$CD4^+$ T CELL MEDIATED TUMOR IMMUNITY FOLLOWING TRANSPLANTATION OF TRP-1 TCR GENE MODIFIED HEMATOPOIETIC STEM CELLS

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DEDICATION

I would like to dedicate this thesis to my family for all their support during my long journey to finishing medical school.

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ABSTRACT

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$CD4^+$ T cell mediated tumor immunity following transplantation of TRP-1 TCR gene modified hematopoietic stem cells

Immunotherapy for cancer has held much promise as a potent modality of cancer treatment. The ability to selectively destroy diseased cells and leave healthy cells unharmed has been the goal of cancer immunotherapy for the past thirty years. However, the full capabilities of cancer immunotherapies have been elusive. Cancer immunotherapies have been consistently hampered by limited immune reactivity, a diminishing immune response over time, and a failure to overcome self-tolerance. Many of these deficiencies have been borne-out by immunotherapies that have focused on the adoptive transfer of activated or genetically modified mature $CD8^+$ T cells. The limitations inherent in therapies involving terminally differentiated mature lymphocytes include limited duration, lack of involvement of other components of the immune system, and limited clinical efficacy. We sought to overcome these limitations by altering and enhancing long-term host immunity by genetically modifying then transplanting HSCs. To study these questions and test the efficiency of gene transfer, we cloned a tumor reactive HLA-DR4-restricted $CD4^+$ TCR specific for the melanocyte differentiation antigen TRP-1, then constructed both a high expression lentiviral delivery system and a TCR Tg expressing the same TCR

genes. We demonstrate with both mouse and human HSCs durable, high-efficiency TCR gene transfer, following long-term transplantation. We demonstrate the induction of spontaneous autoimmune vitiligo and a TCR-specific T_{H1} polarized memory effector $CD4^+$ T cell population. Most importantly, we demonstrate the destruction of subcutaneous melanoma without the aid of vaccination, immune modulation, or cytokine administration. Overall, these results demonstrate the creation of a novel translational model of durable lentiviral gene transfer, the induction of spontaneous $CD4^+$ T cell immunity, the breaking of self-tolerance, and the induction of anti-tumor immunity.

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LIST OF ABBREVIATIONS

ACK	ammonium chloride potassium lysing buffer
AIRE	autoimmune regulator gene
APC	antigen presenting cells
BM	bone marrow
BSA	bovine serum albumin
CD103	cluster of differentiation 103
CD25	cluster of differentiation 25
CD3	cluster of differentiation 3
CD34	cluster of differentiation 34
CD38	cluster of differentiation 38
CD4	cluster of differentiation 4
CD40	cluster of differentiation 40
CD40L	cluster of differentiation 40 ligand
CD44	cluster of differentiation 44
CD45.1	cluster of differentiation 45.1
CD45.2	cluster of differentiation 45.2
CD45RB	cluster of differentiation 45RB
CD62L	cluster of differentiation 62 ligand
CD8	cluster of differentiation 8
cDNA	complementary DNA
CM	complete media
CMV	cytomegalovirus

CLP	common lymphoid precursor cells
cPPT	central polypurine tract derived from HIV-polymerase
CTLA-4	cytotoxic T-lymphocyte antigen 4
DC	dendritic cells
DMEM	Dulbecco’s modified Eagle’s medium
DN	double negative
DP	double positive
EBV	Epstein Barr virus
EDTA	ethylene-glycol-tetra-acetic acid
EF1α	elongation factor 1α
ELISA	enzyme linked immunosorbent assay
ELP	early lymphoid precursor cells
F2A	Furin self-cleaving sequence
FACS	fluorescence activated cell sorter
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
gag	HIV gag gene
gDNA	genomic DNA
GFP	green fluorescent protein
GITR	glucocorticoid induced TNF receptor
GM-CSF	granulocyte macrophage colony stimulating factor
gp100	glycoprotein 100
HA	Hemophilus influenza antigen

HBSS	Hank’s buffered salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HP	homeostatic proliferation
HSC	hematopoietic stem cells
HTLV	human T-lymphotropic virus type I
IFN-γ	interferon γ
IHC	immunohistochemistry
IL-12	interleukin-12
IL-2	interleukin-2
IL-6	interleukin-6
IL-7	interleukin-7
IRES	internal ribosomal entry site
KO	knockout
LAK	lymphokine-activated killer cells
LTR	long terminal repeat
LV	lentivirus
MART-1	melanoma antigen recognized by T cells
MDA	melanocyte differentiation antigen
MHC	major histocompatibility complex
MOI	multiplicity of infection
mTEC	medullary thymic epithelial cells

NCI	National Cancer Institute
NIH	National Institute of Health
NOD	non-obese diabetic
OA1	ocular albinism protein 1
OKT-3	murine monoclonal anti-CD3 antibody
OVA	ovalbumin
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PGK	phosphoglycerate kinase
pol	HIV pol gene
qPCR	quantitative real time PCR
RACE	rapid amplification of cDNA ends
RBC	red blood cells
Rev	regulator of virion protein expression
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
RRE	Rev response element
SCF	stem cell factor
SCID	severe combined immunodeficiency
SFM	serum free media
SIN	self-inactivating

SP	single positive
TCR	T cell receptor
T_{EM}	T effector memory phenotype
Tg	transgenic
Th1	T helper type 1 cells
TIL	tumor infiltrating lymphocytes
TNF	tumor necrosis factor
TPO	thrombopoietin
Treg	regulator T cells
TRP-1	tyrosinase related protein 1
TRP-2	tyrosinase related protein 2
WPRE	Woodchuck hepatitis virus posttranslational regulatory element
VSVG	vesicular stomatitis virus gene
γ-RV	γ retrovirus

I. Introduction

T Cell Development Overview

T lymphocytes, like all cellular components of blood are ultimately derived from and dependent on constant regeneration from hematopoietic stem cells (HSCs) found in bone marrow. HSCs are characterized by their multi-lineage potential and self-renewal capacity. HSCs continuously give rise to lineage-restricted progenitor cells including myeloid progenitors, erythroid progenitors, and common lymphoid progenitor cells. Lymphoid progenitors continue their maturation in secondary lymphoid tissue. Primarily, B cells mature in the bone marrow and T cells mature in the thymus, thus their respective names. Since the crux of this thesis, involves the selection and manipulation of the T cell population, we will focus on T cell development.

Hematopoietic Stem Cells

HSCs are fully capable of self-renewal and differentiation into all the cellular components of blood. Because of their pluripotent nature, HSCs hold great therapeutic promise. In particular, manipulation of the genes involved in HSC development would mean the alteration of all cellular progeny downstream of these early precursor cells. Thus, much research has gone into identifying and characterizing these HSCs and their early decedent progenitor cell populations. HSCs are identified in mice by a distinct surface protein phenotype, $Lin^-/Sca\text{-}1^+/c\text{-}kit^+/CD44^+/CD25^-$. They further differentiate in the bone marrow to become lymphoid biased progenitor cells including LSK CD62L cells ($Lin^-/Sca\text{-}1^+/c\text{-}kit^+/CD62L^+$), early lymphoid precursors (ELP) ($Lin^-/Sca\text{-}1^+/c\text{-}kit^+/Flt3^+/CD27^+$), and common lymphoid precursors (CLP) ($Lin^-/Sca\text{-}1^-/c\text{-}kit^-/IL\text{-}$

7Ra^{+}). A common theme amongst these early progenitor cells is the absence of lineage markers and the expression of c-kit and Sca-1, along with the capacity to traffic to and efficiently proliferate in the thymus, and their propensity to differentiate into lymphoid cells (Haddad et al, 2004; Wu, 2006; Hoebeke et al, 2007; Serwold et al, 2008; Awong et al, 2009).

Thymic Development

Lymphoid progenitor cells subsequently traffic to the thymus, a secondary lymphoid organ devoted to the development, selection, and maturation of T cells. T cell progenitors originating from bone marrow are released in waves into the blood where they travel periodically to the thymus (Miller, 1961; Pullen et al, 1989). These immature thymocytes undergo two phases of selection, both positive and negative. In positive selection, thymocytes recognizing binding components of MHC molecules are promoted and activated while thymocytes that do not recognize self MHC molecules are actively deleted (Bevan and Fink, 1978; Zinkernagel et al, 1978; Pullen et al, 1989). In negative selection, thymocytes that recognize self-antigens are actively deleted thus ensuring removal of mature lymphocytes capable of inducing autoimmunity (von Boehmer et al, 1989; McDonald and Lees, 1990). This process of central tolerance is critical for the prevention of autoimmunity (Schwartz, 1989). Thymic epithelial cells are believed to play a significant role in the negative selection process. In particular, the medullary thymic epithelial cells (mTECs) which express the autoimmune regulator element (AIRE), a regulatory transcription factor which controls the promiscuous expression of a variety of self-antigens in mTECs

allows for the negative selection of self-antigen binding thymocytes. The genes activated by AIRE include genes for a broad spectrum of protein functional classes (growth factors, hormones, structural molecules, and tissue specific proteins that represent essentially every organ) (Gotter et al, 2004; Johnnidis et al, 2005; Anderson et al, 2005).

Immunotherapy

As evidenced above, researchers have taken great strides in uncovering the nature, development and selection of the cells of the immune system. However, this knowledge has not necessarily translated into clinical therapies. The early attempts to harness the vast therapeutic potential of the immune system focused primary on global manipulation of the immune response via application of supraphysiologic levels of cytokines. Initial immunotherapies for cancer focused on the activation of immune cells by administration of high dose interleukin-2 (IL-2). Early studies in the 1980s identified cells capable of directly lysing fresh tumors by incubation of lymphocytes in IL-2 (Yron et al, 1980). Subsequent adoptive transfer of these lymphokine activated killer (LAK) cells in conjunction with IL-2 therapy mediated the regression of some advanced metastatic cancers. Of 55 patients treated with this approach, 21 showed objective clinical regression, and 5 individuals showed complete regression of malignancy (Rosenberg, 1985). These early trials showed promise in stimulating immune response to metastatic melanoma; however, the high doses of IL-2 required for clinical response were intolerable to the majority of patients and mediated toxic side effects, the most common of which was a capillary

permeability leak syndrome that resulted in major fluid retention. Furthermore, the large number of activated cells necessary to mediate tumor regression was cumbersome and limited the practicality of treatment on a large scale (Rosenberg et al, 1985; Rosenberg et al, 1986). Treatment approaches with cancer vaccines and more advanced adoptive immunotherapies have had similarly limited outcomes. Due to these limitations, in the past three decades researchers have sought more efficient approaches to modify the immune response to cancer.

Discovery of Tumor Infiltrating Lymphocytes

In 1986, Rosenberg, Spiess, and LaFreniere at the National Cancer Institute of the NIH discovered a subpopulation of lymphocytes that infiltrate into growing cancers and were 50 to 100 times more potent than LAK cells for use in adoptive transfer therapies (Rosenberg et al, 1986). These tumor infiltrating lymphocytes (TIL) could be expanded *ex vivo* by incubation with IL-2 to sufficient numbers for effective adoptive transfer. Concomitant TIL adoptive transfer, low dose IL-2 treatment and immunosuppression with cyclophosphamide proved to be highly effective in treating hepatic and pulmonary metastases in mice with colon adenocarcinoma (Rosenberg et al, 1986). Unfortunately, early human trials of TIL adoptive cell transfer therapy showed only a limited ability to objectively regress tumor in metastatic cancer patients. However, the isolation of these tumor infiltrating lymphocytes and subsequent clonal expansion of the lymphocytes led to the discovery of T cells specific for many melanocyte differentiation antigens (MDA). $CD8^+$ and $CD4^+$ T cells specific for MDAs have

been identified, and the gene encoding the specific TCR has been sequenced. (Boon and van der Brugges, 1996; Kawakami and Rosenberg, 1997; Jager et al, 1998; Renkvist et al, 2001).

Autoimmunity Versus Tolerance

If tumor antigen specific lymphocytes exist both within the peripheral circulation and at the tumor site, then the question arises, why is there not a consistently observed and fully therapeutic immune response? Although, the exact mechanisms are not clear, the answer lies in the balance between self-tolerance and autoimmunity. Immune tolerance mechanisms have evolved to protect the host from autoimmunity. During the normal development of the T cell repertoire, self-reactive T cells are negatively selected and removed from the total population of T cells within the medulla of the thymus. Medullary thymic epithelial cells (mTECs), under the control of the autoimmune regulator (AIRE) transcription factor, appear to express a range of self-antigens which allows for the deletion of those T cells that recognize self-antigens, a process known as negative selection (Gallegos and Bevan, 2004; Gotter et al, 2004; Anderson et al, 2005; Johnnidis et al, 2005). Thus, T cells potentially capable of reacting against self-antigens undergo apoptosis in the thymus and never enter the peripheral circulation (Anderson et al 2005). The deletion of these self-reactive T cells is mediated by both direct and indirect antigen presentation by mTECs and bone marrow derived antigen presenting cells (APCs) (Gallegos and Bevan, 2004). mTECs promiscuously express a wide range of self antigens under the control of AIRE (Magalhaes et al, 2006). Although, direct presentation of self-peptides by

mTECs can efficiently remove a subset of T cells, bone marrow derived APCs extend the range of clonal deletion by cross-presenting antigen captured from mTECs and removing those T cells. (Gallegos and Bevan, 2005). However, this thymus dependent central tolerization is clearly not absolute as witnessed by self-antigen specific T cells identified in autoimmune disorders and within tumors as TILs and circulating in the peripheral blood of cancer patients (Gallegos and Bevan, 2006).

Central tolerance alone is insufficient to fully prevent autoimmunity. Aside from the normal mechanisms for peripheral anergy, evidence has mounted for the importance of a subset of $CD4^+$ T cells which function as regulatory T (Treg) cells in the periphery (Javia and Rosenberg, 2002; Baecher-Allen and Anderson, 2006). T cells that were able to actively suppress immune responses were described as early as the 1970s (Gershon and Kondo, 1971). In the mid-1990s, these Treg cells, which constitute approximately 10% of $CD4^+$ T cells, were found to co-express the IL-2 receptor α chain (CD25) (Shevach 2002). Subsequent isolation and *in vitro* experiments have shown that Tregs phenotypically express cytotoxic T-lymphoctye antigen 4 (CTLA-4), CD103, and glucocorticoid induced TNF receptor (GITR) family related gene at high levels. The transcription factor Foxp3 appears to act as the master control gene for the development and function of Tregs (Shevach 2002; Fontenot et al, 2003; Nishimura, et al 2004). Unlike, typical $CD4^+$ T cells, Tregs do not appear to undergo activation and proliferation when their TCR recognizes antigen bound to MHC molecules. Instead, these Treg cells are both hyporesponsive and suppressive of normal

$CD4^+$ and $CD8^+$ T cell responses to specific antigens. The exact mechanisms for suppression remains poorly understood. Direct T cell to T cell interaction appears to be a necessary requirement for active suppression by Tregs. However, the function of dendritic cells and other APCs cannot be completely dismissed (Shevach 2002).

In the context of cancer immunity, tolerance to endogenously expressed tumor antigens limits the immune response to tumor and allows for unfettered tumor growth. Tregs isolated from the peripheral blood of metastatic melanoma patients clearly demonstrate a hypoproliferative response to MDA presentation and suppressive capabilities (Javia and Rosenberg, 2003). In one study, Treg cells isolated from MDA peptide vaccinated metastatic melanoma patients were co-cultured with $CD4^+CD25^-$ T cells. Treg cells from 11 of 13 patients suppressed proliferation of the $CD4^+CD25^-$ T cells by an average of 60% when stimulated with a combination of anti-CD3 and irradiated allogeneic PBMC stimulus. The suppression was directly proportional to the number of Treg cells in the co-culture and could be overcome with the administration of high doses of IL-2 (Javia and Rosenberg, 2003).

When the mechanisms of self tolerance are broken, autoimmune responses occur. Autoimmune vitiligo has been observed as a consequence of immunotherapy for melanoma. This selective autoimmune destruction of melanocytes is often observed with objective clinical regression of melanoma. Vitiligo induction has been observed in mice following vaccination with several MDAs including gp100, TRP-1 and TRP-2 (Hawkins et al, 2000; Overwijk et al,

1999; Okamoto et al, 1998). Furthermore, adoptive transfer of gp100 and tyrosinase specific $CD8^+$ T cells has also been observed to cause autoimmune vitiligo in mice (Overwijk et al, 2003). Autoimmune vitiligo appears to be mediated by cytotoxic $CD8^+$ T cells that recognize MDAs which home to the skin and lead to the destruction of pigmented cells as observed in patients with Vogt-Koyanagi-Harada disease (Ogg et al, 1998; Sugita et al, 1996). However, experiments with vaccination of knockout mice revealed a dependence on MHC Class II molecules as would be expected with a $CD4^+$ T cell mediated response, but not MHC Class I in the induction of vitiligo. Taken together these results indicate a central role for $CD4^+$ T cells in the induction of vitiligo (Overwijk et al, 1999).

Myelodepletion Enhances Autoimmunity

The balance between tolerance and autoimmunity can be skewed to favor autoimmunity with lymphodepletion (North,1982; Javia and Rosenberg, 2002; Ghiringhelli et al, 2004). Experiments in mouse models showed that adoptive transfer of tumor specific lymphocytes (North, 1982) and antigen specific vaccination both evoked a Treg mediated immunosupression (Javia and Rosenberg, 2002). Permanent regression of tumors could be achieved by lymphodepletion via administration of cyclophosphamide prior to adoptive transfer of lymphocytes (North 1982). Accordingly, clinical trials with adoptive transfer of TILs demonstrated that modulation of the recipient environment by myelodepletion enhanced clinical responses. In one clinical trial at the NIH, 18 of 35 treated patients with refractory metastatic melanoma showed objective clinical

responses to the combination of lymphodepletion via chemotherapy, adoptive transfer and high dose IL-2 administration, a significantly improved result from prior adoptive transfer trials without lymphodepletion (Dudley et al, 2005). Two mechanisms have been proposed to explain the improved results seen with myelodepletion: 1) the removal of Tregs which otherwise suppress transferred tumor reactive lymphocytes, 2) the decreased competition by endogenous lymphocytes for homeostatic regulatory cytokines such as IL-7 and IL-15 (Dudley et al, 2005).

Limitations of $CD8^+$ T Cells in Cancer Immunotherapy

Given that most tumor cells are MHC Class II negative, the bulk of current research on immunotherapies for cancer has revolved around $CD8^+$ T lymphocytes. Effector $CD8^+$ T cells recognize tumor antigen presented by MHC Class I molecules and directly lyse the offending neoplastic cells. $CD8^+$ T cells induce cell destruction through the release of inflammatory cytokines such as tumor necrosis factor-α and IFN-γ, the induction of apoptosis, and cytotoxic degranulation leading to perforin-mediated lysis. However, the existence of tumor antigen specific $CD8^+$ T cells in metastatic cancer patients indicate that $CD8^+$ T cells alone are insufficient to lead to rejection or regression of cancer (Dudley and Rosenberg, 2003). Researchers have been successful in identifying a multitude of melanocyte differentiation antigen (MDA) specific MHC class I restricted $CD8^+$ T cells including $CD8^+$ T cells specific for gp100, TRP-1, TRP-2 and the immunodominant MART-1 (Pardoll 1994; Boon and van der Brugges, 1996; Kawakami and Rosenberg, 1997; Renkvist et al, 2001). Vaccinations with

these peptide antigens succeeded in routinely generating tumor reactive $CD8^+$ T lymphocytes in patients, but only lead to sporadic regression of tumor (Dudley and Rosenberg, 2003).

Adoptive transfer of autologous T cells derived from patients with metastatic melanoma has proven to be effective in objective tumor regression in a limited number of patients. Subsequent experiments on human patients with refractory metastatic melanoma have shown some clinical response to adoptive transfer of TILs. Tumor reactive T cell subpopulations were selected from TILs by screening for cytokine secretion. TILs were presented with lysates of autologous and established tumor cell lines. T cell subpopulations with high recognition of tumor antigens as measured by cytokine secretion were isolated. TIL cultures that exhibited specific tumor recognition were expanded for treatment using a rapid expansion protocol with irradiated allogeneic feeder cells, anti-CD3 antibody, and IL-2. (Dudley et al, 2002; Dudley and Rosenberg, 2003) In one clinical trial, adoptive transfer of TILs in combination with administration of high dose IL-2, resulted in the objective regression of tumor in 51% of patients (18 of 35 patients) (Dudley et al, 2005).

One limitation of adoptive transfer of TILs is the need to find preexisting tumor reactive colonies. Researchers have addressed this concern by transfer of genetically modified $CD8^+$ T lymphocytes. $CD8^+$ T cells were conferred tumor recognition by transduction with a retrovirus encoding a T cell receptor specific for a MART-1 epitope. Adoptive transfer of these gene modified $CD8^+$ T cells in 15 patients resulted in tumor regression in 2 patients who maintained high levels

of circulating gene modified $CD8^+$ T cells for at least one year post infusion (Morgan et al, 2006).

A second limitation of adoptive cell transfer therapies is the lack of a permanent change in the T cell repertoire. Persistence of antigen specific T cells following adoptive transfer is necessary for effective clinical regression of tumor (Zhou et al, 2004; Robbins et al, 2004; Zhou et al, 2005). Telomere length has been positively correlated with persistence of reactive T cells following adoptive transfer (Zhou et al, 2005; Shen et al, 2007). TILs proliferate extensively following adoptive transfer, but fail to induce substantial telomerase activity, and undergo rapid decreases in telomere length and quickly become replicatively senescent. Those clonotypes with the shortest telomeres at the time of transfer become driven to senescence, while only those with longer telomeres are able to persist and mediate antitumor effects (Shen et al, 2007).

Although the adoptive transfer of $CD8^+$ T cells shows promise, clinical responses have been modest. We believe that the limited success of $CD8^+$ T cells is due in part to the lack of a $CD4^+$ T cell component to the immune response. Successful immunotherapies for cancer will almost certainly require a $CD4^+$ T cell response.

Importance of $CD4^+$ T Cells in Cancer Immunotherapy

$CD4^+$ T lymphocytes play a central role in conducting the initiation and maintenance of the adaptive immune response (**Figure 1**). It has been demonstrated that $CD4^+$ T lymphocytes help the activation and expansion of $CD8^+$ T cells. In particular the release of cytokines such as IL-2, IL-12, and IFN-γ

by Th1 helper cells aid in the activation and expansion of $CD8^+$ cytotoxic lymphocytes. Furthermore, $CD4^+$ T cells appear to be necessary at the time of priming of $CD8^+$ T cells for the effective establishment of $CD8^+$ T cell memory cells and for long term maintenance of antigen-activated $CD8^+$ T cells (Gerloni and Zanetti, 2005; Williams et al, 2006).

Studies on the role of the $CD4^+$ T cells in antitumor immunity have shown the importance of cytokines such as IL-2, IL-12, and IFN-γ to promote activation and expansion of $CD8^+$ cytotoxic T lymphocytes (Gerloni and Zanetti, 2005; Kennedy and Celis, 2008). Further evidence indicates that effective presentation of tumor antigens to $CD8^+$ T cells may require the activation of antigen presenting cells (APC) by $CD4^+$ T cells. Tumor antigen recognition by $CD4^+$ T cells and subsequent interactions between CD40 on $CD4^+$ T cells and the CD40 ligand on APCs activates the APC to present antigen and costimulates the priming of $CD8^+$ T cells. In this model, the activation of $CD8^+$ T cells is achieved through the cross-priming of APCs by $CD4^+$ T cells (Pardoll and Topalian, 1998; Bennettet al, 1998; Toes et al, 1998; Schoenberger et al, 1998).

Cell based vaccine models of antitumor immunity indicates that $CD4^+$ T cells can mediate a $CD8^+$ T cell independent pathway for tumor resistance. In particular, there is evidence that $CD4^+$ T cells can activate and recruit macrophages and eosinophils to tumor sites. Activation of macrophages and eosinophils can produce reactive oxygen intermediates that can directly destroy tumor cells (Hock et al, 1991; Hung et al, 1998; Cohen et al, 2000). Vaccination of mice with a recombinant vaccinia virus encoding the murine TRP-1 antigen led

to autoimmune vitiligo and subsequent rejection of B16 tumor challenge. Vaccination of $CD4^+$ T cell depleted mice did not develop vitiligo and failed to reject the tumor challenge. In contrast, β2m knockout mice which have severely limited numbers of $CD8^+$ T lymphocytes developed autoimmune vitiligo and resistance of B16 tumor challenge at a statistically similar rate as normal C57BL6 mice (Overwijk et al, 1999). In a separate study, vaccination and tumor challenge in nitric oxide synthase knockout mice showed a failure to reject a tumor challenge (Pardoll and Topalian, 1998). Taken together these experiments indicate a $CD8^+$ T cell independent pathway for activated $CD4^+$ T cells in tumor resistance, perhaps through the recruitment and activation of macrophages and eosinophils.

Given the pivotal position that $CD4^+$ T lymphocytes occupy in the adaptive immune response, it is clear that activation of tumor antigen specific $CD4^+$ T cells is necessary for effective long term immune responses to tumor.

Isolation of TRP-1 Specific $CD4^+$ TCR

Researchers in our lab had previously identified an MHC Class II HLA-DR4 restricted $CD4^+$ T cell population capable of reacting against the naturally occurring TRP-1 protein (Touloukian et al, 2002). Human lymphocytes specific and reactive to a 21-residue epitope of TRP-1 (positions 277-297) were isolated from the peripheral blood of a metastatic melanoma patient by limiting dilution. In brief, peripheral blood mononuclear cells (PBMC) were collected by pheresis and cultured in 96 well plates at a concentration of 0.3 cells/well. The cultures were subsequently stimulated with allogeneic PBMC ($5x10^4$ cells/well), IL-2 (50

CU/ml), and murine monoclonal anti-CD3 antibody (OKT-3) (30 ng/ml). The cells were then screened for TRP-1 antigen reactivity by ELISA for cytokine release upon presentation with irradiated APCs loaded with TRP-1 peptide. Positive clones were then expanded by further stimulation with allogeneic PBMC, IL-2, and OKT-3. The selected clones were further screened for cytokine release by ELISA to TRP-1 peptide loaded EBV transformed B cells, tumor lysate, and whole tumor. By this strategy a single clonal population of $CD4^+$ T cells reactive to TRP-1 was identified. The isolated clonal population of $CD4^+$ T cells showed a dosage dependent release of IFN-γ to increasing concentration of peptide. Furthermore, the $CD4^+$ T cells showed a specific response only to TRP-1 epitope presented in the context of the MHC Class II HLA-DR4 molecule.

