TCR Engineering of Natural Killer Cells for Melanoma Treatment

A thesis submitted in partial fulfillment of the requirements

For the degree of Master of Science in Biology

By

Jun Yan

May 2016
The thesis of Jun Yan is approved:

Dr. Zoran Galic

Date

Dr. Jerome Zack

Date

Dr. Randy Cohen

Date

Dr. Cindy Malone, Chair

Date

California State University, Northridge
ACKNOWLEDGMENTS

I would first like to thank Dr. Galic at University of California Los Angeles for the continuous support of my Master thesis research. From the first day I met Dr. Galic, the door to his office was always open whenever I ran into a problem or had a question about my research. He consistently allowed this paper to be my own work, but steered me in the right the direction whenever he thought I needed it.

I would also like to thank my thesis advisor Dr. Malone at California State University for the continuous support of my Master study and research, for directing CIRM Training Program. Without CIRM Training Program, I would not have the great opportunities I had.

Besides my thesis advisor, I would like to thank the rest of my thesis committee Dr. Zack and Dr. Cohen for the assistance they provided at all levels of the research project. I would also like to thank CIRM Training Program and CSUN Thesis/Dissertation/Project/Performance Support Program for supporting my thesis research.

Finally, I must express my very profound gratitude to my family members and to my boyfriend for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them. Thank you.
# TABLE OF CONTENTS

Signature Page .............................................................................................................. ii

Acknowledgement .......................................................................................................... iii

List of Figures ................................................................................................................ vi

Abstract ........................................................................................................................ viii

Chapter 1: Introduction ................................................................................................. 1

  Natural Killer Cells ...................................................................................................... 1
  T Cell Receptor and CD3 ............................................................................................ 4
  Human Embryonic Stem Cells .................................................................................... 5
  Cancer and Melanoma ............................................................................................... 7
  My Project .................................................................................................................. 9

Chapter 2: Material and Methods .................................................................................. 12

  Plasmid Construction ................................................................................................. 12
  Transformation and Infection ...................................................................................... 14
  Derivation of NK Cells from hESCs ........................................................................... 17
  Phenotypic Analysis ..................................................................................................... 18

Chapter 3: Results .......................................................................................................... 19
LIST OF FIGURES

Figure 1.........................................................................................................................10
Phenotypic analysis of NK cells derived from H1 hESCs derived NK cells.

Figure 2.........................................................................................................................10
Testing cytotoxicity of hESCs derived NK cells by killing assay

Figure 3.........................................................................................................................12
Configuration of pCD3- γ/ζ-Puro, pCD3-δ/ε-Neo and F5/Mart-1 TCR-EGFP
lentiviral vectors.

Figure 4.......................................................................................................................16
Schematic representation of the vectors and the protocol used to generate
transgenic hESC.

Figure 5.........................................................................................................................20
Restriction enzyme digests of the CD3 and Mart-1 TCR expressing plasmids

Figure 6.........................................................................................................................21
Testing the CD3 and Mart-1 TCR plasmids expression in 293FT cells

Figure 7.........................................................................................................................22
Phenotypic analysis of genetically modified H1 hESCs

Figure 8.........................................................................................................................24
Phenotypic analysis of genetically modified CD34+ cells
Phenotypic analysis of hESC derived NK-like cells CD3/TCR #1
ABSTRACT

Title:

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Melanoma is the most dangerous form of skin cancer because it is resistant to most common cancer treatments, such as chemotherapy and radiation. In 2014, a novel immunotherapy based on the blockage of the PD-1/PDL-1 signaling pathway was approved for malignant melanoma. However, this treatment is only effective with patients with high-level expression of PD-1/PDL-1. Therefore, developing an alternative melanoma treatment is an urgent mission.

The goal of this proof of principle study was to augment the ability of natural killer (NK) cells to effectively target and kill melanoma cells by engineering them to express a T cell receptor (TCR) specific for melanoma antigen Mart-1. TCRs are not normally expressed on NK cells, although the protein components necessary for TCR signaling exist in these cells. By using this approach, the NK and T cell anti-melanoma properties would be consolidated within one cell type. Furthermore, as melanoma cells generally use different strategies to suppress T and NK cells, the TCR expressing NK cells could compensate for the lack of T cell activity when these cells are absent or
immunosuppressed. In this study, human embryonic stem cells (hESC) were used as the source of genetically modified NKs, as they are relatively easy to genetically manipulate, expand to clinically relevant quantities, and to differentiate into NK cells that are phenotypically and functionally indistinguishable from their blood derived counterparts. TCRs require the CD3 complex of proteins for transport to the cell surface and proper signaling. To provide all the elements needed to support normal TCR function, the coding sequences for the CD3 γ, ζ, δ and ε chains, along with the Mart-1-specific TCR genes, were introduced into H1 hESC. Individual transgenic hESC lines were selected, the expressions of both Mart-1 TCR and CD3 on their surfaces were confirmed, and then they were differentiated into TCR/CD3 NK cells. In the future, Mart-1-specific TCR and CD3 expressing cells will be used in in vitro and in vivo experiments to establish that the presence of the Mart-1 TCR should endow these transgenic NKs with a higher anti-melanoma cytotoxic potential. Importantly, this technology could be adapted and expanded for treatment of other tumors and malignancies.
CHAPTER 1: BACKGROUND AND INTRODUCTION

Natural Killer Cells

Natural Killer (NK) cells were named based on their “natural killing” ability without any prior stimulation by an antigen. Phenotypically, NK cells are characterized by the presence of CD56, and the lack of other T cell or B cell defining markers. Functionally, NK cells are innate lymphoid cells that are important for host immunity defense without relying on antibody-mediated responses (Trinchieri, 1989). NK cells are known to combat the early stages of viral infection and tumor formation by chemically breaking the infected or cancerous cell’s plasma membrane, inducing apoptosis.

NK cells do not randomly kill cells. The immune activities of NK cells are well regulated by the activating and inhibitory receptors on their surfaces. Major activating receptors include NKp46, CD16, NKp30 and CD84, while major inhibitory receptors include KIR-L, LILRB1, CD94 and MHC-I (Vivier, 2011). The overall signaling balance between activating and inhibitory receptors is the key to understanding NK cell function and self-tolerance. Soluble ligands, such as cytokines, chemokines, and cell surface molecules interact and stimulate specific NK cell activating and inhibitory receptors. When cells are healthy, more inhibitory signals are expressed on their surfaces, and NK cell activity is suppressed. For example, healthy cells induce strong inhibitory signals by expressing MHC class I complexes with self-antigens, resulting in NK activity being suppressed, and ignoring these healthy cells. In contrast, many activating ligands are overexpressed in stressed cells, such as tumor and virally infected cells, and NK cells then induce apoptosis of these cells expressing high levels of activating ligands.
T cells and NK cells evolved different mechanisms to detect and kill tumor cells. T cells mainly detect tumorigenic cells by interacting with MHC class I and antigen complexes on tumor cell surfaces. When tumor specific antigen is presented on the cell surface, T cells are activated through interaction between TCR and MHC class I and antigen complexes. On the other hand, NK cells detect abnormally low level of MHC class I (“missing self”), as is in tumor and virally infected cells.

