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EMPLOYING T CELLS FOR ANTIGEN PRESENTATION: ROLE OF NY-ESO-1+ T-APC VACCINE IN MULTIPLE MYELOMA

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**EMPLOYING T CELLS FOR ANTIGEN PRESENTATION:
ROLE OF NY-ESO-1+ T-APC VACCINE IN MULTIPLE MYELOMA**

A

THESIS

Presented to the Faculty of

The University of Texas

Health Science Center at Houston

and

The University of Texas

MD Anderson Cancer Center

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

By

Krina Patel MD

Houston, Texas

August 2016

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Dedication

I dedicate this project to all of my patients. Cancer sucks! Yet, you trust us and fight relentlessly, even though many times treatment comes with significant side effects.

You are the reason for this research!

Acknowledgments

Thank you to the Myeloma SPORE for supporting this project through the CDA program.

I would like to thank my mentor Dr. Laurence Cooper for accepting me into his lab my first year of Hematology/Oncology fellowship. I knew very little about cell therapy then and now it has become an integral part of my career. I truly appreciate your continued support and empathy when I come in before and after rounding and clinics to conduct my experiments. Thank you Dr. Dean Lee for taking over as my committee lead advisor and helping me focus to complete this thesis.

I would like to thank Simon Olivares for teaching me everything (literally everything) and for not laughing too hard when I made dumb mistakes or almost broke expensive equipment. I never would have been able to complete this Masters without you.

Thank you Dr. Harjeet Singh for being an amazing consultant on everything from experimental design to statistics to proper finishing of the figures in this thesis. You are a great friend and I'm lucky to have met you.

Thank you to my clinical mentors Dr. Muzaffar Qazilbash and Dr. Robert Orlowski for your encouragement and aiding my development from fellowship to now as junior faculty. I would also like to thank Dr. Pam Sharma, Dr. Robert Bast and Dr. Partow Kebriaei for being members on my advisory committee.

Thank you to all the members of the former Cooper lab for your friendship and support. Thank you Drs. Lenka Hurton and Aleksandra K Nowicka specifically for the IL-15 plasmid and LAG3 knockout, respectively. I would like to thank Tiejuan Mi for all her help with the mouse studies.

Thank you Dr. Champlin for being a wonderful chair and supporting my time in the laboratory. Thank you to my clinical colleagues for teaching me advanced time management skills to be able to successfully handle all the different hats we must wear daily.

Last, but not least, thank you to my family and friends for dealing with me “always” being on the computer in the evenings and on the weekends.

Abstract

EMPLOYING T CELLS FOR ANTIGEN PRESENTATION: ROLE OF NY-ESO-1⁺ T-APC VACCINE IN MULTIPLE MYELOMA

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Advisory Professor: Dean Lee MD PhD

Introduction: Cellular therapy has shown great potential in early phase clinical trials with persistence of effector cells appearing to lead to improved outcomes. Combined immunotherapy with NY-ESO-1⁺ CAR T cells (TCR mimetic) and an NY-ESO-1 specific T-APC vaccine in myeloma patients may lead to enhanced anti-myeloma efficacy due to improved persistence of the CAR T cells.

Materials and Methods: Using the Sleeping Beauty transposon/transposase system, an NY-ESO-1⁺ TCR and CAR were created as effector cells. Vaccine was produced by electroporation of the NY-ESO-1 gene into T cells to make T cell antigen presenting cells (T-APC). Costimulatory molecules were added to the T-APC to help improve immunization efficacy. *In vitro* studies were conducted to evaluate the ability of T-APC to expand antigen specific effector cells compared to a positive control of K562 artificial antigen presenting cells (aAPC). The ability of effector cells (TCR versus CAR) to kill the U266 myeloma cell line and other targets was also evaluated. An MRD multiple myeloma mouse model using a U266 cell line was used to compare delayed tumor growth for CAR versus CAR+T-APC.

Results: NY-ESO-1⁺ T-APC with mIL-15 were successfully generated with >95% expression of the antigen and co-stimulatory molecule. *In vitro* studies confirmed the NY-ESO-1⁺ CAR expanded upon co-culture with T-APC and lysed the U266 line more efficiently than the NY-ESO-1⁺ TCR. Subsequent *in vivo* studies revealed the NY-ESO-1 CAR delayed tumor growth significantly when compared to the control tumor only group and irrelevant CAR control CD19R⁺ CAR group. The NY-ESO-1⁺ CAR + T-APC group showed similar tumor delay using bioluminescent imaging, but improved tumor control on necropsy compared to the NY-ESO-1⁺ CAR alone group. The NY-ESO-1⁺ CAR + T-APC group also showed increased persistence of CAR⁺ T cells with memory phenotype compared to all other experimental cohorts.

Conclusion: NY-ESO-1⁺ T-APC work similarly to K562 aAPC in expanding NY-ESO-1⁺ CAR T cells *in vitro*, and lead to improved persistence of CAR T cells and anti-myeloma effect *in vivo*.

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CHAPTER 1: Introduction

Combination immunotherapy with CAR T cells and T-APC vaccine post autologous transplantation (auto-HSCT) has great potential for improving outcomes in patients with high risk multiple myeloma (MM). Auto-HSCT alone for MM leads to transient complete responses and extended progression-free survival in a subset (20-30%) of patients.¹⁻³ Even after tandem HSCT, relapse is inevitable in the majority of cases.⁴ Patients with high-risk disease (defined as deletion 17p, t[14;16], or t[14;20]), have an overall survival of 18 to 36 months. Allogeneic HSCT may increase the rate of cure through T cell-mediated graft-versus-MM effect, but conveys increased risk of morbidity and mortality from graft-versus-host disease. After auto-HSCT, decreased anti-tumor immunity leads to suboptimal control of minimal residual disease (MRD) and eventual progression. Furthermore, after high-dose chemotherapy, decreased lymphocyte number and function can last for months to years.⁵ Alternatively, robust recovery of lymphocyte counts predicts improved survival for MM after auto-HSCT, through augmentation of the host antitumor immunity.^{6,7}

Adoptive T cell therapy with antigen specific effector T cells targeting the tumor-associated antigen (TAA) NY-ESO-1 in the context of HLA has great potential for treatment of multiple myeloma. However, persistence of similar effector cells post transplantation in other malignancies is variable. Therefore, the addition of NY-ESO-1⁺ T antigen presenting cell (T-APC) vaccine is a novel approach to improve the persistence of adoptively transferred T cells post autologous transplant as well

as improve endogenous long-term immunity through both direct and cross-priming as described in Figure 1.

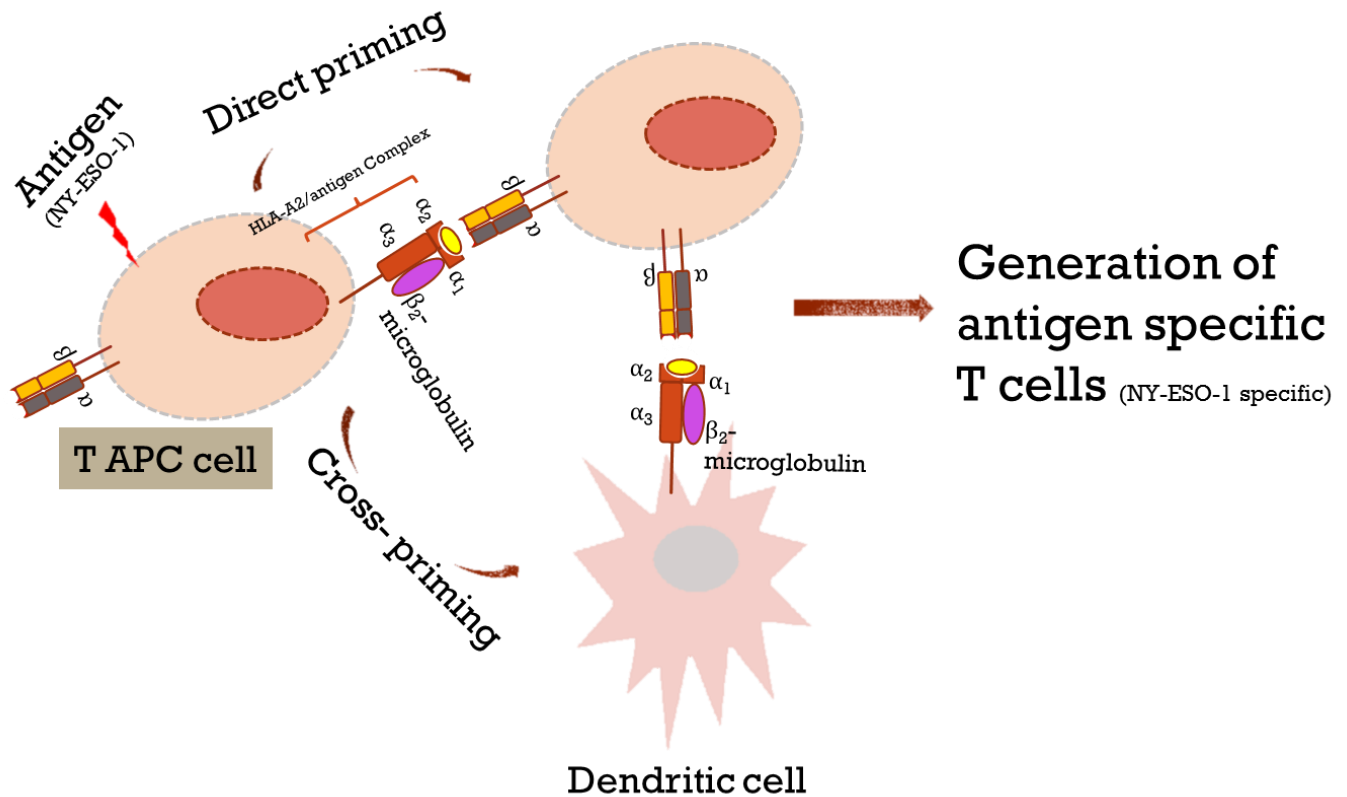


Figure 1. Mechanism of antigen presentation for generation of T-cell immune response. Plasmid DNA is electroporated into the T-APC, processed intracellularly, and presented to an effector T cell in an MHC restricted manner through direct priming. Concurrently, cross presentation of the antigen from the T-APC to professional antigen presenting cells, such as dendritic cells, followed by direct priming may lead to an increased generation of antigen specific T cells. *Courtesy of Simon Olivares*

