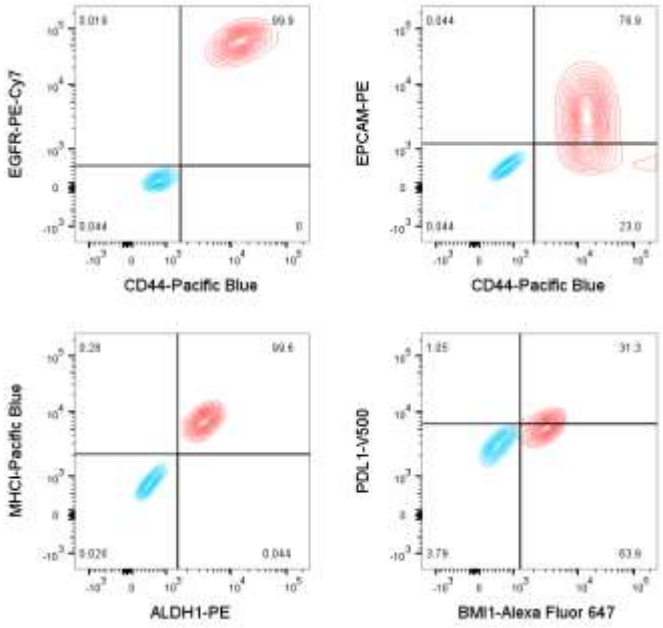
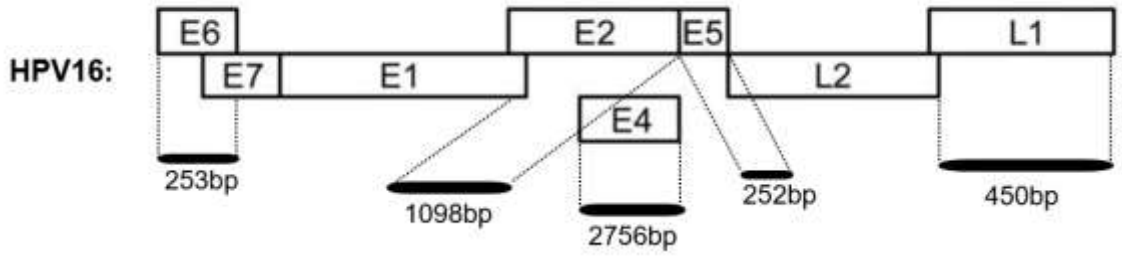


Figure 1. Establishment of conditionally reprogrammed cancer cells from Hu_056. (A) The morphology of the Hu_056 CRC cells are epithelial-like and grow without the necessity of feeder cells. Scale is 100µm. (B) Flow cytometry results of the CRCs looking at epithelial and cancer stem cell markers. The unstained CRC cells are depicted in blue and the stained CRC cells are red.

A



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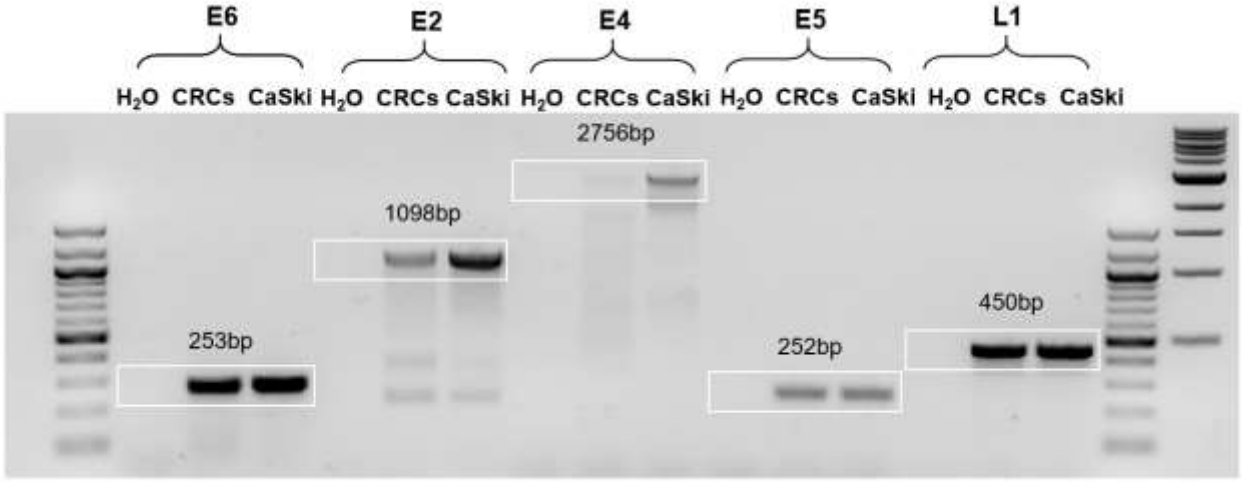


Figure 2. Presence of HPV16 gene in Hu_056 conditionally reprogrammed cells. (A) Schematic showing expected base pair length of the different regions in the HPV16 gene with the primers designed. (B) Gel electrophoresis after PCR with primers amplifying various HPV16 regions (E6, E2, E4, E5, L1). The white boxed regions show the expected DNA length of the amplified regions as well as the predicted base pair length. CaSki, a known cell line containing HPV 16, was used as a positive control and water was used as a negative control.