So, why does cancer still exist? Tumor cells have evolved many mechanisms to escape immune detection. Tumor cells suppress T cell activities by decreasing the expression of MHC class I. Therefore, it is important for NK cells to be able to target those cells that are missing MHC class I. However, tumorigenic cells also develop different mechanisms to deactivate NK cells such as expression of immunosuppressive factors and expression of MHC class I surrogates. TGFβ is a prominent immunosuppressive factor that controls cell cycle. Cancer use TGFβ to control and suppress NK cells activities and proliferation (Massagué, 2008). MHC class I surrogates serve as inhibitory signal for NK cells to avoid NK mediated killing but it cannot be recognized by T cells because it is structurally different from MHC class I (Lisnić, 2010).

In many cases, tumorigenic cells have been shown to deactivate the cancer patient’s antitumor responses by deactivating both T cells and NK cells. Immunotherapy, if successful, by reactivating the patients’ T cells and NK cells has gained a lot of attention recently. Unfortunately, researchers have realized T cell mediated immunotherapy has severe side effects and technical difficulties such as toxicity (June, 2007) and TCR specify (Restifo, 2012). The first challenge is toxicity associated with reactivating inactivated T cells using cytokines (i.e., IL-2). High level of IL-2 can cause
serious and dangerous side effects such as extreme fatigue, low blood pressure and kidney damage. It was also associated with mortality rates of up to 4%. (Schwartz, 2002)

The first T cell medicated immunotherapy clinical trial was conducted using IL-2 *in vivo* to directly activate patient's T cells in 1984 to treat a variety of malignancies (Lotze, 1984). IL-2 mediated high toxicity was a big problem in this and many other subsequent studies (Lotze, 1985; Rosenberg, 1985; SADLACK, 1993). The second challenge is having more than one TCR, a receptor T cells use to recognize specific tumorigenic cells. Each T cell naturally expresses one TCR. Having an additional TCR on a T cell can influence the overall effectiveness of TCRs. By having multiple TCRs on a single T cell, a part of endogenous TCR and a part of transduced TCR can form a hybrid TCR with unknown reactivity (Yin, 2009).

Due to the difficulties associated with T cell mediated immunotherapy, researchers have realized the emerging potential of NK cell based immunotherapy (Miller, 2002) as NK cells also have ability to target and kill cancer cells. Most cancer cells are characterized by low MHC class I expression and high expression level of stress induced surface molecules. NK cells can recognize and kill tumorigenic cells that have these characteristics. Therefore, by strengthening and enhancing NK cell activity, new solutions for cancer treatments may emerge. Different approaches have been taken to strengthen NK cell activities such as expansion of endogenous NK cells, use of stable allogeneic NK cell lines, and use of chimeric antigen receptors (CAR) (Glienke, 2015) and Fc receptors (Min, 2013). Expanding endogenous NK cells to increase anti-tumor activity and treat metastatic melanoma only yielded few positive results to a small fraction of treated patients (Atkins, 1999). Another approach was *ex vivo* activation and
expansion of autologous NK cells. Only 8-20% of NK cells were activated after the adaptive transfer into patients in combination with cytokine IL-2 that promotes NK cell proliferation. However, these clinical trials failed due to poor outcome and similar severe IL-2 related side effects as mentioned above. Because many types of immune cells were activated (i.e., such as T cells, B cells, and macrophages), NK cell activities could have been suppressed. If NK cells can be activated with good tumor cell recognition abilities in cancer patients, there should be better success seen with NK cell immunotherapy.

**T Cell Receptor (TCR) and CDR3**

TCR was first discovered in the early 1980s (Allison, 1982). TCR is a receptor expressed on the surface of T cells that binds and recognizes specific MHC/antigen complex, and most commonly consist of an α subunit and β chain. In healthy cells, MHC presents various self-antigens, which are small parts of degraded proteins synthesized in the cell. When a tumor antigen or any other non-self antigen is present in a cell, MHC binds to the antigen and expresses the MHC/antigen complex on the cell surface. After TCR binds to the MHC/antigen complex, signals are transmitted through CD3 to the downstream pathways. When TCR binds to a foreign antigen, multiple phosphokinase signaling pathways, including Lck and Zap70, lead to T cell activation. Activated T cells proliferate and differentiate into either memory T cells or cytotoxic T cells. Therefore, the TCR is the key receptor for the T cell mediated fight against tumor cells.

Adoptive T cell therapy is a relatively well-established cell mediated immunotherapy that stimulates patient’s T cells to eliminate cancer by genetically engineering TCRs to increase recognition of cancer cells. In the past decade, significant
efforts were made toward engineering TCRs to improve the recognition of antigens to optimize TCR function and sensitivity to improve the efficiency of T cell mediated immunotherapy. Using this method, modified TCRs are reintroduced into autologous T cells and transferred back into the cancer patient. It is one of the more promising immunotherapies with many ongoing clinical trials (Rosenberg, 2015).

There are many studies using genetically modified TCRs on T cells and cells other than T cells (Hall, 1991). However, there has been no research performed using engineered TCR on NK cells. Theoretically, NK cells should be an ideal cell type to engineer TCR because the signaling pathways such as ZAP-70, LAT and Lck phosphorylation already exist in NK cells. In addition, the major technical problem associated with the difficulties of reactivity of endogenous TCR and engineered TCR is nonexistent because there are no endogenous TCRs on NK cells. Multiple studies have shown that chimeric antigen receptors (CARs), which also use CD3 zeta chain as part signal transduction, can enhance NK cell activities (Topfer, 2015) which means TCR might also enhance NK cell activities.

**Human Embryonic Stem Cells**

Human Embryonic Stem Cells (hESCs) are pluripotent stem cells derived from the inner cell mass of an early stage pre-implantation human embryo (blastocyst). Pluripotency is defined by the ability to become any one of the three germ layers: ectoderm, endoderm, and mesoderm. Pluripotency is a unique characteristic found in embryonic stem cells. Martin described mouse embryonic stem cells in 1981 (Martin, 1981), and Thomson established the techniques to isolate and grow hESCs *in vitro*
HESCs are significantly more difficult to culture compared to other cell lines. The hESC proliferation rate is slower, and they are extremely sensitive to stress. In addition, when hESCs were not cultured properly, hESCs were shown to quickly acquire mutations and lose their pluripotency. Until the early 2000s, researchers mostly focused on understanding how to properly and efficiently culture hESCs in vitro. Early culture conditions required co-culture of hESCs with irradiated mouse embryonic fibroblasts (MEF) to support growth. One of the first feeder-free culturing methods was the demonstration of long-term culture of hESCs on tissue culture plates coated with Matrigel in 2001 (Xu, 2001). Researchers have used hESCs as a research tool for almost 20 years as hESCs have become an excellent tool for studying human developmental biology, drug discovery, and transplantation medicine.

HESCs could potentially serve as a great source for cell-based therapies as well. In recent years, stem cells have gained attention as an alternative to tissue transplantation. There are many benefits to use cell-based therapies instead of transplantation, such as eliminating the problems of immune rejection by using immune suppression. Currently, there are a limited number of cell-based therapies available, and these therapies are usually restricted in scope as well. One of the biggest problems for cell based immunotherapy is that the limited resource for non-immunogenic cells which are hard to get from patients, given that most of them have either low immune cell number or their immune cells have become non-functional. HESC may be a potential source of immune cells for treatment of such patients. The FDA approved the first clinical trial using oligodendrocytes derived from hESCs in 2009. Now, there are many more phase I and II
clinical studies using different types of hESC derived cells (Trounson, 2015; Schwartz, 2015; Menasche, 2015).