Cancer vaccines have resulted in some clinical responses, but have fallen short of their potential.^{8,9} Current vaccine strategy shortcomings include difficulty with optimal antigen delivery by professional antigen-presenting cells (APCs), requirement for costimulatory molecules, difficulty with manipulating and expanding

professional APCs *in vitro*, and ineffective delivery to tumor sites.¹⁰ The ability to propagate and genetically modify T cells may help overcome limitations of translating professional APC into T-cell vaccines. Activating T cells *ex vivo* can lead to up-regulation of HLA class I and II, and co-stimulatory molecules, such as CD80 and CD86. Furthermore, endogenous T-cell responses have been observed in patients to transgenes expressed by genetically modified T cells which suggest the ability of T cells to function as APC.¹¹ This new approach will augment immunity against tumor cells by combining adoptive transfer of CAR T cells with the co-administration of a T-cell based vaccine where T cells act as APC (T-APC). T cells and other vaccines have been co-administered to MM patients to improve immunity after auto-HSCT and achieved responses to pneumococcal vaccine and a multi-peptide vaccine including human telomerase reverse transcriptase (hTERT) and survivin.^{12,13} In these early clinical studies, *in vivo* vaccine-primed and *ex vivo* propagated effector T cells were infused during a period of lymphopenia followed by vaccine boost. Therefore we hypothesized that T-APC would expand antigen specific effector cells and lead to improved killing of myeloma, both *in vitro* and *in vivo*. If this approach is deemed successful, translation to anti-myeloma therapy has great potential to improve life for our patients.

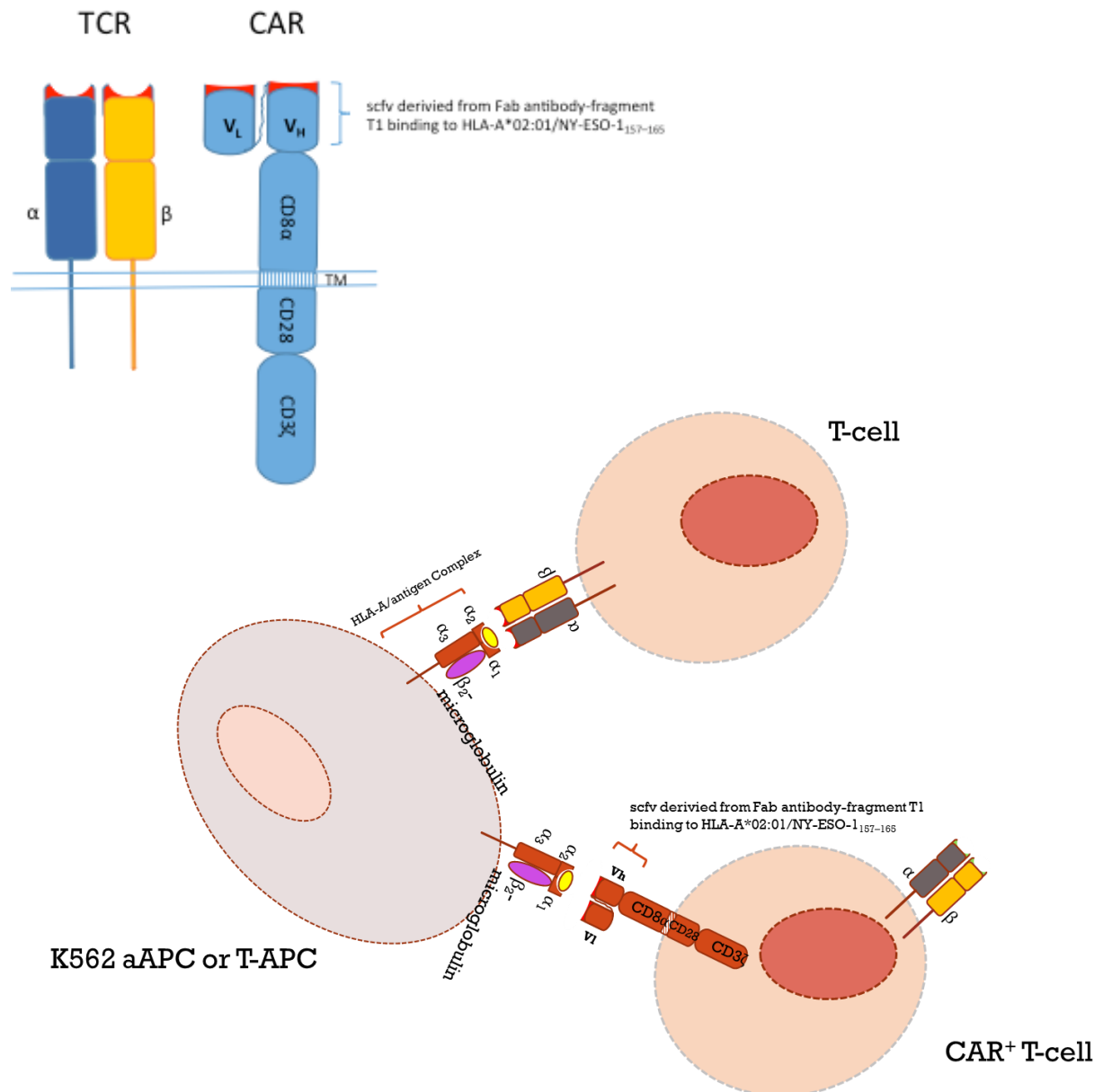


Figure 2. (A) TCRs comprise α/β heterodimers that bind to peptide in a major histocompatibility complex (MHC) restricted manner. TCRs complex with CD3 subunits and co-receptors (CD4 or CD8) associated with Lymphocyte-specific protein tyrosine kinase (Lck). CARs contain single-chain variable fragment (scFv) recognition domains capable of binding to cell surface antigens. CARs contain intracellular signaling domains from CD3 ζ and a co-stimulatory molecule (typically CD28 or 4-1BB). Signaling is initiated by Lck-mediated phosphorylation of immunotyrosine activation motifs (ITAMs) within the cytoplasmic domains of CD3. (B) APC can engage and expand T cells through TCR or CAR. *Courtesy of Simon Olivares*

CHAPTER 2: Materials and Methods

Ethics Statement. All research involving human samples was in accordance with protocols established and approved by the MD Anderson Cancer Center Internal Review Board (IRB). Animals treated in this study were handled in accordance with the strict guidelines established by the MD Anderson Cancer Center Institutional Animal Care and Use Committee (IACUC). Mice were housed in pathogen-free conditions and were monitored daily for welfare-related assessments in accordance to IACUC guidelines.

DNA plasmids

Generation of NY-ESO-1 expression plasmid for T-APC. The sequence for the NY-ESO-1, also known as CTAG1A (GenBank: AJ003149.1), gene was triple flag tagged at the 5' -end and linked to a hygromycin phosphotransferase (Hygro) gene by a foot-and-mouth disease virus 2A self-cleavable peptide. The flag-NY-ESO-1 fusion gene was human codon optimized and synthesized by GeneArt (Germany), and cloned into vector Flag-RhCMVpp65 (CoOp)-F2A-HA-Hyg/pT2SBSO using restriction enzymes ClaI and AfeI at 5' -end and 3' -end respectively. This cloning generated the *Sleeping Beauty* plasmid Flag-NY-ESO-1 (CoOp)-F2A-HA-Hyg/pT2SBSO, which constitutively expresses the fusion gene under the control of the hEF-1 α promoter.

Generation of NY-ESO-1 specific TCR expression plasmid. The 1G4 TCR $\alpha\beta$ sequence was obtained from Robins et al (14). The sequence was codon optimized and synthesized by GeneArt (Germany). The 1G4 TCR $\alpha\beta$ cassette was cloned into

vector GlySer-EGFP (CoOp)/pT2SBSO using restriction enzymes NheI and XhoI at 5' –end and 3' –end respectively. This generated the final sleeping beauty transposon plasmid Anti-NY-ESO-1 TCRs (CoOp)/pT2SBSO, which constitutively expresses the TCR $\alpha\beta$ under the control of the hEF-1 α promoter.