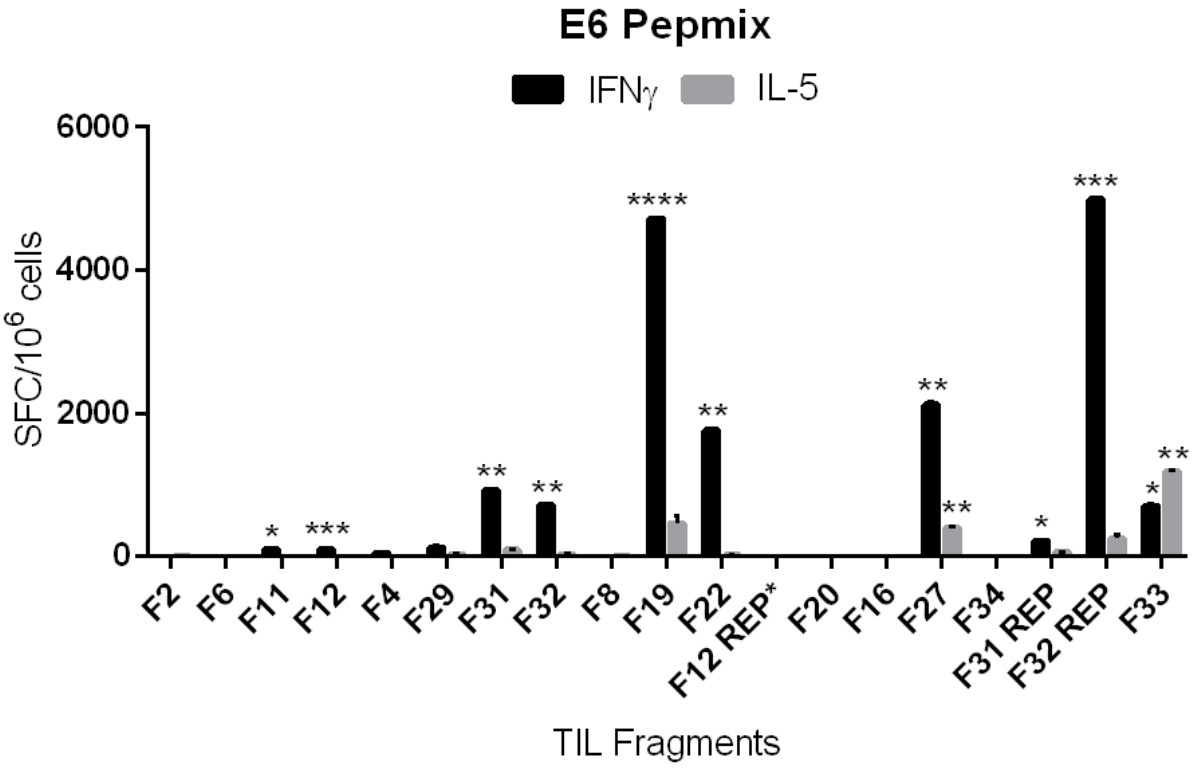


Figure 3. Detection of reactive Hu_056 TIL Fragments against E6 pepmix by EliSpot. Nineteen Hu_056 TIL fragments were tested for reactivity based on secretion levels of IFN-γ and IL-5. The graphs is a combination of several experiments with each fragment having an individual negative (unstimulated) and positive (PHA) controls, which are not shown. SFC stands for spot forming cells. Standard deviation bars are shown with n=3. Statistical significance was determined by a significant change with the unstimulated control. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

*Fragment 12 REP was oversaturated resulting in a negative reading by plate reader.