Over the years, researchers have established protocols to derive many cell types from hESCs, including NK cells (Tabar, 2014). In 2005, the first hESC derived NK cells were produced, expanded and functionally tested by Dr. Kaufman's group (Woll, 2005; Kaufman, 2009; Knorr, 2013). One difficulty in developing these protocols is due to limited understanding on how to mimic in vivo environment in a dish using different combinations of cytokines, chemokines, and various techniques. Any small change may alter the functional properties and quantity of the final target cells, causing the hESCs to differentiate into completely different cell types.

**Cancer and Melanoma**

Cancer cells are genetically altered cells. In healthy humans, there is a well-regulated balance between cell renewal and cell death. Each cell type has its own life span and new cells are produced only when needed. In addition, the cell cycle is normally regulated which means the total cell number is normally maintained. However, there are cells that escape from normal cell cycle control mechanisms and develop the ability to divide indefinitely, producing tumors (Fleit, 2001).

The induction of cancer is normally a multi-step process. A single cell must go through multiple somatic mutations to progressively convert to a cancer cell. These common somatic mutations include loss of tumor suppressor genes and activation of oncogenes that lead to uncontrolled cell growth (Farber, 1984).
Mutations may also include loss and gain of other functions to escape from T cell and NK cell induced killing (Talmadge, 1980). Altered MHC expression is not the only way cancer cells evade the immune system. Tumor cells can also enhance cell growth by antitumor antibodies and antigenic modulation (Fleit, 2001). Antitumor antibodies are produced against tumor specific antigens and block cell mediated lympholysis. Antigenic modulation is common for leukemic T cells, where tumor specific antigens disappear with the presence of antibodies, and tumor specific antigens are displayed on the cell surface when antibodies are not present (Shawler, 1984).

Metastatic melanoma is a type of skin cancer, formed from melanocytes. Though it is a less common form of skin cancer (less than 5%), the mortality rate can be relatively high if not diagnosed early. Both the geographic and ethnical patterns clearly show that the major causes are from UV radiation and genetics (Torre, 2015). Unfortunately, there are only a limited number of treatment options currently available (Jerant, 2000). Chemotherapy is only effective in about 25% of melanoma patients (Eton, 2002). Thus, surgery remains to the best treatment in majority of cases of metastatic melanoma. Other therapies such as the BRAF and MEK inhibitors, IL-2, IFNα2b and cytotoxic chemotherapy have severe side effects and lack effectiveness. Recently, blockage of the PD-1/PDL-1 pathway showed huge promise in clinical studies, but was only shown to be effective in 38% of patients who have high PD-1/PDL-1 expression (Hamid, 2013). Therefore, developing an effective treatment is still an urgent clinical mission.
My Project

As mentioned above, NK cells are one of the few lymphoid cells that were derived from hESCs (Kaufman, 2009). The Galic and Zack labs have successfully cultured NK cells from hESCs with and without gene modification. These hESC derived NK cells were confirmed with both phenotypic characterizations and functionalities. FACS analysis indicates that 61% of hESC derived NK cells expressed both key NK cell markers, including CD45 and CD56 as shown in Figure 1. CD45 and CD56 double positive population also tested positive for other NK cell surface markers, including CD314, CD335, CD336, and CD158b (Figure 1). Using a cell killing assay which evaluated NK cell's ability to target and kill cancer cells, hESC derived NK cells were shown to kill target cells in a dose dependent manner (Figure 2), confirming that these cells are phenotypically and functionally similar to peripheral blood human NK cells.
Figure 1: Phenotypic analysis of hESCs derived NK cells.
FACS analysis was performed on differentiated NK cells derived from hESCs for expression CD45 (Y axis) and CD56 (X axis). The results show that approximately 61% of cells are CD56 and CD45 double positive, confirming that they are NK cell like cells. CD45 and CD56 double positive cells were also tested for NK cell surface markers CD314 (has the ability to co-stimulate multiple NK activation receptors) (middle panel X axis), CD335 (directly involved in target cell recognition and lysis, and is exclusively expressed on CD3-CD56+ NK cells) (middle panel Y axis), CD336 (natural cytotoxicity receptor)(right panel Y axis), and CD158b (right panel X axis)(provides an inhibitory signal on NK cell lytic activity upon interaction with HLAC in an antigen-independent manner).

Figure 2: Testing cytotoxicity of hESCs derived NK cells by killing assay
CD56+ cells derived from hESC were purified and co-cultured with CellTrace Violet labeled target K562 cells at the designated effector to target (E:T) ratios for 4 hours. Cells were then FACS analyzed for cleaved caspase 3 expression which identifies cells actively undergoing apoptosis. Bar graphs show the means of 3 independent experiments with standard deviation for cleaved caspase 3 at different E:T ratios. Statistical analyses performed using One way ANOVA with Bonferroni’s multiple comparison post test. * p<0.05, *** p<0.0001
The goal of this proof of principle study was to evaluate whether I can augment the ability of hESC derived NK cells to recognize and kill melanoma cells by engineering them to express a TCR specific for melanoma antigen Mart-1. To accomplish the goal, three plasmids were cloned to introduce Mart-1-specific TCR and CD3 subunits γ, δ, ε and ζ chains. Multiple plasmids were introduced due to the large number and size of the genes. The TCR used in this experiment is specific for melanoma antigen Mart-1 peptide (aa26-37) presented in the context of HLA-A0201, which was isolated by Dr. Rosenberg’s group. This TCR has been characterized and tested for its function in clinical trials and many other models (Morgan, 2006). HESCs were used rather than peripheral blood derived NK cell lines as the latter is known to be highly resistant to genetic modification. It is technically impossible to introduce multiple genes using multiple plasmids into peripheral blood derived NK cells but much easier in hESC derived NK cells.

My project was the first step to ascertain whether engineered NK cells have a better ability to recognize and kill melanoma cells over non-modified NK cells. Overall, my project used a novel approach to show how hESC derived NK cells could be genetically modified and used in many clinical applications. This project aimed to promote people’s interest in NK cell based anticancer immune therapy, which could contribute to continuing efforts towards making cancer a completely curable disease.
CHAPTER 2: MATERIAL AND METHODS

Plasmid construction

Four CD3 subunits and Mart-1 TCR were cloned into three different plasmids (pCD3-γ/ζ-Puro, pCD3-δ/ε-Neo, F5/Mart-1 TCR-GFP) as shown in Figure 3. I used the F5/TCR specific for the melanoma antigen Mart-1 (peptide aa26-37) presented in the context of HLA-A0201 that was isolated (and kindly provided to Galic and Zack lab) by Dr. Rosenberg’s group at NCI. CD3 δ, ε, γ, and ζ subunits sequences were obtained from NCBI database. PCD3-γ/ζ-Puro plasmid included CD3 γ and ζ subunit DNA sequences, UbicC promoter and Puromycin resistance gene. PCD3-δ/ε-Neo plasmid included CD3 δ and ε subunit DNA sequences, UbicC promoter and Neomycin resistance gene. F5/Mart-1 TCR-GFP plasmid contains Mart-1-specific TCR DNA sequence, UbicC promoter and green fluorescent protein (GFP) DNA sequence.