Efficiency and Safety of Lentiviral Gene Delivery

Over the past decade γ-retroviruses (γ-RVs) have been used widely in both animal models and human clinical trials to introduce gene products into peripheral T cells, dendritic cells (DCs), and HSCs (Schnell et al, 2000; Cavazanna-Calvo et al, 2000; Yang et al, 2002; Moeller et al, 2005; Morris et al, 2005; Morgan et al, 2006). Despite their widespread use, γ-RVs appear to have limited intrinsic value because of several factors, including requirement of a long-term *in vitro* preactivation period, limited transgene expression in early progenitor cells, attenuation of gene expression after progenitor differentiation and/or long-term culture and engraftment, and ongoing fears regarding biosafety (Miller et al, 1990; Roe et al, 1993; Gothot et al, 1998; Glimm et al, 2000; Klug et al, 2000; Baum, 2007). To achieve higher levels of gene transfer, greater infectivity in

metabolically inactive target cells, and improved biosafety profile, recent methods have shifted to lentiviral (LV) technology (Woods et al, 2002; Bartosch and Cosset, 2004). Unlike native HIV or HTLV, current generation lentiviral vectors lack the accessory components necessary for viral self-replication and have been modified to contain a chimeric Rous sarcoma virus/HIV 5' LTR enhancer and a heterologous internal promoter (e.g. CMV, EF1α, PGK) (Woods et al, 2002). The use of a stronger promoter has been shown to eliminate the need for the HIV Tat protein (Sastry et al, 1996). Lentivectors have also been made self-inactivating (SIN) by deleting the majority of the U3 region in the 3' LTR thereby inhibiting viral RNA production in target cells (Zufferey et al, 1998; Dull et al, 1998). Current lentiviral vectors have also been successfully used to deliver complex gene products into non-dividing (mitotically quiescent, G_0/G_1 phase) HSCs. Most importantly, there is increasing evidence of enhanced persistence and biosafety of both genomically integrated and non-integrated vector DNA, inducing theoretically lower rates of insertional mutagenesis (Montini et al, 2006; Yanez-Munoz et al, 2006). For these reasons, we chose a lentiviral construct as our gene delivery vector.

Summary

As outlined above, T cell selection is a two step mechanism involving both positive and negative selection. Lymphocytic precursor cells migrate from the bone marrow to the thymus. T cells mature in the thymus which provides structural support for the sequence of steps necessary for T cell selection (Miller, 1961). Immature T cells are located in the cortex of the thymus and progress

through a series of alterations which can be traced by the differing expression of surface antigens (CD3, 4, 8, and the TCR) using surface marker specific antibodies. The vast majority of thymocytes undergo apoptosis unless successfully selected by positive selection (McDonald and Lees, 1990).

Positive selection is mediated by thymic cortical epithelial cells that select only those T cells with TCRs that recognize self-MHC molecules (Bevan and Fink, 1978; Zinkernagel et al, 1978; Pullen et al, 1989). Furthermore, positive selection promotes the survival of only those T cells with proper matching of MHC molecules and co-receptor. Thus, T cells with TCRs that recognize MHC class I molecules and express the CD8 co-receptor go on to mature into cytotoxic T cells, while those binding MHC class II molecules express CD4 and mature into cytokine releasing T cells (Bevan and Fink, 1978; Zinkernagel et al, 1978; McDonald and Lees, 1990; McDonald and Lees, 1990). The overall effect of positive selection is the survival of only those T cells with TCRs that bind self-MHC molecules and express the corresponding co-receptors. The surviving maturing T cells then progress deeper into the thymic medulla where negative selection occurs.

Negative selection is mediated for the large part by medullary thymic epithelial cells expressing AIRE and bone marrow derived dendritic cells located in the corticomedullary junction of the thymus and macrophages scattered throughout the medulla (Gallegos and Bevan, 2004; Gotter et al, 2004; Anderson et al, 2005; Johnnidis et al, 2005). T cells that have been positively selected are presented with a wide array of self-peptides bound to MHC molecules by these

antigen presenting cells. Those T cells that recognize self-antigen are lead to undergo apoptosis, thus removing any T cells that recognize self-antigens (McDonald and Lees, 1990; von Boehmer et al, 1989; Schwartz, 1989; Anderson et al, 2005). The remaining T cells are released from the thymus into the periphery and remain small inactive mature T cells until activated by binding to non-self-antigens bound to MHC molecules. The overall effect of positive and negative selection is two-fold. Positive selection results in the peripheral circulation of only those T cells that recognizes self-MHC molecules with expression of the appropriate co-receptor leading to MHC restriction (Bevan and Fink, 1978; Zinkernagel et al, 1978; von Boehmer et al, 1989; Nikolic-Zulgic and Bevan; 1990). Negative selection results in the removal of T cells that recognize self-antigen bound to self-MHC molecules leading to self-tolerance. (von Boehmer et al, 1989; Schwartz, 1989; McDonald and Lees, 1990; Gotter et al, 2004; Johnnidis et al, 2005; Anderson et al, 2005)

The central tolerance mechanisms of positive and negative selection are not absolute as evidenced by the existence of T cells specific for self antigens in melanoma patients (Knuth et al, 1992; Anichini et al, 1993; Houghton, 1994; Topalian et al, 1994; Cox et al, 1994; Bowne et al, 1999; Bouneaud et al, 2000). Experiments with high doses of IL-2 have shown the existence of lymphocytes capable of recognizing tumor cells. These tumor-infiltrating lymphocytes have been successfully cultivated in the presence of high concentrations of IL-2. TILs are an oligoclonal expansion of T cells with specificity for tumor antigens that surround and invade the tumor. In melanomas a series of melanocyte

differentiation antigens (MDA) have been identified that are specifically targeted by T cells. These MDAs which in large part are associated with the membrane of melanosomes are glycoproteins. Many potential melanosome antigens exist, yet only a few seem to be recognized by the immune system. Furthermore, evidence indicates that MDAs such as MART-1, OA1 and TRP-1 are capable of reversing tolerance to self-antigens and inducing the proliferation of specific T cells reactive to self-antigens leading to autoimmune vitiligo (Okamoto et al, 1998; Overwijk et al, 1999; Hawkins et al, 2000; Garbelli et al, 2005).

T cells capable of recognizing melanoma antigens have clearly been demonstrated by the presence of MDA recognizing T cells. TIL isolated from melanoma patients have yielded T cells with specificity for several MDAs including MART-1, gp100, tyrosinase, TRP-1 and TRP-2 (Boon and van der Brugges, 1996; Kawakami and Rosenberg, 1997; Kawakami, Dudley et al, 2001). Early immunotherapies for melanoma have taken advantage of these tumor specific T cell populations either by intrinsic expansion with administration of cytokines, vaccinations or by *ex vivo* expansion and adoptive transfer. However, clinical efficacy has proven elusive. Current therapeutic approaches have focused primarily on $CD8^+$ cytotoxic T cells, which have limited durability and capability to recruit the other components of the immune response. In particular, $CD4^+$ T cells play a central role in a robust immune response including induction and maintenance of $CD8^+$ T cells, activation of APCs, upregulation of MHC molecules, and in limited cases the direct lysis of tumor cells (Hock et al, 1991; Hung et al, 1998; Cohen et al, 2000; Pardoll and Topalian, 1998; Bennettet et al,

1998; Toes et al, 1998; Schoenberger et al, 1998; Gerloni and Zanetti, 2005; Williams et al, 2006; Kennedy and Celis, 2008).

The promise of cancer immunotherapy is the permanent modification of the immune system to recognize tumor antigens. A powerful tool in achieving this goal has been the development of gene therapy by viral vector delivery systems. Recent clinical applications have shifted to lentiviral technology because of their safer integration profile and greater infectivity in mitotically quiescent cells. Implementing a lentiviral construct, I was able to create a gene delivery system capable of efficiently and permanently alter the T cell repertoire to express a highly avid and function T cell receptor capable of recognizing and destroying melanoma tumor cells in recipient mice.

II. Research Goals

Immunotherapeutic approaches to treat melanoma have become increasingly sophisticated with attempts to manipulate the immune response via high dose IL-2, adoptive transfer, and gene therapy. Although, many of these approaches show promise, clinical response appears modest, with adoptive transfer of melanoma antigen specific $CD8^+$ T cells showing the most impressive results. $CD8^+$ cytotoxic lymphocyte therapies are limited by lack of support from the remaining cellular components of the immune system such as dendritic cells, memory cells, B cells, and $CD4^+$ T cells. Increasing evidence indicates that $CD4^+$ T helper cells play a crucial function as a facilitator in the anti-tumor response, particularly through the induction and maintenance of $CD8^+$ T cell immunity (Ossendorp et al, 1998; Overwijk et al, 1999; Langlade-Demoyen et al, 2003; Wang et al, 2004: Sun Et al, 2004; Williams et al, 2006). Yet despite their important role there have been limited efforts to exploit $CD4^+$ T cells to enhance the immune response to cancer.

Based on the synthesis of the current research given above, the overarching goal of this research was to gain a better understanding of lentiviral transduction on hematopoietic stem cell transplantation, development and selection, and potential anti-tumor immunity of $CD4^+$ T cells specific for melanocyte differentiating antigens. In particular, I was interested in the kinetics of bone marrow transplantation in terms of engraftment, reconstitution and long-term viability and function of gene modified $CD4^+$ T cells specific for a naturally occurring self-antigen, TRP-1. Furthermore, I was seeking to better understand

the role of central and peripheral selection in the proliferation and expansion of a tumor antigen/self-antigen specific $CD4^+$ T cells in both the non-transplant and post-transplant setting. Finally, we hoped to discover anti-tumor capacities of this novel TRP-1 specific T cell.

To address this goal, I first identified, characterized, and isolated a TRP-1 peptide specific TCR. I then created a lentiviral delivery system optimized to transfer the TRP-1 TCR gene to hematopoietic stem cells. In parallel, I created a novel TRP-1 TCR Tg mice expressing a chimeric TCR with our TRP-1 TCR variable region and a murine constant region. I then conducted a series of primary and secondary bone marrow transplant experiments. By analyzing the kinetics, expression, viability, and function of the transduced HSC population and its derivative cells I sought to identify the modalities for cancer immunotherapy that address some of the shortcomings of current clinical approaches.

The overall strategy consisted of identification and isolation of a TRP-1 specific TCR from a human peripheral blood mononuclear cell (PBMC) colony, construction of a lentiviral construct encoding the TRP-1 TCR, subsequent transduction of HSCs and bone marrow transplantation of gene modified hematopoietic stem cells into myelodepleted recipients. I sought to address several basic questions regarding engraftment and development of gene modified HSCs in bone marrow transplants and to further investigate the anti-tumor capacity of lentivirus transferred $CD4^+$ T cells. Initially, I investigated the kinetics of engraftment and development of lentivirus transduced HSCs in bone marrow transplants. Secondly, I investigated the role of MHC molecules in

central and peripheral selection of a lentivirally delivered self-antigen specific TRP-1 TCR in the post transplant setting. Finally, I sought to determine the functional capacity of TCR gene transferred $CD4^+$ T cells to mediate autoimmunity and anti-tumor immunity.

III. Materials and Methods

Animal and Cell Lines

TRP-1 TCR Tg mice were generated in collaboration with the Restifo Lab at the National Cancer Institute at the NHI (Bethesda, MD). The α and β genomic variable domains of the human TRP-1 TCR were PCR amplified from the same full length sequence (described below) and TA-cloned into pCR4TOPO (Invitrogen) validated by sequencing, sub-cloned into TCR cassette vectors provided by Dr. D. Mathis (Harvard Medical School, Boston, MA (Kouskoff et al, 1995)). The TRP-1 TCR cassette vectors were then sent to the Restifo Lab and co-injected into fertilized C57BL/6 embryos yielding 9 TCR transgenic founder lines. The founder mice were shipped to the Indiana University LARC animal facilities where the founder mice were screened by PCR for the presence of the transgene, and then crossed with themselves and with DR4 Tg mice to generate experimental colonies. Progeny were screened for allelic copies of the TCR and DR4 genes by PCR, or by flow cytometry using a PE-labeled DR4 Ultimer^{TM} containing the TRP-$1_{277\text{-}297}$ epitope (ProImmune Ltd, Oxford, UK). Murine class II-deficient, DR4-IE transgenic mice (DR4 Tg) (Ito et al, 1996), fully backcrossed onto a C57BL/6, were purchased from Taconic Farms. B6.SJLPtprcaPepcb/BoyJ (or Boy-J) and NOD/SCID/IL2rγKO (NOD/SCID-2) mice were purchased from Jackson Labs. All mice were 6-10 weeks old for experiments, and housed and bred at the IU LARC barrier facility under an established study. Murine tumor lines B16 (ATCC, Manassas, VA), MC-38 (ATCC); human melanoma lines 624 and 1102 Mel, human EBV-B cell line 1088 EBV-B (provided by S.A. Rosenberg,

Surgery Branch, NCI/NIH), and human lymphoid cells line Sup-T1 (ATCC) were maintained in complete media (CM) (Touloukian et al, 2000). The HLA-DRB1* genotypes of human tumor lines used in the following experiments included 624 Mel (0401,0701); 1102 Mel (0401,1502), and 1088 EBV-B (0301,0401), (Toulokian et al, 2001). Human melanoma line 1102 Mel was transduced with the retrovirus pLXSN (Clontech, Mountain View, CA) expressing either GFP or TRP-1 (provided by P. Robbins, Surgery Branch, NCI/NIH). Murine tumors B16 and MC-38 were transduced with a lentivirus expressing DRA and DRB1*040. DR4 expressing tumors were periodically enriched by flow sorting to maintain expression at >80%.

Limiting Dilution

T cell clones specific for melanocyte differentiating antigens were isolated using limiting dilution. Human peripheral blood mononuclear cells harvested from HLA-$DR4^+$ donors were harvested by pheresis and donated by the Rosenberg Lab (NCI/NIH). PBMCs were plated in 96 well U-bottom culture plates at a concentration of 0.3 cells/well co-cultured with $DR4^+$ 1088 EBV B cells pulsed with target peptides and cultured for 7 days. The T cell clones were subsequently stimulated with allogeneic PBMCs from at least three different donors ($5x10^4$ cells/well) in human culture media supplemented with IL-2 (50 CU/ml) and OKT3 (30 ng/ml). Cells were cultured from 7-14 days and screened for growth and antigen reactivity via ELISA for IL-2 and IFN-γ. Selected clones were expanded using a previously described rapid expansion protocol in T25 culture flasks with allogeneic PBMC ($2.5x10^7$ cells/flask supplemented with IL-2

50 CU/ml, and OKT3 30 ng/ml). Clones expanded by rapid expansion were screened for target antigen specificity by ELISA with co-cultures for peptide, tumor lysates and whole tumor.

Detection of Cytokines Using ELISA

Purified peptides ($HA_{306-318}$; PKYVKQNTLKLAT and human $TRP\text{-}1_{277-297}$; ISPNSVFSQWRVVCDSLEDYD) were purchased from BioSource (Invitrogen, Carlsbad, CA. Monoclonal antibodies (mAbs) were used to block T cell interactions (at 50 μg/ml) and included: L243 (against HLA-DR; IgG2a; eBioscience, San Diego, CA), W6/32 (against HLA-A, B, C; IgG2a; eBioscience), L3T4 (against human CD4; IgG1; eBioscience), and HIT8a (against human CD8; IgG1; eBioscience). Isolated T cells were washed and plated in triplicate on round bottom 96 well plates at a concentration of 10^5 cells/well. T cells were stimulated with either medium alone or co-cultured with $DR4^+$ 1088 EBV B cells alone, 1088 EBV B cells pulsed with human TRP-1 (amino acids 277-297), murine TRP-1 (amino acids 277-297) and 2H hTRP-1 (amino acids 266-477) proteins, a control OVA recombinant peptide all at a concentration of 50μg/ml, murine (B16-F10) and human (1088 and SK23 Mel) melanoma lysates, along with control 1088 EBV-B cell lysate, and $DR4^-$ B cells loaded with 1088 Mel lysate. To up-regulate MHC class II, 624 Mel was pre-treated with IFN-γ x 48 hr at 200 U/ml). All targets (10^5) and T cells (105) were cocultured in duplicates in U-bottom 96-well plates for 24 h. Culture supernatants were assayed for IFN-γ and IL-2, using commercially available ELISA kits (BD Bioscience, San Jose, CA). In brief, 96 well immunosorbent plates were coated with IL-2 and IFN-γ

specific capture antibody plates for 12 hours at 4^{o}C in a 2% BSA PBS assay diluent buffer. Plates were subsequently washed three times in ELISA wash buffer (0.1% Tween-20 in PBS) and blocked for two hours at room temperature in assay diluent buffer. Diluent buffer was removed and plates were loaded with 50 μl of supernatants and cytokine standards and incubated for two hours at room temperature. Plates were washed five times with wash buffer to remove excess supernatants and incubated at room temperature with 100 μl of biotinylated detection antibodies dissolved in assay diluent buffer for 1 hour. Plates were washed five times with wash buffer and incubated with streptavidin alkaline phosphatase in diluent buffer for 1 hour. The reactions were stopped with a 50 μl of stop buffer (2M hydrosulphuric acid) and the plates were read with 10 minutes of stopping the reactions. Cytokine levels were determined by measuring the absorbance at 415 nm. All assays were performed at least twice with similar experimental results.

Cloning of TRP-1 TCR

T cell clones specific and highly avid for TRP-1 were isolated by limiting dilution. From these clones the gene for the TCR was sequenced using a 5' rapid amplification of cDNA ends (RACE) assay kit (R&D Systems). Total RNA was extracted from the T cell clone, and each TCR subunit was amplified. The PCR products were subsequently TA-cloned and sent to the NCI for sequencing to determine the variable regions of the α and β chains. After determining the specific α and β chains, the full-length chain was amplified, TA-cloned, and resequenced yielding specific, fully amplified TCR chains. The amplified TCR

chains were sent back to our laboratory wherethe genes were TA cloned using a TOPO TA cloning kit from Invitrogen. The gene for the α and β chains were subsequently PCR cloned into an Invitrogen Gateway entry plasmid (pENTR) separated by either an internal ribosomal entry site (IRES) or a Furin self-cleaving sequence (F2A). The TCR alpha and beta chains were re-amplified with an optimal Kozak initiation sequence (Kozak, 2002) and restriction sites using Platinum-Pfx polymerase (Invitrogen). To generate the IRES vector, the α-chain was cloned into an IRES-containing derivative of pENTR1a (Invitrogen) from DraI to a SpeI site 5' to IRES. The β-chain was cloned 3' from an NcoI site at the 13th ATG of IRES to XbaI. Both chains were then sequenced and LR-clonased, into a modified Gateway (Invitrogen) destination lentiviral plasmid (pRRL-Dest-Wsin) (Dull et al, 1998). These modifications included a central polypurine tract derived from HIV-pol(cPPT) sequence inserted between the REV response element (RRE), the CMV promoter, and the Woodchuck hepatitis virus posttranslational regulatory element (WPRE) element proximal to the 3' SIN-LTR. To generate the F2A vector, Furin-2A peptide sequence was exchanged through a series of intermediates for the IRES element, transferred into the pENTR plasmid, then LR-cloned from the pENTR plasmid into pRRL-Dest-Wsin. Similarly constructed vectors which contain GFP (LV-GFP) and (LV-DR4) were also prepared.

Other Plasmids Used

Lentivirus component plasmids and a lentivirus encoding for the green fluorescent protein (pCSCGW-GFP) were donated by the Cornetta Lab and

included packaging plasmid pMDLgpRRE (containing the gag-pol accessory genes), rev expressing plasmid pRSV-rev, and envelope plasmid pMDG-VSVG. Once again employing the Invitrogen Gateway System, our collaborators in the Cornetta Lab were able to construct a bicistronic lentiviral plasmid encoding for the α and β chains of the human HLA-DRA-DRB1*0401 MHC class II molecule (pCSCGW-DR4 IRES) using the same cloning methods described above.

Lentiviral Production

LV-GFP and LV-TRP1-IRES or LV-TRP1-F2A lentiviral supernatants were prepared by transient transfection of 293T cells in T-150 flasks with vector DNA (26.4 µg), packaging plasmid pMDLgpRRE (containing the gag-pol accessory genes) (13.2 µg), rev expressing plasmid pRSV-rev (6.6 µg), and envelope plasmid pMDG-VSVG (9.2 µg) using a Promega ProFection Mammalian Transfection System-Calcium Phosphate Kit. Culture medium was replaced 16-18h after transfection with GIbco OptiPro SFM. Viral supernatants were then harvested at 24 and 48 hours after media exchange, centrifuged at 3500 rpm, filtered through 0.45mm cellulose acetate filter, and concentrated with a CentriconPlus-70 device (Millipore) at 3500 rpm x 30 minutes. The concentrated viral supernatants were aliquoted and frozen at -80^{o}C before calculation of titer.

Lentivirus Titering

To determine the titer of frozen viral supernatants, 293 cells were plated at 10^5 cells/well in 6 well plates, and serial dilutions (10^{-3}, 10^{-4}, 10^{-5}) of concentrated vector supernatants were prepared in DMEM media and incubated with cells for 4 hours with polybrene at 8µg/ml (5% CO_2, 37^{o}C). Cells were maintained in

culture for 72 hours, trypsinized and aliquoted, stained with a PE conjugated TCR β-chain antibody, and analyzed by flow cytometry. Titers were calculated using the following formula: titer = (F x Co/V) x D. F is the frequency of GFP-positive or TCR-β chain positive cells determined by flow cytometry; Co is the total number of target cells infected; V is the volume of the inoculum; D is the virus dilution factor.

Collection of Bone Marrow, Peripheral Blood Cells, and Splenocytes

Mice were euthanized by cervical dislocation and bone marrow cells were harvested by bilateral surgical resection of the tibia and femur. Collected tibia and femur specimens were collected in RPMI complete media (5% FBS in RPMI 1640 supplemented with 1% L-glutamine, 1% non-essential amino acids, 1% sodium pyruvate, 1% penicillin/streptomycin) to minimize cell death. Harvested tibia and femurs were thoroughly removed of excess muscle and connective tissue. Bone marrow was then washed out of the bones by insertion of a 27 gauge syringe needle and flushing with RPMI complete media from both proximal and distal ends of the bones. The crude bone marrow cell collection was then collected in 50 ml conical tubes and centrifuged at 1200 rpm and excess media discarded. Erythrocytes were lysed using an RBC lysing buffer (Qiagen, Inc.) for 10 minutes at room temperature. The cells were then filtered through a 75 mm nylon filter to remove clumps and ensure a single cell solution. The cells were centrifuged at 1200 rpm for 5 minutes and washed twice with appropriate media or buffer.

Peripheral blood cells were harvested by tail vein bleeds. Mice were inserted into specially designed metal mouse holders which immobilized the mice while allowing access to the tails. Using surgical scalpels, superficial incisions were made as distally as possible to nick the tail veins. The tail veins were then bled by external manipulation and peripheral blood collected in heparin treated microcapillary tubes. Collected peripheral blood was subsequently transferred to 1ml of 2% heparin solution in flow cytometry tubes to prevent clotting. Cells were then centrifuged and excess heparin discarded. Peripheral blood cells were treated were RBC lysis buffer and filtered as described for bone marrow cells. Spleens were collected by surgical resection and homogenized by mechanical mastication with a sterile glass slide and a blunt instrument. Crudely disaggregated splenocytes were then treated with RBC lysis buffer, filtered to yield a single cell suspension, and washed as previously described.

Collection of Human Cord Blood Cells

Cord blood cells were collected by fractionation using Ficoll gradient. Cord blood was graciously donated by the Broxmeyer Lab. Only cord blood less than 24 hours old were used in these experiments. The cord blood was mixed with an equal volume of EDTA Wash Buffer (2mM EDTA in PBS) solution to prevent clotting and lyse RBCs. This cord blood mixture was carefully layered over 20 ml of Ficoll plaque in 20 ml aliquots in a 50 ml conical tube. The tubes were then centrifuged at 1500 rpm for 30 minutes and the white cell layer formed between the Ficoll and aqueous layer was collected. Collected cells were washed with 40 ml of EDTA Wash Buffer to remove excess Ficoll plaque and

centrifuged at 2000 rpm for 20 minutes. Excess buffer was discarded and the remaining cord blood cells washed twice with appropriate media or buffer.

Magnetic Bead Separation

Murine bone marrow cells and human cord blood cells collected as previously described were treated with antibody labeled magnetic microbeads to extract murine Lin^- cells and human $CD34^+$ cells, respectively, using Miltenyi Biotec magnetic microbead separation kits (Miltenyi Biotec). Cells were washed with MACS buffer (2% bovine serum albumin in PBS) and labeled with an appropriate amount of specific Biotin-Antibody Cocktail for 10 minutes at 4^oC. The cells were than incubated with an anti-biotin microbead solution for an additional 20 minutes at 4^oC. The cells were washed with MACS buffer, centrifuged at 1200 rpm and excess buffer discarded. Samples were then suspended in MACS buffer and run through a MACS column (LS column) and MACS separator (Miltenyi Biotec). Collected cells were centrifuged, washed with MACS buffer and counted. A small aliquot was set aside for antibody staining and analysis by flow cytometry to check the efficiency of separation and purity of the collected samples. The remaining cells were cultured in the appropriated pre-stimulating media for 12 to 36 hours prior to lentiviral transduction. Murine Lin^- cells were prestimulated for 12 to 36 hrs in a murine prestimulating media (Gibco StemPro 34 SFM Media, 1% Penicillin/Streptomycin, 1% L-Glutamine, 100 ng/ml SCF, 10 ng/ml TPO, 50 ng/ml Flt-3 Ligand). Human $CD34^+$ cells were prestimulated for 12 to 36 hrs in a human prestimulating media (Gibco StemPro 34 SFM Media, 1% Penicillin/Streptomycin, 1% L-Glutamine, 100ng/ml SCF, 50

ng/ml TPO, 100 ng/ml IL-6). Pre-stimulated cell populations were then transduced with lentivirus.

Transduction Protocol

Cells were collected and plated at $5x10^5$ cells per well in 24 well flat bottom culture plates and incubated overnight in appropriate media. Cells well centrifuged and excess media removed. Cells were then spinoculated with concentrated lentiviral supernatant in appropriate media supplemented with 8 μg/ml of polybrene at 1200 rpm for at least 2 hours at 25°C. Spinoculated cells were immediately incubated for a further 2 hours at 37°C. Following this 4 hour spinoculation/incubation period the cells were centrifuged at 1200 rpm for 5 minutes and excess media was removed by aspiration. All cells were transduced at multiplicity of infections ranging from 10:1 to 100:1 depending on the experiment. Following remove of the viral supernatant/media fresh media appropriate for the cell type was added to the wells. The transduced cells were incubated at 37°C for 72 hours. Transduced cells were then either antibody stained for analysis by flow cytometry or prepared for bone marrow transplantation.