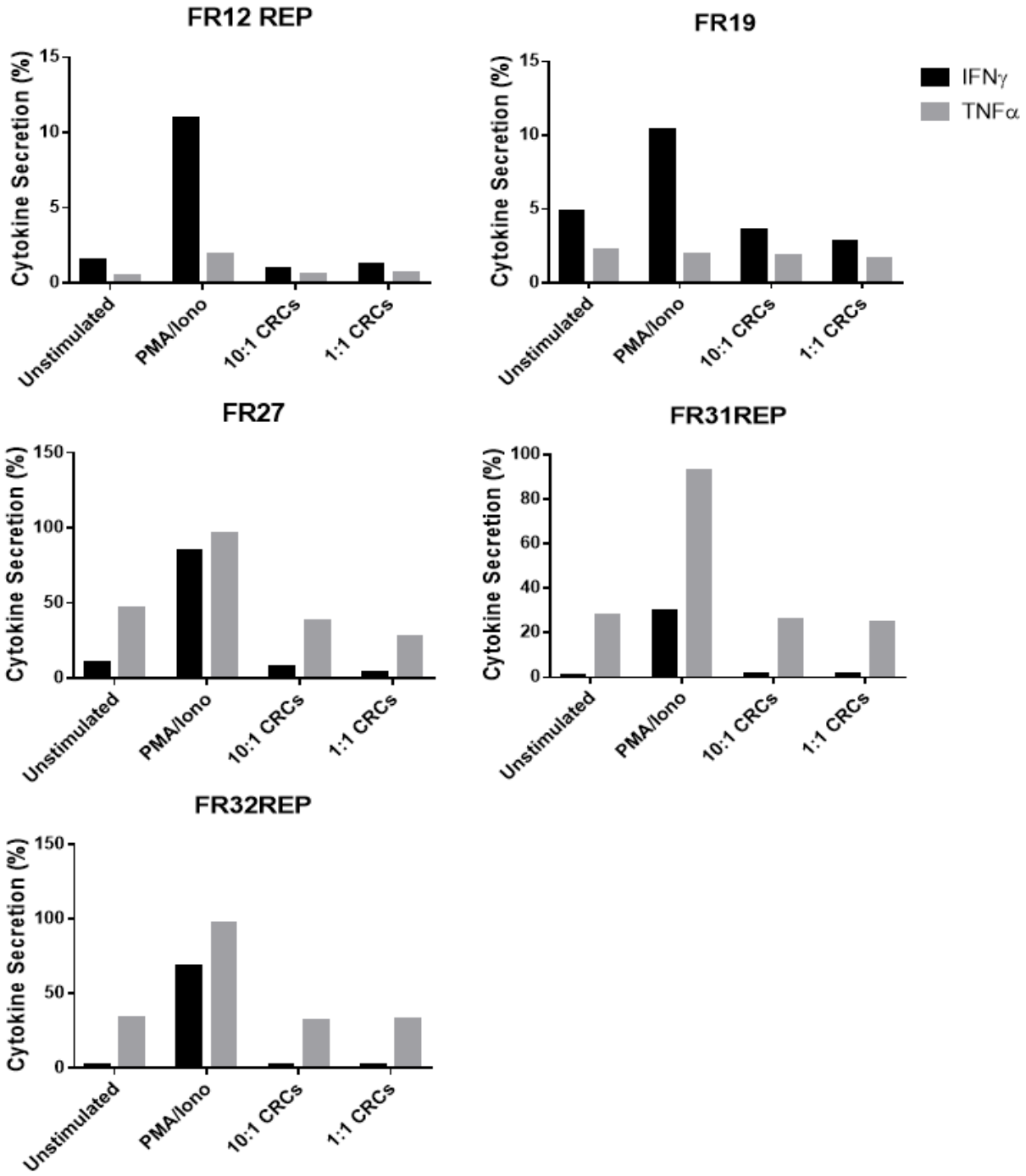
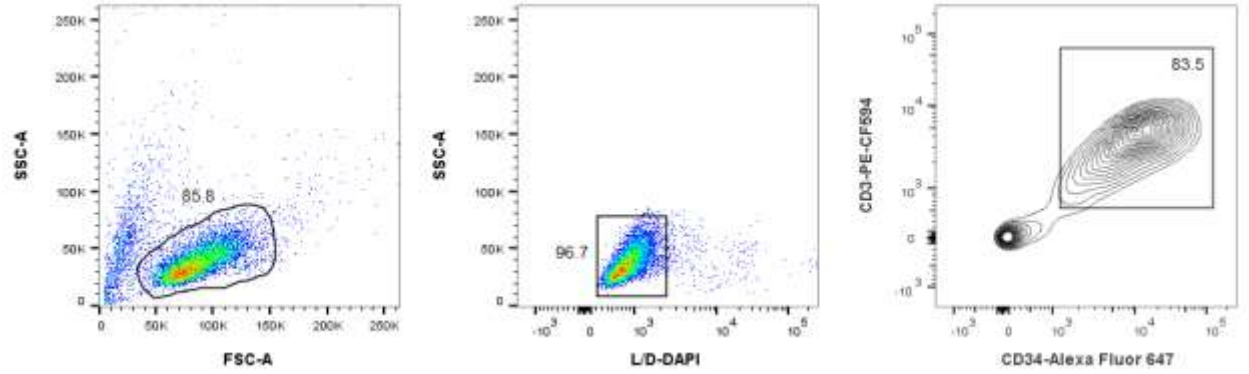


Figure 4. E6 pepmix-specific Hu_056 TIL do not recognize Hu_056 CRC. TIL co-cultured with CRCs do not show an increase in secretion of IFN-γ or TNFα compared to the unstimulated controls by flow cytometry. Cytokine secretion percentages were gated for $CD3^+$ cells. The CRCs were pretreated for 48 hours with 10nM of IFN-γ. Ratio is effector: target. Due to limited samples, experiments were performed n=1.