![Figure 3: Configuration of pCD3-γ/ζ-Puro, pCD3-δ/ε-Neo and F5/Mart-1 TCR-EGFP lentiviral vectors.](image)

pCD3-γ/ζ-Puro vector contains Ubiquitin C promoter (UbiC), CD3 γ and ζ subunits, and puromycin resistant gene. pCD3-δ/ε-Neo vector contains UbiC, CD3 δ and ε subunits, and neomycin resistant gene. F5/Mart-1 TCR-EGFP vector contains UbiC, TCR α and β subunits and green florescent protein (GFP).
The plasmids (pUC57-CD3-δ-ε, pUC57-CD3-γ-ζ) containing CD3 subunits and corresponding restriction enzyme cleavage sites were manufactured by GenScript. The plasmid pUC57-CD3-δ-ε included a DNA fragment with cloning sequences for CD3-δ-ε flanked by restriction enzyme cleavage sites for AgeI-HF (New England BioLabs) and SbfI-HF (New England BioLabs) restriction enzymes. This fragment was cloned into corresponding restriction sites of the lentiviral vector plasmid F12-PUR, which was previously constructed in Zack laboratory. This vector contains UbiC promoter and the puromycin resistance gene. CD3-δ-ε and F12-Puro were both digested with AgeI and SbfI and gel purified. The CD3-δ-ε was then ligated with F12-PUR using T4 DNA ligase (Invitrogen) overnight at 4°C. The ligation product (pCD3-δ/ε-Neo) was transformed into competent cells using the heat shock method, and resulting individual bacterial colonies were amplified overnight in Luria broth (LB) with Ampicillin. The positive clones were confirmed using restriction enzyme digests and gel electrophoresis along with the DNA 2-log ladder (New England BioLabs). Large quantity of the plasmid was prepared using maxiprep plasmid purification kit (Qiagen). CD3-γ-ζ was ligated with F12-Neo, which contains UbiC promoter and the neomycin resistant gene, and then ligation product (pCD3-γ/ζ-Neo) isolated using the same methods and restriction digestion enzymes along with the DNA 2-log ladder. Large quantity of ligation product was produced using the same methods as well.

As mentioned above, Dr. Rosenberg’s Lab provided Mart-1-specific TCR and the plasmid that was slightly modified by adding green fluorescent protein (GFP) gene and removing the puromycin gene. The GFP fragment was produced using PCR amplification of the GFP fragment from F12-PURO plasmid. The forward primer was
TGCGATCGCCGCACCATGGTGAGCAAGGGCGAGGAGCTG, and the reverse primer was TGCTAGCTTTACTCTTGTACAGCTCGTCCATGC. A few base pairs were added to the end of each primer to create restriction enzyme cleavage sites for enzymes AsiSI and NheI. Both Mart-1-specific TCR and PCR amplified GFP fragments were digested with enzyme AsiSI (New England BioLabs) and NheI (New England BioLabs), and Mart-1-specific TCR fragment was isolated by gel extraction. TCR and GFP were ligated using T4 DNA ligase (Invitrogen) overnight at 4°C. Ligation was confirmed using both enzyme digestion along with a DNA 2-log ladder and DNA sequencing (Eurofins Scientific). The ligation product (F5/Mart-1 TCR-EGFP) was transformed into competent cells using the heat shock method and amplified overnight in LB with Ampicillin. Large quantity of Mart-1 TCR-GFP plasmid was prepared using maxiprep plasmid purification kit (Qiagen) for transfection.

Transformation and infection

Transformation of all three plasmids (pCD3-γ/ζ-Puro, pCD3-δ/ε-Neo, F5/Mart-1 TCR-EGFP) was performed using Lipofectamine 2000 (Invitrogen), Opti-MEM Medium (Thermo Fisher Scientific) and 293FT cells with 90-95% confluency. Negative control was 293FT cell with F5/Mart-1 TCR-EGFP. Transformation was performed following the manufacturer’s protocol. The cells were analyzed using Fluorescence-activated cell sorting (FACS) analysis after 48 hours with HLA-A*02:01 Mart-1 Tetramer-ELAGIGILTV-PE (Medical and Biological Laboratories) and anti-CD3 conjugated to Pe-cy7 (eBioscience).
Four CD3 subunits and Mart-1 TCR-GFP coding sequences were delivered into H1 hESC line using a replication defective lentivirus-based transduction method. Lentivirus was produced using 90-95% confluency 293FT cells, Lipofectamine 2000 (Invitrogen) and Opti-MEM Medium (Thermo Fisher Scientific). PCD3-δ/ε-Neo plasmid, VSVG plasmid, and delta8.2 plasmid were used to create the lentivirus for CD3 δ and ε. VSVG plasmid forms backbone envelope and the delta8.2 plasmid is for packaging of lentivirus formation. Lentivirus containing CD3 γ/ζ and Mart-1 TCR-GFP were created using the same method as the CD3 δ/ε plasmid virus. After 12 hours, media was changed to DMEM (Thermo Fisher Scientific) with 10% Fetal bovine serum (FBS) (Corning) and 1% Penicillin-Streptomycin (Thermo Fisher Scientific). Then, 48 hours after media change, each lentivirus was collected and concentrated via ultracentrifugation.

H1 hESC line was cultured in DMEM/F12 (Gibco) supplemented with 20% KnockOut Serum Replacement (Gibco), 1x Glutamax (Gibco), 1x MEM Non-Essential Amino Acids (NEAA) (Gibco), 100μM 2-Mercaptoethanol (βME), bFGF (Stemcell technologies), on mouse embryonic fibroblast (MEF) feeder cells. H1 hESCs colonies were digested with Collagenase IV (Gibco) to obtain smaller cell aggregates prior to infection, and incubated for two hours after infection with lentivirus. Infection was performed in a sequential order as shown in Figure 4. The first infection was done using CD3-δ/ε-Puro lentivirus, and puromycin dihydrochloride was added to the hESC media for 4 days to select CD3-δ/ε-Puro lentivirus infected hESC cells. Then, the second infection of Puromycin selected cells was performed the CD3- γ/ζ-Neo lentivirus, and Neomycin Sulfate was added to the hESC media for 2 weeks. To establish three individual cell lines, individual hESC clones were selected and expanded. Each cell line
was infected with F5/Mart-1 TCR-EGFP lentivirus, and GFP positive cells were sorted in the UCLA Broad Stem Cell Center Flow Cytometry Core. One clone from each cell line was then selected and expanded (CD3/TCR #1, CD3/TCR #3, CD3/TCR #5). Expression of Mart-1 TCR and CD3 were tested with HLA-A*02:01 Mart-1 Tetramer-ELAGIGILTV-PE (Medical and Biological Laboratories) and anti-CD3 conjugated to Pe-cy7 (eBioscience). After each infection and selection, aliquots of hESCs were cryopreserved for future studies.