Cell Staining and Analysis Using Flow Cytometry

Isolated cells were placed in flow cytometry tubes and washed twice with FACS buffer (2% fetal bovine serum in PBS). Following centrifugation at 1,200 rpm for 5 minutes at 4°C, wash buffer was discarded and 1μl/tube of anti-mouse F_cIII/II receptor antibody (F_c Block; BD Biosciences) was added to each tube and incubated for at least 15 minutes at 4°C. When staining with the TRP-1 Ultimer

(ProImmune, Inc.), Ultimer was added (5 to 10 μl/tube depending on cell count), vortexed, and incubated in darkness at room temperature for 4 hours. The cells were then washed twice with FACS buffer and excess wash buffer discarded. For antibody staining, specific flourochrome conjugated antibody was added (1 to 10 μl depending on the antibody), vortexed, and incubated in darkness at 4^{o}C for at least 30 minutes. The cells were then washed twice with FACS buffer and excess buffer discarded. If the analysis was to occur on the same day as collection and staining, samples were kept at 4^{o}C in darkness until flow cytometric analysis. If the analysis was to occur the following day or later, the cells were fixed by addition of a 2% formaldehyde in PBS fixation buffer (200 μl/tube) and storage at 4^{o}C in darkness. Samples were analyzed by flow cytometry on FACScan, FACS Calibur, or LSR II machines and data was analyzed using BD CellQuest (BD Biosciences). To determine gene specific T cell frequency a combination of antibodies against CD45 (FITC, eBioscience), CD45.1 (FITC, eBioscience, CD3 (PE Cy5, BD Bioscience), CD4 (APC, BD Bioscience), CD8 (Pacific Blue, eBioscience, and the DR4 Ultimer^{TM} containing either the TRP-$1_{277-297}$ epitope (TRP1-Ultimer) or the control $gp100_{44-59}$ epitope (both PE, ProImmune) were used. To determine T cell activation status, a combination of antibodies against CD4 (APC), TRP1-Ultimer (PE), CD44 (PE Cy5, eBioscience), CD45RB (FITC, eBioscience), and CD62L (Pacific Blue, eBioscience) were used. To determine the frequency of post-transplant human mononuclear cells following transplantation into NOD/SCID-2 mice, peripheral blood samples were stained with mCD45.2 (FITC, eBioscience), hCD45 (PE

Cy5, BD Bioscience), hCD4 (APC, BD Bioscience), hCD8 (Pacific Blue, eBioscience), and the TRP1-Ultimer (PE). Statistical significance in gene expression between groups was determined using a Student T-test.

Bone Marrow Transplantation

Transduced cells were incubated for three days and subsequently collected, washed in PBS, and counted. Recipient mice were placed on doxycycline feeds four days prior to transplantation and lethally irradiated (950 rads) one day prior to transplantation. Each mouse was administered $5x10^4$ of either the LV-TCR transduced or TRP-1 TCR Tg Lin^- bone marrow cells along with $5x10^5$ either HLA-DR4 Tg or C57BL/6 competitor unfractionated bone marrow cells via tail vein injection. The mice were carefully placed in immobilizing containers and using a 27 gauge needle, a syringe preloaded with the proper concentration of both the HSCs and the competitor rescue whole bone marrow cells was threaded into the lateral dorsal tail vein.

Immunohistochemistry

Skin or tumor samples were both fixed in formalin and embedded in paraffin or snap frozen in liquid nitrogen. The fixed or frozen samples were sent to the Indiana University Pathology Laboratories where standard H&E staining was done on all specimens. For skin samples, blocks were stained with S100 (rabbit polyclonal 1:1000, Dako, Glostrup, Denmark; Envision Rabbit Polymer DAB), CD3 (rabbit polyclonal 1:200, Dako; Envision Rabbit Polymer DAB), or human- TCR-β (mouse IgG1 1:50, Santa Cruz Biotechnology, Santa Cruz, CA; Envision Rabbit Polymer DAB). Skin sample were also frozen in liquid nitrogen

and stained with antibodies to CD4 (RM4-5 1:500, Serotec, Oxford, UK; Donkey anti-Rat DAB) and CD8 (169.4 1:200, Serotec; Donkey anti-Rat DAB). For tumor samples, blocks were stained with CD3 (Envision Rabbit Polymer NovaRed), or human- TCR-β (Envision Rabbit Polymer NovaRed).

Determination of LV-TCR Gene Expression

Total RNA was isolated from $1x10^5$ BM cells obtained from 6 month secondary transplant recipients (both LV-TCR and Control) and converted to cDNA. A qPCR analysis using ABI One-Step Real-Time PCR System (Applied Biosystems - ABS, Foster City, CA) was then performed using a custom designed α-variable-chain probe (ABS) specific for the LV-TRP-1 vector. Relative TCR gene expression (RQ) was based on the amplification ratio of LV-TCR/Control Txp using β-actin as an endogenous control. The PCR reactions were set up with TaqMan PCR Core Reagents (ABS) using about 100 ng of cDNA (50 μl total reaction volume), and amplified at 95°C for 10 minutes plus 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Lentivirus Integration Analysis

Genomic DNA (gDNA) was prepared (Qiagen, Valencia, CA) from $1x10^5$ BM cells obtained from 6 month secondary transplant recipients (both LV-TCR and Control). Concentration and purity of the gDNA was determined by spectrometry. All samples showed a 260/280 ratio of 1.7 or greater indicating a high purity. Sample concentrations varied from 0.400 to 0.0500 mg/ml. The gDNA from transplant recipients were diluted to a concentration of 250 ng/ml. The gDNA samples were then sent to the Cornetta Labe where the samples

were then individually digested with Tsp5091 (New England BioLabs Ipswich, MA) at 65°C for 2 hours. Following digestion, gDNA fragments were purified using a MinElute Reaction Cleanup Kit (Qiagen). In short, 150 uL of Elution Reaction Cleanup (ERC) Buffer was added to each sample and transferred to spin columns. Columns were centrifuged for 1 minute at >10,000 x G and the pass-through was discarded. 750 mL of Buffer PE was added to each column and spun for 1 minute at > 10,000 x G. Pass-through was discarded and the column was dried by spinning for 2 min. at 10,000 x G. The purified DNA samples were eluted from the columns with 12 ml of EB buffer. Primers specific for the LTR region of the lentiviral plasmid were biotinylated with native Pfu DNA polymerase (Invitrogen). The primer extension products were subsequently purified using the ERC kit. Microbeads with covalently attached streptavidin (Dynabeads M-280, Invitrogen) were washed and 40 ml of the bead slurry was added to each biotinylated primer. Beads were subsequently washed and blunt end adaptors were ligated with T4 DNA ligase. Integration sites were then identified by nested PCR: master mix consisting of 2 x Extensor HiFi PCR mix (Invitrogen) and gDNA fragments were aliquoted into 0.2 mL PCR reaction tubes. 1 ml of the microbead-primer bead slurry was added and PCR amplified (94°C x 2 min, 95°C x 15 sec, 60°C x 30 sec, 68°C x 2 min for 30 cycles; 68°C x 10 min, 4°C x hold). After the 1st round of nested PCR a 1:50 dilution was done from each PCR reaction tube and a 2nd round of nested PCR was performed. Each PCR sample was analyzed by agarose gel electrophoresis.

Tumor Treatment Assays

Six month old secondary DR4 Tg transplant recipients involving an experimental arm (LV-TCR-F2a) and a control arm were subcutaneously implanted with B16-DR4 melanoma cells ($5x10^5$ cell/200 ml HBSS) at 5 mice per group (2 groups). Perpendicular tumor diameters were blindly recorded every 3 days. Mice were sacrificed at 20 days post-implantation and tumors were excised for IHC. The statistical significance between groups was based on tumor size (using a Student T-test). Experiments were performed twice in a blinded, randomized fashion with similar results.

IV. Results

Identification of a TRP-1 Specific TCR

Using a clonal population of $CD4^+$ T cells specific for the TRP-$1_{277-297}$ epitope (Touloukian, et al 2002), the gene sequence of the individual α and β subunits of the TCR were identified from these lymphocytes via a 5' RACE strategy at the NCI (Invitrogen GeneRacer Kit). In short, total RNA was extracted from the T cell clone, and each TCR subunit was amplified. The PCR products were subsequently TA-cloned and sequenced to determine the variable regions of the α and β chains. After determining the specific α and β chains, the full-length chain was amplified, TA-cloned, and resequenced, yielding specific, fully amplified chains: TCR-α: Vα13-2*01, J22*01; TCR-β: V5.4*01, J1-1*01, D1*01. The VDJ regions were analyzed using the V-QUEST search engine from the IMGT web site (http://imgt.cines.fr/IMGT_vquest/vquest?livret=0&Option=humanTcR). (**Figure 2**)

Construction of a Bicistronic Lentivirus

Next, I constructed a bicistronic lentivirus containing the genes for the α and β subunits. I chose to implement a bicistronic lentivirus to deliver the α and β chains of the TCR because the co-expression and successful pairing of the two chains is essential for the proper expression of the TCR. Previous experiments have shown efficient transduction of hematopoietic stem cells with multicistronic viral systems (Cui et al, 2003; Yang et al, 2002; Yang and Baltimore, 2005; Morgan et al, 2006). I ultimately chose to implement a lentiviral vector because of their reported advantage at transducing mitotically quiescent cells such as

HSCs and clinically safer genetic integration profile (Schroder et al, 2002; Wu et al, 2003). Two approaches were used to link the two target genes together. I constructed both an internal ribosomal entry site (IRES) and Furin self cleavage protein (F2A) bicistronic lentiviruses. Schematic diagrams for our lentiviral constructs are shown in **Figure 3**.

Efficiency of Transduction

To evaluate the efficiency and functionality of TCR gene transfer, I tested the newly generated LV-TCRs (LV-TCR-IRES or LV-TCRF2A) on human PBMCs prestimulated with IL-7. I used a TRP-$1_{277–297}$–specific PE-labeled pentamer (Ultimer; TRP-1 Ultimer) to measure specific cell surface expression, to avoid confusing true expression levels of the introduced α/β heterodimer with levels that result from mispairings with endogenous T cell α and β chains. As shown in **Table 1**, I observed statistically significant differences in TCR gene expression using the LV-TCR-F2A construct at increasing viral titers (37.5% vs. 56.4%; P = 0.0008 at MOI 100). Both the IRES and F2A constructs efficiently transduced JRT3 cells and PBMCs. However, transduction with the target TCR proved to be more efficient using the F2A construct in both JRT3 cells (p=0.022 at an MOI of 10:1) and PBMCs (p=0.0008 at MOI of 100:1) particularly at higher MOIs (**Figure 4**). Thus, the remainder of the experiments focused on the F2A construct.

I next tested the efficiency of the lentiviral construct to transduce both murine and human HSCs. Murine HSCs were harvested from bone marrow and enriched by depletion of lineage positive cells using a Miltenyi Biotec Lineage Depletion Microbead Kit. Murine HSCs were prestimulated for 12 to 36 hrs in a

prestimulating media (Gibco StemPro 34 SFM Media, 1% Penicillin/Streptomycin, 1% L-Glutamine, 100 ng/ml SCF, 10 ng/ml TPO, 50 ng/ml Flt-3 Ligand). Human HSCs were harvested from human cord blood. Following fractionation the cells were enriched using a Miltenyi Biotec CD34+ Microbead Kit. Human HSCs were prestimulated for 12 hrs in a prestimulating media (Gibco StemPro 34 SFM Media, 1% Penicillin/Streptomycin, 1% L-Glutamine, 100ng/ml SCF, 50 ng/ml TPO, 100 ng/ml IL-6). Following prestimulation HSCs were spinoculated with lentivirus at MOIs from 10:1 to 30:1 with 8 μg/ml of polybrene at 1200 rpms at 25^{o}C for 2 hrs. The cells were then incubated for an additional 2 hrs at 37^{0} C. Following incubation, the media was changed for fresh prestimulating media and incubated for 72 hrs.

Results as measured by flow cytometry using a peptide-antibody complex (TRP-1 Ultimer provided by ProImmune Inc.) showed approximately 10-50% transduction efficiency in human CD34^{+} cells and approximately 10-20% in murine Lin^{-} cells at MOIs of 15:1 to 30:1 (**Figure 5)**. I conducted multiple repeat experiments with Lin^{-} HSC enriched populations with lentivirus encoding green fluorescent protein (LV-GFP) as a control group (**Figure 6**). Lin^{-} cell transduction with the LV-TRP-1 TCR showed high expression at 28.7% ± 3.0 as measured by flow cytometry. Overall, the results demonstrate a high level of expression of the complete TRP-1 TCR in transduced Lin^{-} cells.

Transduced Cells Retain Functionality of TCR

To evaluate the *in vitro* functionality of the LV-TCR-F2A lentiviral construct, we transduced IL-7 prestimulated PBMCs at an MOI of 15. After

overnight incubation, transduced PBMCs were co-cultured with $DR4^+$ 1088 EVB B cells loaded with a panel of control and experimental peptides. Transduced cells were found to recognize the specific TRP-$1_{277-297}$ peptide, melanoma tumor lysates (B16), and TRP-1 positive tumor lines (B16-DR4, 624 Mel pretreated with IFN-γ, and 1102 Mel stably transduced with TRP-1) only in the context of the proper MHC class II HLA-DR4 restriction element as measured by specific IFN-γ production on ELISA (**Figure 7**). No increase in IFN-γ was seen with the control peptide HA306-318, control lysate (TRP-1 negative 1102 melanoma tumor lysate), or control intact tumor (1102 melanoma tumor cells transduced with a vaccina virus encoding GFP). Moreover, interactions with peptide pulsed targets were found to be both DR-restricted (blocked by L243) and CD4-specific (blocked by L3T4), but were not effected by control class I and CD8 specific antibodies.

Development of a TRP-1 TCR Transgenic Mouse

To help evaluate the efficiency and effects of lentiviral transduction on HSC development our collaborators in the Restifo Lab created a transgenic mouse strain which constitutively expressed the same variable α and β genetic components as the LV-TCR (TRP-1 TCR Tg). The variable regions of the α and β chain of the TRP-1 TCR were cloned into TCR cassette vectors which encoded the murine constant regions of the α and β chains. Our collaborator Dr. Nicholas Restifo at the National Cancer Institute, injected the TCR cassette vector encoding this chimeric TRP-1 specific TCR into the embryos of fertilized C57BL/6 mice. The founder mice were screened by PCR for the transgene, specifically

the variable region of the α chain. Initial characterization of the naïve TRP-1 TCR Tg mice by flow cytometry showed very limited presence of TRP-1 TCR specific lymphocytes in the peripheral blood or spleen using the TRP-1 Ultimer. The low or absent levels of TRP-1 specific T cells in the periphery of TRP-1 Tg mice is not surprising given the lack of expression of the proper MHC Class II restriction element in this transgenic mice. In the native TRP-1 Tg mice levels of Ultimer positivity was near that of negative controls and only 6.4% ± 1.0 of peripheral blood $CD4^+$ T cells were specific for the TRP-1 Ultimer (**Figure 8**). I subsequently bred the TRP-1 TCR Tg mice with the MHC Class II HLA-DR4 Tg mice to obtain the proper restriction element. In this double transgenic mouse which expresses the proper MHC restriction element, peripheral blood antibody and Ultimer staining revealed that 79% ± 8.3 of all circulating $CD4^+$ T cells are specific for the TRP-1 Ultimer (**Figure 8**). Mice crossed onto a HLA-DR4 background strongly expressed specific TCR ($CD4^+$/$Ultimer^+$, 79% ± 8.3), while those on a C57BL/6 background expressed low levels only ($CD4^+$/$Ultimer^+$, 6.4% ± 1.0; $P < 0.0001$). These results strongly suggested the necessity of aproper restriction element (HLA-DR4) in positive T cell selection. To control for experimental specificity and background (< 1%), I used a Ultimer specific for a $gp100_{44\text{-}59}$ DR4-restricted epitope. This multimeric antibody control was used for all subsequent flow cytometry analysis.

Transplantation of Lentivirus Gene Modified HSCs

To better understand the cellular dynamics and kinetics of lentivirally transduced HSCs to home, engraft, and reconstitute the immune system

following transplantation I began a series of transplantation experiments. I initially sought to understand the basic mechanics of transplantation with lentivirally manipulated HSCs by conducting a pilot lentiviral transplant experiment. Using a lentiviral construct encoding a gene for green fluorescent protein (LV-GFP), I transduced Lin^- HSCs from Boy/J donor mice. $2.5x10^4$ transduced Lin^- cells were transplanted per mice along with $5x10^5$ competitor rescue bone marrow from unmanipulated C57BL/6 mice into 30 C57BL/6 recipients. Five mice were subsequently euthanized and the peripheral blood, splenocytes, thymus, and bone marrow were analyzed by flow cytometry for CD3, CD4, CD8, CD45.1, CD45.2, and GFP expression (**Figure 9**). Transplanted, transduced Lin^- cells maintain GFP expression in the peripheral blood ($CD45.1^+/GFP^+$), developing into a high percentage (over 20% by 12 weeks post transplant) of mature T cells ($TCR^+/CD3^+$) and $CD4^+$ T cells. Gene modified cells are also present and remain stable within spleen, thymus, and bone marrow for up to 6 months post transplant. Furthermore, transduced HSCs expressing GFP appear to undergo nor thymic development progressing from double positive thymocytes to single positive mature thymocytes.

Lentiviral Transduction of HSCs Leads to Stable Long Term Integration

Several lentiviral protocols have already been described for gene transfer into transformed cells, lymphocytes, DCs, and HSCs (Cui et al, 2003; Cavalieri et al, 2003; Lizee et al, 2004; He et al, 2005; Mostoslavsky et al, 2005). Although different heterologous promoters (PGK, CAG, EF1α, and CMV), vectors (both mono and bicistronic), pseudotyped envelopes, and inoculation protocols have

been used, the vast majority involve the use of cytokine prestimulation (Miyoshi, 2004). Because HSCs are relatively resistant to even high concentrations of lentiviral particles (Sutton et al, 1998) and susceptible to cell death following viral manipulation, I based the transduction protocol on both an overnight prestimulation with SCF, TPO and Flt-3L, a spin/rest inoculation at an MOI of 15, followed by an overnight recovery phase in cytokine media prior to transplantation (Barrette et al, 2000; Mostoslavsky et al, 2005).

To quantify the engraftment potential, gene transfer efficiency, and long-term stability of transduced HSC, I employed a standard CD45.1/CD45.2 competitive isocongenic repopulation (Tao et al, 2007). I initiated a series of transplant experiments into DR4 Tg and C57BL/6 mice to determine the engraftment efficiency of unmanipulated TRP-1 Tg HSCs and LV-TCR transduced Lin^- HSCs. To assess the impact of lentiviral transduction on the engraftment and development of HSCs we established a CD45.1/CD45.2 competitive transplant experiment with either TCR Tg or LV-TCR transduced Lin^- BoyJ (CD45.1) HSCs and DR4 Tg (CD45.2) competitor bone marrow cells. Bone marrow was harvested from the femur and tibia bilaterally and treated with ACK lysing buffer to remove RBCs. The cells were subsequently disaggregated into a single cell solution and filtered through a 100μm nylon filter. The bone marrow cells were then depleted of lineage positive cells by using a Miltenyi Biotec Murine Lineage Depletion Microbead Kit. BoyJ HSCs were prestimulated overnight in a prestimulation cytokine media, and transduced the following morning with a concentrated LV-TCR supranatant at an MOI of 30 to 1.

Transduced cells were incubated for three days and subsequently collected, washed in PBS, and counted. Ten DR4 Tg recipient mice and five C57BL/6 recipient mice were placed on doxycycline feeds four days prior to transplantation and lethally irradiated (950 rads) one day prior to transplantation. Each mouse was administered $5x10^4$ of either the LV-TCR transduced or TRP-1 TCR Tg Lin^- bone marrow cells along with $5x10^5$ DR4 Tg competitor unfractionated bone marrow cells via tail vein injection. Five DR4 Tg recipient mice received the LV-TCR transduced HSCs (LV-TCR/DR4). Five DR4 Tg recipients were given HSCs from the TRP-1 TCR Tg donors (TCR Tg/DR4). Five C57BL/6 mice were transplanted with LV-TCR transduced HSCs (LV-TCR/BL6). A control transplant group of 5 DR4 Tg mice were established which were administered bone marrow cells from a C57BL/6 mouse. Peripheral blood was obtained at 1, 4, 6, and 9 month time points and stained for murine CD3, CD4, and CD8 along with the TRP-1 Ultimer complex. By 4 months, immune reconstitution appears robust with successful repopulation of both $CD8^+$ and $CD4^+$ T cells. **Figure 10** shows a representative flow cytometry analysis, which schematically outlines our analysis. Each mouse was analyzed, by gating initially around $CD3^+CD4^+$ cells. The Ultimer positivity within this population of gated cells was then determined. As can be seen in this example, a high percentage of circulating lymphocytes in both experimental transplant groups were positive for the specific TCR with 43.3% and 43.8% of mature $CD3^+/CD4^+$ lymphocytes exhibiting specificity for the TRP-1 Ultimer in the LV-TCR and TRP-1 Tg mice, respectively.

I further analyzed the entire pool of circulating lymphocytes in the peripheral blood, to measure the percentage of all lymphocytes that were specific for the TRP-1 Ultimer (**Figure 11**). At 1 month post-transplant, TRP-1 specific mature $CD3^+CD4^+$ T cells constituted less than 5% of all lymphocytes in all the transplant groups. However, by 4 months post transplant, differences in $CD3^+CD4^+$ $Ultimer^+$ populations were evident in the LV-TCR HSCs transplanted into DR4 Tg recipients (LV-TCR/DR4) versus those transplanted into the C57BL/6 recipient mice (LV-TCR/BL6) (15.8% ± 2 versus 8.2% ± 2, $p<0.05$). Statistically similar to the LV-TCR/DR4 group, 11.6% ± 2.5 of mature $CD3^+CD4^+$ lymphocytes from the TRP-1 TCR Tg HSCs transplanted into DR4 Tg recipients (TCR Tg/DR4) were specific for the TRP-1 TCR.

As seen on Table 2, by 6 months post-transplant this difference in TRP-1 TCR positivity between the LV-TCR/DR4 and LV-TCR/BL6 groups had narrowed and was no longer statistically significant (33% ± 4.6 vs 38% ± 7.5; $P > 0.05$). All groups showed continued increases in TRP-1 TCR positive T cells in the mature T cell population. TRP-1 TCR positivity in the peripheral mature $CD3^+CD4^+$ T cell populations was 33% ± 4.6 in the LV-TCR/DR4 transplants, 25% ± 4.7% in the LV-TCR/BL6 transplants, and 38% ± 7.5 in the TRP-1 Tg/DR4 transplants.

I subsequently performed two additional repeat competitive transplantation experiments with TRP-1 TCR lentivirus transduced HSCs and DR4 Tg recipient host mice (5 mice in each group) and performed similar peripheral blood and splenocytes analyses at various time points up to 12 months. My results demonstrate that the unmanipulated TRP-1 Tg HSC transplants on average had

a higher level of TRP-1 Ultimer positivity, particularly after the 4 month time point in all cellular compartments analyzed as compared to the lentivirally transduced TCR transplants. However, the LV-TCR transduced transplants demonstrated robust high level expression of the TRP-1 TCR up to 12 months post transplant. I also learned some valuable information regarding the timing of the emergence of lymphocytes into the periphery and the kinetics of reconstitution of the lymphocyte population in our TRP-1 TCR model. Lymphocytes expressing the TRP-1 TCR first emerge into the periphery around 4-6 weeks post transplant and steadily increase. I analyzed several different compartments within the peripheral mononuclear cell population including the total peripheral blood mononuclear cells, all $CD3^+$ cells, and finally the mature $CD3^+CD4^+$ T cell population. As a percentage of all cells in the peripheral blood, TRP-1 TCR positive cells steadily increased from about 2.5% at 2 months to 5% in 4 months for both the LV-TCR and the TRP-1 Tg transplants. However, by 12 months, the TRP-1 Tg transplants were significantly higher in the TRP-1 Ultimer positivity than the LV TCR transplants (8% ± 1.7 versus 22.6% ± 1.5; $P = 0.001$). (**Figure 12)**. When analyzing only the $CD3^+$ lymphocyte population, a similar pattern of emergence and steady expansion is seen in both the LV-TCR and TRP-1 Tg groups. However, at all time points the TRP-1 Tg group exhibited significantly higher levels of TRP-1 TCR expression peaking at 12 months, 17.8% ± 2.3 versus 36.7% ± 2.4; $P = 0.006$ (**Figure 13**). Finally, I analyzed the mature $CD3^+CD4^+$ T cell population (**Figure 14**). In this population, TRP-1 TCR expression peaked around 6 months at 75.4% for the TRP-1 Tg transplant and

48.1% in the LV-TCR transplants, and slightly decreased at 12 months. Even at 12 months post transplants, both groups maintained a high level of TRP-1 TCR express (65.2% ± 6.3 in the TRP-1 TCR group and 26.1% ± 2.4 in the LV-TCR group; $P = 0.004$).

Transduced T Cells Undergo Development in the Thymus

The vast majority of early lymphoid progenitors travel to the thymus and undergo a complex multi-step process of differentiation, proliferation, and repertoire selection (Walker and Abbas, 2002; Ohashi, 2003; Heinzel et al, 2007). Although much of this process occurs early in mammalian life, there also appears to be increasing clinical and experimental evidence that thymopoetic activity undergoes substantial re-amplification (or "thymic rebound") well into late adult life (Hakim et al, 2005). These findings are particularly true in patients successfully treated for HIV or following chemotherapy-induced lymphopenia and subsequent transplantation (Sfikakis et al, 2005). Given the results that both an appropriate restriction element (HLA-DR4) and co-receptor expression (CD4, not CD8) are critical for TCR expression, I sought to determine both the cellular density and compositional makeup of individual thymic subpopulations in 12 month old post-transplant recipients (approximately 14 months old overall).

The thymus of lentivirus transplanted mice were collected and analyzed. The thymii were surgically removed, mechanically homogenized, filtered and stained with TRP-1 Ultimer and antibodies against CD3, CD4, CD8 and CD45. Despite the old age of the mice, a high percentage of TCR specific gene expression was observed in double negative (DN), double positive (DP), and

single positive (SP) populations, these findings appear consistent with continued HSC production and thymic replenishment of gene-specific T cell progenitors. Analysis by flow cytometry revealed TRP-1 TCR expression at all stages of thymocyte development from the immature DN to DP and SP state (**Figure 15**). At 12 months 36% of DN thymocytes, 45% of DP thymocytes, 18% of $CD8^+$ thymocytes, and 13% of $CD4^+$ thymocytes expressed the TRP-1 TCR (**Figure 16**). Also observed was a steep and significant drop off (DP 45% ± 7.3; SP CD4 13% ± 3.1; $P = 0.002$) in TCR gene expression as thymocytes transitioned from DP to SP, results consistent with partial central elimination of auto-reactive TRP-1277-297-specific DR4-restricted specific T cells. Overall these findings appear in agreement with existing models of self-tolerance (Bouneaud et al, 2000; Gallegos and Bevan, 2004; Anderson et al, 2005), as well as those unique to TRP-1 in which higher avidity T cells are generated in mice genetically deficient in TRP-1 (Brandmaier et al, 2009).

Transduced $CD4^+$ T Cells Retain Specificity and Function

Splenocytes harvested from LV-TCR tranplants were tested *ex vivo* for specificity and function by ELISA measuring IFN-γ release. Splenocytes were fractionated for $CD4^+$ T cells by magnetic microbead separation as previousy described then co-cultured overnight with $DR4^+$ 1088 EBV B cells preloaded with a panel of TRP-1 specific and non-specific peptides and tumor lysates (**Table 3**). $CD4^+$ T cell populations showed dramatic release to cytokine in response to the TRP-1 peptide in a dose dependent manner. Furthermore, these T cells

recognized 624 melanoma tumor lysate, but not 1102 melanoma lysate which does not express high levels of TRP-1.