A



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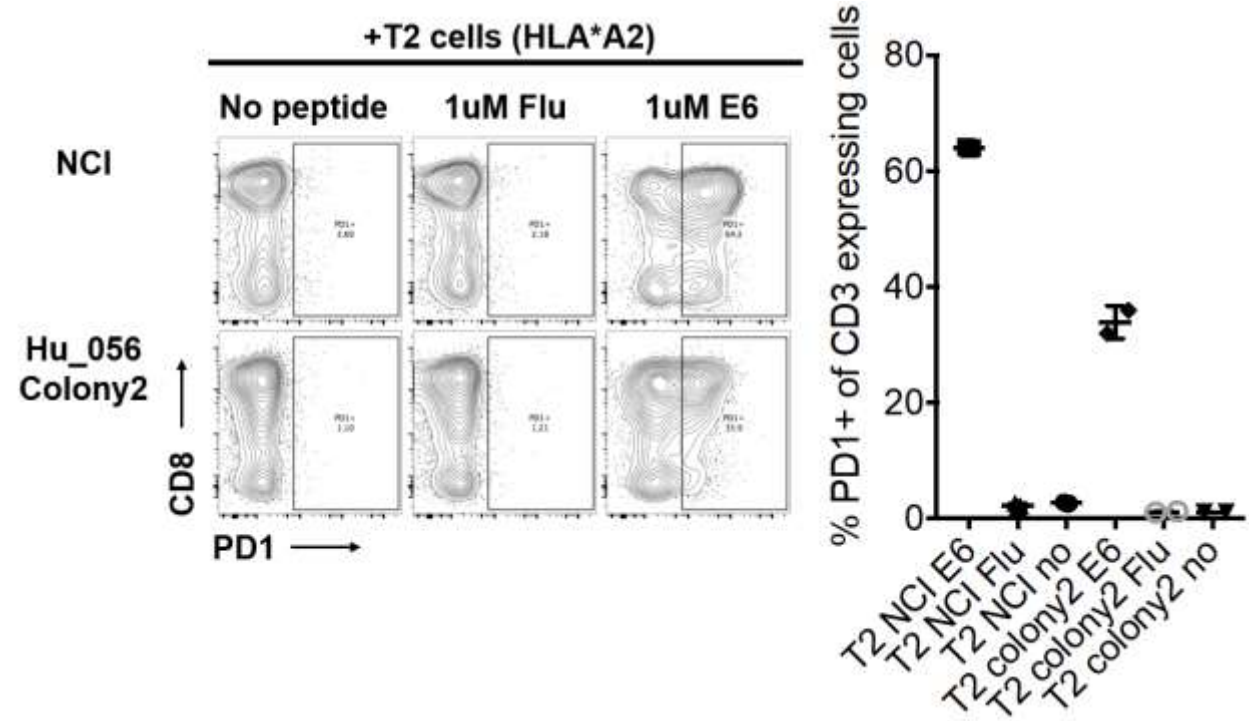
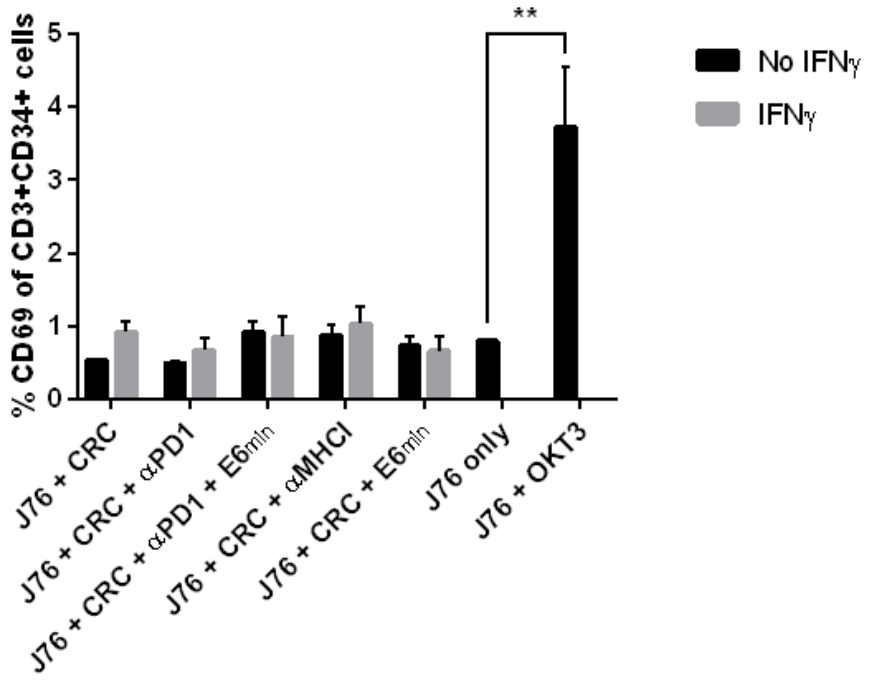


Figure 5. Transduced E6$_{29-38}$-specific J76s recognize E6$_{29-38}$ peptide presented by T2 cells. (A) J76s were successfully transduced with lentivirus containing a HLA*02:01-restricted HPV16 E6$_{29-38}$-specific TCR plus a tCD34 marker as shown by flow cytometry. (B) Transduced E6$_{29-38}$-specific J76s were co-cultured with E6$_{29-38}$ peptide presented by T2 cells. 'NCI' denotes an E6$_{29-38}$-specific TCR obtained from the National Cancer Institute and was used as a positive control. Standard deviations are shown and were performed n=2. 'Hu_056 colony2' depicts Hu_056 CRC cells.