Figure 4: Schematic representation of the vectors and the protocol used to generate transgenic hESC. Lentiviruses were used to sequentially transduce hESC and selected with puromycin, neomycin and GFP.
Derivation of NK cells from hESCs

I utilized Dr. Ni's published NK cells differentiation protocol that uses embryoid bodies (EBs) approach to promote differentiation of hESC into the three germ layers (Ni, 2013). HESC derived hematopoietic stem cells were selected using the cell surface maker CD34, and further differentiation was promoted by different cytokines to obtain mature NK cells with CD3 and Mart-1-specific TCR.

HESCs were maintained on irradiated MEF cells before making EBs. To initiate differentiation, hESC colonies were dislodged using 0.5mg/ml dispase (StemCell technologies) and allowed to fold into EBs. EBs were cultured in IMDM media (Gibco) supplemented with 15% FBS (Corning), 1x Glutamax (Gibco), 1x MEM NEAA (Gibco), 200µM βME, 1x Penicillin-Streptomycin (Gibco), 10ng/ml BMP4 Recombinant Human Protein (Gibco), 50ng/ml FLT3 Recombinant Human Protein (Gibco), 300ng/ml SCF Recombinant Human Protein (Gibco). EBs were cultured for 15 days to initiate spontaneous differentiation and promote mesoderm formation. After 15 days the EBs were harvested by treating with 0.5x trypsin (Gibco) with 2% chicken serum to generate a single cell suspension. The CD34 positive cells were purified using CD34 MicroBead kit (Miltenyi Biotech). The majority of CD34+ cells were precursors of different hematopoietic lineages. CD34+ cells were then co-cultured with mouse fetal liver stromal cells (AFT024) and differentiated to NK cells using NK media containing 56.6% DMEM (Gibco), 28.3% Ham/F12 (Gibco), 1x Glutamax (Gibco), 25uM 2-mercaptoethanol (Gibco), 5ng/ml sodium selenite, 50uM ethanolamine, 28.95ug/ml ascorbic acid, 1x penicillin-streptomycin (Gibco), 15% HI human AB (Gibco), 20ng/ml SCF Recombinant Human Protein (Gibco), 5ng/ml IL-3 Recombinant Human Protein (Gibco), 20ng/ml IL-7.
Recombinant Human Protein (Gibco), 10ng/ml IL-15 Recombinant Human Protein (Gibco) and 10ng/ml Flt-3 Recombinant Human Protein (Gibco) for 30-40 days. IL-3 was used only in the first week because IL-3 was known to promote early development of NK cells. All other cytokines were used throughout the 30-40 day NK cell differentiation.

**Phenotypic Analysis**

Expression of Mart-1-specific TCR and CD3 subunits was tested by FACS analysis on hESCs, CD34 positive hematopoietic stem cells, and mature NK cells. For adherent cells, single cells suspension was obtained by treating the cell cultures with StemPro Accutase Cell Dissociation Reagent (Gibco) for 5 to 10 minutes. Single cell suspension was stained with HLA-A*02:01 Mart-1 Tetramer-ELAGIGILTV-PE (Medical and Biological Laboratories) and anti-CD3 conjugated to Pe-cy7 (eBioscience). Cells were fixed with 1% formaldehyde before FACS analysis. Single color controls and corresponding negative control were also prepared using the same method.

Other cell surface antigens unique to different differentiation stages were also analyzed using FACS analysis. Expression of CD34 and CD45 was tested on CD34 positive hematopoietic stem cells with anti-CD34 conjugated to PE-Cy7 (eBioscience) and anti-CD45 conjugated to BV421 (eBioscience). CD45, CD56, CD158b, CD335 were used to evaluate the maturity of hESC derived NK-like cells. Human ESC-derived NK-like cells without Mart-1-specific TCR were used as the negative control. Single color controls were prepared using the same method.
CHAPTER 3: RESULTS

Three plasmids (pCD3-γ/ζ-Puro, pCD3-δ/ε-Neo, F5/Mart-1 TCR-EGFP) were cloned and confirmed using restriction digestions and gel electrophoresis as shown in Figure 5. For pCD3-γ/ζ-Puro plasmid, restriction enzyme AgeI-HF and SbfI-HF were used to perform restriction digestion and a 1130bp band for CD3 γ and ζ genes and a 10kb band for rest of the vector inserts along with the DNA 2-log ladder were observed as shown in the left side of Figure 5. For pCD3-δ/ε-Neo plasmid, restriction enzyme AgeI-HF and SbfI-HF were used to perform restriction digestion and a 1230bp band for CD3 δ and ε genes and a 10kb band for rest of the vector inserts along with the DNA 2-log ladder were observed as shown in the middle of Figure 5. GFP gene fragments were PCR amplified and sequenced (data not shown). After constriction of the F5/Mart-1 TCR-EGFP plasmid, AsisI and NheI were used to perform a restriction digest and a 730bp band for the GFP gene and a 11kb band for the Mart-1-specific TCR and other vector inserts, along with the DNA 2-log ladder, were observed as shown in the right side of Figure 5.
Figure 5: Restriction enzyme digests of the CD3 and Mart-1 TCR expressing plasmids
Restriction enzyme digests of the CD3 and Mart-1 TCR expressing plasmids used in this project (pCD3-γ/ζ-Puro, pCD3-δ/ε-Neo, F5/Mart-1 TCR-EGFP). To verify the presence of the genes for CD3 γ/ζ, δ/ε and GFP, each plasmid was digested with appropriate restriction enzymes. PCD3-γ/ζ-Puro plasmid and pCD3-δ/ε-Neo plasmid were digested with AgeI-HF and SbfI-HF. F5/Mart-1 TCR-EGFP plasmid was digested with AsisI and NheI. DNA fragments were separated using gel electrophoresis and visualized using EtBr. Size of each DNA fragment was determined based on DNA 2-log ladder (0.1-10.0 kb).

Three plasmids (pCD3-γ/ζ-Puro, pCD3-δ/ε-Neo, F5/Mart-1 TCR-EGFP) were also transfected into 293FT cells, and the cell surface expression of the CD3 and Mart-1-specific TCR were detected using FACS analysis as shown in Figure 6. 293FT cells with the TCR-GFP plasmid was used as the negative control since Mart-1-specific TCR cannot be expressed without CD3. A Mart-1 tetramer is an MHC class I tetramer, which is composed of a complex of four HLA MHC class I molecules each bound to Mart-1 antigen. A Mart-1 tetramer was used to detect the expression of Mart-1-specific TCR.
The results indicated that there was neither CD3 nor Mart-1-specific TCR expression on 293FT cells transfected with only F5/Mart-1 TCR-EGFP plasmid, and 95.8% co-expression of CD3 and Mart-1-specific TCR when the cells were co-transfected with all of the plasmids. The linear relationship between expression of Mart-1-specific TCR on the X-axis and CD3 antigen expression on the Y-axis indicated an expression ratio of 1:1.

![Figure 6: Testing the CD3 and Mart-1 TCR plasmids expression in 293FT cells](image)

A plasmid carrying the genes for F5/Mart-1 TCR was introduced into 293FT cells either by itself (left panel) or in combination with plasmids that carry the coding sequences for the CD3 γ, δ, ε and ζ chains (right panel) by transfection of 293FT cells. FACS analysis was performed on both transfected samples to determine the expression of Mart-1-specific TCR (X-axis) and CD3 (Y-axis) on 293FT cell surface. The results show that 95.8% coexpression of the Mart-1 TCR and CD3 was observed only in the presence of the CD3 complex, while F5/Mart-1 TCR-EGFP plasmid transfected cells indicated <1% CD3 and Mart-1 TCR double positive cells, confirming the functionality of CD3 proteins in the TCR trafficking process.