Development of Autoimmune Vitiligo

A result of the LV-TCR transduced HSC transplantation has been the development of spontaneous autoimmune vitiligo (**Figure 17**). Between 8-16 weeks post transplantation, LV-TCR mice were consistently found to develop progressive vitiligo (**Table 4**). This pattern of depigmented skin bordered by areas with no apparent signs of pigment loss resembled vitiligo development in other models of TRP-1 autoreactivity (Overwijk et al, 1999; Bowne et al, 1999; Leitner et al, 2003; Muranksi et al, 2008), as well those observed in patients that have responded to IL-2 treatment (Rosenberg and White, 1996), or those treated with myeloablative chemotherapy and the adoptive transfer of TIL (Dudley et al, 2002). A high proportion of transplant recipients regardless of DR4 background developed vitiligo when transplanted with either the LV-TCR HSC (16 of 19 mice) or the TRP-1 TCR Tg HSC (26 of 37 mice) and the DR4 Tg competitor bone marrow regardless of recipient background, while none of the control transplants including those with the LV-GFP and went on to develop autoimmune vitiligo. The emergence of spontaneous vitiligo in the C57BL/6 recipient mice indicates that thymic expression of HLA-DR4 and positive selection in the thymus is not necessary for autoimmune vitiligo. However, the lack of vitiligo in all mice receiving C57BL/6 competitor bone marrow indicates the necessity for a peripheral antigen presenting cell expressing the proper MHC restriction element. Similar vitiligo development was also observed in mice transplanted with HSCs

derived the TCR Tg, but none in the endogenous, non-transplanted TCR Tg (despite the higher gene-specific frequency), nor in those lacking expression of HLA DR4. These results suggested that myelodepletion and transplantation combined with specific immune reconstitution (either from LV gene-modified or Tg HSCs in combination with HLA-DR4 expression) provided the appropriate conditions for the development of autoimmune vitiligo.

To address the pathogenesis of autoimmune vitiligo, I analyzed both paraffin embedded and liquid nitrogen frozen skin samples from LV-TCR and control transplants by immunohistochemistry (IHC). A small patch of the skin sample was fixed in paraffin and stained for immunohistology using markers for CD3 and S-100 (a marker specific for neurocrest cells such as melanocytes). Sub-dermal hair follicle centered melanocytes were nearly completed obliterated with effacement of the normal architecture based on staining with H&E. Furthermore, affected skin samples demonstrated a dense increase in $CD3^+$ T cell lymphocytic infiltration in the vitiliginous dermal space surrounding the hair follicles but not in the control mice (**Figure 18**). Frozen sections of the vitiligo afflicted skin patches stained for CD4 and CD8 revealed that the lymphocytic infiltrate consisted of both $CD8^+$ and $CD4^+$ T cells (**Figure 19**). To determine if TCR gene-specific T cells were invading the sub-dermal compartment, sections were stained with an exclusively human TCR β-chain specific antibody (**Figure 20**). As observed, staining was both identical in pattern and location with the CD3 staining and distribution. To verify antibody specificity, human tonsil and

mouse spleen samples were similarly analyzed and found to be appropriately positive and negative.

Activation Status and Functionality of Transplanted T Cells

To further understand the pathogenesis behind post-transplant vitiligo, I investigated the activation status of TCR-specific CD4+ T cells. I had already observed that non-transplanted endogenous TCR Tg mice failed to develop vitiligo. Based on these early observations, I hypothesized that post-transplant specific T cells would exhibit surface bound phenotypic features consistent with spontaneous immune activation (Dutton et al, 1998; Klebanoff et al, 2005; Yang et al, 2006; Stockinger et al, 2006). To capture the phenotype of the TRP-1 TCR expressing $CD4^+$ T cells, I collected splenocytes from 6 month old transplanted mice and separated $CD4^+$ T cells using a no touch murine $CD4^+$ magnetic Microbead separation kit (Miltenyi Biotec). The $CD4^+$ T cells were then stained with the TRP-1 Ultimer and antibodies for CD4, CD44, CD45RB, and CD62L and analyzed by flow cytometry. Untreated, unmanipulated age matched DR4 Tg mice splenocytes were used as a control group. In the native DR4 Tg control group, the $CD4^+$ T cell population was $CD44^{low}$, $CD45RB^{high}$, and $CD62L^{high}$ indicative of a naïve phenotype. By comparison, the control transplant group exhibited a more generalized effector phenotype with $CD44^{high}$, $CD45RB^{high}$, $CD62L^{int}$ activation profile. In contrast, the activation profile of $CD4^+$/$Ultimer^+$ T cells from LV-TCR transplants was $CD44^{high}$, $CD45RB^{low}$, and $CD62L^{low}$ indicative of a T effector memory phenotype (T_{EM}). Importantly, the $CD4^+$/$Ultimer^-$ subpopulation was similar in its expression profile to that of the

control transplant population ($CD44^{high}$, $CD45RB^{high}$, $CD62L^{int}$), suggesting the divergence of two distinct T cell populations within the same host, the clonal-like T_{EM} $CD4^+$/$Ultimer^+$ vs. the polyclonal effector $CD4^+$/$Ultimer^-$, independent of the effects of transplantation (**Figure 21**).

Given the spontaneous T_{EM}-status of $CD4^+$/$Ultimer^+$ cells, I then addressed whether or not T cells from LV-TCR transplants, would immediately react to TRP-1 specific targets. To test this question, splenocytes from 6 month old transplants (both LV-TCR and control) were harvested then stimulated *ex vivo* for 24 hours with peptide or tumor. As seen **Figure 22**, cells obtained from the LV-TCR group responded to TRP-1 specific targets, secreting IFN-γ in response to TRP-$1_{277-297}$ at titering peptide concentrations and to B16-DR4 tumor, but not to control targets $HA_{306-318}$ or to control tumor MC38-DR4 tumor. These results appear consistent with a specific and high avidity (recognition was observed at 1 nM) T_{H1}-polarized T cell population.

Secondary Transplant Experiments and Integration Analysis

Following the successful primary transplantation of LV-TCR transduced HSCs, I performed three secondary transplant experiments using the bone marrow from the primary transplants. Bone marrow was harvested from the donor mice at the time of euthanization, in the LV-TCR transplant experiments as previously described. $5x10^5$ whole bone marrow cells were non-competitively transplanted per mouse into DR4 Tg mice (5 mice per group). Since these mice had previously received DR4 Tg competitor rescue bone marrow, a second competitive reconstitution was deemed unnecessary. All mice were followed and

analyzed at 6 months post transplant for CD3, CD4, CD8, CD45 and TRP-1 TCR expression (**Figure 23**). As shown, the levels of $CD4^+$ T cells expressing the TRP-1 TCR closely matched that of the primary transplants and remained stable for the duration of the analysis. TCR specific cells were identified at a level of 47.5% ± 8.3 ($CD45^+/CD3^+/Ultimer^+$) within the global CD3 compartment and at a level of 53.1% ± 10.7 within the CD4 sub-compartment ($CD45^+/CD3^+/CD4^+/Ultimer^+$). These results were notable for the higher expression levels observed within both populations compared with the 12 month expression levels observed in the parental primary transplant group (12 m primary vs. 6 m secondary, P = 0.02).

Having now demonstrated stable and high level TCR gene expression 18 months after a primary transplantation, I sought to further understand the genetic stability of the 6 month old secondary transplant recipients. Total RNA and gDNA was prepared from $1x10^5$ BM cells (from both LV-TCR and control transplants) and analyzed by both qPCR for relative TCR gene expression and by standard PCR using a vector specific probe to determine the number of lentiviral integrants. As seen in **Figure 24A**, using a probe specific for the variable chain of the humanized α subunit, TCR gene expression from LV-TCR transplants was found to be more than 1000-times greater than the background signal produced in control transplants but 43-fold less than that observed in the TCR-Tg group (P = 0.01 for LV-TCR vs. TCR-Tg). These results were consistent with differences in protein level TCR expression. Using a probe specific for the LV vector backbone, 4-6 integration sites and internal control fragment were

identified by nested PCR in 3 separate LV-TCR transplant samples, compared with control transplant (0 integrants) (**Figure 24B**). Both results clearly demonstrate long-term post transplantation genetic integration and stability.

Secondary LV-TCR Transplants Reject Subcutaneous Tumors

Immune reconstitutions after BM transplantation, dendritic cell vaccination, or following the adoptive transfer of antigen-specific lymphocytes have been shown to enhance anti-tumor immune responses (Borrello et al, 2000; Gattinoni et al, 2005; Moyer et al, 2006). Given the high level TCR gene expression, spontaneous autoimmunity, T_{EM} activation, and *in vitro* anti-tumor activity, I asked if long-term secondary transplant recipients would induce protective tumor immunity. To address this question, transplant recipients (both LV-TCR and control, 5 mice per group) were subcutaneously injected with B16-DR4 tumor cells. Because of the preexisting "activated" T_{EM} status of the transplanted animals no additional cytokines, immune modulators (such as α-CTLA-4) or vaccinations were added to enhance the anti-tumor effect. Beginning three days after injection, and every 3 days thereafter, the product of the perpendicular tumor diameters were recorded. As seen in **Figure 25**, secondary LV-TCR transplants significantly rejected tumor growth compared with control transplants over a 20 day time period. Tumor size between d12-d20 in the LV-TCR arm was also non-significant ($P = 0.09$) suggesting cessation of tumor growth.

Based on IHC staining of paraffin-embedded specimens, tumors from LV-TCR transplants but not controls were found almost entirely necrotic and associated with hemorrhagic infiltrates (**Figure 26**). Tumors were also found to

contain dense trafficking populations of $CD3^+$ and human TCR-β^+ (gene-specific) tumor-infiltrating lymphocytes (**Figure 26**), suggesting a similar infiltrative pattern as that observed within the autoreactive vitiligo skin specimens. The nova-red substrate was used instead of DAB to more effectively differentiate between necrotic tumor/pigment and the cellular infiltrate.

Gene Modification and Transplantation of Human HSCs

Running in parallel with the murine HSC transplant studies I conducted a series of transplant experiments to elucidate the characteristics and kinetics of human $CD34^+$ HSCs in non-obese diabetic/severe combined immunodeficient IL-2 receptor γ chain knock out (NOD/SCID-2) mice. $CD34^+$ HSCs were extracted from fresh cord blood by fractionation as described in **Materials and Methods**. In brief, fresh cord blood (less than 24 hours old) was treated with an equal volume of 2mM EDTA in PBS solution. 20ml aliquots of the cord blood/EDTA/PBS mixture were carefully layered on top of 20 ml of Ficoll plaque in 50 ml conical tubes. The cord blood was centrifuged at 1500 rpm for 30 minutes to fractionate the lymphocyte layer. The cell layer was collected and washed with 30 mls of the 2mM EDTA/PBS solution and centrifuged at 2000 rpm for 20 mins at 25^oC. The supernatant was discarded leaving behind the mononuclear cells. The $CD34^+$ cells were enriched for using a Miltenyi Biotec $CD34^+$ Microbead magnetic bead separation kit. Following selection of $CD34^+$ cells, the $CD34^+$ HSCs were incubated overnight at $5x10^5$ cells per well in 6 well plates with human prestimulation media (StemPro SFM34 Media, 1% L-glutamine, 1% penicillin/ streptomycin, 10ng/ml SCF, 100ng/ml TPO, 100ng/ml

IL-6). The following morning the cells were transduced using our established transduction protocol with the TRP-1 TCR lentivirus. Three days post-transduction, the cells were collected and a sample of the transduced $CD34^+$ cells was analyzed by flow cytometry using a CD34 antibody and the TRP-1 Ultimer. After confirmation of successful transduction, the transduced $CD34^+$ cells were transplanted into previously irradiated (350 rads) NOD/SCID-2 mice at a concentration of $2.5x10^4$ cells/mice along with $2.5x10^5$ DR4Tg BM cells. Peripheral blood lymphocytes were analyzed by flow cytometry with antibodies for human CD45, CD3, CD4, CD8, HLA-DR, murine CD45.2 and the TRP-1 Ultimer at set intervals up to 6 months post transplant.

Human lymphocytes were isolated by magnetic microbead separation for CD45 (Miltenyi Biotec) and analyzed for TRP-1 TCR expression. At 3 months post transplant, I initially observed a higher than normal number of double positive immature T lymphocytes in the peripheral blood (**Figure 27**). These double positive lymphocytes exhibited high levels of TRP-1 TCR expression (78%). By 4 months post transplant, the number of double positive T cells had drastically decreased to 2% of all $CD4^+$ T cells (**Figure 28**). At 4 months, 10.3% of all mature single positive $CD3^+CD4^+$ T cells expressed TRP-1 TCR as measured by Ultimer staining and flow cytometry.

TRP-1 TCR Confers Tumor Immunity

Five of the NOD/SCID-2 mice transplanted with LV-TCR modified HSCs were enlisted in a tumor rejection assay. A human melanoma line that had previously been transduced with a retrovirus encoding the gene for the MHC

class II transcription factor CIITA, 624 Mel CIITA was injected subcutaneously into the transplanted mice and 5 control NOD/SCID-2 mice. The engraftment and growth of the melanoma was carefully monitored and measured for 24 days post injection (**Figure 29**). As observed, none of the LV-TCR transplants developed measurable tumors. However, all of the control transplants allowed for the engraftment and growth of the melanoma. Tumors first became apparent in the control transplants at day 9 and continued to steadily grow up to 24 days post transplant.

V. Discussion

While most gene-therapy strategies have focused on the introduction of defective genes or antigens or used fluorescent gene products to track long-term development, few have attempted to introduce TCR genes into HSCs (Dudley et al, 2000). In those examples that do exist, TCR genes introduced with RVs have targeted only experimental antigens (OVA) (Morgan et al, 2003; Zhao et al, 2005). Herein, I report a new translational model of TCR-based immunotherapy directly applicable to patients with metastatic melanoma. I demonstrate both spontaneous autoimmunity and the destruction of subcutaneous melanoma that occur following long-term transplantation with gene-modified HSCs. My studies, based on the use of a high expression lentiviral system to deliver to HSCs a potent class II-restricted $CD4^+$ TCR, were assisted by neither the use of vaccines, cytokines, or immune modulators to either induce a population of autoreactive memory T cells or to achieve its antitumor effects.

To design a model encompassing features that would address both self-tolerance and tumor immunity and simultaneously serve as a potential source of preclinical information, I utilized a previously identified tumor-reactive, self-reactive, TRP-1-specific, DR4-restricted T cell clone. I then engineered both a high expression LV and a TCR Tg expressing the same TCR genes. As mentioned earlier, I focused on current generation SIN lentivectors, not only because of their enhanced capacity to stably infect metabolically dormant HSCs, but because of their improved biosafety profile compared with standard γ-RVs (Sinn et al, 2005; Levine et al, 2006). These experimental observations have

been further supported by clinical trials either ongoing or those in preparation for treatment of patients with HIV, β-thalassemia, Parkinson's disease, and Wiskott-Aldrich syndrome (Hargrove et al, 2008; Isacson and Kordower, 2008; Galy et al, 2008; D'Costa et al, 2009). With a functional LV-TCR and TCR Tg in hand, we demonstrate engraftment, thymic trafficking and differentiation, long-term TCR gene expression in both murine and humanized transplants, spontaneous post-transplant autoimmunity, and hemorrhagic necrosis of subcutaneous melanoma. Although experimental answers obtained from well-constructed transgenic models are largely precise and elucidating, they are also fraught with the potential for over-simplicity and a failure to translate into beneficial human medical care (Rivera and Tessarollo, 2008). In this model, I used the TCR Tg solely to gauge the efficiency of gene-transfer. The information gleaned from the TCR Tg was, therefore, designed to be in service to the greater translational value served by the LV-TCR.

Using these building blocks I propose a novel melanoma based murine model to examine the role of $CD4^+$ T cells in cancer immunology using a lentiviral delivery system to modify hematopoietic stem cells. The model involves four key components: 1) isolation of a T cell receptor reactive to a specific melanocyte differentiation antigen, tyrosinase related protein 1 (TRP-1); 2) development of a lentiviral construct for the delivery of the gene encoding the TRP-1 TCR; 3) creation of a constitutively TRP-1 TCR expressing transgenic mouse (TRP-1 TCR Tg) to study gene transfer efficiency; 4) transplantation of gene modified HSCs.

Effects of Lentiviral Transduction on HSC Transplantation

Through the transplantation experiments I was able to elucidate the kinetics of transplantation following manipulation of HSC with lentiviral constructs. Lentiviral transduction, although a traumatic and toxic event appears to have little or no deleterious effects on the ability of HSCs to home, engraft and reconstitute a lethally irradiated recipient. The initial experiments with lentivirus encoding GFP allowed me to approximate the kinetics of transplantation and effectively set the stage for the larger, more ambitious LV-TRP-1 TCR experiments. The general kinetics of transplantation of gene modified HSCs were consistent across all of the transplantation experiments. Early peripheral blood mononuclear cells began emerging from the bone marrow at around 6-8 weeks post transplant (**Figure 8, 9 and 11**). Total cell number increases dramatically from 2 months to 4 months, then levels off until it reaches a steady state around 6 months post transplant. These time points are mirrored in both the lentivirus transduced transplants and the unmanipulated TRP-1 Tg transplants (**Figure 11**) indicating that lentiviral transduction did little harm to the capacity of the HSCs to home, engraft and differentiated into blood component cells.

A permanently reconfigured immune system of durable high-frequency lymphocytes has long been a goal of immunologists and clinicians in pursuit of treatments for viral pathogens and cancer (Parish, 2003). Concerns persist, however, regarding the feasibility of delivering such a treatment within a narrow enough time-frame. Most clinical therapies and translational models have

focused on fully actualized therapies that deliver their effect with little delay, such as vaccines (peptide, virus, DCs, heat shock proteins, gene-modified tumor cells), cytokine infusions (IL-2, IL-7, IL-12, GM-CSF), immune modulators (myeloablative chemotherapy, anti-CTLA-4) or adoptive immunotherapies (TIL, cloned T cells, gene-modified T cells) (Parish, 2003; Rosenberg, 2004). Limitations and delays in immune reconstitution are a well described physiologic consequence of autologous HSC transfer, and the kinetics of individual mononuclear populations have been shown to favor the early return of granulocytes and B cells, with $CD4^+$ T cells often being the last subpopulation to return to normal levels (Talmadge et al, 1997; Laurenti et al, 2004). In this model, such observations were also found to be true. To improve T cell recovery, IL-7 administration has been successfully used in non-human primates (Storek et al, 2003) and patients in with cancer (Rosenberg et al, 2006). Nevertheless, the Achilles heel of such a potential treatment in patients pertains to the relative late emergence of the $CD4^+$ T cell subpopulation. Despite these concerns, the timing of maximum (or even normal) T cell recovery may not be the most meaningful physiologic end point of gene-modified HSC transfer. Although gene-specific T cells peaked 4-6 months after transplantation, low levels were detected after 6-8 weeks and chronologically correlated with the development of vitiligo (occurring between 8-12 weeks). These findings suggested that the magnitude of the TCR-specific frequency was less an important indicator of functionality than their qualitative behavior or the amplitude of their emergence into the periphery. Such observations have been supported by other models in

which small numbers of T cell clones differing only by activation status or T helper phenotype more effectively induce autoimmunity when compared with control populations (Lanzavscchia and Sallusto, 2002; Minang et al, 2010).

Given the lack of information pertaining to the kinetics of engraftment and development of lentivirus transduced HSCs, I was able to address several critical questions regarding the use of lentivirally transduced HSCs in bone marrow transplantation. These experiments indicate that the time from transplantation, engraftment, and emergence from the bone marrow and thymus to the periphery for lymphocytes is around 6-8 weeks. Furthermore, lymphocyte reconstitution happens briskly between 2-6 months then stabilizes after the 6 month time point and decreases slightly, but remains relatively stable for the duration of the experiments up to 12 months in almost all transplanted groups.

Peripheral Homeostatic Proliferation Leads to Expansion of Selective T Cell Clones

Homeostatic proliferation (HP) represents a naturally occurring multi-log expansion of lymphocytes in response to depletion or ablation followed by either BM reconstitution or adoptive transfer (Mackall et al, 1997; King et al, 2004). Immune responses that occur during HP appear to favor the dramatic expansion of T cells and lead to the precipitation of autoimmune events (King et al, 2004; Kruprica et al, 2006). These observations have been shown associated with a number of factors, including the increased availability of homeostatic cytokines (IL-7, IL-15), the depletion or inhibition or Tregs, generalized host inflammation, and the availability of self-ligands (Mackall et al, 1997; Fry et al, 2001; Boyman et

al, 2007). IL-7 availability, in particular, has also been found to be a critical factor in T helper and thymocyte survival along with the promoting of HP of both naïve and memory T cell subpopulations (Fry et al, 2001).

There are a number of possible explanations for the HP of TCR-specific $CD4^+$ T cells. Even though the importance of IL-7 has been clearly stated, I found that IL-7 receptor expression by flow cytometry on thymic DPs and SP CD4s as well as peripheral blood and splenic CD4s to be statistically no different within either the $Ultimer^+$ and $Ultimer^-$ population. On the other hand, although generalized antigen may be limiting to the vast majority of T cells, TRP-1 loaded DCs may be uniquely available to the $CD4^+/Ultimer^+$ subpopulation. Unlike most antigens, a fragment of the TRP-1 protein (the highest expressed of all melanocyte differentiation antigens) containing the 277-297 epitope has been shown to be secreted into the peripheral circulation (Xu et al, 1997). In theory, circulating TRP-1 could be captured by immature APCs (either in the skin or within peripheral lymphoid organs), that in turn become activated during the course of systemic injury from lethal irradiation (Hill et al, 1997; Zhang et al, 2002). Expansion of $CD4^+$ T cells has been tied to not only the relative load of antigen, but to the prolongation of that antigen load well after initial exposure (Obst et al, 2005; Jelley-Gibbs et al, 2005). With all this being said, the magnitude of the burst size may be a simple function of mass effect given the high frequency of gene specific HSCs and T cell progenitors introduced at the time of primary transplantation. Lin^- HSCs examined by flow cytometry 3 days

after transduction had TCR-specific levels that ranged between 35-70% (*data not shown*).

Other explanations may also account for both the magnitude of the burst size and the unique T_{EM} status of the $CD4^+$/$Ultimer^+$ subpopulation. In general, two competing models of memory T cell development have been proposed. The first involving a separate lineage from effectors, and the second proposing that T cells pass initially through an effector stage (Hu et al, 2001; Opferman et al, 1999). As a consequence of either scenario, T cell clones can then give rise to multiple effector and memory T cell populations, by means of asymmetrical cell division. After antigen exposure, memory cell expansion is classically followed by contraction, and stable persistence. Although I showed activation status at 6 months post primary transplantation (similar profiles were found at all time points), I found a sharp increase in the total $CD4^+$/$Ultimer^+$ T cell population between the 12 month primary and 6 months secondary transplants, consistent with both memory decay and re-expansion. I also found a splitting of two distinct T cell populations within the same host (the clonal-like T_{EM} $CD4^+$/$Ultimer^+$ vs. the polyclonal effector $CD4^+$/$Ultimer^-$) irrespective of the effects of transplantation. In the end, the $CD4^+$/$Ultimer^+$ T cell population may have had a competitive survival advantage and propensity for memory differentiation based on the relative high availability of TRP-1.

Transplantation of LV-TCR Transduced HSCs Breaks Self-Tolerance

The proposed TRP-1 TCR model allows for the novel study of a self-antigen specific TCR in the post-transplant setting. Although researchers in the

past have successfully transduced HSCs using viral systems none have explicitly characterized the fate of HSCs modified to express an endogenous self-antigen specific TCR. Dr. David Baltimore and colleagues transduced HSCs to express an OVA specific $CD4^+$ TCR. However, because of the highly immunogenic non-self nature of the antigen chosen it left unclear the effects of thymic selection on a normally occurring self antigen specific TCR such as TRP-1 (Yang and Baltimore, 2005). As noted in the **Results**, engraftment and development of HSCs transduced with LV-TCR appeared robust up to 12 months post transplantation with only slightly reduced numbers of circulating TCR specific T cells in transplants without the expression of the proper MHC Class II restriction element in the thymus (**Figure 9**). Initially, the LV-TCR/BL6 transplant group lagged behind the LV-TCR/DR4 transplants in terms of CD4+ T cell expression of TRP-1 TCR. The lag in TRP-1 TCR positive T cells in the LV-TCR/BL6 group is not altogether surprising given the lack of expression of HLA-DR4 in the thymic epithelium of C57BL/6 mice. As we saw previously (**Figure 7)**, in the non-transplanted native setting, TRP-1 TCR Tg mice on a C57BL/6 background exhibit little to no T cells in the peripheral with TRP-1 TCR expression. However, in the post-transplant setting a far higher number of TRP-1 specific T cells are found in the periphery. By 6 months, there is statistically no difference in TRP-1 TCR expression in the DR4 Tg and C57BL/6 recipients. Most likely, the initial depression in TRP-1 specific T cells in the C57BL6 transplants is due to central deletion. The lack of the proper HLA-DR4 restriction molecule in these recipient mice in conjunction with the expression of a self-antigen specific TCR leads to

the apoptosis and deletion of the T cell. However, some TRP-1 TCR expressing T cells do escape deletion and appear in the periphery as detected by flow cytometry. Considering that the sheer number of lymphocytes expressing the TRP-1 TCR has been artificially increased many fold higher than any naturally occurring TCR by gene modification, it is easy to imagine that the normal thymic deletion mechanisms might become overwhelmed. Once, these self-antigen specific T cells escape into the periphery, they would be confronted with a microenvironment rich in homeostatic cytokines due to irradiation of the recipient mice and subsequent elimination of competing lymphocytes. Further, these self-antigen specific T cells would encounter antigen presenting cells loaded with the TRP-1 antigen. In such a favorable stimulation- rich environment, the TRP-1 T cells could readily expand to fill the empty niche. This homeostatic and antigen driven peripheral expansion would readily explain the transplantation kinetics I have observed in the transplant experiments.

In TRP-1 model, vitiligo developed in C57BL/6 recipients (along with a corresponding similar level of TCR specific cells **Table 3**), not just in DR4 Tg recipients. These findings underscore a broader range of effects by the gene-specific $CD4^+$ T cell population, regardless of the relative magnitude of circulating cells present. The polarized T_{H1} production of IFN-γ may have produced multiple effects, including direct melanocyte cytotoxicity, up-regulation of MHC molecules that enhance target recognition, and an enhancement of APC activation leading to further amplification of the T helper response. Such functions have been well associated with the unique, but underutilized properties of $CD4^+$ T cells. While

long-term cellular persistence was an initial objective of this model, the amplitude of gene-specific T cell expansion (between 8-24 weeks) and T_{EM} activation were clearly dramatic, and we propose associated with both autoimmunity and antitumor activity.

These transplant experiments hint that in the myelodepleted irradiated state engraftment and selection of TRP-1 specific $CD4^+$ T cells does occur as indicated by the observance of spontaneous autoimmune vitiligo in these HLA-DR4 negative recipient mice. I thus, hypothesize that although central selection in the thymus may be a significant factor in maximizing the selection of TRP-1 TCR specific $CD4^+$ T cells, mechanisms of selection and homeostatic proliferation exist that either bypass or overwhelm the normal selection of T cells in the thymus.