A



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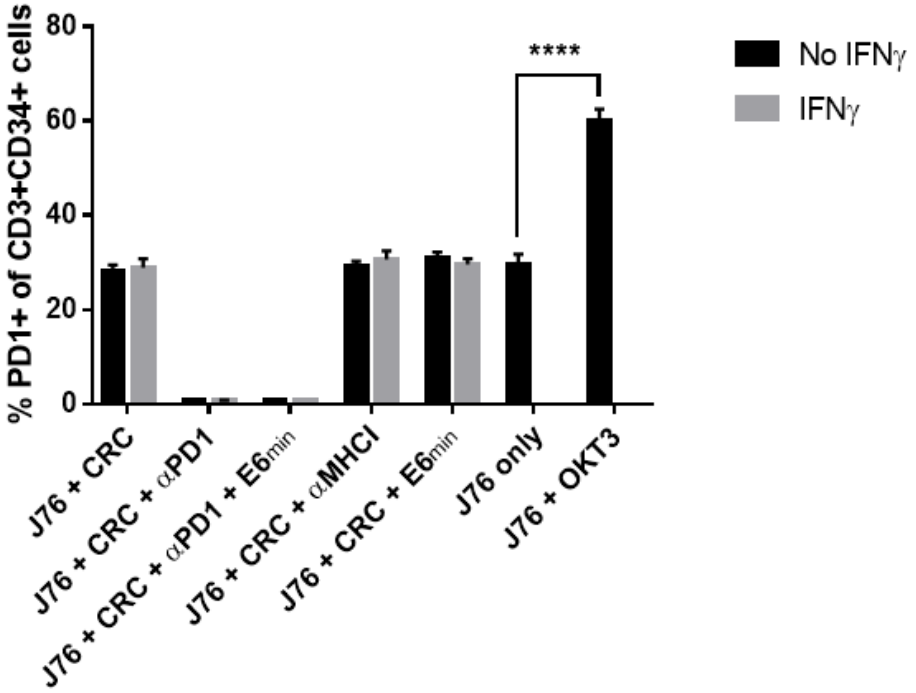


Figure 6. Lack of recognition of Hu_056 CRCs by transduced E6$_{29-38}$-specific J76s. Co-culture with the transduced J76s and CRCs with the different conditions show no significant increase in PD1 or CD69 expression when compared to the 'J76 only' control by flow cytometry. The CRCs were treated with 10nM of IFN-γ. Standard deviation bars are shown, n=3. **p<0.001, **** p<0.0001

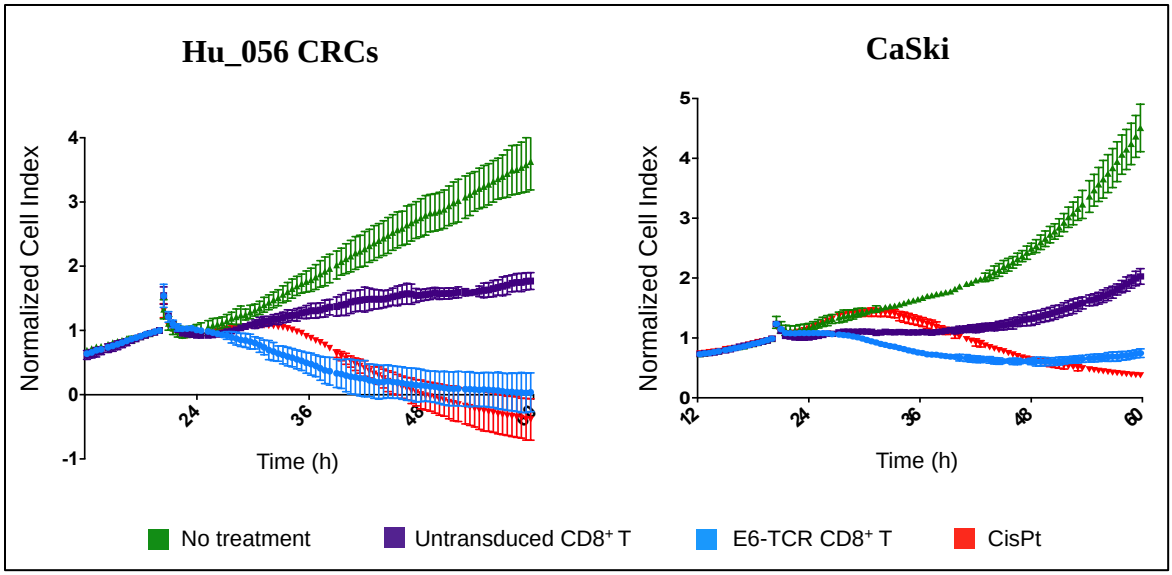
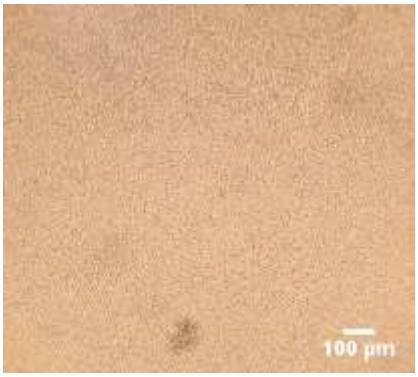


Figure 7. Cytotoxic recognition of Hu_056 CRC and Cervical Carcinoma (CASKI) cell lines by human T cells expressing E6$_{29-38}$/A2.1-specific TCR. Human primary PBMC were transduced with pHAGE lentivirus encoding a HLA*02:01-restricted HPV16 E6$_{29-38}$-specific TCR plus a tCD34 marker (or control LV alone). CD34^{+} CD8^{+} cells were isolated by positive selection and used as effectors in an impedance-based cytotoxicity assay using the ACEA XCelligence system with either an autologous PDX cell line generated from the patient's own HPV16+ HNSCC tumor or an established HPV16+ A2.1+ Cervical Cancer cell line, CASKI. Impedance measurements were taken longitudinally every 15 minutes for the indicated time period. Figure 7 is co-authored by Martin Naradikian.