Generation of NYESO1-CD28 CAR (TCR mimetic) transposons. The CARs NYESO1-IgG4-CD28 and NYESO1-CD8 α -CD28, were constructed using the Fab antibody-fragment T1, previously described (15), which binds to the HLA-A2/NY-ESO-1_(peptide 157-165) complex. The scFv sequence was comprised of the V_H and V_L sequences of the Fab antibody-fragment T1, along with a Kozak consensus ribosome binding sequence and the human GM-CSF receptor alpha-chain leader peptide. The V_L was connected to V_H using the Whitlow linker peptide (amino acid, GSTSGSGKPGSGEGSTKG). The scFv was further connected in frame to the hinge and transmembrane regions of the human CD8 α chain (residues 115 – 189 of the mature protein), or to a modified human IgG₄ hinge and Fc regions (residues 161 – 389 of the mature protein), and a CD28 transmembrane domain. The hinge on the Fc, was mutated from amino acids CPSC and CPPC (single letter code) to enhance stability of the dimerized IgG₄ heavy chain. The transmembrane regions were fuse in frame to the CD28 endodomain which was connected in frame to the CD3- ζ cytoplasmic domain. Both CARs were codon optimized and synthesized by GeneArt (Germany), and cloned into the clinical sleeping beauty plasmid CD19R-CD8a-CD28 (CoOp)/pSBSO-SIM using restriction enzymes NheI and XhoI at 5' -end and 3' –end respectively. This procedure generated the two CAR plasmids NY-ESO-1R- CD8 α -

CD28 (CoOp)/pSBSO-SIM and NY-ESO-1R-IgG4-CD28 (CoOp)/pSBSO-SIM, which constitutively express the CAR under the control of the hEF-1 α promoter.

SB11 Plasmid

The SB transposase SB11 was expressed from plasmid pCMV-SB11 as previously described.¹⁶

Membrane bound IL-15 (mIL15)

IL-15 fused to IL-15R α (membrane bound) was constructed as previously described.^{17,18}

Electrotransfer of plasmid vectors

Generation of T-APC

To manufacture T-APC's, normal donor human PBMC's were genetically modified by electroporation using the Sleeping beauty system as previously described¹⁶. Briefly, PBMCs were thawed, washed and rested for 2-4 hours. They were then resuspended in hypo-osmolar buffer at 100×10^6 cells/mL. Aliquots were added to 0.2 cm cuvettes containing 15 μ g of the NY-ESO-1/HLAA2 plasmid, 15 μ g of the IL-15 plasmid, 5 μ g of CRISPR-Cas9-Lag3 plasmid (CRISPR-Cas9 based plasmid specific for Lag3, GenBank: NM_002286, gene targeting at exon 2. This was a kind gift from Dr. Aleksandra K Nowicka), and 10 μ g of sleeping beauty transposase SB11 plasmid, in a total volume of 400 μ L. Each cuvette received a single 40-microsecond pulse of 250V after which the cells were rested in 20% FBS phenol free RPMI

overnight. The next day, the cells were co-cultured with OKT3 aAPC (K562 aAPC loaded with anti-CD3 antibody, OKT3, via CD64 Fc γ receptor) and IL-21. Cytocidal levels of hygromycin at 0.2 μ g/mL were added on day +5 and subsequently weekly.

Generation of K562 aAPC

K562 artificial antigen presenting cells, K562-A2⁺ NY-ESO-1 aAPC , was generated through enforced co-expression of truncated CD19, CD64, CD86, CD137L and HLA-A2 on K562 cells genetically modified with lentiviral vectors and was a gift from Carl June (University of Pennsylvania)¹⁹. This aAPC was further modified using SB system to co-express the _{Flag}-NY-ESO-1-F2A-HA-Hygro fusion protein, the cells were grown in cytocidal levels of hygromycin at 0.4 μ g/mL, to eliminate the non-integrants, and to enforce the expression of the NY-ESO-1 protein. The K562-A2⁺ NY-ESO-1 aAPC uniformly and stably co-expressed CD19, CD32, CD64, CD86, CD137L, HLA-A2 and NY-ESO-1 (detected by intracellular flag staining).

Generation of NYESO-1 TCR and NYESO-1 CAR T cells 1G4 TCR⁺ and CAR⁺ T cells were propagated based on modifying standard operating protocols as previously described^{20,21}. Cryopreserved PBMC, obtained from healthy donors after informed consent, were thawed the day of the electroporation (designated day 0) and rested for 2 hours at 37°C. PBMC for electroporation were spun at 200g for 10 minutes and 10x10⁶ cells were mixed with supercoiled DNA plasmids (2.5 μ g SB11 transposase and 7.5 μ g SB transposon) in Human T cell Nucleofector Solution (cat#VPA-1002, Lonza), added to a cuvette, and electroporated on the U-014 program of Amaxa Nucleofector II (Lonza). Electroporated cells were transferred to a

6-well plate containing phenol-free RPMI, 20% FBS, and 1x Glutamax-100. The following day, electroporated T cells were phenotyped and stimulated with either irradiated (30Gy) NY-ESO-1 T-APC or irradiated (100Gy) K562-A2⁺ NY-ESO-1 aAPC at a ratio of 1:1 (CAR or TCR⁺ T cell : aAPC). Each co-culture was supplemented with IL-21 (cat# AF20021; Peprotech, Rocky Hill, NJ; 30 ng/mL) starting at initiation of culture and every 2–3 days thereafter. TCR and CAR expression was evaluated weekly to determine the number of aAPC to add to co-cultures every 7 days. If contaminating NK cells reached >10% of the total population, they were depleted from co-cultures with paramagnetic CD56 microbeads (Miltenyi Biotec, Auburn, CA) and LS columns (Miltenyi Biotec). Phenotyping and functional analyses were performed between days 21 to 29.

Cell lines. K562, EL4 and U266 cell lines were acquired from American Type Culture Collection (Manassas, VA), and were maintained in complete media (RPMI, 10% FBS (Hyclone, Logan, UT), and 1x Glutamax-100). All tumor cells were free of mycoplasma and other microbial pathogens.

Flow cytometry. All mAbs were purchased from BD Biosciences (San Jose, CA), except for CCR7 mAb (eBioscience, San Diego, CA), IL-15 mAb (R&D Systems, Minneapolis, MN), Fc mAb used to detect CAR (Invitrogen), and R-PE labeled Pro5 MHC Pentamer NY-ESO-1, (ProImmune). Staining was performed as described²². Samples for flow cytometry were acquired on FACS Calibur (BD Biosciences) and analyzed using FCS Express (version 3.00.0612).

***In vitro* cytotoxicity and cytokine production.** *In vitro* specific lysis was assessed using a standard 4-hour chromium release assay, as previously described²¹. Expression of cytokines was evaluated by intracellular staining and flow cytometry. CAR⁺ T cells were incubated with an equal volume and number of target cells for 6 hours at 37°C in the presence of Brefeldin-A (GolgiPlug; BD Biosciences) to block exocytosis and secretion of cytokines. Co-cultures were then (i) stained for surface markers, e.g., CD3 and CAR, (ii) fixed and permeabilized with BD Cytofix/Cytoperm (cat# 555028, BD Biosciences), (iii) stained for intracellular IFN γ , and (iv) analyzed by flow cytometry. Positive control were T cells treated with Leukocyte Activation Cocktail (BD Biosciences).

Mouse *In Vivo* Model. U266-ffLuc-EGFP tumor cells were generated by electroporating U266 cells during log phase growth with linearized ffLuc-EGFP-pcDNA. Transduced U266 myeloma cells were sorted for uniform EGFP expression by FACS to obtain cells with ffLuc activity suitable for non-invasive BLI. Immunocompromised female NSG mice (6–12 weeks of age; NOD.Cg-Prkdc^{scid}Il2r^{gtm1Wjl}/SzJ; Jackson Laboratory, Bar Harbor, ME) were intravenously (i.v.) injected with 1.25×10^4 U266-ffLuc-EGFP cells on day 0 (total n = 30), were mixed together, and then randomly distributed into 6 groups, which were treated with (i) no treatment (n = 5), (ii) NYESO1CD28⁺ CAR T cells (n = 5), (iii) NYESO1CD28⁺ CAR T cells + NYESO1 T-APC (n = 5), (iv) CD19RCD28⁺ CAR T cells, (v) CD19RCD28⁺ CAR T cells + NYESO1CD28⁺ CAR T cells, and (vi) NYESO1 T-APC alone. CAR T-cell doses (10^7 total cells per mouse) were administered on day 1 while T-APC doses (10^7) were infused on days 1 and 5 for group 3 and 5. CAR expression on

infused T cells was 89% for the NYESO1CD28⁺ CAR and 90% for the CD19CD28⁺ CAR. The T-APC flag expression was 80% on day of infusion. BLI from tumor ffLuc was monitored weekly. Student t test [1 tailed, type 2] was used for statistical analysis between groups of mice (n = 5 per group). At the end of the experiment, mice tissue (blood, spleen and bone marrow) was harvested and analyzed for the presence of tumor (GFP) and T cells (CD3).

CHAPTER 3: Results

NY-ESO-1 T-APC were successfully constructed with the NY-ESO-1 and IL-15 co-stimulatory molecule plasmids. It has been previously shown that T cells increase expression of CD80 and CD86 (co-stimulatory molecules also on professional antigen presenting cells) upon activation¹¹. However, to increase the T-APC potency, several costimulatory adjuvants were evaluated. IL-15 regulates T and NK cell activation and proliferation, provides survival signals to maintain memory T cells in the absence of antigen, and suppresses apoptosis in T cells by inducing Bcl-2 and Bcl-xL²³⁻²⁵. CD40L, a member of the TNF superfamily, augments anti-cancer T cell immune responses by triggering effective activation and maturation²⁶. IL-7 is important for B and T cell development and homeostasis and may decrease CD4⁺CD25⁺Foxp3⁺ regulatory T cells²⁷. The goal was to find the best combinations of co-stimulatory molecules added to the T-APC which would lead to increased immunogenicity. Even though mIL-15 was successfully electroporated into the T-APC, unfortunately the CD40L and IL-7 plasmids failed to integrate for unknown reasons despite multiple attempts. Therefore, the rest of the studies were continued with IL-15 alone as an adjuvant for the T-APC. As shown in Figure 3, T-APC successfully expressed both the NY-ESO-1 (flag) antigen on intracellular staining as well mIL-15 on surface staining. There are no commercially available flow antibodies for cancer testes antigens like NY-ESO-1, therefore a flag was added to the plasmid for which intracellular staining could be done to confirm integration. Furthermore, because this antigen is MHC restricted, HLA-A2⁺ T cells were used for the study. T-APC are expanded on OKT3 aAPC; when comparing T-APC with no co-stimulatory

molecules to T-APC with mIL-15, the latter expanded slightly better, but this was not significant (Figure 4).