Conclusion

These results highlight several important findings regarding reconstitution of the hematopoietic cells in the post transplant setting: 1) the expression of the proper MHC Class II restriction element, HLA-DR4, in the thymic epithelium may be necessary for the maximal central selection of T cells. However, peripheral homeostatic expansion of peripheral lymphocytes in the context of HLA-DR4 can compensate for central deletion, 2) the LV-TCR transduced HSCs competed, engrafted, and developed comparably to the unmanipulated TCR Tg HSCs indicating that lentiviral transduction did not adversely affect the capacity and function of the HSCs, 3) the lentiviral gene delivery system successfully led to a high level of circulating T_{EM} $CD4^+$ lymphocytes expressing the TRP-1 TCR.

The most clinically significant evidence I present is the near total liquefaction of tumor following subcutaneous implantation in long-term secondary transplants. These results demonstrate not only potent antitumor activity 18 months after initial transplantation, but the dense cellular trafficking of LV-TCR specific T cells. While many adoptive immunotherapies have either maximized their therapeutic effect or been totally dependent upon the addition of cytokine administration, vaccination, myelodepletion (lethal irradiation or RAG KOs) or the use of immune modulators (α-CTLA-4) I elected to demonstrate this effect without such assistance (Overwijk et al, 2003; Quezada et al, 2010). I propose that these freestanding antitumor effects were likely secondary to both the HP and the spontaneous T_{EM} activation of TCR-specific cells. In conclusion, I suggest that this novel translational model of immunotherapy, utilizing the unique properties of HSC transplantation, LV technology, and $CD4^+$ T cells, has the opportunity to bring new medical care to patients with metastatic melanoma.

VI. Limitations and Future Directions

Increased Efficiency of TCR Expression

A major potential limitation is the inefficient expression of our bicistronic TCR gene *in vivo*. I have shown in preliminary optimization studies that the F2A construct is superior to the IRES construct in expression of the TCR; however, the successful transcription and translation of such a long single protein chain might be limiting and reduce the expression of both gene products. Moreover, a single general promoter such as CMV might not be sufficient to drive the expression of high levels of our bicistronic gene in developing lymphoid cells. Along with inefficient expression of both chains of the TRP-1 TCR, mismatched pairing of the α and β chain to endogenously expressed α and β chains may further reduce the functional efficiency of transduced lymphoid cells to recognize TRP-1.

To address these concerns I have investigated the possibility of constructing a dual promoter lentiviral construct, with each chain of the TCR under the control of two separate promoters. Although, the implementation of the natural lymphocyte specific CD2 promoter would be favorable, the CD2 promoter has never been shown to be a strong enough promoter to drive the expression of a TCR taken out of its natural context. As such, I have focused my attention on two potent, general promoters, EF1α and CMV. The use of independent dual internal promoters in a bicistronic lentiviral construct has shown increased expression of the transgenes in $CD34^+$ HSCs as compared to a single promoter construct. In particular, the dual promoter bicistronic constructs appear to be

advantageous in the expression of the downstream cistron (Yu et al, 2003; Ben-Dor et al, 2006). Furthermore, I am seeking to increase the efficiency of transduction by implementation of several other lentiviral envelope proteins such as the feline endogenous virus RD114 glycoprotein and the murine leukemia virus glycoprotein. One or both of these alternative envelope proteins might prove to be either more effective or less toxic in transducing HSCs.

Mechanisms of Autoimmune Vitiligo

These transplantation experiments with donor HSCs from both TRP-1 Tg mice and TRP-1 TCR lentivirus transduced HSCs have demonstrated consistent spontaneous autoimmune vitiligo. Transplantation of GFP lentivirus transduced HSCs into irradiated mice did not develop vitiligo in prior experiments indicating that this phenomenon is an antigen specific event associated with the TRP-1 TCR. My data indicates that the myelodepletion of recipient mice coupled with the homeostatic proliferation of highly self-reactive TRP-1 TCR $CD4^+$ T cells in the post transplant setting breaks the normal tolerance mechanisms leading to autoimmunity. Furthermore, no other autoimmune responses were observed in TRP-1 TCR transduced HSC transplanted mice. The autoimmunity appears to be a melanocyte specific response to the transplantation of TRP-1 TCR expressing HSCs. The breaking of self-tolerance is a significant step in priming the immune system to promote tumor regression, and numerous studies have demonstrated autoimmune vitiligo as a side effect of melanoma immunotherapy in both mice and humans (Garbelli et al, 2005; Okamoto et al, 1998; Overwijk et al 1999). The cellular mechanism of autoimmune vitiligo in melanoma immunity

is unknown. Although, melanocyte destruction might ultimately depend on lysis by $CD8^+$ effector T cells, Ihypothesize that the breaking of tolerance and development of vitiligo is a TRP-1 antigen specific $CD4^+$ T cell dependent response. The elucidation of the cellular mechanisms of autoimmune vitiligo is a future direction of the project. I will establish a series of transplants with lentivirally transduced HSCs. Both C57BL/6 and DR4 Tg mice will be transplanted with TRP-1 TCR lentivirus transduced HSCs. Recipient mice developing autoimmune vitiligo will be analyzed at set intervals (onset of vitiligo, 2, 4, 6, 8, 12 wks post onset of vitiligo). Lymphocytes harvested from peripheral blood, spleen, bone marrow, and thymus will be characterized for phenotypic markers (CD45.1, CD45.2, CD3, CD4, CD8, CD25, Foxp3, human $\alpha\beta$ TCR, and TRP-1 Ultimer) by antibody staining and flow cytometry. As described previously, the epidermis of these mice will be fixed in formalin and sent for histologic staining for CD3, S-100, IL-2 and IL-17 to observe the lymphocytic infiltrations typically found in vitiligo. We will seek to characterize the cytokine profile of the lymphocytes by co-culture and subsequent ELISA and ELISPOT for IFN-γ, IL-2, and IL-17 as previously described.

Lymphocytes harvested from the spleen of recipients developing vitiligo will be analyzed for activation status. $CD4^+$ T cells will be selected by using a Miltenyi Biotec CD4 Microbead Kit. The $CD4^+$ T cells will then be stained with antibodies for activation status markers CD44, CD45RB, CD62L, CD69, and CCR7 and phenotype markers CD4 and the TRP-1 Ultimer. Analysis by flow cytometry should allow us to capture the activation status of the $CD4^+$ T cells in

general and the TRP-1 TCR^+ T cells in specific. I have previously run this activation status experiment on naïve BoyJ and DR4 Tg mice splenocytes and will use the activation status marker profile from these prior experiments as the naïve control group for comparison to the vitiligo transplant mice. *In vitro* experiments to test for melanocyte recognition and destruction will be established by directly placing splenocytes to an established HLA-DR expressing melanocyte culture with or without administration of various cytokines such as IL-2 and IL-17.

After characterization of the lymphocytes and timing of the development of vitiligo in the transplanted mice we will establish two transplant groups (6 DR4 Tg mice per group) which will undergo either $CD4^+$ or $CD8^+$ T cell depletion by specific anti-CD4 and anti-CD8 antibodies from hybridoma cell lines (ATCC, Manassas, VA, USA). Following irradiation and transplantation of either TRP-1 TCR Tg HSCs or TRP-1 TCR lentivirus transduced HSCs, recipient mice will receive a total of six intraperitoneal injections of either rat anti-mouse CD4 (clone GK1.5) or rat anti-mouse CD8 (clone 2.43) monoclonal antibodies in a PBS solution. Normal rat IgG will be used as a control group (Miyazaki et al, 2005). The exact timing of the injections will be set to occur prior to and during the peak occurrence of vitiligo as observed in the previous transplant group. The transplanted mice will be carefully monitored for the development of vitiligo and lymphocytes collected from the peripheral blood will be analyzed by flow cytometry and antibody staining for CD45.1, CD45.2, CD3, CD4, CD8, and TRP-1 Ultimer on weeks 4, 6, 8, 10, 12, 16, 20 and 24.

Spontaneous autoimmune vitiligo is a significant development in our mouse melanoma model, and an understanding of the cellular mechanisms involved in the development of vitiligo in the transplanted mice could have potential implications for the ultimate goal of treating melanoma by transplantation of HSCs. I fully expect that many of the lentivirus transduced HSC transplant recipients will develop vitiligo as in the previous experiments. In the irradiated lymphodepleted mice, I suspect that the high expression of the TRP-1 TCR in the transplanted HSCs will overcome the normal tolerance mechanisms allowing for the selection and proliferation of these TRP-1 self-peptide specific T cells. The exact timing of the development of vitiligo and kinetics of lymphocyte development and activation will allow me to properly time the injections for $CD4^+$ and $CD8^+$ T cell depletion *in vivo*. In the earlier transplants, low levels of lymphocytes became detectable around 4-6 weeks. The development of vitiligo will lag behind the reconstitution of the bone marrow following irradiation and transplantation. My data indicates vitiligo to occur between 6-10 weeks post transplant

Due to the limitations of formalin fixed paraffin embedded immunohistology, our analysis of lymphocytes in the skin has been limited to staining for CD3, S-100, and the cytokines IFN-γ, IL-2 and IL-17. I am currently working to circumvent this limitation by developing a method to directly analyze the lymphocytes in the skin by flow cytometry. The preliminary experimental approach involves the harvesting of lymphocytes from the skin of vitiliginous mice by disruption and enzymatic digestion by dispase and collagenase similar to

protocols used in the extraction of thymocytes from whole thymus used by other researchers. In brief the procedure involves the harvesting of the entire epidermis from the mice, and subsequent disruption using a surgical scalpel. Following disruption, the tissue undergoes a series of digestions with collagenase and dispase. The cellular mixture is then carefully layered over Ficoll plaque and centrifuged at 1200 rpm for 20 minutes. The cellular layer is than subsequently collected for analysis. Lymphocyte yields from this approach have been limited to approximately 50,000 cells per mice, which has been sufficient to perform simple flow cytometric analysis. However, the results have been inconclusive. I will continue to refine this technique to increase the yield and better characterize the phenotypes of the lymphocytic infiltrates demonstrated in the skin of the vitiligo mice.

The $CD4^+$ and $CD8^+$ T cell depletion experiments will allow for a better understanding of the roles that these two cells play in the development of autoimmune vitiligo. Most likely, the $CD8^+$ T cells act as the effector T cells that directly destroy the melanocytes and will be found to be necessary for the development of vitiligo. However, I suspect that the TRP-1 specific $CD4^+$ T cells are equally necessary for the development of vitiligo, either by release of IL-17 and subsequent recruitment of inflammatory cells, or perhaps by activation of dendritic cells and subsequent increased presentation of antigen to naïve $CD8^+$ T cells as in the cross presentation model discussed earlier. Furthermore, it is possible that these TRP-1 specific $CD4^+$ T cells might directly lyse melanocytes.

Skewing the T Cell Repertoire

Ther overall strategy of this thesis was to conduct a series of lentivirus transduced HSC bone marrow transplants as described above. In future experiments I will seek to manipulate the reconstitution of the T cell repertoire by early vaccination of the transplanted mice with a recombinant vaccina virus encoding a gene for the human TRP-1 peptide (rVV-hTRP-1). The optimal immunization schedule is unknown, therefore, I will implement immunization at two different time points (8 wks and 12 wks post transplant). To measure the kinetics and effects of immunization I will analyze the transplanted mice at various time points (2, 4, 6, 8, 12, 16, 20, and 24 wks) and in various organs (thymus, spleen, inguinal lymph nodes, peripheral blood, and bone marrow). As previously, I will concurrently transplant TRP-1 Tg Lin^- cells into DR4 Tg recipient mice to use as a comparison group to the lentivirus transduced transplant group.

I will analyze the lymphocytes from the various organs by flow cytometry implementing fluorescent antibodies to CD45.1, CD45.2, CD4, CD8, CD3, human $\alpha\beta$ TCR, and TRP-1 Ultimer. In addition I will seek to capture the activation status of the immune response by flow cytometry of various markers for naïve T cells (TRP-1 Ultimer, $CD45.1^+$, $CD4^+$, $CD25^{low/int}$, $CD44^{low}$, $CD45RB^{high,}$ $CD62L^{high}$, and $CD69^{low}$), effector T cells (TRP-1 Ultimer, $CD4^+$, $CD25^{high}$, $CD44^{high}$, $CD45RB^{high}$, $CD62L^{int}$, $CD69^{high}$), and memory T cells (TRP-1 DR4 Ultimer, $CD4^+$, $CD25^{low}$, $CD44^{high}$, $CD45RB^{low}$, $CD62L^{low}$, $CD69^{low}$).

I will seek to capture the functional capacity of the TRP-1 TCR $CD4^+$ T cells to specifically recognize TRP-1 peptide in the context of HLA DR4 MHC

Class II molecule by *ex vivo* co-culture and ELISA and ELISPOT analysis for release of cytokines to peptide loaded APCs, tumor lysates, and whole tumors. Co-cultures will be established following the protocols previously described. ELISA and ELISPOT will be conducted by standard established protocols.

I will further seek to manipulate the post transplant host environment to favor the repopulation of the T cell repertoire by administration of the homeostatic proliferation promoting cytokine, IL-7. Increasing evidence indicates that post-transplant administration of IL-7 increases T cell regeneration (Mackall et al, 2001). I will establish TRP-1 TCR lentivirus transduced HSC transplants as described above and test the effects of IL-7 on T cell survival and proliferation. IL-7 will be administered via intraperitoneal injection (10μg/injection) twice a day for seven consecutive days and started immediately after the first immunization, or not given at all (control arm). To control for non-specific mimicking of TCR signaling through IL-7, I will also incorporate a control immunogen using the MDA, gp100 (gene-gun gp100 plasmid or DC's transduced with an adenovirus encoding gp100). Fourteen days after the 3rd immunization with/without IL-7, animals will be sacrificed and tissues harvested (lymph nodes and spleen). Samples will then be assayed by flow cytometry for changes in frequency, absolute numbers, and activation status as previously described. As before, changes in T cell function will be assessed using ELISPOT and ELISA assays for specific responses to TRP-1 peptide, tumor lysates, and whole tumor cells to measure changes in T cell avidity.

Tumor Immunity

While this study has established the protective effects of LV-TCR transduced HSC transplantation to reject subcutaneous tumor, I have not tested the potential to treat established tumors. I will test the capacity of the TRP-1 TCR $CD4^+$ T cells to mediate tumor immunity by a series of adoptive transfer experiments into recipient mice with previously established melanoma tumors. Subcutaneous tumors will be established in DR4 Tg mice by the injection of $1x10^4$ B16 tumor cells suspended in 100 μl of HBSS into the right and left flanks of 5 DR4 Tg mice 14 days prior to adoptive transfer. Lymphocytes harvested from the spleen of TRP-1 TCR lentivirus transduced HSCs transplanted mice will be adoptively transferred at $1.5x10^7$ splenocytes per mice via tail vein injection. Tumor volume will be assessed twice weekly using calipers to measure tumor growth or regression. Tumor area will be calculated as the product of width and length.

I will further seek to elucidate the cellular mechanisms of any observed tumor regression by selective adoptive transfer of either $CD4^+$ or $CD8^+$ T cells from the splenocytes of TRP-1 TCR transduced HSC transplant mice. $CD4^+$ and $CD8^+$ T cells will be selected using a Miltenyi Biotec $CD4^+$ and $CD8^+$ Microbead Selection Kit and adoptively transferred into previously established melanoma injected in DR4 Tg recipient mice as described above. Similarly, I will deplete individual T cell populations starting 3 days post transfer using antibodies specific for $CD4^+$, $CD8^+$, and $CD25^+$ T cells. Adoptive transfer experiments will be performed as described above with splenocytes from TRP-1 TCR lentivirus

transduced HSC transplant recipient mice. Anti-CD4, anti-CD8, and anti-CD25 antibodies from hybridoma cell lines will be administered as intraperitoneal injections (0.1 mg/mouse) every 3 days post adoptive transfer until the day of analysis. Tumor width and length will be measured to assess tumor regression.

Active, specific, persistent tumor immunity is the ultimate goal. As observed with the development of autoimmune vitiligo in the transplanted mice, the TRP-1 TCR is fully capable of recognizing TRP-1 peptide and actively mediating the destruction of TRP-1 expressing melanocytes *in vivo*. The cellular mechanism and the role of $CD4^+$ and $CD8^+$ T cells in this antitumor response are unclear. However, I hope to elucidate some of the basic roles played by these T cell populations with the T cell population depletion and specific $CD4^+$ and $CD8^+$ T cell adoptive transfer experiments. Furthermore, I seek to better understand the post transplantation host conditions that will promote the survival and proliferation of our gene modified HSCs by immunization and administration of IL-7.

Building on prior experiments investigating the basic effects of lentiviral transduction on HSC transplantation and elucidating the mechanisms of autoimmune vitiligo, I hope to address essential questions regarding enhancing tumor immunity by antigen presentation and transplantation of gene modified HSCs. Ultimately, I hope that these findings can be the basis for a clinical trial with human patients with metastatic melanoma.

	MOI	TCR-IRES	TCR-F2A	GFP	p=
JRT3	3:1	35.6	48.7	92.0	0.023
	10:1	76.7	94.1	98.0	0.022
PBMC	1:1	3.0	2.0	43.0	NS
	3:1	6.0	3.0	86.0	NS
	10:1	18.0	14.0	92.0	NS
	30:1	26.3	37.3	ND	0.012
	100:1	37.5	56.4	ND	0.0008

Table 1. Comparison of IRES and F2A lentiviral transduction experiments in Jurkat (JRT3) and human peripheral blood mononuclear cells (PBMC). A lentiviral construct encoding the green flourescent protein (GFP) was used as a control group to test the efficiency of the transduction protocol. Both the IRES and F2A constructs efficiently transduced JRT3 and PBMCs, however, the F2A construct proved to be more efficient, particularly in PBMCs at high multiplicities of infection (MOI) (p=0.012 at MOI of 30:1, p=0008 at MOI of 100:1).

Txp Groups	All CD4	CD45.1/CD4
LV-TCR/DR4	33% +/- 4.6	64% +/- 4.1
LV-TCR/BL6	25% +/- 4.7	ND
TCR-Tg/DR4	38% +/- 7.5	ND
LV-GFP	23% +/- 4.2	ND

Table 2. Analysis of $CD4^+$ T cell compartment in LV-TCR transduced and TCR-Tg Lin^- HSC transplants into either irradiated DR4 or C57BL/6 recipient mice (5 mice per group). Peripheral blood mononuclear cells were collected and stained with fluorescent antibodies for murine CD3, CD4, CD8, CD45.1 and the TRP-1 pentamer at 6 months post transplantation. Antibody binding to surface markers was measured by flow cytometry as previously described. At 6 months transplantation there was no statistical difference between the LV-TCR transduced and TRP-1 Tg transplant groups (33%+/-4.6 vs 38%+/-7.5). The LV-TCR transplants into C57BL/6 recipients also exhibited Ultimer specificity at 25%+/-4.7. LV-GFP was used as a transduction and transplant control group. In the LV-TCR/DR4 transplant group, 64%+/-4.1 of $CD45.1^+/CD3^+/CD4^+$ T cells were specific for the TRP-1 Ultimer.

Targets	IFN-γ (pg/ml)
CM	2
HA 100 uM	17
TRP1 100 uM	1390
TRP1 10 uM	912
TRP1 1.0 uM	255
TRP1 0.01 uM	61
1102 Mel	19
624 Mel + IFN	856

Fractionated $CD4^+$ splenocytes

Table 3. IFN-γ release as measured by ELISA in splenocytes harvested from LV-TCR transplants 12 months post transplantation. Cell media (CM), negative control peptide (HA), TRP-$1_{277-297}$ epitope at increasing concentrations (TRP1), control 1102 melanoma cells (1102 mel), and 624 melanoma whole tumor cells pretreated with IFN (624 Mel + IFN).

Primary HPSC	Competitor BM	Txp Host	Vitiligo	Onset	Vitiligo/Total
Txp LV-GFP	DR4 TG	DR4 TG	No	NA	0/15
Txp LV-TCR-F2A	DR4 TG	DR4 TG	Yes	8-16 weeks	11/14
Txp LV-TCR-F2A	DR4 TG	C57BL/6	Yes	8-16 weeks	5/5
Txp LV-TCR-F2A	C57BL/6	C57BL/6	No	NA	0/5
Txp TCR TG	DR4 TG	DR4 TG	Yes	6-10 weeks	17/25
Txp TCR TG	DR4 TG	C57BL/6	Yes	6-10 weeks	9/12
Txp TCR TG	C57BL/6	C57BL/6	No	NA	0/5
Non-Txp TCR+/DR4+	NA	NA	No	NA	NA
Non-Txp TCR+/DR4-	NA	NA	No	NA	NA

Table 4. Summary of development of spontaneous autoimmune vitiligo in all transplant groups. The development of vitiligo as measured by external phenotypic changes in coat color was recorded for all transplant groups. As shown in the table the development of vitiligo was dependent on the presence of the TRP-1 TCR (via lentiviral transduction or the TCR transgenic) and the proper MHC class II DR4 element (via the competitor BM).

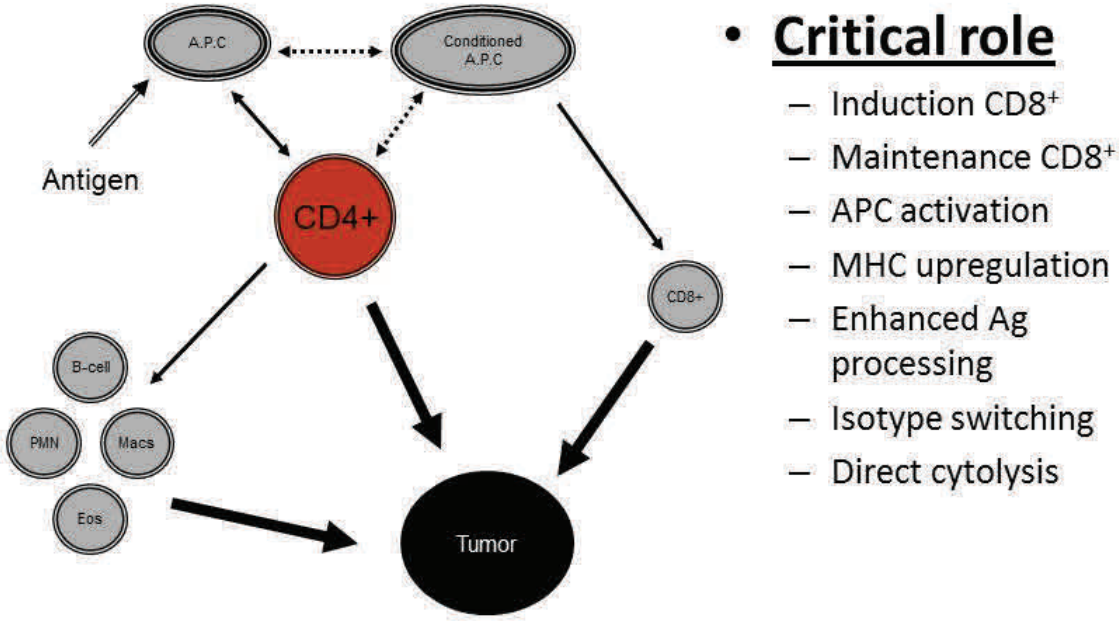

Figure 1. Central role of $CD4^+$ T cells in tumor immunity. $CD4^+$ T cells are involved in: 1. The induction and maintenance of $CD8^+$ T cells. 2. The activation of antigen presenting cells (APC) 3. the upregulation of MHC molecules. 4. Enhanced antigen processing. 5. B-cell isotype switching. 6. Direct cytolysis of tumor cells.

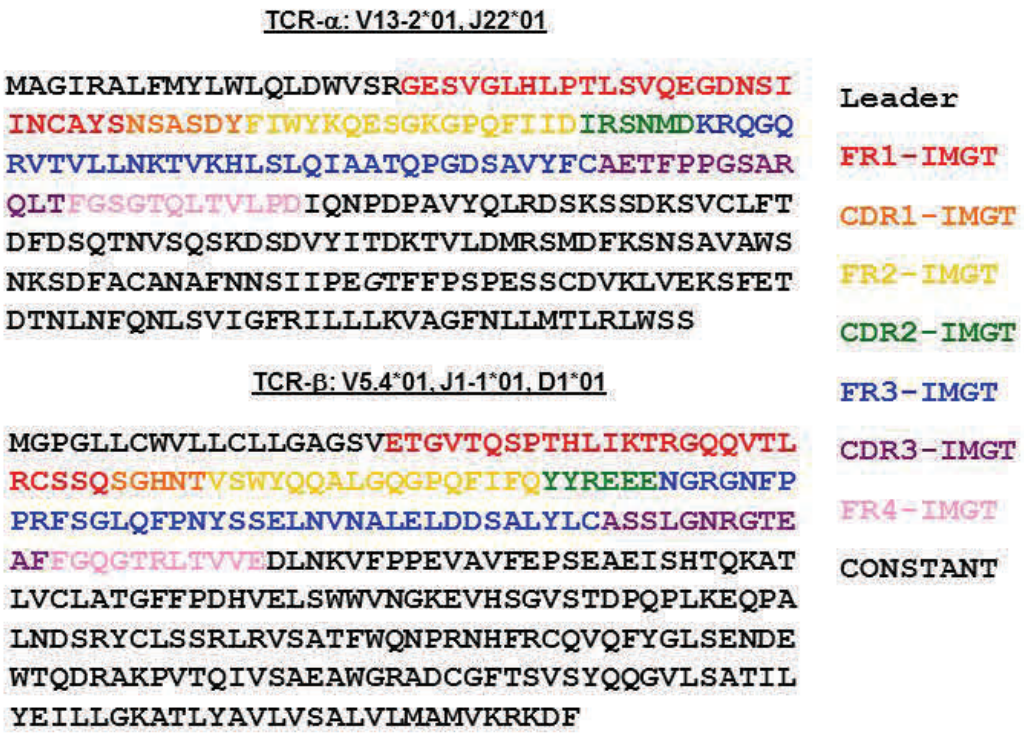

Figure 2. Cloned α and β TCR subunits of TRP-1/DR4 Clone-2**.** The specific VDJ regions of the α and β chains were analyzed and identified using the V-QUEST search engine from the IMGT website (http://imgt.cines.fr/IMGT_vquest/vquest?livret=0&Option=humanTcR).