SUPPLEMENTAL FIGURES

A



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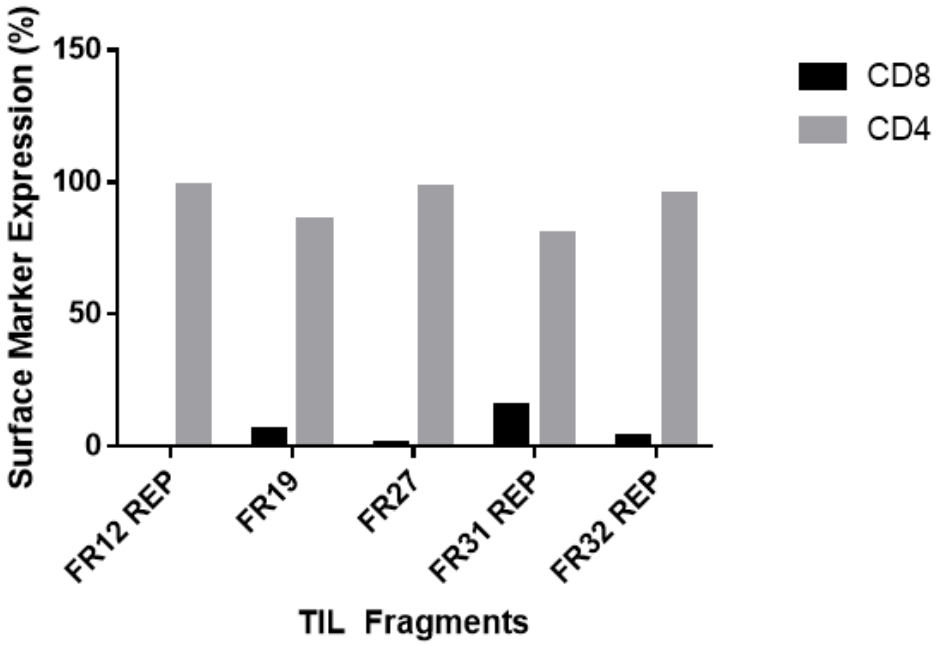
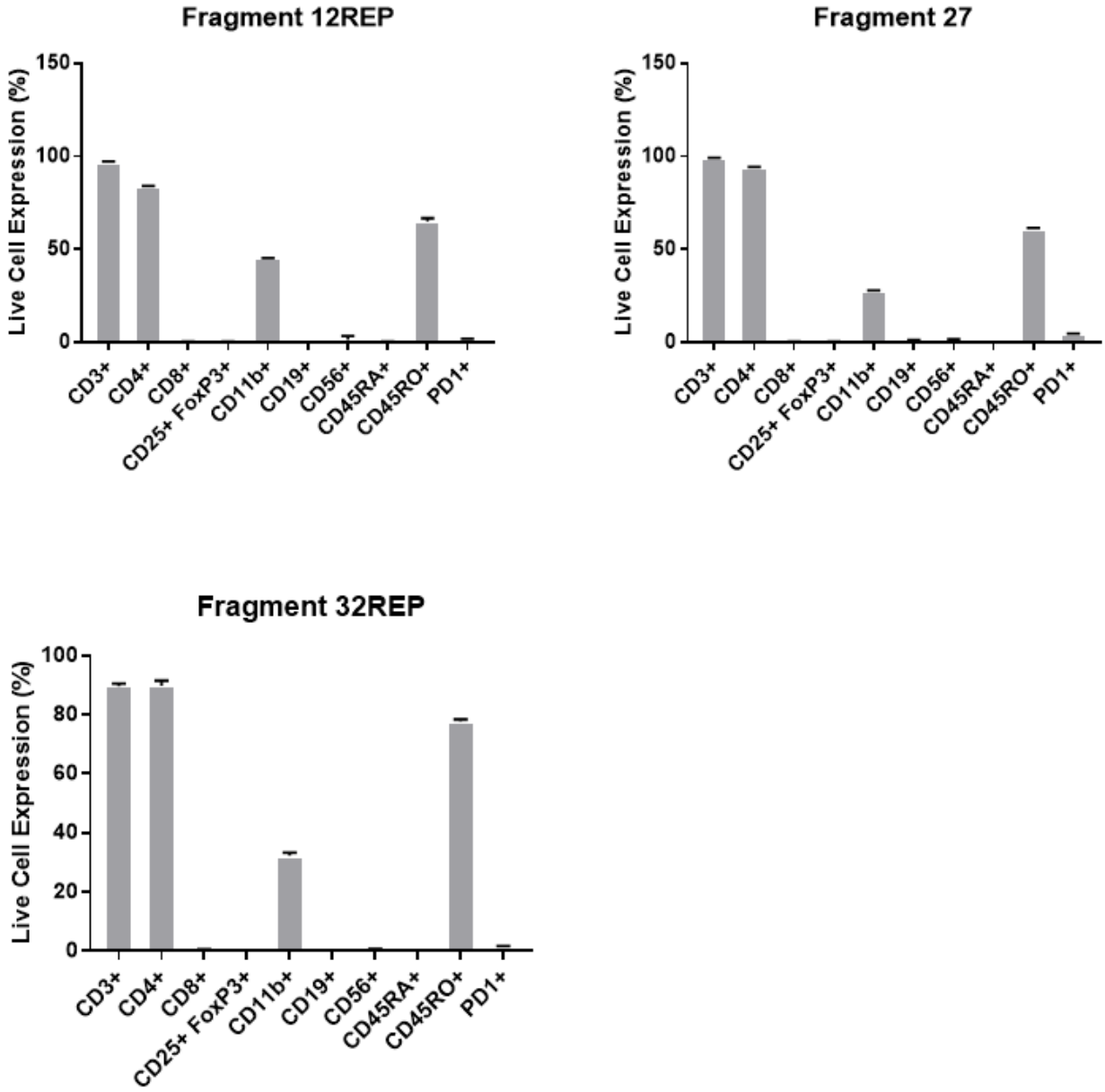