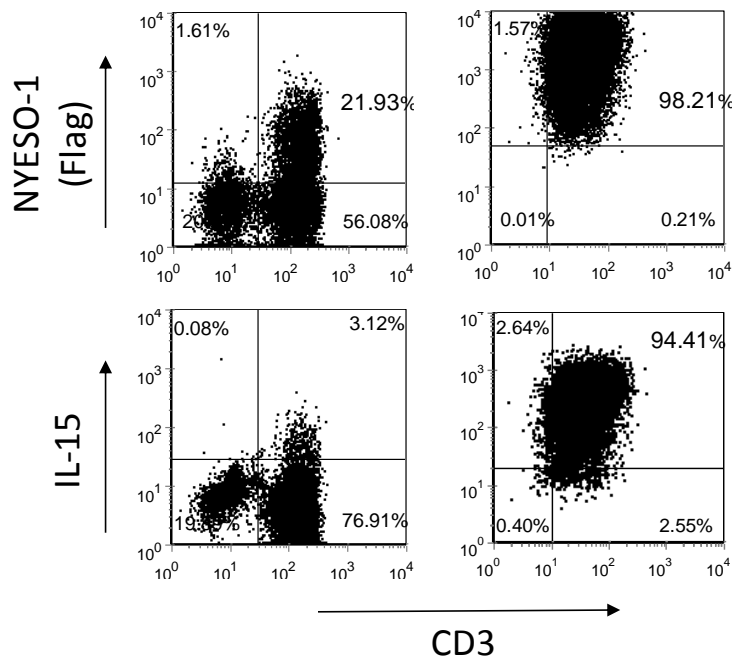


Figure 3. T-APC Phenotype. T-APC were electroporated with mIL-15 and NYESO1 antigen and cultured on OKT3 aAPC. After 4 weeks of stimulations, T-APC had greater than 90% Flag⁺ (intracellular stain for detection of the NY-ESO-1) and IL-15 (surface stain) expression.

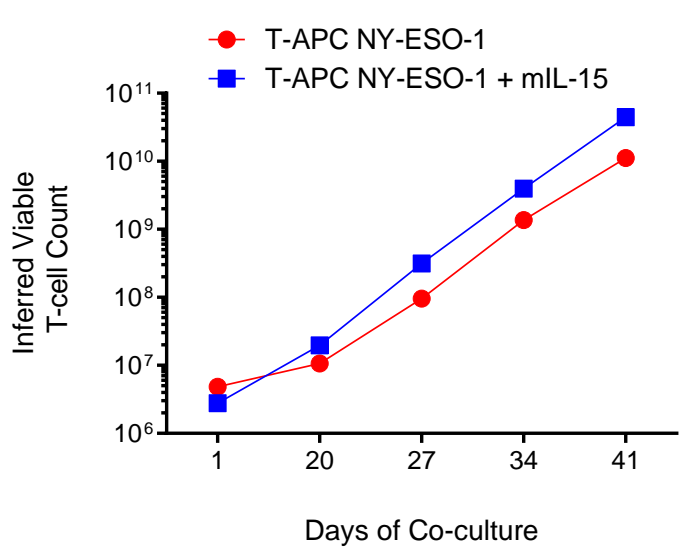


Figure 4. T-APC Expansion Kinetics. T-APC were expanded on OKT3 aAPC in the presence of IL-21. T-APC without mIL-15 also had IL-2 added to the culture. T-APC with mIL-15 had a trend towards increased expansion compared to T-APC with no costimulatory molecules, however it was not significant (p value 0.3, 2 way ANOVA).

The T-APC were able to propagate the NY-ESO-1 CAR as well as the NY-ESO-1 K562 aAPC, *in vitro*. K562 aAPC have been used to expand CAR T cells and TCR⁺ T cells by our group¹⁶. Therefore, experiments were conducted comparing the ability of the NY-ESO-1 T-APC (experimental) versus the K562 aAPC to expand antigen specific effector T cells. These effector T cells included NY-ESO-1⁺ CAR T cells (TCR mimetic), NY-ESO-1⁺ TCR T cell and non-modified T cells to evaluate both APC types' ability to truly vaccinate in an antigen specific manner. Figure 5A demonstrates that T-APC is able to induce NY-ESO-1 specificity in TCR⁺ and non-modified T cells by day 14. However as shown in Figures 5B and 6, TCR⁺ T cells and non-modified T cells do not expand in the presence of T-APC. TCR⁺ T cells expand with K562 aAPC, however non-modified T cells do not expand with either APC. NY-ESO-1⁺ CAR T cells however expand with both T-APC and K562 in a comparable manner.

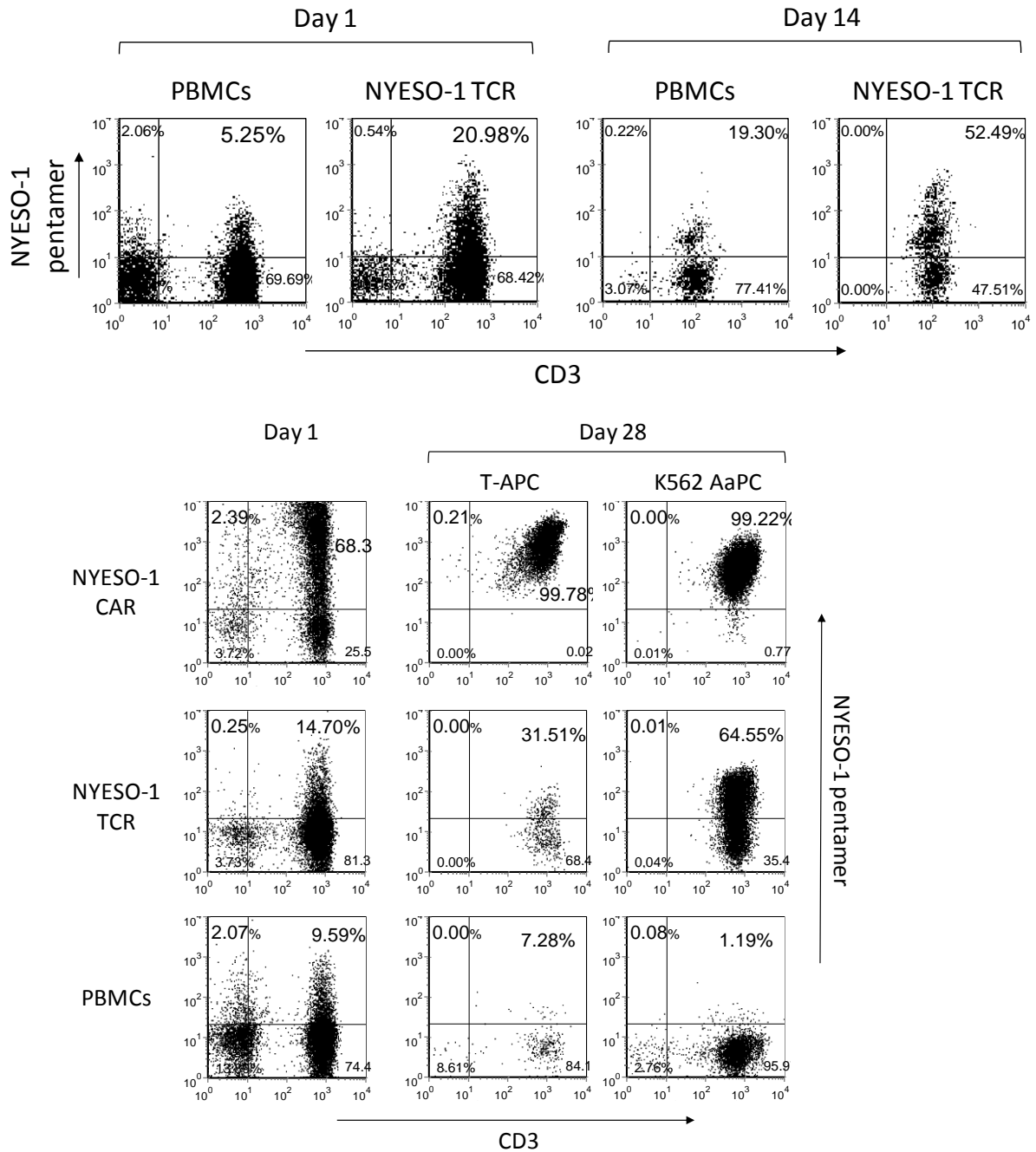


Figure 5. Expansion of effector cells (CAR, TCR, PBMCs) on T-APC versus K-562 aAPC. Unmodified PBMCs or PBMCs electroporated with NYESO-1 CAR/NYESO-1 TCR were co-cultured on T-APC or K562 aAPC expressing the NYESO-1 antigen and evaluated for TCR/CAR expression using NYESO1 pentamer. The TCR and PBMC had increased NY-ESO-1 expression at day 14 with the T-APC (A), however, not as well as compared to the CAR T cells when cultured for 28 days(B). T-APC expanded the CAR just as well as the K562 aAPC (positive control).