Three lentivirus preparations (CD3-δ-ε, CD3-γ-ζ, Mart-1 TCR-GFP) were performed using these plasmids, and H1 hESCs were sequentially infected using these
lentiviruses as shown in Figure 4. After hESCs were infected with all three lentiviruses, three CD3/TCR cell lines (CD3/TCR #1, CD3/TCR #3, CD3/TCR #5) and three corresponding CD3 control lines (CD3 #1, CD3 #3, CD3 #5) were individually established. For each line, expression of CD3 and Mart-1-specific TCR were confirmed using FACS analysis (Figure 7). Based on the results, more than 98% co-expression of both CD3 and Mart-1-specific TCR were observed in all three lines. Even though there was a slight variation in expression levels, there were very few CD3 and Mart-1-specific TCR double negative cells in any cell line. In addition, FACS analysis was performed 3 months and 6 months after infection, and continuous and stable expression of CD3 and Mart-1-specific TCR was confirmed (results not shown).

![Figure 7: Phenotypic analysis of genetically modified H1 hESCs](image)

HESCs were infected with lentiviral vectors produced from plasmids pCD3-Υ/ζ-Puro, pCD3-δ/ε-Neo and F5/Mart-1 TCR-EGFP. Three different lines, CD3/TCR #1, CD3/TCR #3 and CD3/TCR #5 were generated. FACS analysis was performed on each genetically modified H1 hESC line for expression Mart-1 TCR (X-axis) and CD3 (Y-axis). The results show that 98.3% co-expression of Mart-1-specific TCR and CD3 on CD3/TCR #1 (left panel), 98.9% co-expression of Mart-1-specific TCR and CD3 on CD3/TCR #3 (middle panel) and 98.8% co-expression of Mart-1-specific TCR and CD3 on CD3/TCR #5(right panel).

From each genetically modified hESC line, hematopoietic stem cells were derived using the previously mentioned method, and the CD34 expressing cells were selected.
FACS analysis was performed to analyze the purity of selected CD34 positive cell as shown in Figure 8. The CD34 and CD45 double positive cells are considered true hematopoietic stem/progenitor cells (HSPCs), and CD34 positive CD45 negative cells are mostly likely endothelial cells because CD34 antigen is also a marker for these cells (Asahara, 1997). The top half of Figure 8 shows HSPCs produced from both hESCs CD3/TCR #1 and #3 lines. There were 8.2% HSPCs in the control (CD3 #1) and 11.6% HSPCs in CD3/TCR #1. CD3/TCR #3 showed similar results but was produced at a different time point. There were 6.2% HSPCs in the control (CD3 #3) and 9.5% HSPCs in CD3/TCR #3. In the bottom half of Figure 8, expression of CD3 and Mart-1-specific TCR for both CD3/TCR #1 and #3 were confirmed using FACS analysis. In addition, no CD3 or Mart-1-specific TCR expression was detected on corresponding control lines.
After EB mediated differentiation, CD34 positive cells were selected using CD34 antibody and magnetic beads. Using FACS analysis, isolated CD34 positive cells were analyzed for CD34 (top panel, Y-axis), CD45 (top panel, X-axis), Mart-1 TCR (bottom panel, X-axis), and CD3 (bottom panel, Y-axis) before co-culturing with AFT024. Upper half of the figure shows that CD3#1 control sample had 9.7% CD34+/CD45+ double positive where CD3/TCR #1 sample had 13.8% CD34+/CD45+ double positive, and CD3#3 control sample had 6.2% CD34+/CD45+ double positive where CD3/TCR #3 sample had 9.5% CD34+/CD45+ double positive. CD34+/CD45+ double positive cells are hematopoietic stem/progenitor cells (HSPCs). Even though not all CD34 positive cells were HSPCs, we did have significant concentration of HSPCs in our population in CD3#1 control, CD3/TCR#1, CD3#3 control, and CD3/TCR#3. The bottom half of the figure shows that both CD3/TCR#1 and CD3/TCR#3 had the most co-expression of Mart-1 TCR and CD3, where their controls had neither expressed on the cell surface.

The HSPC mixtures were cultivated and differentiated to mature NK cells. HESC derived NK-like cells were collected and analyzed using FACS analysis. The expression levels of CD45 and CD56 were used to evaluate the characteristics of hESC derived NK-like cells. Subset of cells that had both CD45 and CD56 receptors were most likely NK cells. Cells with both CD45 and CD56 receptors were further tested for CD3 and Mart-1-specific TCR expression. Almost all cells from CD3 #1 had no CD3 or Mart-1-specific
TCR cell surface expression, and 87.4% of cells from TCR/CD3 #1 had expression of both CD3 and Mart-1 TCR as shown in Figure 9.

**Figure 9: Phenotypic analysis of hESC derived NK-like cells CD3/TCR #1**

Both CD34+ TCR/CD3 #1 and CD3 #1 cells were cultured on AFT024. After 33 days of differentiation on AFT024, FACS analysis was performed for expression CD45 (top panel X-axis) and CD56 (top panel Y-axis). The results show that 27.4% (CD3/TCR #1) and 30.9% (CD3 #1) are CD56 and CD45 double positive. CD56 and CD45 double positive indicated mature NK cells. CD45+/CD56+ NK cells were further analyzed for CD3 (bottom Y-axis) and Mart-1 TCR (bottom X-axis) expression. TCR/CD3 #1 indicated 87.4% CD3 and Mart-1 TCR double positive, while CD3 #1 indicated <1% CD3 and Mart-1 TCR double positive cells.
CHAPTER 4: DISCUSSION AND SIGNIFICANCE

The Goal of This Project

The ultimate goal for this study is to establish a proof of principle that human NK cells engineered to express a melanoma specific TCR can be used to treat metastatic melanoma skin cancer in animal models of this disease. Metastatic melanoma accounts for the majority of deaths due to skin cancer, despite the fact that it is a less common form of skin cancer. One of the main reasons is the lack of effective treatment options. Therefore, it is critical to develop and establish new treatments for metastatic melanoma.

Immunotherapy is the new and promising cancer therapy that stimulates and boosts the patient’s immune system. In recent years, the FDA approved more than 20 antibody-based immunotherapies. In 2015 alone, FDA approved three checkpoint inhibitors and the first oncolytic virus therapies (Mullard, 2016). Immunotherapy provides new therapeutic approaches to treat many advanced cancers, including metastatic melanoma. For example, in 2014, the FDA approved a new immunotherapy using PD-1/PDL-1 inhibitors to treat metastatic melanoma. The goal of this thesis project was to create a new NK cell based immunotherapy approach by engineering Mart-1-specific TCR and CD3 to increase NK cell's antitumor activities.