Supplemental Figure 1. Establishment of tumor infiltrating lymphocytes from Hu_056. (A) Morphology of the established Hu_056 TIL resembles lymphocyte-like suspension cells. Scale bar is 100µm. (B) The Hu_056 TIL fragments are predominantly CD4 T-cells with very few CD8 T-cells.



Supplemental Figure 2. Hu_056 TIL Fragments are predominantly CD4$^+$ effector T-cells. Flow Cytometry demonstrated that TIL Fragments 12REP, 27 and 32REP were predominantly CD4$^+$ T-cells. The presence of CD45RO$^+$ suggested that these were effector CD4 T-cells. There was also some expression of CD11b$^+$, a marker for macrophages. Standard error bars are shown with n=3.

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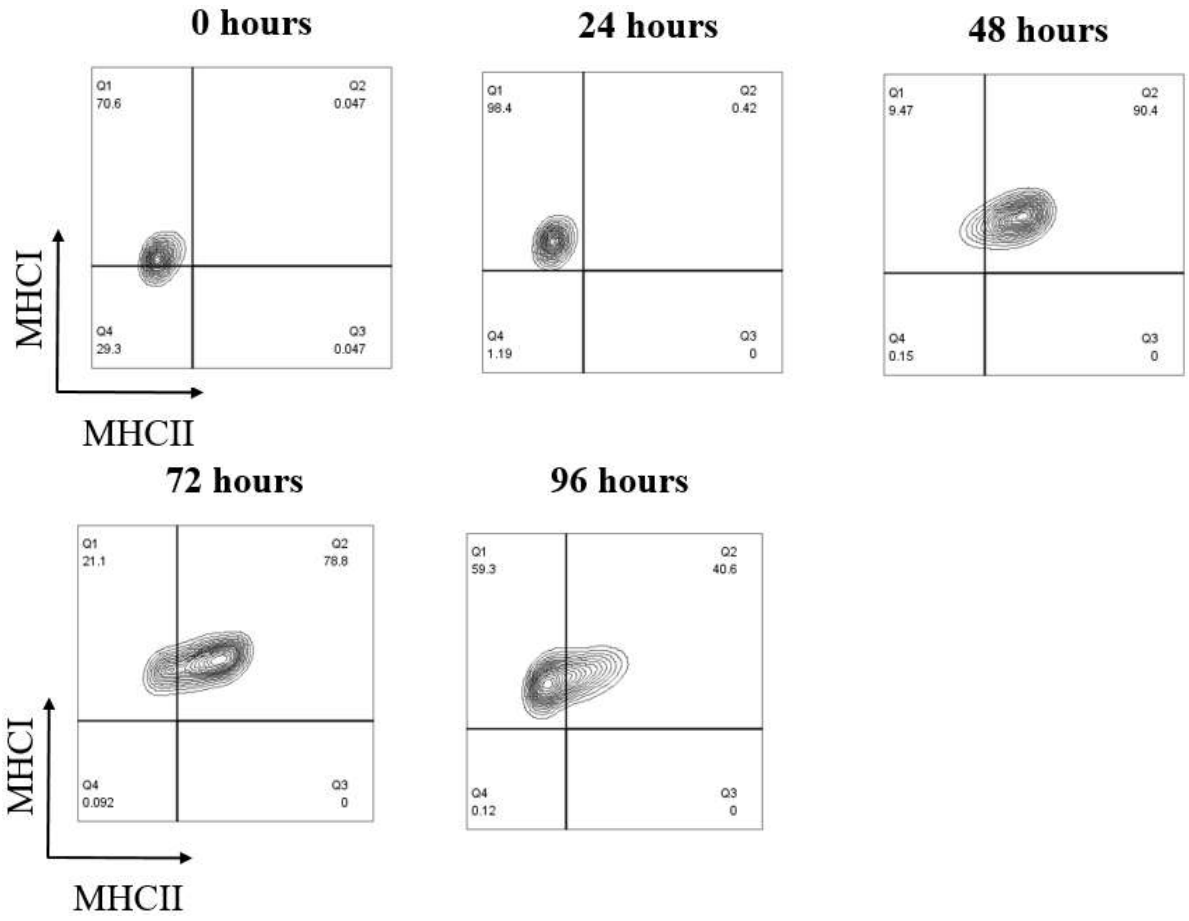