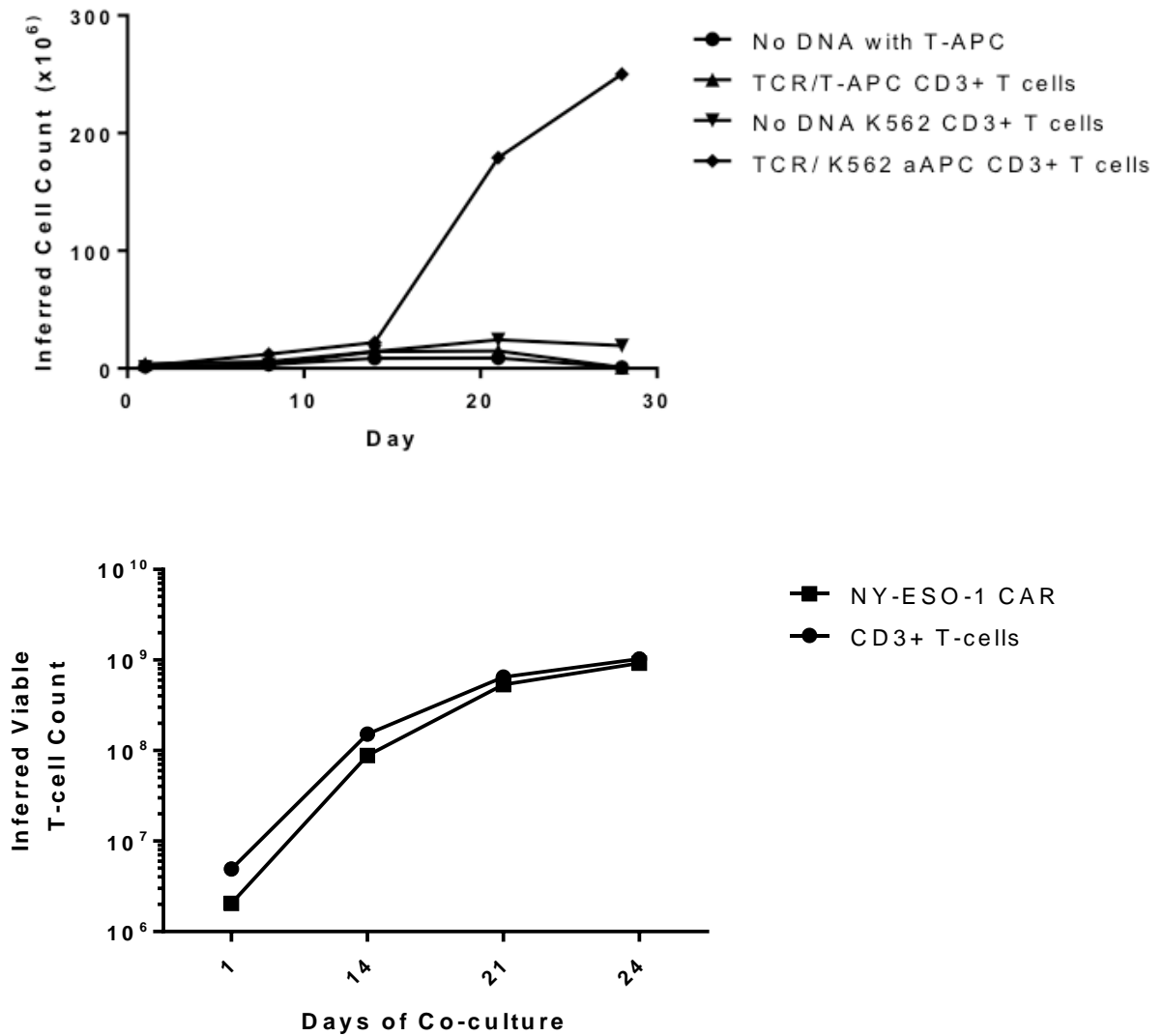


Figure 6. Expansion kinetics for CAR/TCR⁺ T cells. Unmodified or, CAR⁺/TCR⁺ T cells were co-cultured on K562 aAPC or T-APC and enumerated every week to evaluate their proliferation. (A) Non-modified T cells (No DNA CD3⁺ T cells) do not expand with either K562 aAPC or T-APC. NY-ESO-1⁺ TCR expand well with the K562 aAPC, but not the T-APC. (B) The CAR T cells expand just as well on K562 aAPC (shown) and T-APC (data not shown).

To evaluate specificity of the NY-ESO-1 T-APC, a soluble form of NY-ESO-1 CAR was produced and conjugated to PE-Cy5 (Creative Biolabs) for flow cytometric

detection of NYESO-1 antigen on APCs and tumor cells. As in figure 7, testing showed background expression of 3.7% on control T cells. The T-APC had a surprisingly low 13.8% expression while the U266 myeloma cell line, known to have high levels of NY-ESO-1 expression by IHC, showed 57% binding to the soluble CAR. T-APC from only one donor was tested and it had only 79% expression of flag.

Due to the lack of expansion of the NY-ESO-1 TCR⁺ and non-modified T cells by the NY-ESO-1 T-APC, both co-stimulatory and inhibitory molecules were checked on the T-APC prior to each stimulation. The NY-ESO-1 T-APC had fewer co-stimulatory molecules and more inhibitory molecules expressed compared to the NY-ESO-1 K562 aAPC (figures 8 and 9). This surprisingly revealed that T-APC, which are expanded on OKT3 aAPC, had increasing levels of LAG3 with each expansion and decreased expression of most other co-stimulatory molecules, contrary to previous data¹¹.

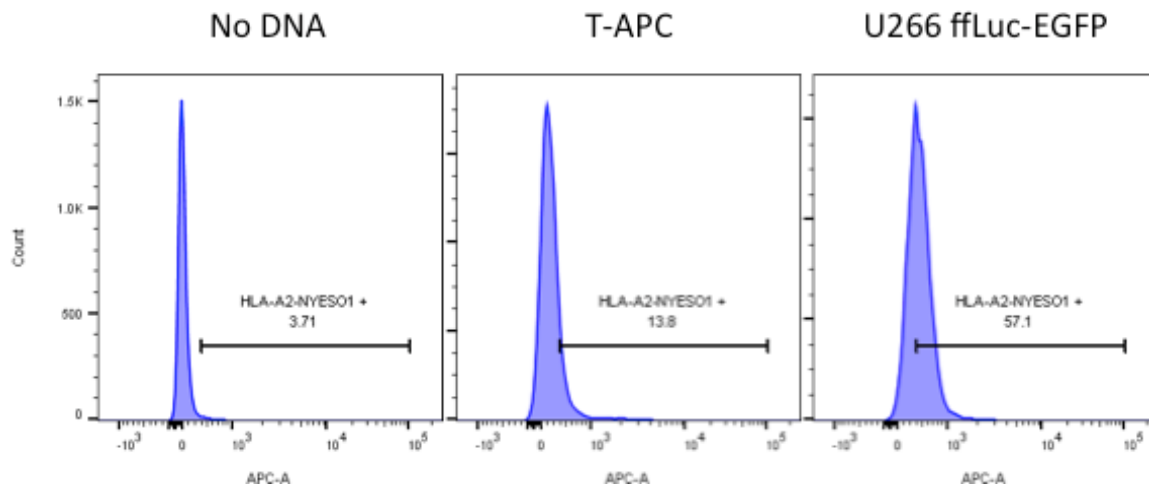


Figure 7. NY-ESO-1 Expression. An NY-ESO-1 HLA-A2 soluble CAR linked to (PE-Cy5) was obtained from (Creative Biolabs). Unmodified T cells, T-APC and U266 cells were stained with soluble CAR for 30 min and analyzed using flow cytometry. NY-ESO-1 T-APC expressed __ 10% more than the negative control of non-modified T cells. The positive control, the U266 cell line, demonstrated 57% expression.

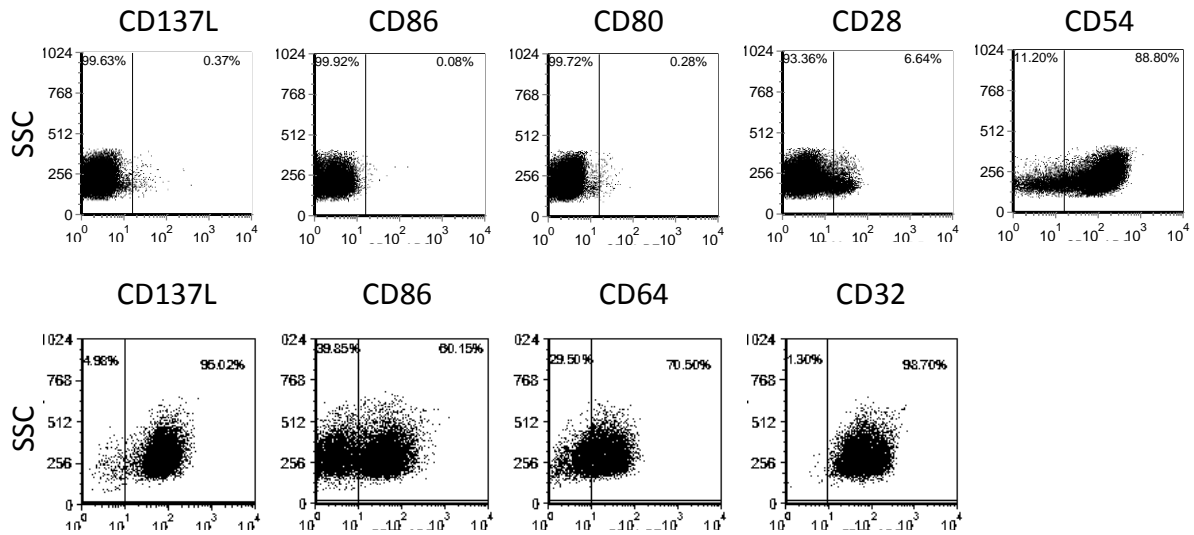


Figure 8. Co-stimulatory molecules expressed on (top) T-APC and (bottom) K562. T-APC showed minimal levels of CD28 and high levels of CD54, while the K562 aAPC expressed CD137L, CD86, CD64, and CD32.