TCR Engineering of NK Cells

Mart-1 is a commonly expressed melanoma antigen (Kawakami, 1994). The Mart-1-specific TCR used in this study is a well-studied TCR, which used in many clinical trials (Rosenberg, 1998; Panelli, 2000). Therefore, it was the perfect TCR to use in this proof of principle study. Mart-1-specific TCR was engineered onto the surface of
NK cells aiming to enhance NK cells ability to detect and kill melanoma cells that usually cannot be recognized by any immune cell. The idea was to engineer the Mart-1-specific TCR, which is usually only present on T cells, into NK cells in order to merge both T cell and NK cell antigen recognition properties into one cell type. In addition, engineering of the Mart-1-specific TCR also serve as an activating signal for NK cells. Therefore, NK cells that express Mart-1-specific TCR should elicit more efficient anti-melanoma responses, and kill melanoma cells better than an NK or T cell alone.

Even though there is not any previous research using TCR engineered NK cells, this approach is potentially feasible given that some of the signaling pathways (ZAP-70, LAT and Lck) that are found in T cells and associated with TCR signaling also present in NK cells (Zhang, 1998). In addition, researchers have shown that different types of Chimeric Antigen Receptors (CARs) work in NK cells (Glienke, 2015; Schönfeld, 2015) and T cells (Ritchie, 2013; Lorentzen, 2015). CAR is a form of an artificial T cell receptor that utilizes TCR/CD3ζ chain for signaling (Moritz, 1995). CAR has been used in multiple T cell mediated immunotherapy clinical trials which reported more than 90% remission rate for certain forms of leukemia (Maude, 2014). After the successes of CAR-based approach in T cells, researchers engineered a CAR on NK cells (Glienke, 2015). Researchers have also engineered a CAR on hESC and iPSC derived NK cells, and have shown it to be effective in killing malignant cells (Hermanson, 2015). TCR has been shown to use CD3ζ chain for the signaling pathways (Sloanlancaster, 1994). Therefore, when the Mart-1-specific TCR on NK cells is activated, these signaling pathways would most likely activate NK cells. For that reason, Mart-1-specific TCR and CD3 expressing
NK cell would likely be functional and have an increased ability to recognize and kill Mart-1 expressing melanoma cells.

Since this was a novel approach to engineer a TCR on NK cells, there were two fundamental questions. The first question was whether Mart-1-specific TCR and CD3 could properly be expressed on NK cells and another question was whether the presence of this protein complex would affect the hESC viability and differentiation status. Answering these two questions were the main focus of my study. Previous studies have shown that many signaling pathways that TCRs use on T cells are also present on NK cells, with the exception of the CD3 antigen (Vivier, 2004). Therefore, both the CD3 antigen and a Mart-1-specific TCR were engineered so that they would be properly expressed on NK cells. As a result of this project, Mart-1-specific TCR was expressed on NK cells by engineering CD3 and Mart-1-specific TCR in hESCs, and then performing NK cell differentiation.

The first phase of this project was constructing three plasmids for CD3 and Mart-1-specific TCR, and testing their function. Construction of the three plasmids (pCD3-γ/ζ-Puro, pCD3-δ/ε-Neo, F5/Mart-1 TCR-EGFP) was confirmed by the expression and function of the CD3 and Mart-1-specific TCR complex. The results indicated that CD3 is capable of transporting Mart-1-specific TCR onto the cell surface in cell types other than T cells, and that Mart-1-specific TCR itself does not have the capability to be expressed on the cell surface without assistance by CD3. This is also supported by a previous study performed Dr. Hall which has also shown expression of CD3 and TCR complex on non-T cells required all of the six subunits including CD3 δ, ε, γ, and ζ subunits and TCR α and β subunits using Western Blot (Hall, 1991). The 1:1 ratio expression of CD3 and Mart-1-
specific TCR further demonstrated that TCR and CD3 are co-dependent and co-localization, which is consistent with previous study (Birnbaum, 2014). Dr. Hall also showed the 1:1 ratio expression of CD3 and Mart-1-specific TCR using 2D sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Hall, 1991). Combining previous results and 293FT cell transfection results confirmed that engineering of both CD3 and Mart-1-specific TCR are necessary to express Mart-1-specific TCR on hESCs.

After proper plasmids construction was confirmed, phenotypic analysis confirmed that both CD3 and Mart-1-specific TCR were successfully integrated into the hESC genome and highly expressed in three individually established H1 hESC CD3/TCR lines. In this study, lentivirus was used to genetically modify hESC, and previous gene therapy research has shown that the integration via lentivirus can affect expression of integrated genes and host gene expression (Themis, 2005). Therefore, three lines were established to confirm that the random integration sites did not affect the expression of Mart-1-specific TCR, CD3 and genes that are expressed on hESCs. Linear one to one ratio expression of CD3 and Mart-1-specific TCR suggested co-dependent and co-localized expression of CD3 and TCR on H1 hESCs which is consistent with previous studies (Hall, 1991; Birnbaum, 2014).

Multiple studies have shown genetic manipulation on hESCs did not alter hESCs viability or their ability to be differentiated along the hematopoietic lineage including T cell and NK cells. In a previous study, Dr. Vizcardo generated Mart-1-specific TCR iPSCs, and the study aimed to expand antigen-specific T cell using Mart-1-specific TCR iPSCs as the source. The results from the study showed expression of both CD3 and
Mart-1-specific TCR on iPSCs do not interfere with pluripotent markers such as TRA-1-60, TRA-1-81, NANOG, OCT3/4, SSEA3, and SSEA4 (Vizcardo, 2013). Even though iPSCs and hESCs are different cell types, they are both pluripotent stem cells and they are phenotypically and functionally very similar (Puri, 2012). Studies have also demonstrated the generation of T cell lineage cells from GFP engineered hESCs for T cell adaptive therapy (Galic, 2009). Dr. Ni used a similar approach and generated CD4 ζ NK cells using hESCs for HIV treatment (Ni, 2014). Both studies performed extensive phenotypic analysis of pluripotent markers expressed on hESCs, as well as hematopoietic markers such as CD34, CD 45, CD43, CD133, CXCR4 and CD38 on hESCs derived HSPCs. The expression of these markers on hESC derived HSPCs suggested hESC derived HSPCs are phenotypically similar to isolated HSPCs. These results support our findings that the expression of CD3 and Mart-1-specific TCR in hESCs did not change hESCs viability or their ability to be differentiated along the hematopoietic lineage.

HSPCs were derived using each genetically modified CD3/TCR line, and successful integration of CD3 and Mart-1-specific TCR was confirmed. Between two lines, some part of the result was consistent and others were not. For example, it was clear that CD3/TCR lines had higher CD34/CD45 double positive population compared to CD3 lines. This might be due to the interaction between CD3 and TCR with hESC differentiation process. HSPCs were about 6-11 % from a CD34 positive cell population. In a previous study, Dr.Galic generated 85.7% HSPCs from a CD34 positive cell population from GEP expressing hESCs using a feeder free differentiation protocol (Galic, 2009). This suggests that the differentiation protocol used in this project can be further optimized to achieve a higher yield of HSPCs. Even though there is no previous
research indicating whether CD3 or Mart-1-specific TCR interfere with hESC differentiation process, TCR and CD3 were connected to downstream signaling pathways and activating some of the downstream signaling pathways may have influenced the differentiation process, leading to differences in HSPCs. Interestingly, CD3/TCR #1 and CD3 #1 had higher CD34/CD45 double positive populations compared to CD3/TCR #3 and CD3 #3 even though exact same protocol was used. This inconsistency may have been due to slight differences caused by reagents from different lot number and perhaps irregularities in human culturing techniques. Nonetheless, the phenotypic characterization of the fully differentiated cells in this study confirmed the presence of NK-like cells with CD3 and Mart-1-specific TCR.