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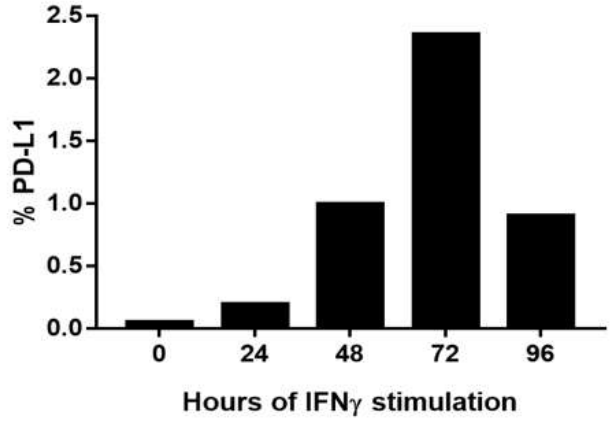
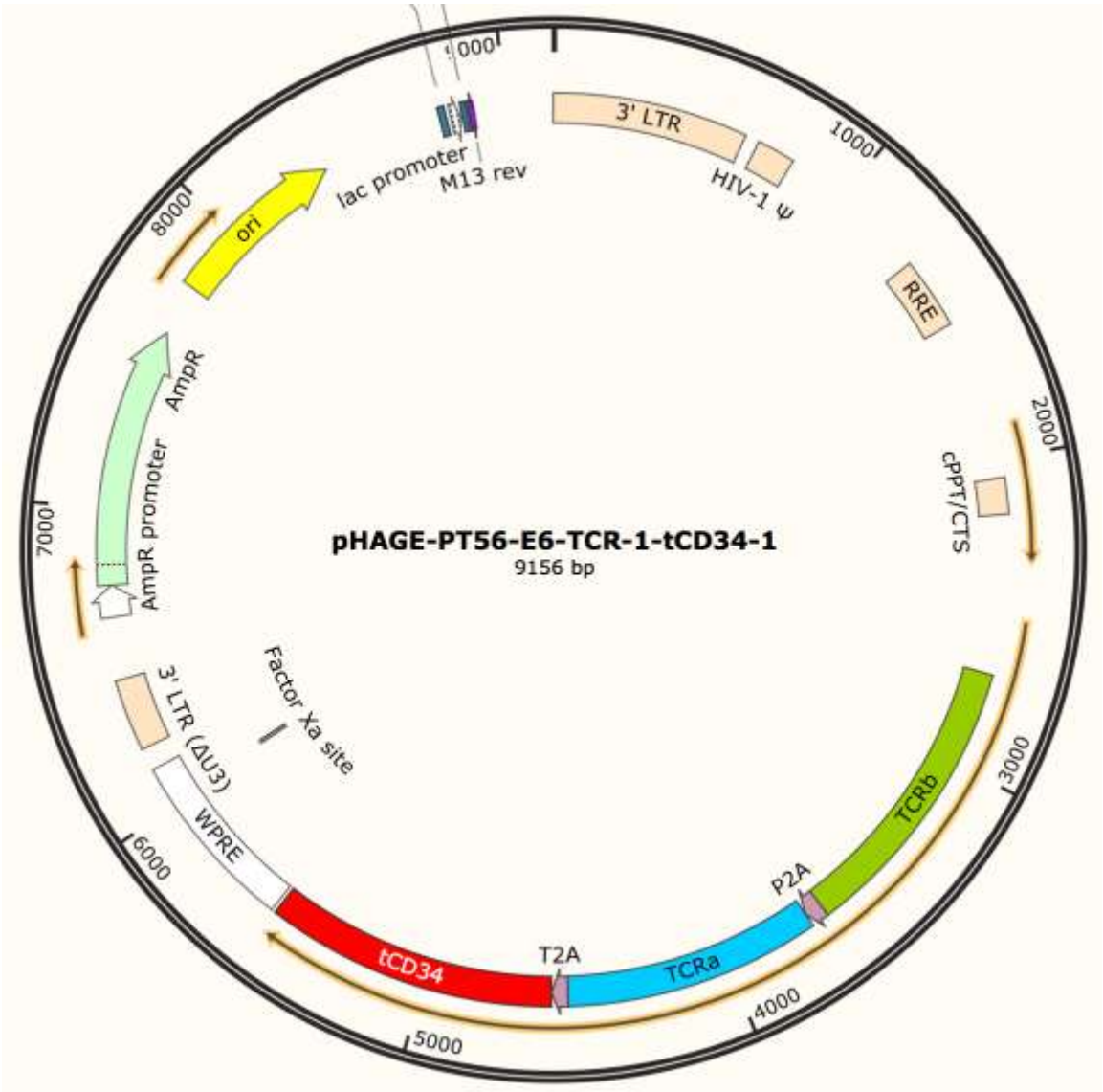


Supplemental Figure 3. IFN-γ stimulation of CRCs promotes expression of MHC-II. (A) 10nM of IFN-γ was added to Hu_056 CRCs at various time points and 48 hours of stimulation showed the highest expression of MHC-I and MHC-II. (B) PD-L1 expression of the CRCs peaked at 72 hours.



Supplemental Figure 4. Plasmid map encoding the $E6_{29-38}$-TCR from Hu_056 TIL FR12REP. The plasmid was designed with the TCRα and TCRβ chain from a $CD8^+$ HPV16 $E6_{29-38}$-specific TCR in the Hu_056 TIL Fragment 12. A truncated CD34 marker was inputted to allow for identification of successfully transduced cell.

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