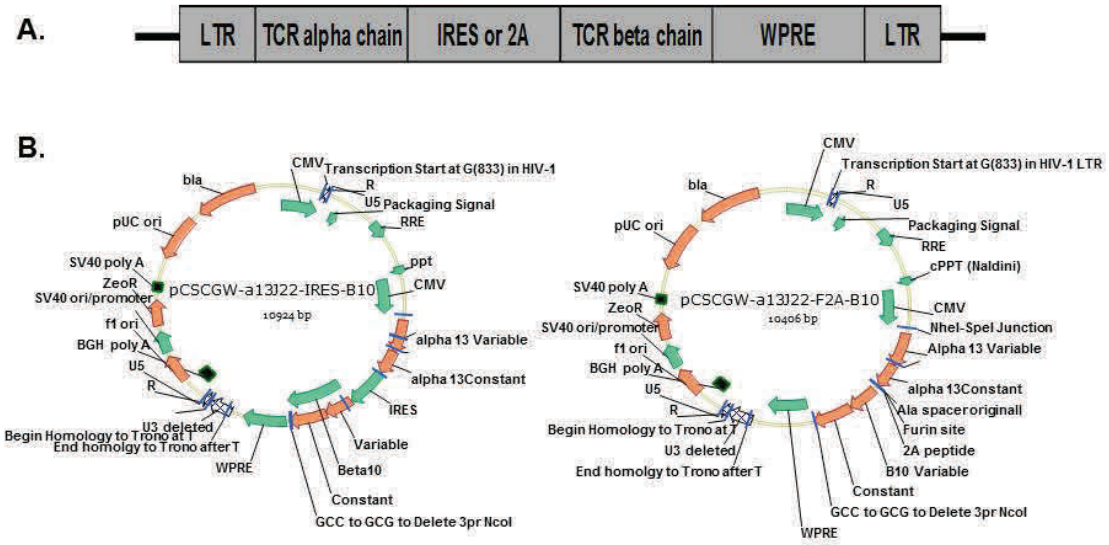

Figure 3. Schematic diagrams of the TRP-1 TCR lentiviral constructs. **A.** Schematic of lentiviral constructs implementing either an internal ribosomal entry site (IRES) or Furin self-cleavage site (2A) between genes encoding the a and b chains of the TRP-1 epitope specific TCR. **B.** Detailed diagrams of the TRP-1 TCR lentiviral constructs. The non-specific general promoter of the cytomeglovirus (CMV) was chosen to drive the expression of the downstream a and b chains.

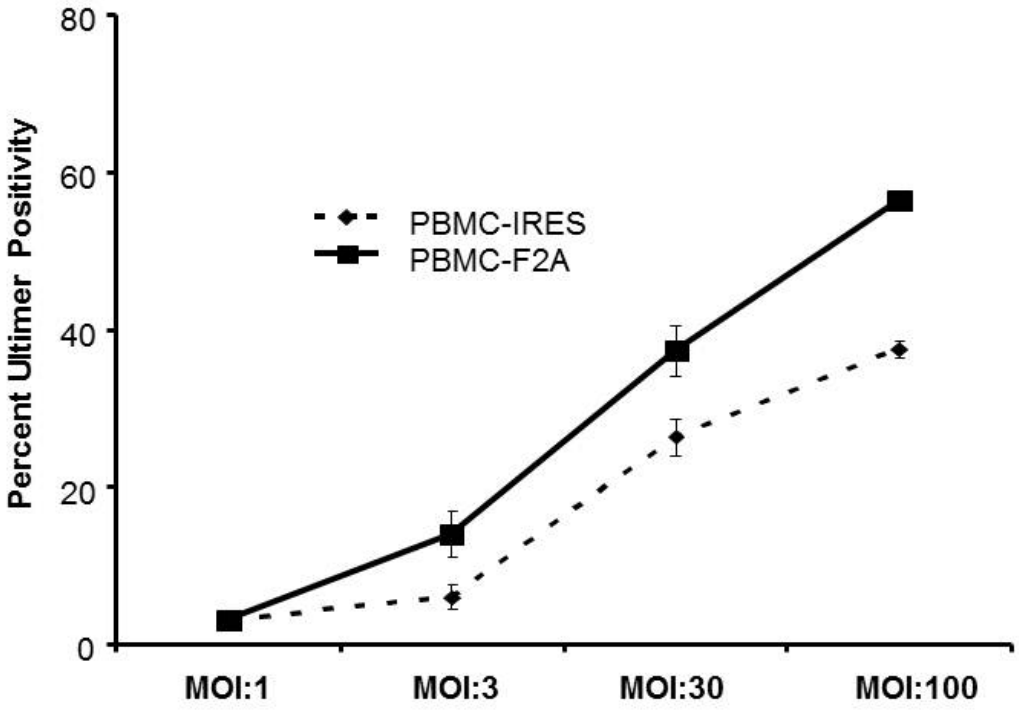

Figure 4. Comparison of IRES and F2A lentiviral transduction experiments in human peripheral blood mononuclear cells (PBMC). Both the IRES and F2A constructs efficiently transduced PBMCs, however, the F2A construct proved to be more efficient, particularly in PBMCs at high multiplicities of infection (MOI) ($p=0.012$ at MOI of 30:1, $p=0008$ at MOI of 100:1).

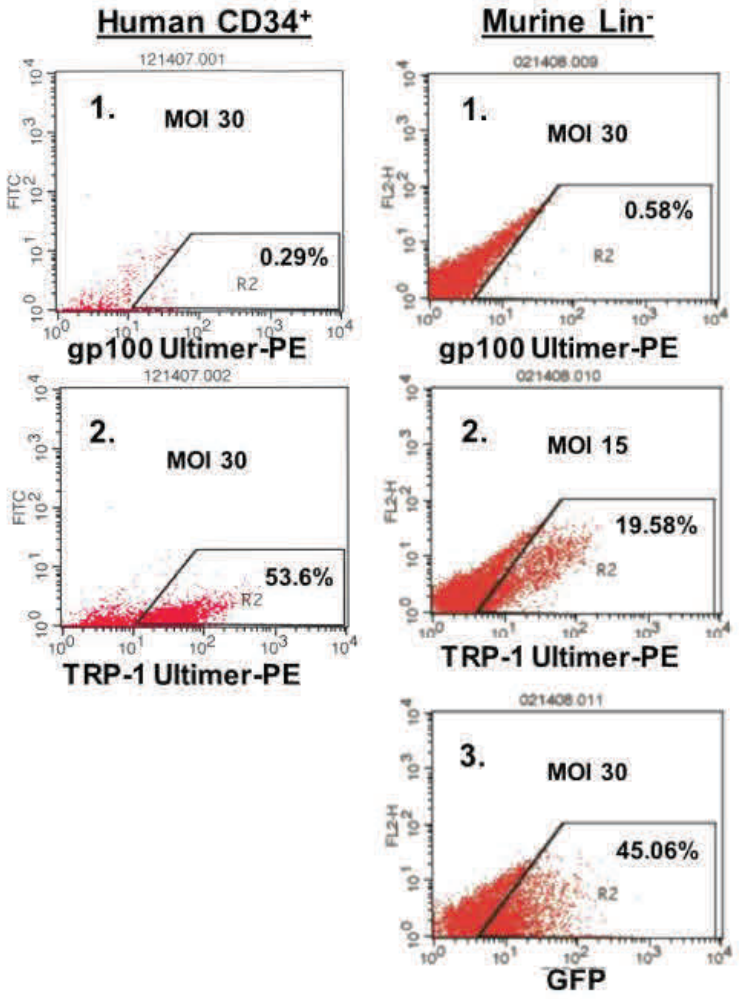

Figure 5. Representative flow cytometry results for lentiviral transduction of human CD34^{+} and murine Lin^{-} cells with the TRP-1 TCR F2A lentiviral construct. A gp100 specific pentamer was used as a negative control to gauge the background non-specific binding of pentamer complexes, shown in row 1. A lentivirus encoding the green fluorescent protein (GFP) was used as a positive control for the transduction protocol as shown row 3. Flow cytometric analysis was performed 72 hours post transduction using a TRP-1 TCR specific Ultimer (Proimmune, Inc.).

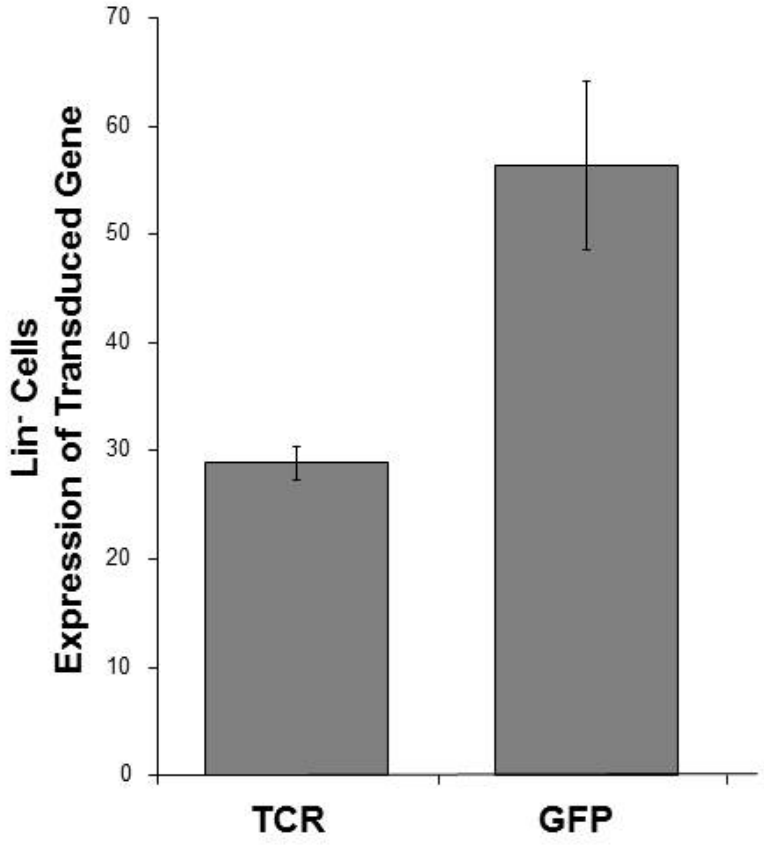

Figure 6. Lentiviral constructs efficiently transduce murine Lin- cells and leads to high expression of transduced gene (28.7%±3) A lentivirus encoding the green fluorescent protein (GFP) was used as a positive control for the transduction protocol (56.2%±8) Flow cytometric analysis was performed 72 hours post transduction using a TRP-1 TCR specific Ultimer (Proimmune, Inc.). A gp100 specific Ultimer was used as a negative control to gauge the background non-specific binding of pentamer complexes. The results represent five separate transduction experiments with at least 3 wells of independent cells in each group.

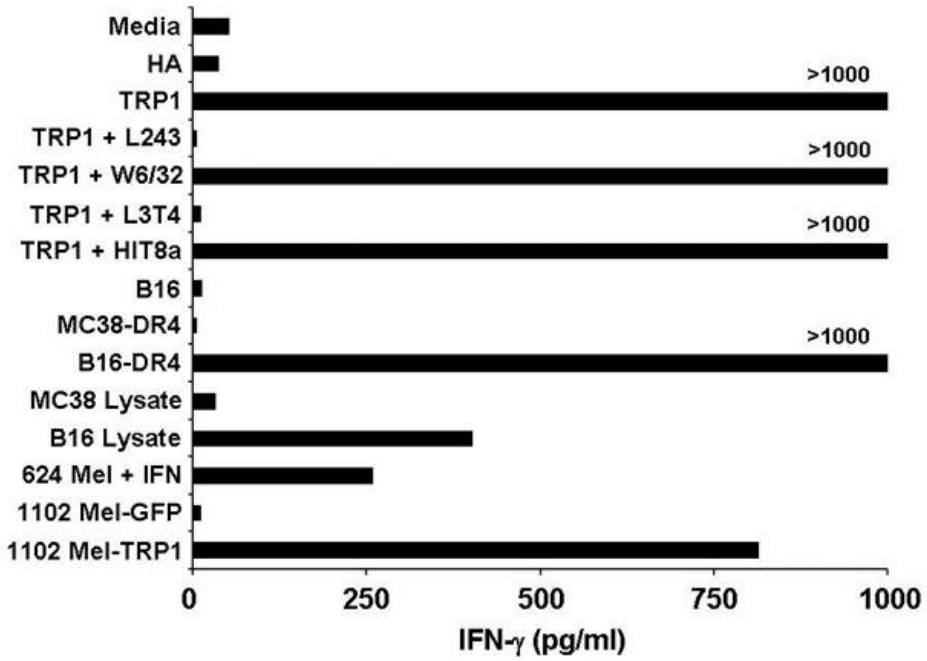

Figure 7. Peripheral blood mononuclear cells transduced with LV-TRP1-F2A (LV-TCR) are functionally specific. Specific IFN-γ production detected by ELISA to the specific peptide (TRP-$1_{277-297}$), lysate (B16) and intact tumors (B16-DR4, 624 Mel pretreated with IFN-γ, and 1102 Mel stably transduced with TRP-1), but not to the control peptide ($HA_{306-318}$), control lysate (MC38) or control tumors (wild type B16, MC38-DR4, and 1102 Mel stably transduced with GFP). Interactions with TRP-$1_{277-297}$ pulsed targets were blocked by L243 (HLA-DR) and L3T4 (CD4), but not by control W6/32 (class I) or HIT8a (CD8) antibodies. All peptides were pulsed onto $DR4^+$ 1088 EBV-B cells at 50 μM x 3 hrs.

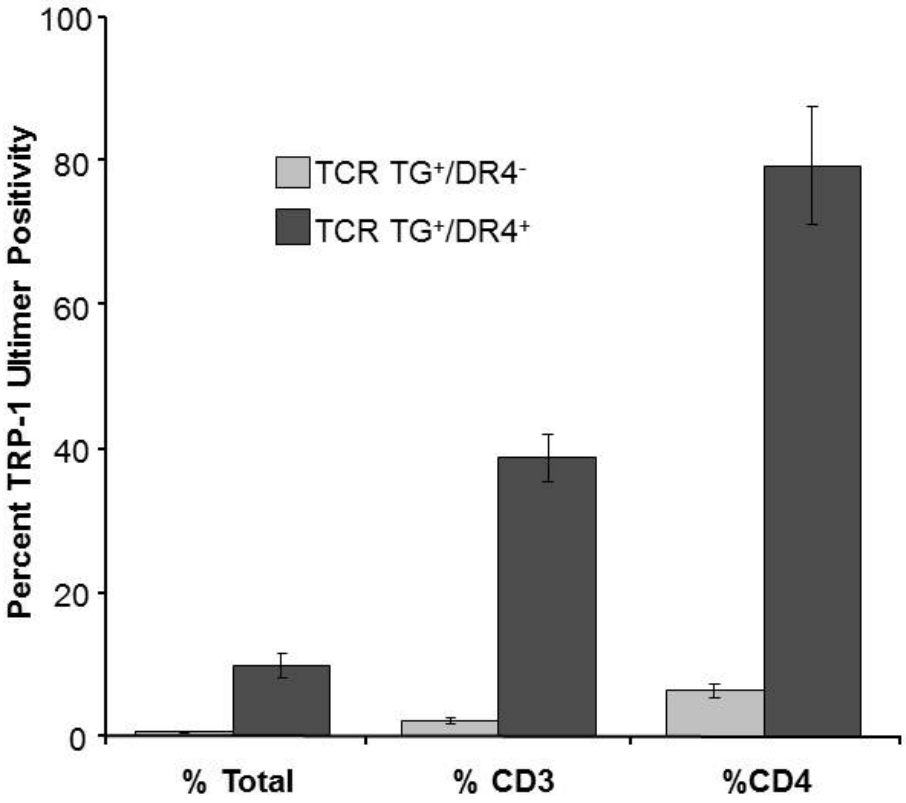

Figure 8. Positive selection of the TRP-1 TCR following crossbreeding into DR4 Tg mice**.** Peripheral blood cells were stained for CD3, CD4 and the TRP-1 Pentamer in both TRP-1 TCR Tg mice and TRP-1 TCR/DR4 Tg mice. Each group contains at least 5 mice that were individually bled, stained and analyzed by flow cytometry. In the TRP-1 TCR Tg mice, levels of pentamer positivity was near that of negative controls and ranged from 2-7% in CD4 T cells (Lighter shaded bars). Once crossed on to the HLA-DR4 Tg mice, nearly 80% of CD4 T cells in the peripheral blood were specific for the TRP-1 Ultimer (Darker shaded bars). Within the CD4 cells, 79% ± 8.3% versus 6.4% ± 1.0%; *P < 0.0001.* Data (mean ± SEM with P value; n = 5–7 per group)

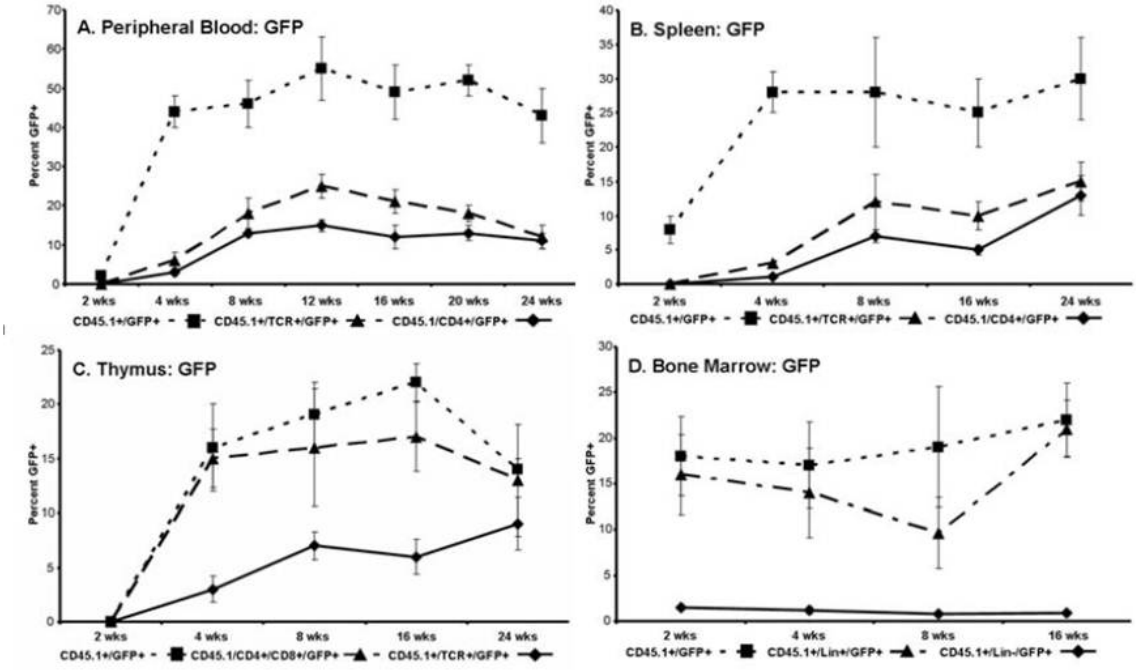

Figure 9. Gene modified HSCs successfully engraft and repopulate transplant host over 6 months. CD45.1/CD45.2 competitive repopulation involving co-transplantation of GFP transduced Lin^- BoyJ (CD45.1) HSCs and unmanipulated congenic bone marrow cells from a C57BL/6 mouse (CD45.2) transplanted into irradiated DR4 Tg recipient mice. **A.** Transplanted, transduced Lin^- cells maintain GFP expression in the peripheral blood ($CD45.1^+/GFP^+$) , developing into a high percentage of mature T cells ($TCR^+/CD3^+$) and $CD4^+$ T cells. **B.** Gene modified cells area also present and remain stable in within the spleen. **C.** Developing double positive ($CD4^+/CD8^+/GFP^+$) thymocytes carrying GFP are also identified and maintained in the thymus. **D.** Gene modified donor cells ($CD45.1^+/GFP^+$) are also stable in the bone marrow. Percentages represent 5 mice in each group, for each time point.

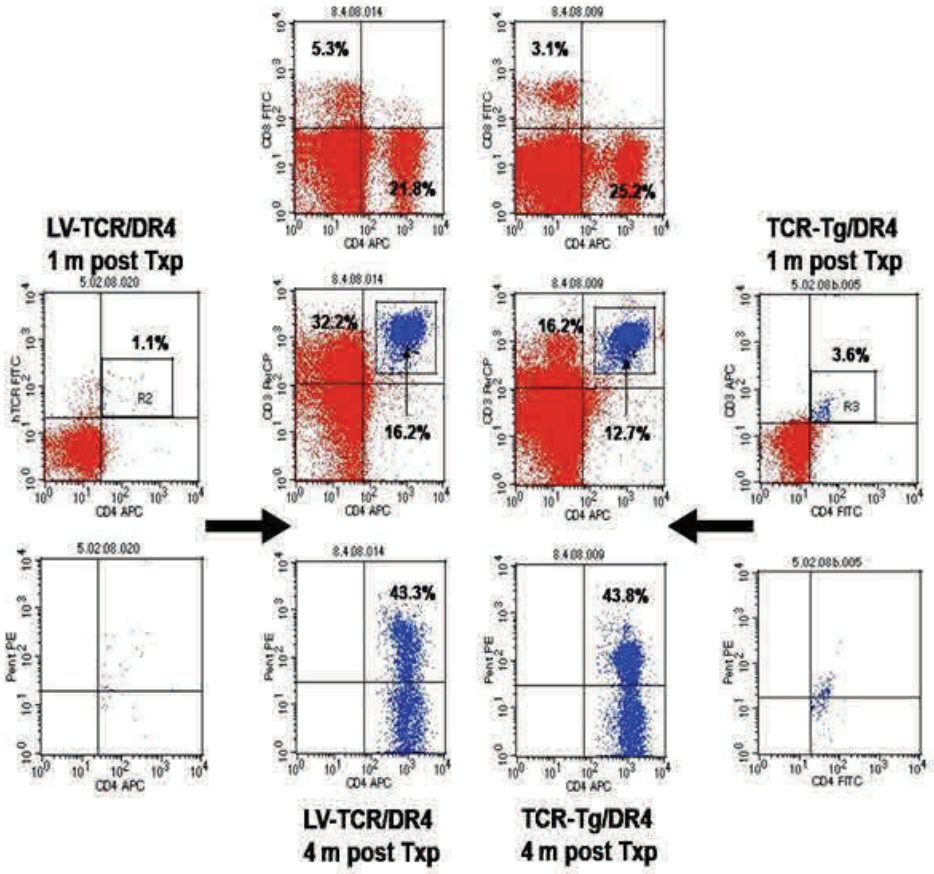

Figure 10. Representative flow sample shows successful engraftment and emergence of lymphocytes 1 and 4 months post transplantation with either LV-TCR transduced Lin- HSCs (LV-TCR/DR4) or TCR-Tg Lin$^-$ HSCs (TCR-Tg/DR4) into irradiated DR4 recipient mice. At 1 month post transplant, few CD4 staining cells are seen in the peripheral blood (1.1% in the LV-TCR/DR4 and 3.6% in the TCR-Tg/DR4). By 4 months post transplantation lymphocytes (both CD4$^+$ and CD8$^+$) have reconstituted the peripheral blood. Of the mature CD3$^+$/CD4$^+$ T cells 43.3% and 43.8% stain positive for the TRP-1 Ultimer in the LV-TCR/DR4 and TCR-Tg/DR4 groups, respectively. Data are representative of flow cytometry studies done on 4 separate transplant experiments.

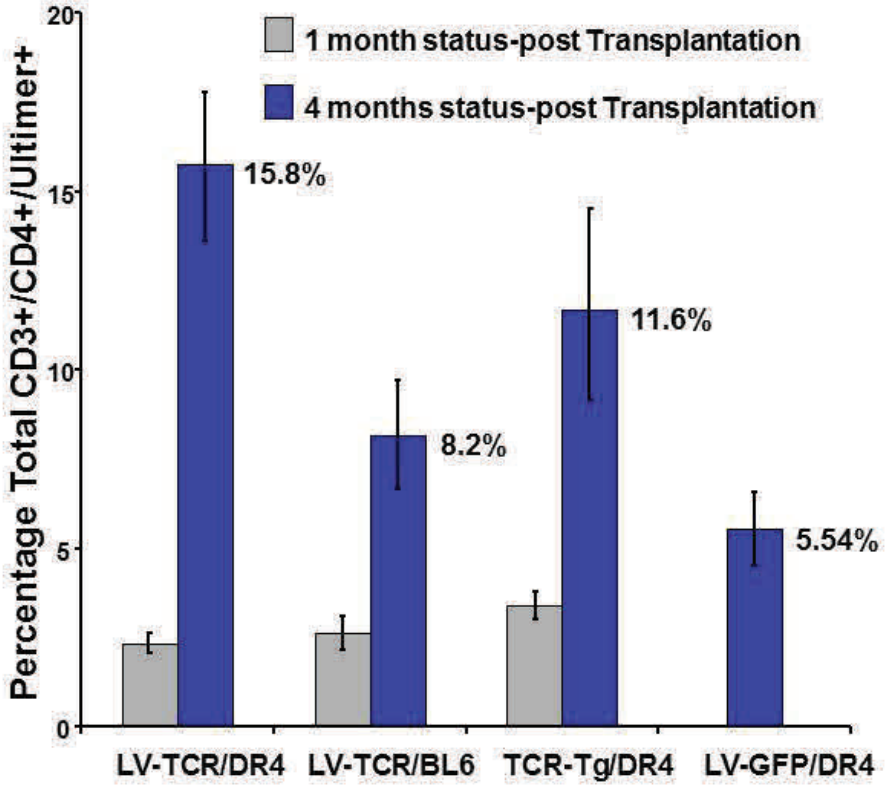

Figure 11. Transplantation of LV-TCR and TRP-1 TCR Tg Lin^- HSCs results in high expression of TRP-1 specific TCR. As previously described, competitive bone marrow transplants were established with LV-TCR transduced and TRP-1 Tg lineage depleted BM cells and transplanted into irradiated recipient DR4 Tg and C57BL/6 mice. Peripheral blood was obtained at 1 and 4 months and stained with PE labeled TRP-1 pentamer, APC labeled anti-CD4 antibody, and PE-CY5 labeled anti-CD3 antibody. At 1 month post transplantation (grey bars), TRP-1 pentamer specific lymphocytes constituted less than 5% of the total circulating $CD3^+/CD4^+$ lymphocytes in all transplant groups. At 4 months post transplantation (blue bars), the LV-TCR in DR4 Tg recipients (LV-TCR/DR4) had the highest percentage of circulating TCR specific $CD3^+/CD4^+$ T cells at 15.8%+/-2, followed closely by the TCR-Tg in DR4 Tg recipients (TCR-Tg/DR4) at 11.6%+/-2.5. The LV-TCR in C57BL/6 group only expressed the specific TCR in 8.2%+/-2 of $CD3^+/CD4^+$ cells, statistically less than the LV-TCR/DR4 group ($p<0.05$). A control group of LV-GFP transduced HSCs in DR4 Tg recipients (LV-GFP/DR4) only expressed GFP in 5.5%+/-1 of $CD3^+/CD4^+$ T cells at 4 months.