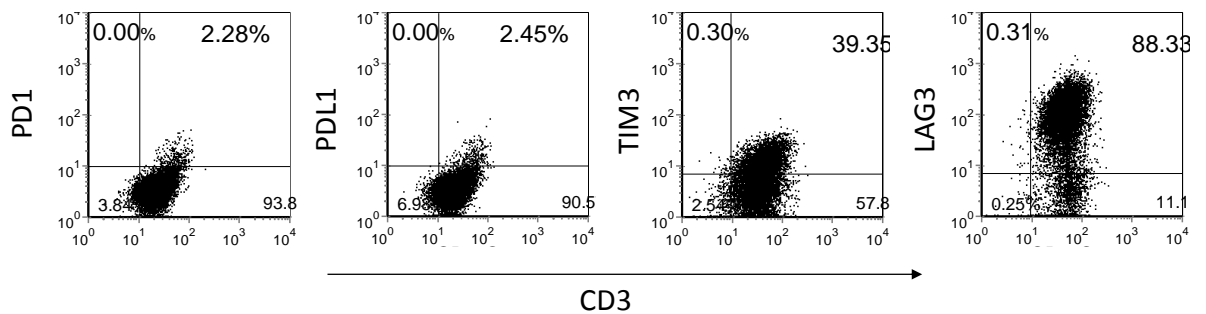


Figure 9. Inhibitory molecules expressed by T-APC. Expression of PD1, PDL1, TIM3 and LAG3 was measured after 7 days of culture by flow cytometry. Increased expression of LAG3 was observed with each stimulation of T-APC with OKT3 aAPC.

LAG3 is known to bind to MHC II with higher affinity than CD4 and negatively regulate cellular proliferation, activation and homeostasis of T cells^{28,29}. It also helps maintain CD8⁺ T cells in a tolerogenic state. Therefore the next set of experiments evaluated the effects of inhibiting LAG3. Experiments were initially conducted using a LAG3 monoclonal antibody which was added to the T-APC and TCR cultures; however all the cell cultures died after 2 weeks. Because the monoclonal antibody was cumbersome and expensive, a LAG3 knockout (KO) version of the T-APC was created utilizing the CRISPR-Cas9 system³⁰. Experiments using the LAG3 KO versus wild type (WT) T-APC were conducted to evaluate effector cell expansion capabilities. Although initially a difference was seen, after 3 weeks of co-culture, all

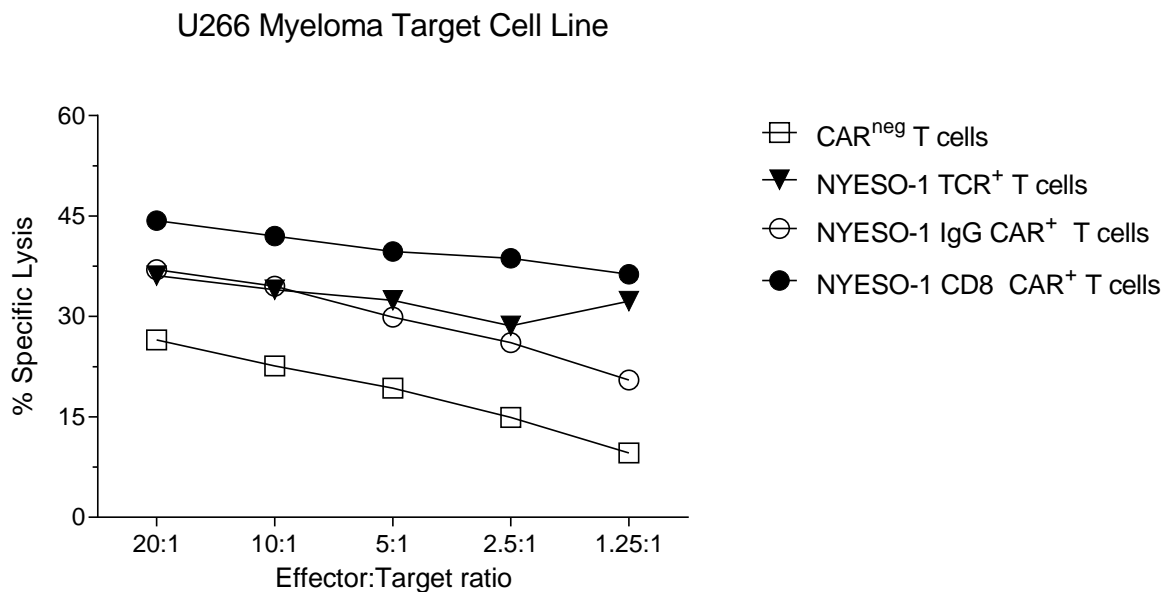


Figure 10. Redirected lysis of CAR/TCR T cells using chromium release assay. The U266 cell line was co-incubated with different effector cells including non-modified T cells (CAR^{neg}), NY-ESO-1+ TCR, NY-ESO-1+ CAR with the IgG stalk, and NY-ESO-1+ CAR with CD8 α stalk. The NY-ESO-1 CAR with CD8 α stalk killed the U266 cell line most.

cells died off and no conclusions could be inferred. Evaluation of LAG3 KO versus WT did reveal decreased IL-2 and increased IL-10 production in the WT group (data not shown). These studies will need to be repeated in the future.

All NY-ESO-1 specific effector cells were able to kill the U266 myeloma cell line better than non-specific T cells, however the CD8alpha CAR demonstrated the most efficient *in vitro* killing. Due to increased immunogenicity, non-specific activation and clearance via binding to Fc γ receptors of CARs containing the IgG₄ stalk we switched to a CD8 α stalk for all CAR T cells. Therefore, we repeated the chromium release assay shown in figure 10, which demonstrated improved cytotoxicity by the new CD8 NYESO-1 CAR compared to the IgG CAR.

Owing to superior expansion on NY-ESO-1 T-APC and improved lytic capabilities of the NY-ESO-1⁺ CAR with CD8 α stalk, an *in vivo* experiment with this combination was performed.

Five cohorts were studied in this minimal residual disease model *in vivo* experiment: tumor only, NY-ESO-1⁺ CAR, NY-ESO-1⁺ CAR and T-APC, CD19R CAR, CD19R CAR and NY-ESO-1⁺ CAR, and T-APC alone. Tumor cells were given on day -1, followed by CAR T cells +/- T-APC on day 0. Repeat T-APC were given to the two groups getting vaccine on day +5 as a boost (Figure 11). Mice were imaged weekly and sacrificed on day +43 for T cell and tumor evaluation (Figure 12). Any group of mice that received the NY-ESO-1⁺ CAR, had the most delayed tumor growth. Interestingly, the only cohort that had CAR still persistent at time of sacrifice,

were the mice that received NY-ESO-1⁺ CAR and T-APC. Further investigation revealed that some of the persisting CAR T cells had memory phenotype as well.