In a previous study, researchers engineered HIV-1 Gag Protein, SL9 Epitope-Specific TCR on CD34 positive hematopoietic stem cells using fetal liver as the cell source for adaptive T cell therapy (Kitchen, 2009), instead of hESCs as in this study. The main concern with using fetal liver is that it is extremely difficult to use fetal liver in clinical applications, even though fetal liver is an accessible source of CD34 positive HSPCs for research purposes. Even though small numbers of hematopoietic stem cells are found in peripheral blood (Körbling, 1981), the bone marrow is the major source of HSPCs in our body (THOMAS, 1970). Because HSPCs in bone marrow are a rare cell population, HSPCs must be expanded in vivo or differentiated from other cell types for clinical applications. Since the ultimate goal of this study is to develop a new immunotherapy for melanoma patients, hESCs are a better source for HSPCs than the fetal liver. In the future, it may be possible to establish protocols for expanding HSPCs in vivo, which will be better than using hESCs as the source for HSPCs (Kang, 2016).
Because peripheral blood NK cells are known to be fairly resistant to genetic manipulation, we need alternative sources for genetically modified NK cells for clinical application. This study demonstrated that hESC is a good source of genetically modified NK cells for future immunotherapies. Even though peripheral blood NK cells are used in research, the engineered DNA is usually directly introduced by transfection, which is not feasible in clinical applications. (Topfer, 2015) Currently, the most commonly studied NK cell line for clinical applications has been the NK-92 cell line. It has also been used in a clinical study (Tonn, 2013). NK-92 cells lack some of the important NK activating receptors such as CD16, NKp44, and NKp46 (GONG, 1994). NK-92 cells also have multiple cytogenetic abnormalities (MacLeod, 2002). Therefore, we definitely need to find better sources of NK cells for therapeutic use, and this study along with previous studies of hESCs derived HSPCs (Ni, 2013) has shown that hESCs derived NK cells can be one of them.

There are huge benefits of engineering TCR on NK cells, instead of on T cells, because NK cells do not have endogenous TCR. If there were no endogenous TCR subunits on T cells, an engineered TCR would be expressed more efficiently without the competition with endogenous TCR, and it would eliminate complications caused by having multiple TCRs on a given cell. In T cells, high expression levels of endogenous TCRs could lead to abnormal placement of engineered TCRs due to the presence of both endogenous and engineered TCRs on the surface of the cells. For example, the α subunit of endogenous TCRs and the β subunit of engineered TCRs can form a hybrid TCR with unknown and possible self-reactive functionalities (Roncarolo, 2007). In T cells, in order to avoid the formation of hybrid TCR and to enhance T cell function, studies have tried to
increase the expression level of engineered TCRs by suppressing endogenous TCR expression (Cohen, 2006; Voss, 2008). Another way to enhance T cell function is to increase engineered TCR affinity. One study showed that single and dual amino acid changes in the third complementarity-determining region (CDR3) α or β chains increased the affinity and recognition of TCRs (Robbins, 2008), because CDR3 of a TCR is critical for antigen recognition and binding. It is important to find the optimal TCR affinity as previous research suggested that both extremely high and low affinity can be harmful to patients (Stone, 2009). Extremely high affinity TCRs may lead to loss of peptide specificity (Zhao, 2007), and low affinity TCRs have shown to produce T cells with poor function (Dudley, 2008). Unfortunately, the optimal TCR affinity remains unknown. By engineering TCR on NK cells, many of the complications caused by having multiple TCRs can be solved.

**Future Directions**

This research was the first step toward testing whether we can augment the ability of NK cells to recognize and kill melanoma cells. To test whether CD3 and Mart-1-specific TCR NK cells would augment the ability of endogenous NK cells to recognize and kill melanoma cells, more functionality tests need to be performed in the future. TCR signaling should be tested to confirm that the engineered TCRs are compatible with ZAP-70, LAT and Lck signaling pathways in NK cells. Cytokine production and cytotoxicity tests are needed to show that hESC-derived NK cells have the same function as peripheral blood isolated NK cells in their ability to produce and release cytokines, and to target melanoma cells for destruction. Because CD3 and Mart-1 specific TCR NK cells
have the necessary signaling transducing molecule (CD3 ζ) for TCR activation, and CARs with CD3 ζ as signaling transducing molecule have shown to enhance NK cell killing abilities, CD3 and Mart-1 specific TCR expressing NK cells are expected to enhance the NK cell killing abilities towards Mart-1 expressing melanoma. Since the previous data from Galic and Zack labs have demonstrated that hESC derived NK cells are phenotypically and functionally similar to peripheral blood isolated NK cells, CD3 and Mart-1 specific TCR NK cells are expected to be phenotypically and functionally similar to peripheral blood isolated NK cells, but with better recognition and killing abilities of Mart-1 expressing melanoma cells. After in vitro functional tests, in vivo killing assays will be required to confirm the enhanced hESC-derived CD3 and Mart-1-specific TCR NK cells function. Conducting NK functional tests in animal models can be challenging. The Mart-1 specific TCR is HLA-A0201-restricted, and HLA-A0201 is a MHC class I expressed exclusively in humans. Therefore, the reactivity with a murine MHC class I remains unknown. In addition, melanoma is a solid tumor, and solid tumors have less accessible to NK cells as compared to monolayer cells tested in vitro. The CD3 and Mart-1 specific TCR NK killing abilities in animal models are hard to predict because of the reasons listed above.

Technical difficulties were a main reason why there are fewer efforts made toward creating NK cell based immunotherapies compared to T cell based immunotherapies. For a long time, peripheral blood NK cells were the only potential source of NK cells for clinical application (Woll, 2005). Major genetic manipulation on peripheral blood NK cells used for immunotherapy had two major problems. First, peripheral blood NK cells are known to be extremely resistant to genetic manipulation.
Second, peripheral blood NK cell expansion required high concentration of cytokines (IL-2), and high levels of IL-2 have been shown to cause many adverse reactions in the body (Dutcherd, 1991). Using hESCs as the cell source for engineering NK cells solved both problems. hESCs are much easier to genetically manipulate, and hESCs have the ability to proliferate over prolonged periods of time without the need of cytokines. By demonstrating that TCR engineering can also be applied to hESC derived NK cells to create new immunotherapies, studies should make significant advances toward creating NK cell based immunotherapies.

Recently, NK cell mediated immunotherapies have developed rapidly since the discovery and creation of iPSC (Takahashi, 2006). IPSCs have greatly accelerated NK mediated immunotherapy research by eliminating problems associated with immune rejection. In addition, iPSC can be derived from patients' skin cells, and they are easily accessible compared to hESCs. This proof of principle study can now be replicated using iPSC as they have been shown to be more practical for clinical immunotherapy applications. Because both iPSC and hESC are pluripotent stem cells, they are expected to have very similar results from the CD3/TCR iPSC derived NK cells.

In the future, other researchers should be able to simply change the Mart-1-specific TCR, which is specific to metastatic melanoma, to other well-studied TCRs to treat other types of cancer using the same principle, and this proof of principle study demonstrated a new approach for NK cell mediated immunotherapy.
REFERENCE