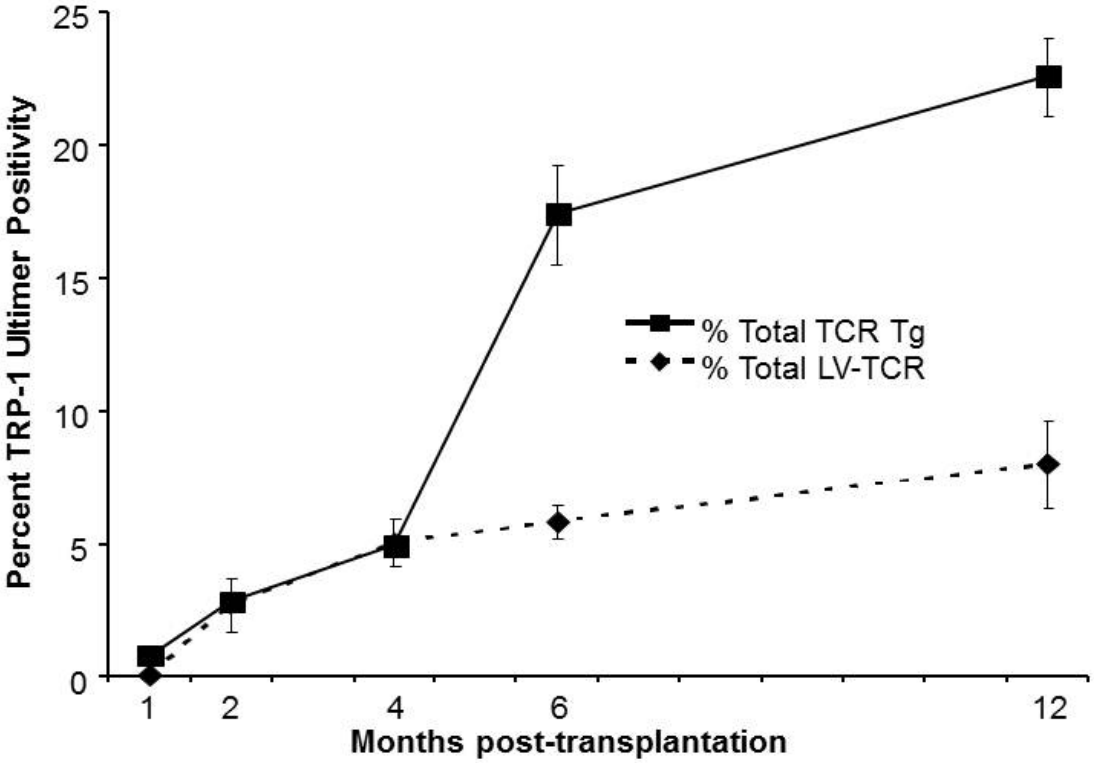

Figure 12. 12 month analysis of LV-TCR and TRP-1 TCR Tg Lin$^-$ HSCs transplanted into DR4 Tg mice (n=10 for each group). Percent TRP-1 Ultimer positive as a function of total number of peripheral mononuclear cells. Results show high expression of TRP-1 specific TCR in both groups with TRP-1 TCR Tg transplants expressing higher numbers after 4 months. At 12 months, 8% ± 1.7% versus 22.6% ± 1.5%; P = 0.001. As previously described, competitive bone marrow transplants were established with LV-TCR transduced and TRP-1 Tg lineage depleted BM cells and transplanted into irradiated recipient DR4 Tg mice. Peripheral blood was obtained at 1,2,4,6, and 12 months and stained with PE labeled TRP-1 Ultimer, FITC labeled anti-CD8 antibody, APC labeled anti-CD4 antibody, and PE-CY5 labeled anti-CD3 antibody.

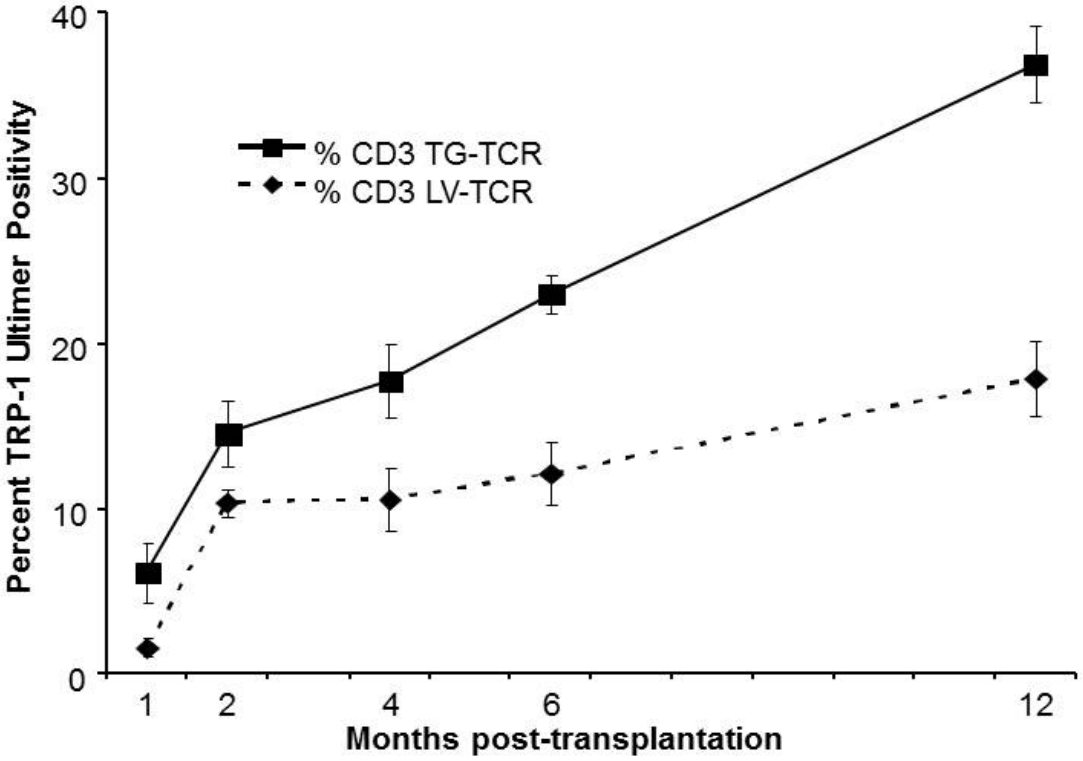

Figure 13. 12 month analysis of LV-TCR and TRP-1 TCR Tg Lin$^-$ HSCs transplanted into DR4 Tg mice (n=10 for each group). Percent TRP-1 Ultimer positive as a function of CD3$^+$ peripheral mononuclear cells. Results show high expression of TRP-1 specific TCR in both groups, but TRP-1 TCR Tg transplants expressed higher percentages at all time points. At 12 months, 17.8% ± 2.3% versus 36.7% ± 2.4%; *P = 0.006.* As previously described, competitive bone marrow transplants were established with LV-TCR transduced and TRP-1 Tg lineage depleted BM cells and transplanted into irradiated recipient DR4 Tg mice. Peripheral blood was obtained at 1,2,4,6, and 12 months and stained with PE labeled TRP-1 Ultimer, FITC labeled anti-CD8 antibody, APC labeled anti-CD4 antibody, and PE-CY5 labeled anti-CD3 antibody.

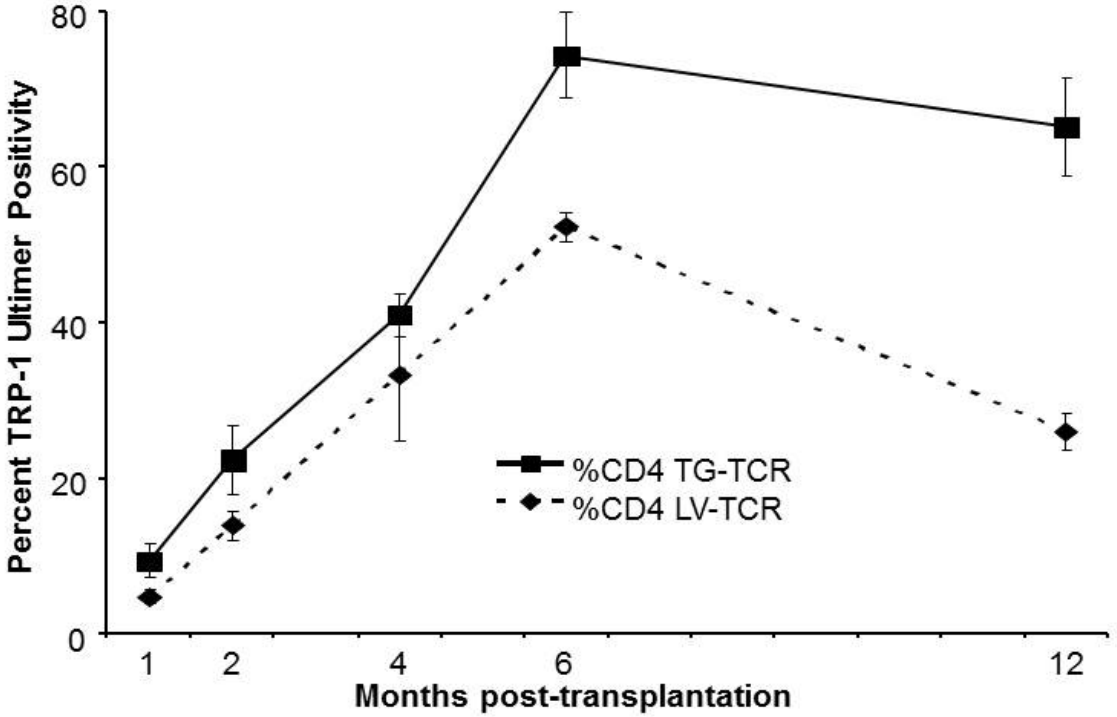

Figure 14. 12 month analysis of LV-TCR and TRP-1 TCR Tg Lin$^-$ HSCs transplanted into DR4 Tg mice (n=10 for each group). Percent TRP-1 Ultimer positive as a function of CD4$^+$ peripheral mononuclear cells. Results show high expression of TRP-1 specific TCR in both groups, but TRP-1 TCR Tg transplants expressed higher percentages at all time points. At 12 months, 26.1% ± 2.4% versus 65.2% ± 6.3%; *P = 0.004.* As previously described, competitive bone marrow transplants were established with LV-TCR transduced and TRP-1 Tg lineage depleted BM cells and transplanted into irradiated recipient DR4 Tg mice. Peripheral blood was obtained at 1,2,4,6, and 12 months and stained with PE labeled TRP-1 Ultimer, FITC labeled anti-CD8 antibody, APC labeled anti-CD4 antibody, and PE-CY5 labeled anti-CD3 antibody.

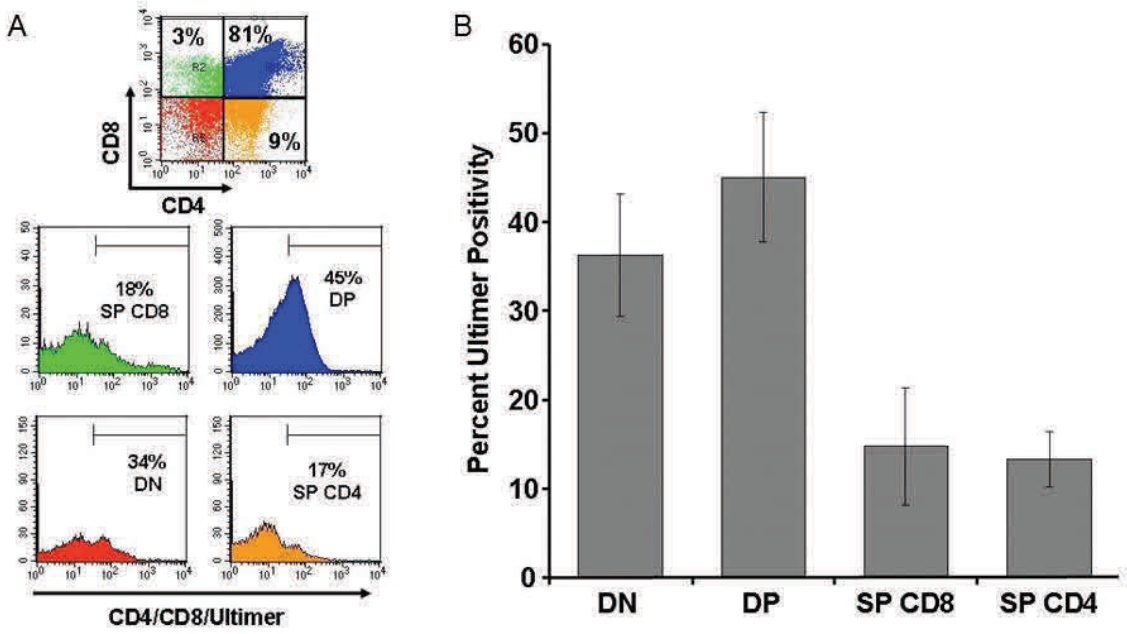

Figure 15. Analysis of thymocytes in LV-TCR HSC transplanted DR4 Tg mice. At 12 months the thymus from LV-TCR transplants were harvested and analyzed by flow cytometry. **A.** This representative flow sample exhibits our analysis technique. Thymocytes were stained for TRP-1 Ultimer, CD3, CD4, and CD8. Each compartment of the maturing thymocyte population (double negative, double positive, and CD4 and CD8 single positive) were analyzed for TRP-1 Ultimer positivity. **B.** Summary of TCR gene expression in DN, DP, SP CD8, SP CD4 subpopulations. 36% of DN thymocytes, 45% of DP thymocytes, 15% of $CD8^+$ thymocytes, and 13% of $CD4^+$ thymocytes expressed the TRP-1 TCR. As expected, a large percentage of cells are deleted from the double positive stage to the mature single positive stage of central thymic selection. DP 45% ± 7.3% versus SP CD4 13.3% ± 3.1%; *P = 0.002. Data (mean ± SEM* with *P value; n = 4–6 per group).*

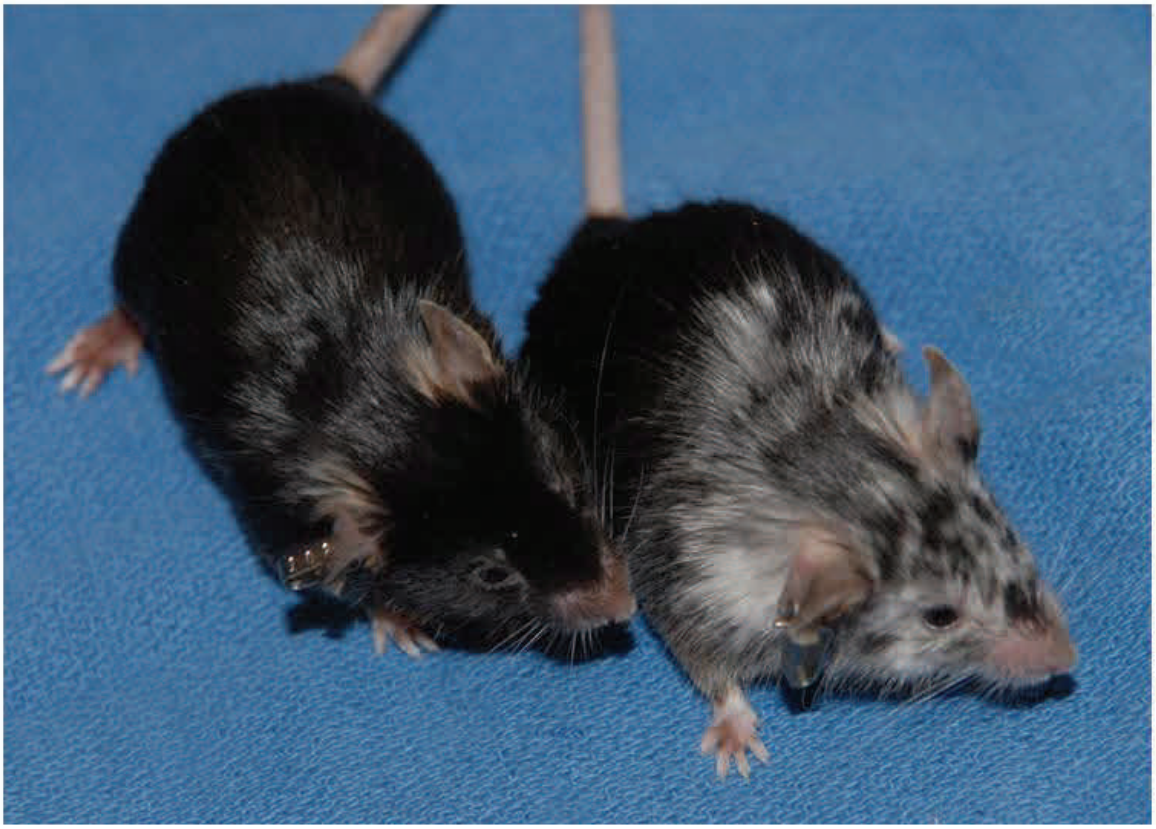

Figure 16. Development of spontaneous autoimmune vitiligo in mice transplanted with LV-TCR Lin- bone marrow cells. Depigmentation of the hair follicles in the vitiliginous mouse (right) exhibits an irregular, spotted pattern. A control transplant mouse (left) is shown for comparison.

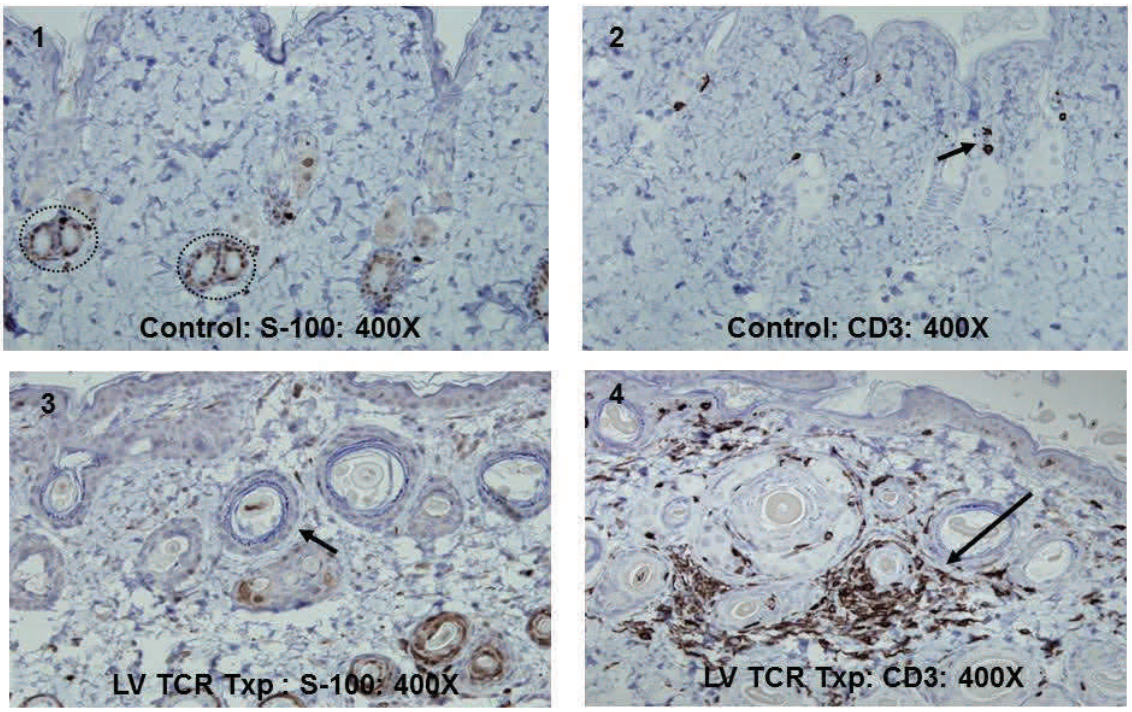

Figure 17. Skin samples of LV TRP-1 TCR BM transplanted mice that developed spontaneous autoimmune vitiligo. The samples were fixed in paraffin and stained for S-100 (a marker for neurocrest derived cells such as melanocytes) and mouse CD3. The top two panels show the staining pattern in the skin of a normal immune replete control mouse. Melanocytes typically reside around the hair follicles as highlighted by the dotted circles (Slide1), and few lymphocytes are found in normal skin (Slide 2). In the vitiliginous mice, there is a clear loss of melanocytes around the hair follicles in affected skin areas as indicated by the arrow in Slide 3. Unlike normal skin in vitiliginous skin, a profuse lymphocytic infiltration particularly around the hair follicles is observed (Slide 4).

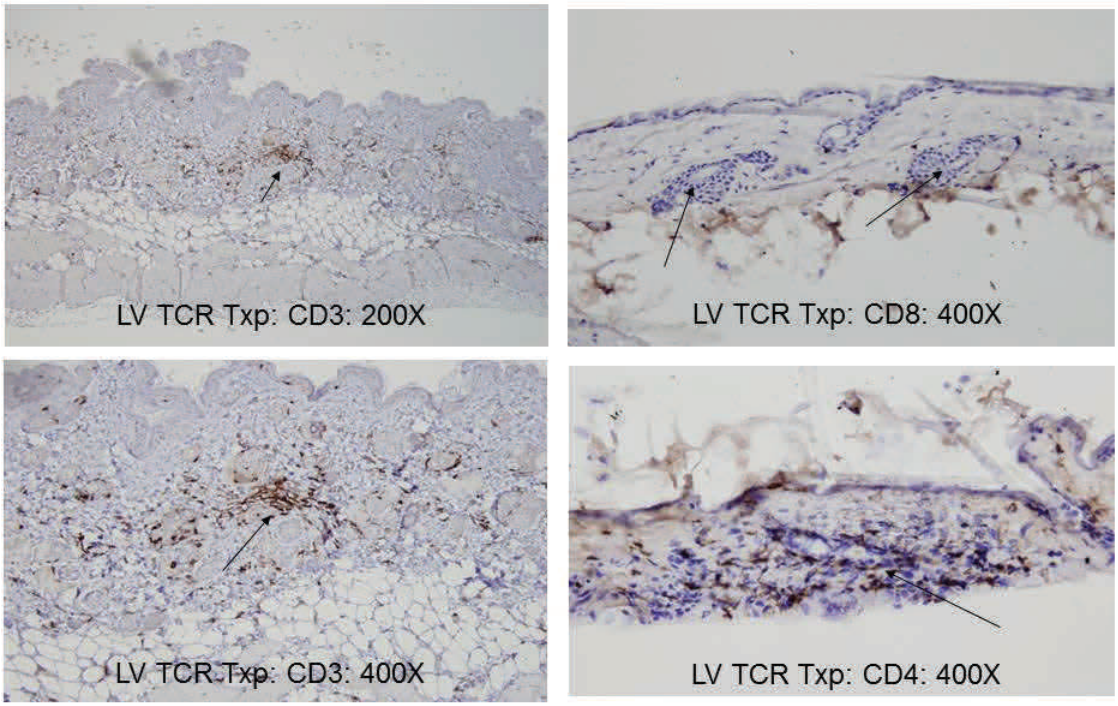

Figure 18. Skin samples of LV TRP-1 TCR BM transplanted mice that developed spontaneous autoimmune vitiligo. The left two panels show paraffin embedded staining for S-100 and CD3 as previously described. The right two panels show frozen section samples of vitiliginous skin stained with antibodies specific for murine CD4 and CD8, respectively.

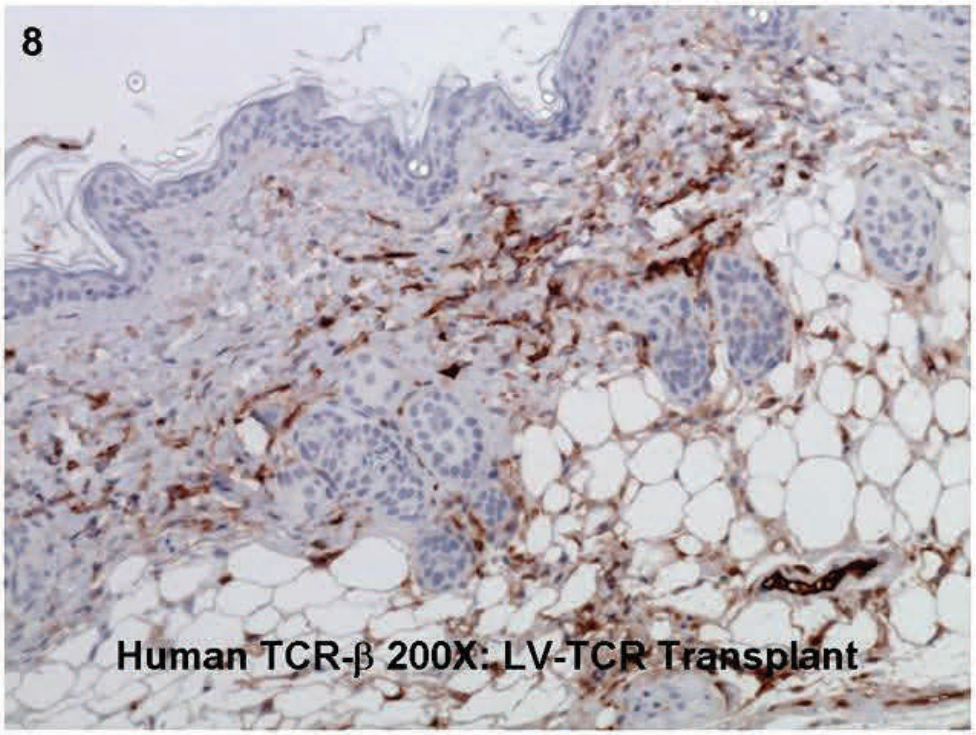

Figure 19. Vitiligo is associated with gross distortion of the natural skin architecture, melanocyte destruction, and TCR gene specific $CD4^+$ T cell infiltration. Immunohistochemistry staining at 6 month post-transplantation of paraffin-embedded skin sample from LV-TCR transplant with antibody specific for human-TCR-β.

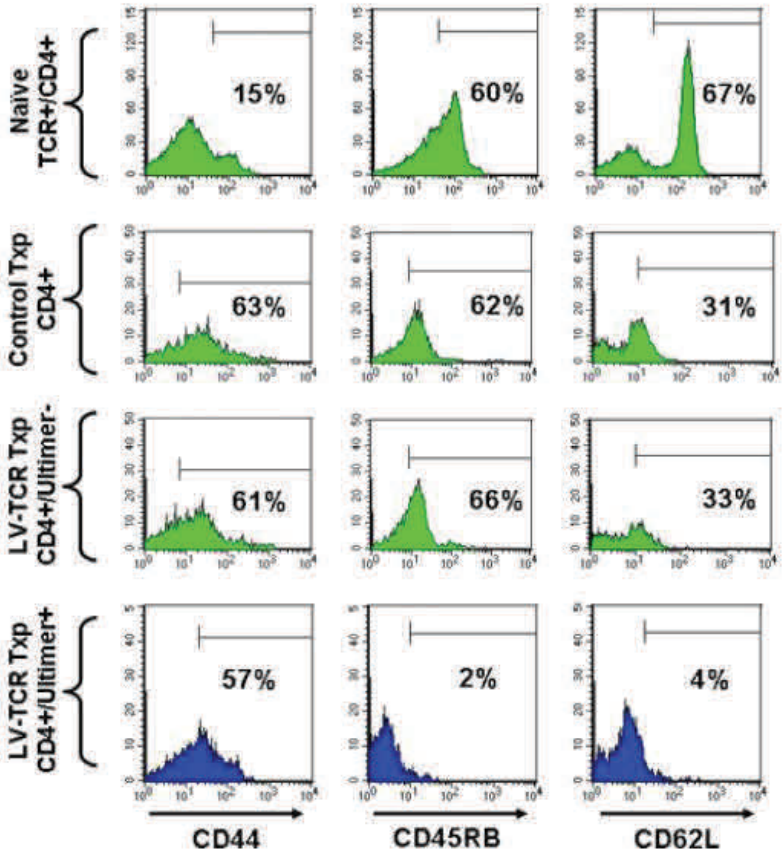

Figure 20. Representative flow cytometry analysis of splenocytes from transplant experiments. Transplant recipients exhibit a T_{EM} phenotype and react spontaneously to tumor *ex vivo*. Peripheral blood samples at 6 months post-transplantation were obtained and stained with antibodies against CD4, TRP1-Ulimter, CD44, CD45RB, and CD62L. LV-TCR transplant CD4+/Ultimer+ cells exhibit a memory-effector (T_{EM}) phenotype ($CD45RB_{low}$, $CD62L_{low}$, $CD44_{high}$), while the polyclonal CD4+/Ultimer- and CD4+ control population exhibit a more generalized effector profile ($CD45RB_{high}$, $CD62L_{int}$, $CD44_{high}$), contrasting with naïve CD4+/Ultimer+ cells ($CD45RB_{high}$, $CD62L_{high}$, $CD44_{low}$) from endogenous non-transplanted TCR Tg.