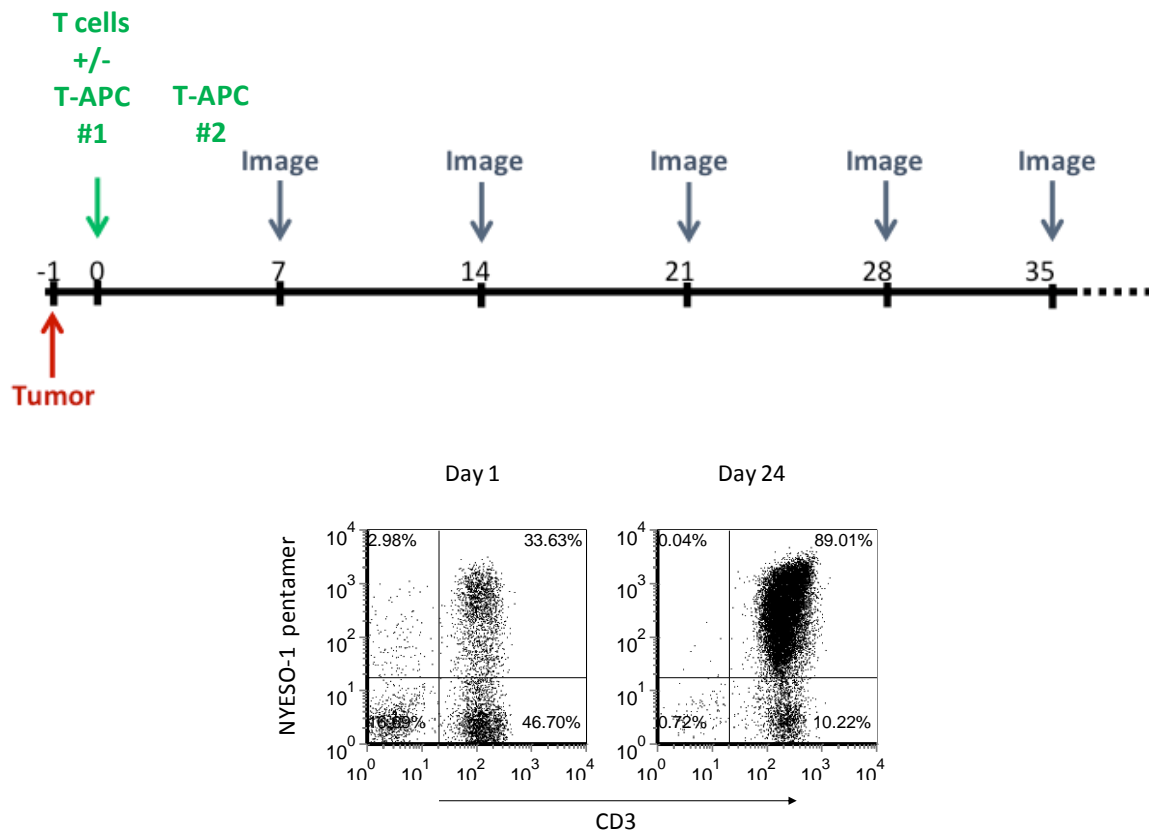


Figure 11. *In vivo* MRD mouse model. Six groups of mice (n=5 per group) were injected with tumor on day -1 followed by CAR T cell injections on day 0. Two groups of mice had T-APC injected at day 0 and day +5. The six groups were tumor only, tumor + NY-ESO-1⁺ CAR, tumor + NY-ESO-1⁺ CAR + NY-ESO-1 T-APC, tumor + NY-ESO-1⁺ CAR + CD19⁺ CAR, tumor + CD19⁺ CAR, and tumor + NY-ESO-1 T-APC. 89% of NY-ESO-1⁺ CAR T cells were NY-ESO-1 pentamer positive on day 24, which was day of infusion.

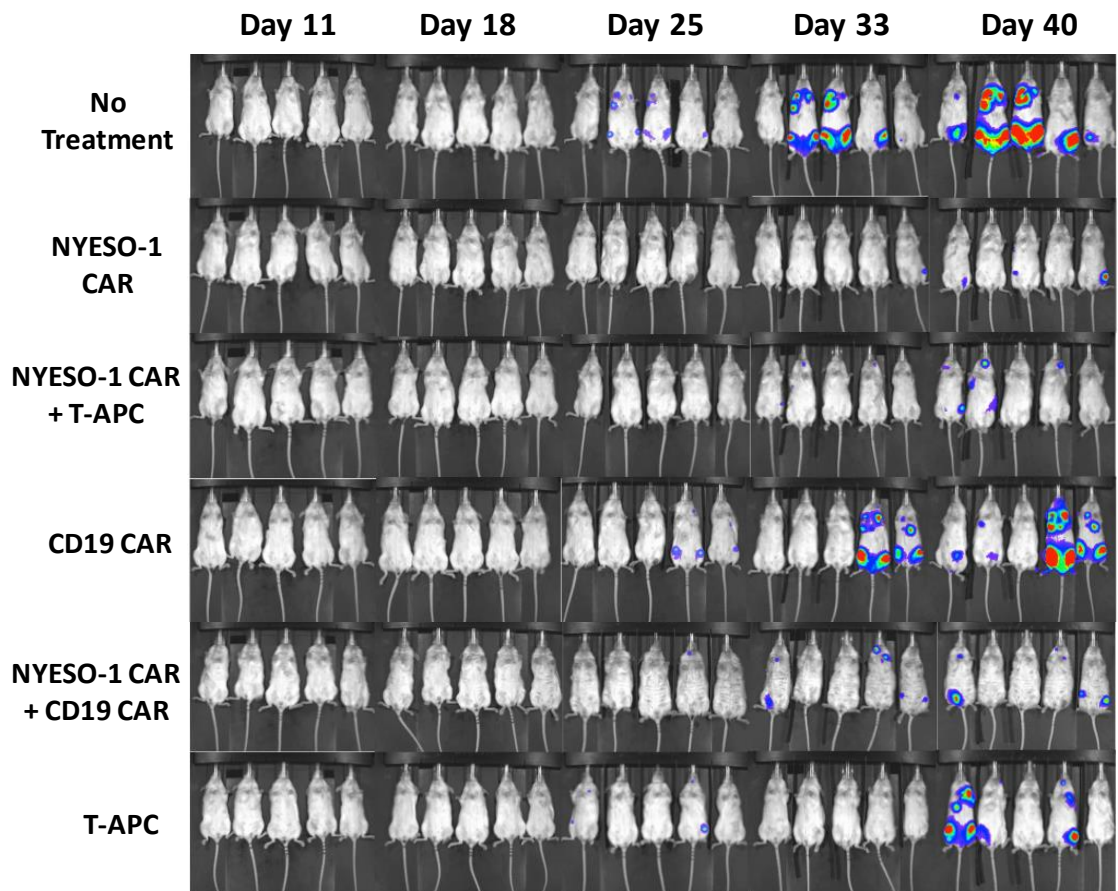
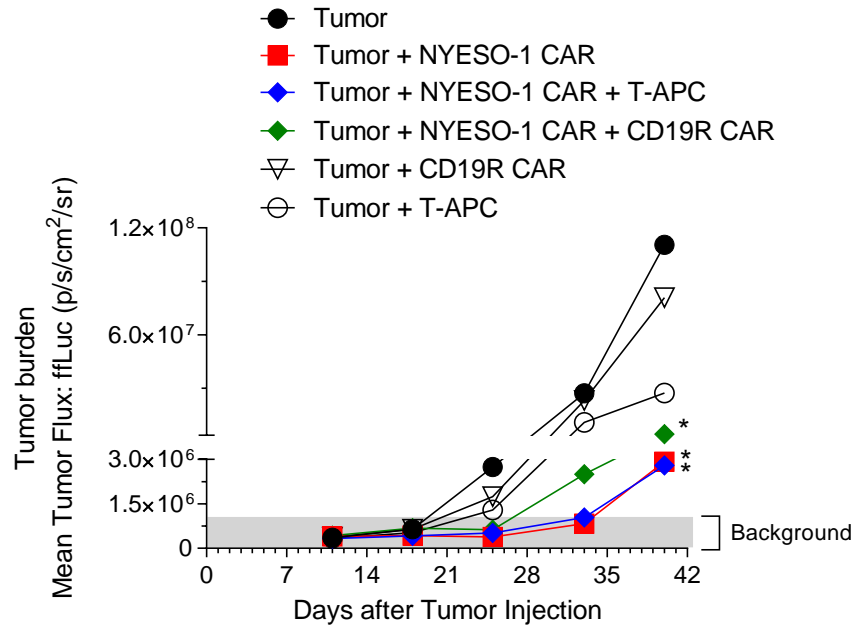


Figure 12. *In vivo* MRD mouse model imaging. After injections of tumor and T cells, mice were imaged weekly to monitor BLI from tumor ffLuc. Mice with NY-ESO-1⁺ CAR T cells had the least tumor growth per imaging.

Table 1. Summary of Tumor and T cells in mice at days 43-45.

Experimental Group	Mouse	Total Cells (%)						CD3+ T cells (%)					
		Tumor (CD138+)			T cells (CD3+)			NYSEO-1 CAR (NYSEO-1 pentamer ⁺)			T-APC (Flag+)		
		Blood	Spleen	BM	Blood	Spleen	BM	Blood	Spleen	BM	Blood	Spleen	BM
Tumor + NYESO-1 CAR	1	0	0	0.2	0.1	0	0	80.8	0	0			
	2	0	0	0	0	0	0	0	0	0			
	3	0	0	0	0	0	0	0	0	0			
	4	0	0	0	0	0	0	0	0	0			
	5	0	0	1.0	0.2	0	0	50	0	0			
Tumor + NYESO-1 CAR + T-APC	1	0	0	0	7.2	4.6	2.4	4.9	7.3	13.1	93.2	93.2	96
	2	0	0	0	4.4	14.8	1.9	17	34.2	26.2	39.8	40.3	53.7
	3	0	0	0.1	0.6	0.3	1.8	0.8	7.3	5.8	92.3	90.0	90.6
	4	0	0	0	9.4	20.4	5.9	5.8	16.2	10.5	45.5	43.9	75.5
	5	0	0	0	6.2	21	2.1	10	20.8	26.8	76.5	65.6	76.3
Tumor+ CD19RCD28	1	0	0	0.1	0	0	0	0	0	0			
	2	0	0	0	0	0	0	0	0	0			
	3	0	0	0	0	0	0	0	0	0			
	4	0.3	0	15	0	0	0	0	0	0			
	5	0	24.5	0.4	1.4	0	0	33.3	0	0			
Tumor+ CD19RCD28+ NYESO-1 CAR	1	0.1	0	2.39	3.4	4.1	0.8	23.3	32.1	33.9			
	2	0	0	0	4.9	2.5	0.3	34.7	26.6	45.2			
	3	0	0	0	0.1	0	0	33.4	4.6	2.3			
	4	0	0	0	2.5	2	0.5	10.8	28.9	26.8			
	5	0	0	0.2	0.75	0	0	20.7	0	0			

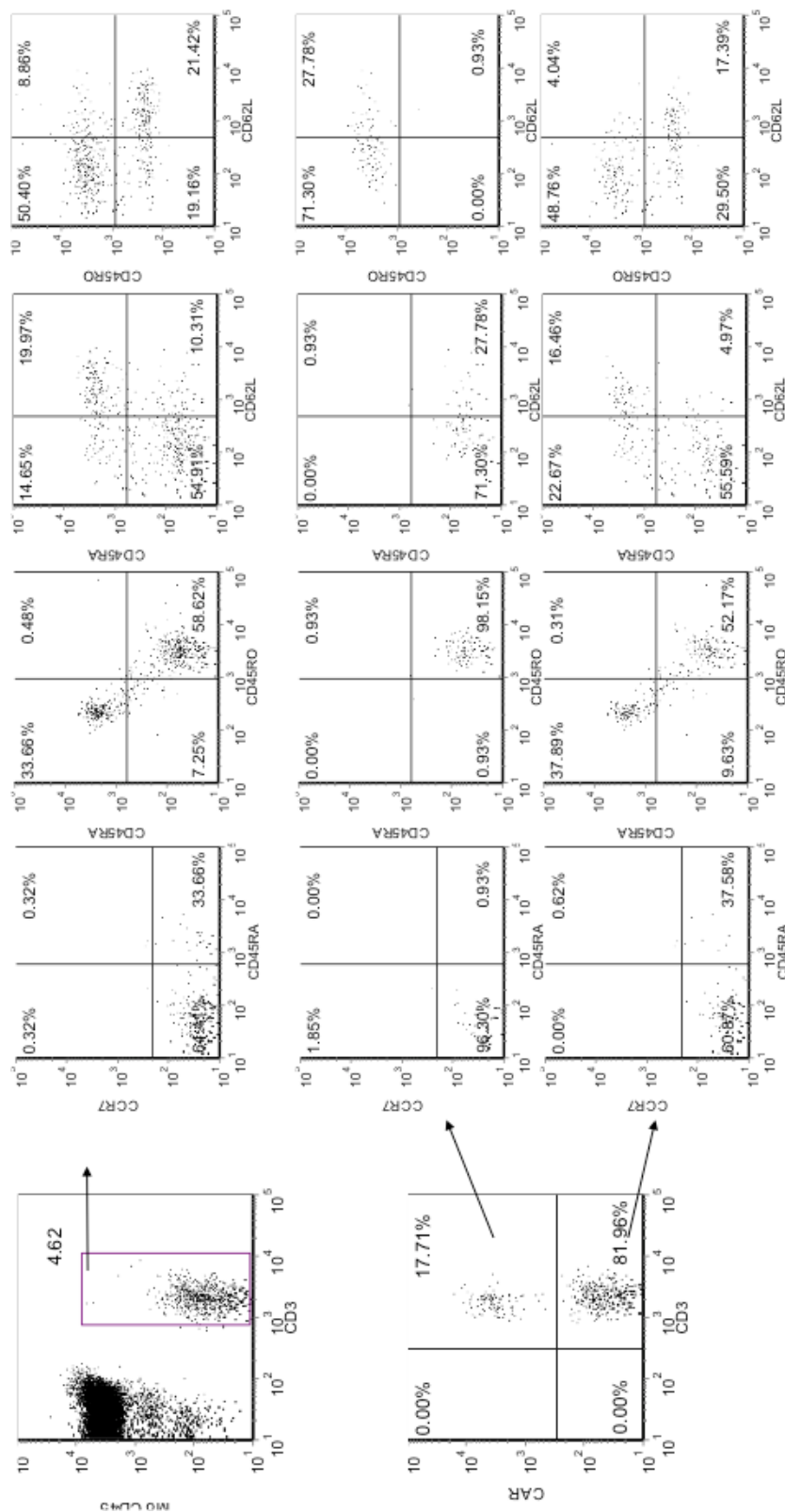


Figure 13. Immunophenotyping of CAR T Cells and T-APC in a mouse from the CAR+T-APC group. CD3+ T cells were seen only in mice with CAR+T-APC (shown) and T-APC only (data not shown). T-APC were mostly effector phenotype, however CAR T cells showed a range of cell types from naïve to memory to effector.

CHAPTER 4: Discussion

Despite the surge of FDA approved novel therapies for multiple myeloma patients in recent years, the disease is still considered largely incurable. However, with more patients reaching a minimal residual type state, introducing immunotherapy at this point (i.e. post autologous stem cell transplant) may be our best hope to lead to cure. Cell therapy has shown great potential in CD19+ diseases such as acute lymphoblastic leukemia (ALL) and diffuse large B cell lymphomas (DLBCL), but studies in myeloma patients are very early. Longer persistence of these cells seems to be associated with improved outcomes. Therefore, using a vaccine such as T-APC which is easy to make and manipulate, inexpensive, and easy to give, in conjunction with CAR T cells, may lead to improved persistence and therefore outcomes.

Because NY-ESO-1 is an intracellular antigen which needs to be processed and presented in an MHC restricted manner, we were limited to testing our hypothesis in HLA-A2⁺ donors. The T-APC were easily produced with >90% Flag and IL-15 expression with most donors; however, two major issues still remain: (1) we have not been able to show specificity of the T-APC and (2) we were unable to add any other co-stimulatory molecules besides IL-15, to help improve function. We also were not able to show an increase in CD80 or CD86 co-stimulatory molecules with the activation of the T-APC as our previous study had shown.

Our effector T cell studies showed that the T-APC with IL-15 were able to produce NY-ESO-1 specific T cells from un-manipulated PBMC while NY-ESO-1

K562 aAPC were not able to do so. However, the T-APC could not expand these effector cells or the NY-ESO-1⁺ TCR cells numerically. The NY-ESO-1⁺ CAR T cells (TCR mimetic) expanded in an antigen specific manner with both the T-APC and K562 aAPC. Furthermore, the NY-ESO-1⁺ CAR T cells killed the greatest percentage of the U266 myeloma cell line *in vitro*. Thus, the CAR T cell and T-APC combination was taken forward.

In our MRD *in vivo* model, we demonstrated that the mice which received the NY-ESO-1 CAR T cells (tumor + NY-ESO-1⁺ CAR T cells, tumor + NY-ESO-1⁺ CAR T cells + NY-ESP-1 T-APC, and NY-ESO-1⁺ CAR T cells + CD19R⁺ CAR T cells) had the lowest tumor burden when all the mice were sacrificed between days 43-45. The T cells persisted only in the two groups which received T-APC and in the group which received the NY-ESO-1 CAR and CD19R CAR. The group which received NY-ESO-1⁺ CAR T cells and T-APC had the most effector T cells present at time of sacrifice with the lowest tumor burden on necropsy. The CAR T cells had the greatest distribution of subtypes as well, from naïve to memory to effector T cells.

As seen in our *in vivo* studies, we would predict that giving T-APC and CAR T cells to myeloma patients would lead to persistence of effector cells of memory and effector subtypes along with decreased tumor burden than giving CAR T cells alone. We hope that this will translate into longer progression free survival and overall survival for our patients.

CHAPTER 5: Limitations

This study has a novel approach for improving CAR T cell persistence and potentially providing an alternate vehicle for anti-cancer vaccines. More studies need to be conducted, however, to prove this.

The biggest limitation to this study is lack of true specificity of the T-APC. The NY-ESO-1 antigen unfortunately does not have a commercially available antibody which we could use to detect its expression on the T-APC. We therefore purchased a soluble NY-ESO-1+ CAR (TCR mimetic) attached to a known flow color. Unfortunately we only tested this on one donor T-APC which was only 79% flag positive at that time. The soluble CAR seems to work with negative control showing background expression of 3.8% and positive control (U266 myeloma cell line) showing 57% expression.

Further limitations include the MHC restricted status of this antigen. Further work is being done in our lab to make “off the shelf” cell therapy products for this antigen as well as other cancer testis antigens.

IL-15 has the potential to be leukemogenic. Therefore switch technology is being developed to be able to turn IL-15 on T-APC off if need be.

CHAPTER 6: Future directions

We are currently completing *in vivo* mouse survival studies to further investigate the clinical translational potential of NY-ESO-1⁺ CAR and T-APC versus NY-ESO-1⁺ CAR alone.

We will also thaw our previous 4 T-APC products from different donors and repeat flow evaluation with the NY-ESO-1⁺ soluble CAR to test specificity. We will also re-evaluate the expression of other co-stimulatory molecules such as CD80 and CD86 on all these donor T-APCs with new antibodies.

We will attempt to increase immunogenicity of the T-APC with the addition of CD40L +/- IL-12 with new plasmids.

Finally we hope to conduct a phase I/II clinical trial using NY-ESO-1⁺ TCR mimetic with T-APC in patients with multiple myeloma.

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Vita

Krina K. Patel is the daughter of Kiritkumar Patel (NASA engineer born and raised in Gujarat, India) and Indira Patel (accountant born and raised in Mombasa, Kenya) and was born and raised in Friendswood, Texas. She graduated from Clear Brook high school in 1999 and attended college at the University of Pennsylvania (UPenn) in Philadelphia, PA where she received a double major in Biological Basis of Behavior and South Asian Regional Studies. Her 3 years of conducting research in a surgical oncology lab under Drs. Douglas Fraker and Chandrakala Menon at UPenn convinced her to obtain a medical degree to help in the fight against cancer. She received her MD from the University of Texas Southwestern Medical school in Dallas, Texas and completed Internal Medicine residency at the University of Texas Houston Medical School where she stayed for an extra year to serve as Assistant Chief of Service. She then received her Hematology/Oncology fellowship training at MD Anderson Cancer Center where she served as Chief Fellow during her second and third years of training. She joined on as assistant professor in the department of Stem Cell Transplantation and Cellular Therapy in 2014 where her research focus is improving outcomes of patients with multiple myeloma and other hematological malignancies with stem cell transplantation and novel cell therapies. She joined the Graduate School of Biomedical Sciences in 2012.