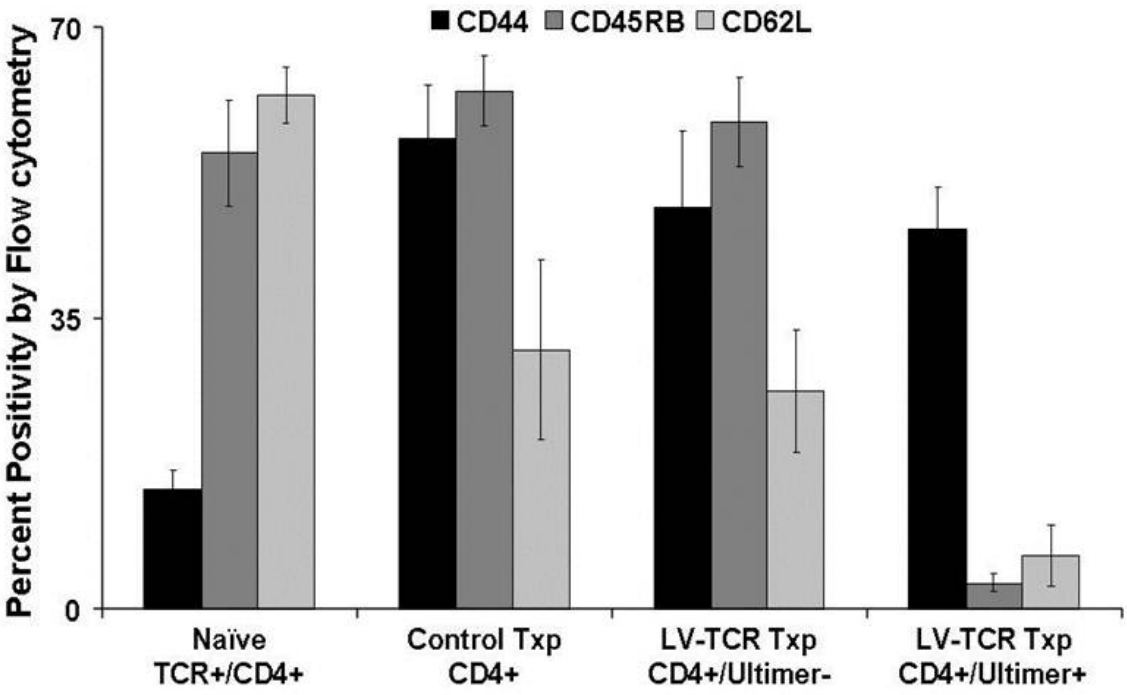

Figure 21. Summary of activation status of all CD4+ subpopulations. Transplant recipients exhibit a T_{EM} phenotype and react spontaneously to tumor *ex vivo*. Peripheral blood samples at 6 months post-transplantation were obtained and stained with antibodies against CD4, TRP1-Ulimter, CD44, CD45RB, and CD62L. LV-TCR transplant CD4+/Ultimer+ cells exhibit a memory-effector (T_{EM}) phenotype ($CD45RB_{low}$, $CD62L_{low}$, $CD44_{high}$), while the polyclonal CD4+/Ultimer- and CD4+ control population exhibit a more generalized effector profile ($CD45RB_{high}$, $CD62L_{int}$, $CD44_{high}$), contrasting with naïve CD4+/Ultimer+ cells ($CD45RB_{high}$, $CD62L_{high}$, $CD44_{low}$) from endogenous non-transplanted TCR Tg. Data (mean ± SEM; *n = 5 per* group).

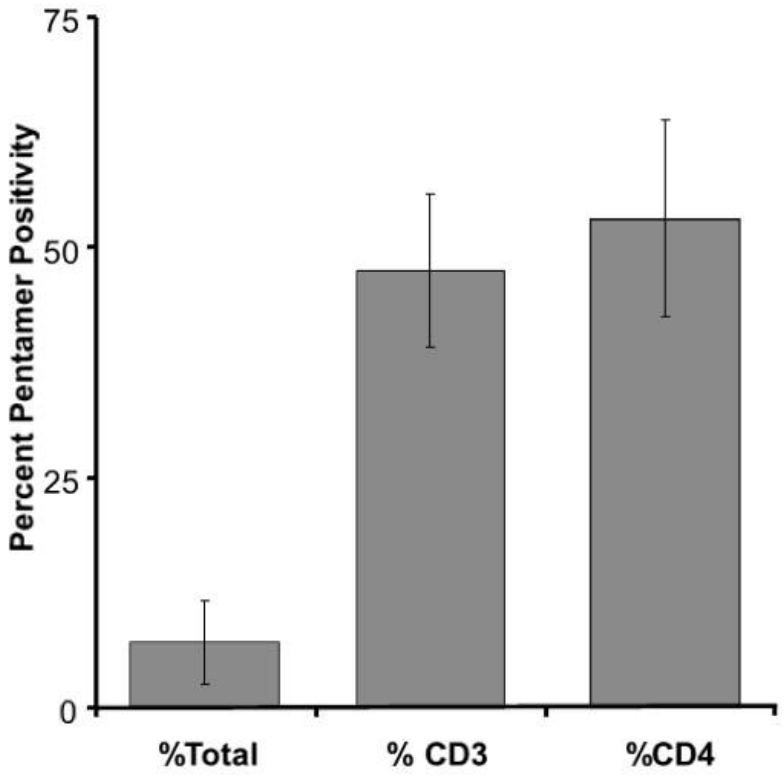

Figure 22. $CD4^+$ T cells from secondary transplant recipients exhibit high level gene specific TCR expression. Peripheral blood from 6 month secondary transplants were stained with antibodies against CD4, CD8, CD3 and the TRP -1 pentamer and analyzed by flow cytometry. Total, CD3, and CD4 populations: 7.1% ± 4.5% within the total cellular compartment (CD45+Ultimer+), 47.5% ± 8.3% within the global CD3 compartment (CD45+CD3+Ultimer+), and 53.1% ± 10.7% within the CD4 subcompartment (CD45+CD3+CD4+Ultimer+). Data (mean ± SEM;*n = 5 per group)*

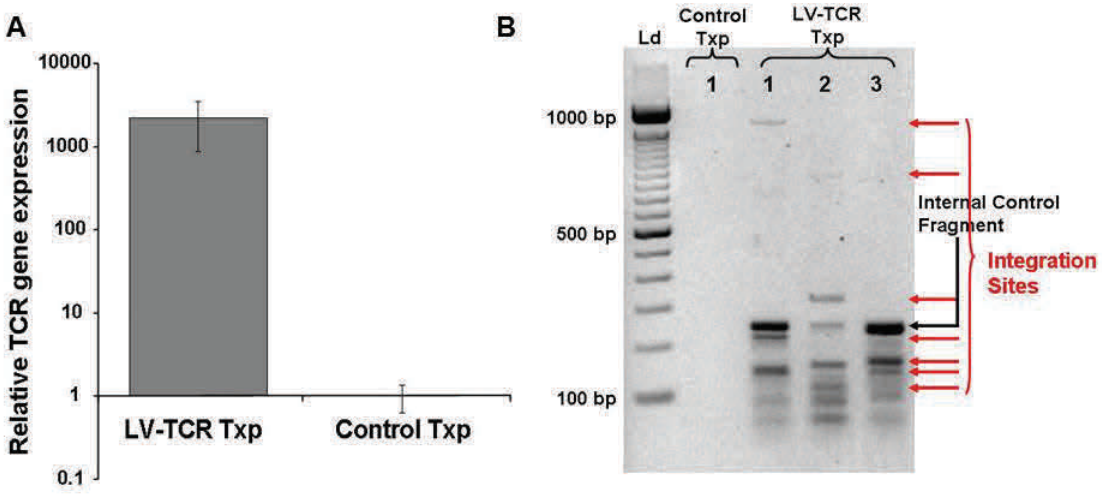

Figure 23. CD4$^+$ T cells from secondary transplant recipients exhibit high level gene specific TCR expression and multi-copy integration of the LV-TCR gene product. **A.** Relative TCR gene expression is >1000 times greater than control transplants (*P=0.01*). Total RNA was prepared from BM obtained from 6 month old secondary LV-TCR and Control transplants and analyzed by qPCR for relative TCR gene expression using an α-chain specific probe. Relative gene expression (RQ = LV-TCR/Control) is displayed. **B.** Multicopy integration of the LV-TCR gene product is found in secondary transplant recipients. Using a vector backbone specific probe, 4-6 integrants were identified in three separate LV-TCR transplant samples compared with Control.

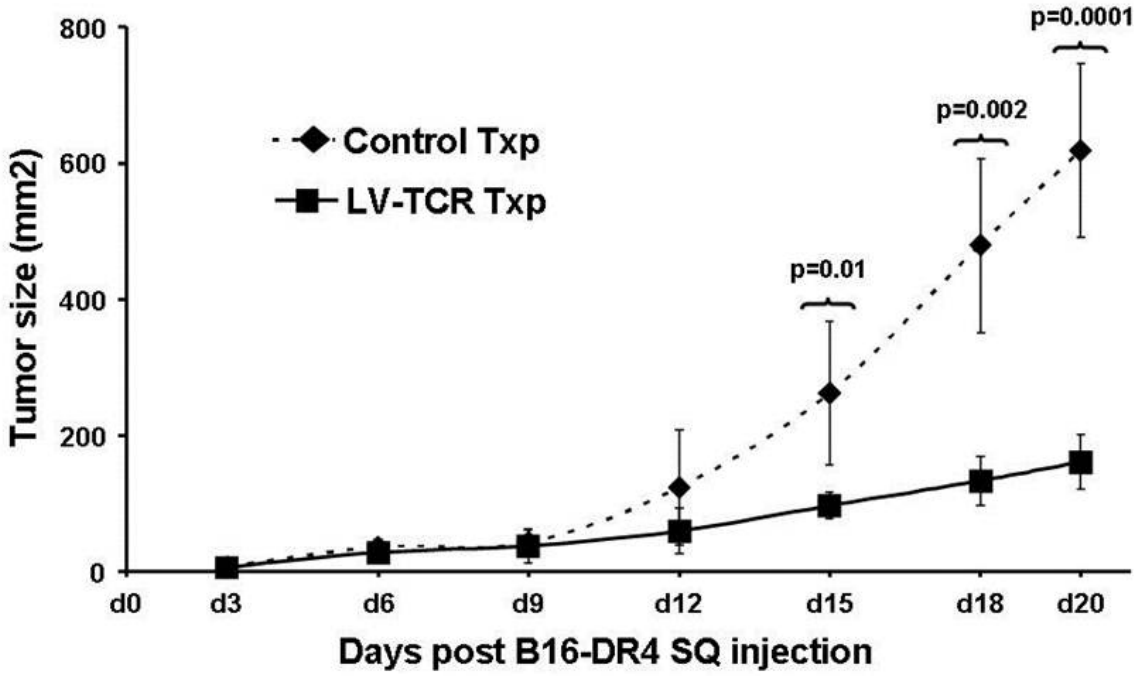

Figure 24. Six month secondary TCR transplant recipients reject subcutaneous melanoma. LV-TCR and control transplants (5 mice per group) were subcutaneously injected with $5x10^5$ B16-DR4 tumor cells. Three days after injection, tumor size was recorded. Data are displayed as mean ± SEM with *P value; n = 5* per group. Statistical significance between groups was based on tumor size using a Student T-test. Experiments were performed in a blinded, randomized fashion, and executed independently 3 times.

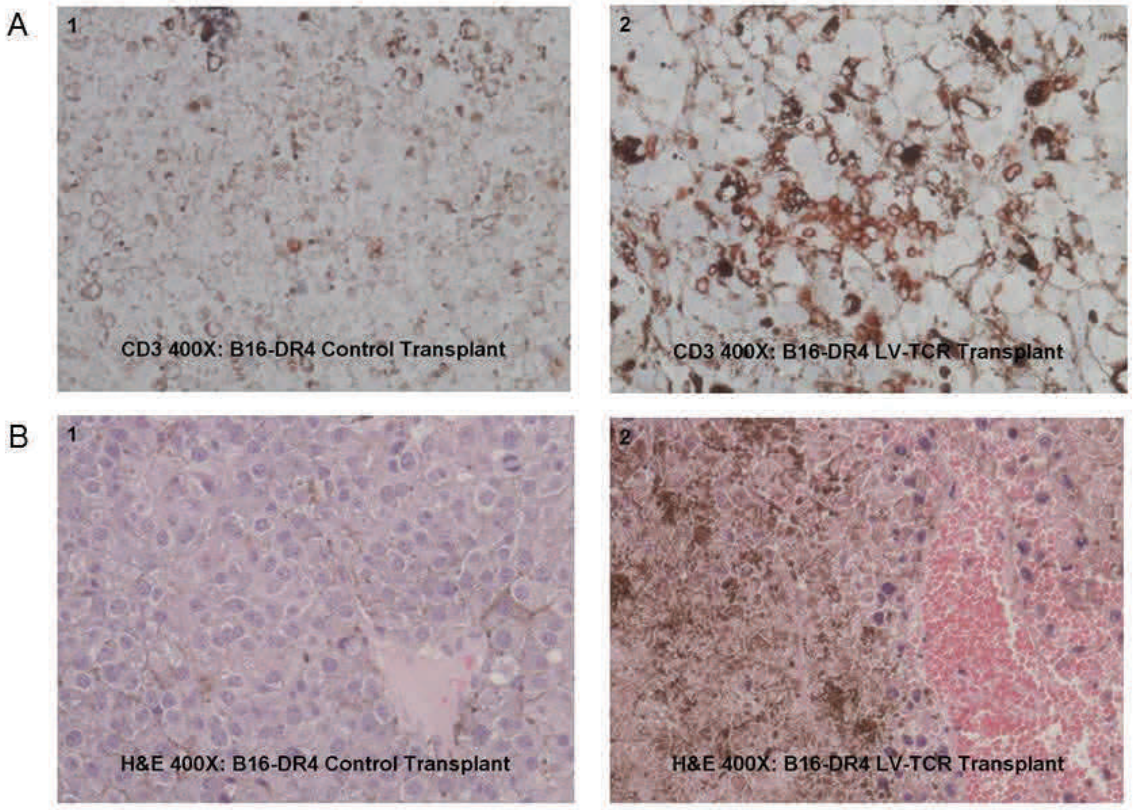

Figure 25. Tumors from LV-TCR secondary transplants are necrotic. Immunohistochemistry staining of paraffin-embedded tumors resected from 6 month transplants. **A.** Resected tumor cells were stained with antibody specific for murine CD3. Panel 1 shows B16-DR4 tumors from control transplant. Panel 2 shows tumor from LV-TCR transplant. **B.** H&E staining of tumor resections from control transplant (Panel 1) and LV-TCR (Panel 2) shows necrotic changes in LV-TCR transplant.

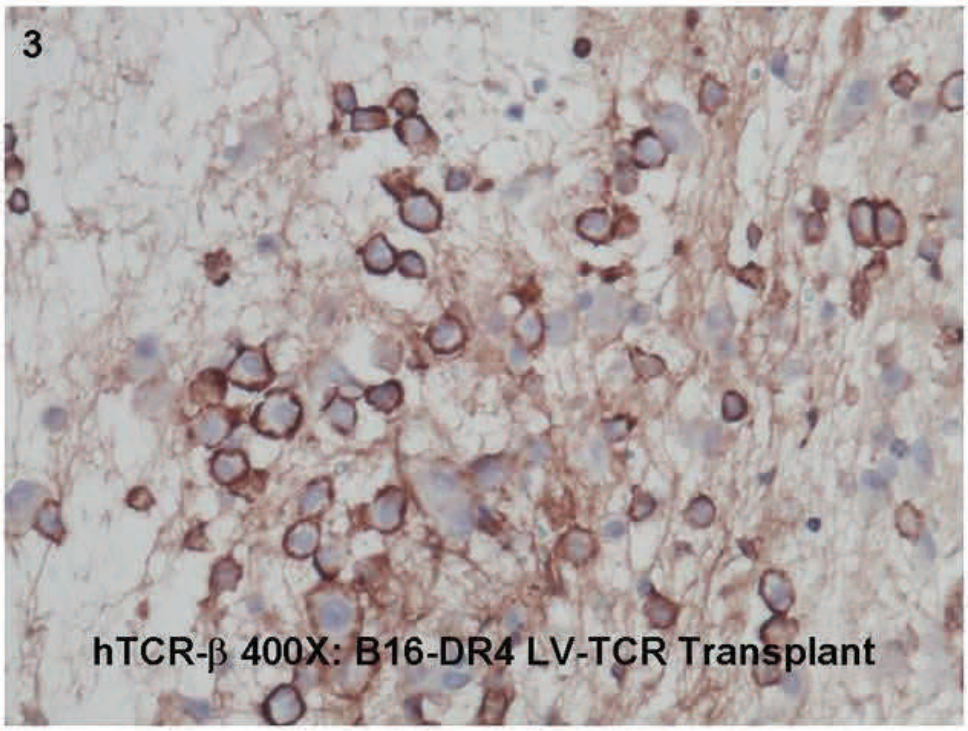

Figure 26. Paraffin-embedded tumor resection from LV-TCR secondary transplant with immonhistochemistry staining specific for human TCR-β chain expression. Tumors from LV-TCR secondary transplants exhibit lymphocytic infilitration expressing human TCR-β

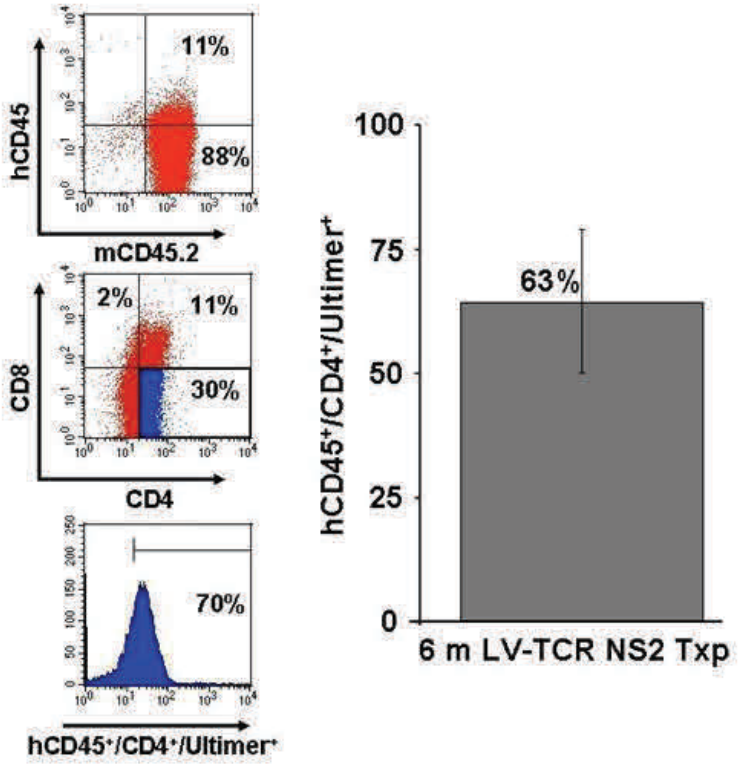

Figure 27. LV-TCR gene-modified human $CD34^+/CD38^-$ HPCs obtained from cord blood functionally engraft and repopulate 6 month old NOD-SCID-IL2rγ KO transplant recipients. **A,** Flow cytometry analysis of peripheral blood demonstrates high level TCR gene expression: hCD45/mCD45.2/CD4/CD8/ Ultimer staining. **B,** Six month transplant summary of TCR gene expression within the $hCD45^+/CD4^+/Ultimer^+$ subpopulation. Data are displayed as mean ± SEM; *n* = 5 per group.

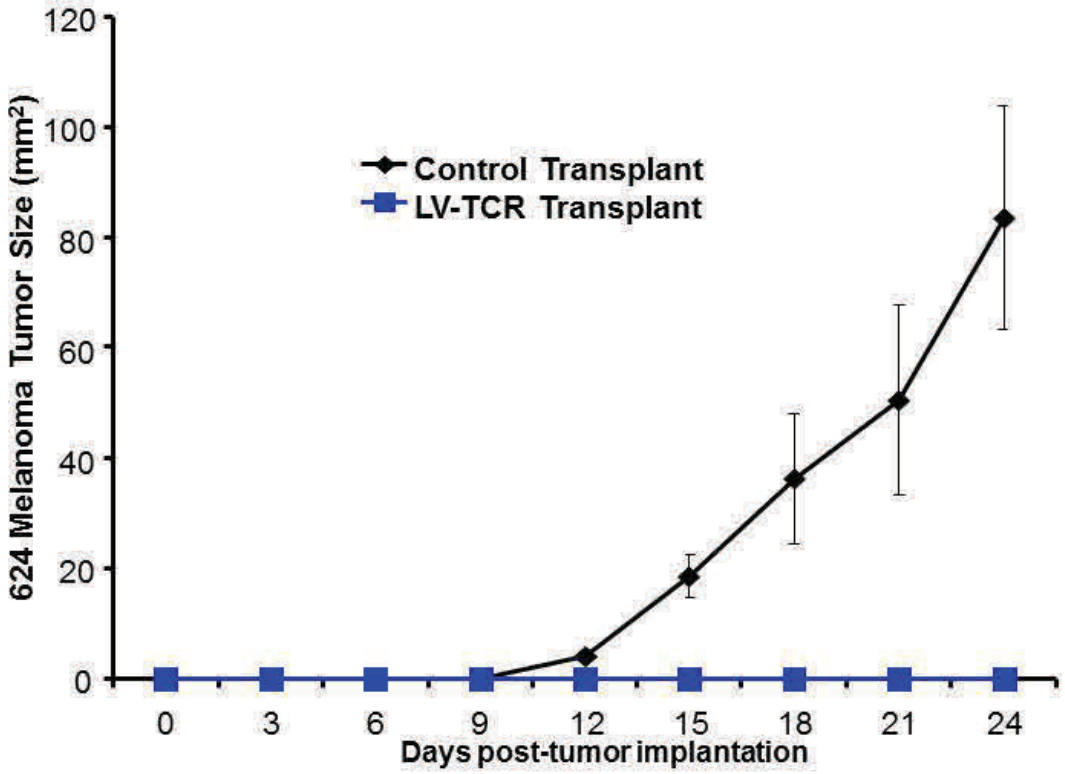

Figure 28. LV-TCR transplants in NOD-SCID-IL2rγ KO recipients reject human melanoma implantation. 6 month old control and LV-TCR transplants (5 mice in each group) were injected subcutaneously with $5x10^5$ human 624 melanoma cells at Day 0. Tumor size was measured every 3 days for 24 days. No tumor was visibly observable in any of the LV-TCR transplants through 24 days post-tumor implantation, while all 5 mice in the control transplants developed visibly observable tumors between 9 and 12 days.

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CURRICULUM VITAE

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EDUCATION

Indiana University, Indianapolis, IN Ph.D. in Microbiology and Immunology	2012
Indiana University School of Medicine, Indianapolis, IN M.D. in Medicine	2010
Yale University, New Haven, CT M.P.H in Health Economics and Management	2003
Yale University, New Haven, CT B.S. in Molecular Biochemistry and Biophysics	2000
West Lafayette High School, West Lafayette, IN Diploma	1996

AWARDS

Roy H. Behnke Scholarship, Indiana University	2003-2007
Dr. Wright Sr. Scholarship, Walther Cancer Institute	2006
National Merit Scholarship, Yale University	1996

PUBLICATIONS AND ABSTRACTS

Sung P Ha, Nicholas D Klemen, Garrett H Kinnebrew, Andrew G Brandmaier, Jon Marsh, Giao Hangoc, Douglas C Palmer, Nicholas P Restifo, Kenneth Cornetta, Hal E Broxmeyer, Christopher E Touloukian. 2010. Transplantation of mouse HSCs genetically modified to express a CD4-restricted TCR results in long-term immunity that destroys tumors and initiates spontaneous autoimmunity. *Journal of Clinical Investigation.* 102(12):4273-4288.

Andrew G Brandmaier, Wolfgang W Leitner, Sung P Ha, John Sidney, Nicholas P Restifo, Christopher E Touloukian. 2009. High-avidity autoreactive CD4+ T cells induce host CTL, overcome Tregs and mediate tumor destruction. Journal of Immunotherpay. 32(7):677-688.

Michael C Lipke, Sung P Ha, Christopher D Fischer, Jonas Rydberg, Stephen M Bonsib, Chandru P Sundaram. 2007. Pathologic characteristics of exophytic renal masses. *Journal of Endourology.* 21(12):1489-1492.

Sung P Ha, Kenneth Cornetta, Hal E Broxmeyer, Nicholas P Restifo, Christopher E Touloukian. 2008. CD4+ T cell immunity following gene transfer into hemaopoietic stem cells. *4th Annual Academic Surgical Congress*.

WORK EXPERIENCE

University of Wisconsin Hospitals and Clinics, Madison, WI
Neurosurgery Resident 2010-2012
Neurosurgery resident.

Nicaragua Medical Internship, Nicaragua
Intern 2004
Eight week summer internship in various rural and urban hospitals in Nicaragua sponsored through the University of California Medical Schools. Participated in the public health assessment and daily clinic duties of third in urban and rural clinics in Nicaragua.

NERA/Mercer Management Consulting, New York, NY
Research Associate 2000-2001
Assessed damage analysis for a variety of securities cases ranging from 10b-5 cases to customized long-term studies of restatements and earnings surprise impact on stock price.

Neurobiology Laboratory, Yale University, New Haven CT
Researcher 1999-2000
Conducted independent electrophysiological studies in memory and learning developing mathematical models of neural networks.

Trumbull News, Yale University, New Haven, CT
Co-Editor 1998-1999
Co-wrote and edited production of weekly newsletter.

Orthopedic Oncology Department, MGH, Boston, MA
Summer Intern 1998
Organized and conducted clinical study of Ewing's Sarcoma collecting and building databases. Observed patient rounds and surgical rotations.

Biomedical Engineering, Purdue University, West Lafayette, IN
Researcher 1997
Coordinated and conducted experiments in nerve and skin regeneration on extracellular matrices.

MEMBERSHIPS

Congress of Neurological Surgery, Member 2010-2012

Medical School Student Council, Class Vice President	2006-2007
Rock for Riley, Executive Committee Member	2003-2010
Evening of the Arts, Co-Host	2005-2009
Yale Young Authors' Club, Co-Founder	1999-2000
Korean American Students at Yale, Member	1998-2000
Yale Emergency Medical Trainers Club, Presiding Officer	1998-2000