Using Tcr Transgenic, Gp100 Reactive T Cells and Checkpoint Inhibition to Target Lymphangioleimyomatosis

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USING TCR TRANSGENIC, GP100 REACTIVE T CELLS AND CHECKPOINT INHIBITION TO TARGET LYMPHANGIOLEIMYOMATOSIS

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to my family and friends
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ABSTRACT

Lymphangioleiomyomatosis (LAM) is a low-grade neoplastic disease affecting primarily women. It is characterized by cystic lung disease as well as renal and retroperitoneal tumors called angiomyolipomas and lymphangiomas. Tumor cells have smooth muscle features as well as neuroendocrine cell surface markers, and the disease can be diagnosed by HMB45 staining of tumor cells. We questioned whether expression of melanocytic antigens, specifically gp100, creates an opportunity to treat LAM by adoptive T cell transfer. LAM lung lesions demonstrate poor immune surveillance, therefore adoptive T cell transfer could offer benefits in this disease.

A link was made between melanoma and depigmenting vitiligo lesions as a source of gp100-reactive T cells. These T cells were first isolated from a vitiligo patient and the resulting gp100-reactive T cell receptor-bearing T cells were measured for reactivity towards relevant targets. The vitiligo lesion-derived T cell receptor, SILv44, was cloned into a lentiviral vector and introduced into magnetically sorted primary human CD8+ T cells. Transgenic T cells were combined 5:1 with melanoma cells, and LAM10224, a patient-derived LAM cell line, and responses were measured by ELISA detecting IFN-γ, after 48 hrs. Cytotoxicity was measured by CD107a expression. Transgenic T cells conferred specific reactivity to HLA-A2+, gp100+ cells including LAM10224 HLA-A2 transfected cells in vitro. The SILv44 T cell receptor demonstrated specific IFN-γ release in response to gp100, HLA-A2+ LAM10224 and melanoma cells, as well as comparable CD107a expression to that of PMA-ionomycin stimulated cells with minimal off target reactivity. Because LAM10224 has been shown to form tumors in
immunodeficient mice, we challenged groups of 2 SCID mice with LAM10224 cells transfected to express HLA-A2. One group was treated with SILv44 transgenic T cells, the remaining two groups were treated with SILv44 transgenic T cells in combination with anti-PD1 antibody and untransduced T cells respectively. Both SILv44 alone and in combination with antiPD1 effectively reduced tumor burden in mice when compared to control. These data provide preclinical evidence for the efficacy of adoptive T cell as a treatment, combined with checkpoint inhibition, to curtail the burden of LAM disease in human patients.
Lymphangioleiomyomatosis (LAM) is a rare cystic lung disease that predominantly affects women of middle age (Darling, Pacheco–Rodriguez, Gorio, Lesma, Walker & Moss, 2010). The disease manifests with the destruction of the lung with progressive pulmonary dysfunction (Hirama et al., 2007). Additionally, LAM is frequently found in patients with tuberous sclerosis complex (TSC). TSC is mediated by an autosomal dominant mutation in either TSC1 (hamartin) or TSC2 (tuberin) genes that inhibit the complex formation of the two encoded mTOR regulating proteins (Darling et al., 2010). The result of this mutation is the generation of hyper proliferative smooth muscle-like LAM cells as a result of an unregulated mTOR pathway. Additionally, it is unknown whether LAM cells originate in lung tissue or elsewhere in the body (Darling et al., 2010). It is known, however, that LAM cells express CD44 splice variant 6, a common marker for metastasis, suggesting that tumors developing in pulmonary tissues can give rise to metastases (Darling et al., 2010). This observation is also supported by multi organ involvement in patients with the disease (Matsumoto et al., 1999).

The morphology and localization of the LAM cells suggest a smooth muscle origin. This observation is supported by LAM cell expression of α-smooth muscle actin (Hirama et al., 2007).
The definitive marker for LAM cells is recognition by the antibody HMB45, which is specific for the melanosomal differentiation marker gp100 (Krymskaya, 2008). Expression of gp100 suggests melanocytic differentiation of LAM cells. Additionally, the expression of gp100 in LAM cells creates a link to possible therapeutic innovation. The target of existing immunotherapy is often melanosomal associated proteins, such as gp100. The use of immunotherapeutics is now widely studied for the treatment of metastatic melanoma (Roszkowski, 2005). The expression of these melanocytic markers on LAM cells render LAM an ideal target for adoptive T cell therapy. In vitro work has shown that LAM cells are susceptible to melanoma reactive T lymphocytes and that T lymphocyte infiltration in patients suffering from LAM is absent (Klarquist et.al., 2009). It has been demonstrated via immunohistochemistry that the levels of CD4+ and CD8+ T lymphocytes are decreased in LAM patient lungs when compared to healthy controls (Krymsikya pd-l1 in lam). These data suggest that the adaptive immune response is directly hindered by the presence of LAM cells and that LAM cells may have the ability to suppress adaptive immune response in vivo. TSC2(+/−) mouse models of LAM disease have shown that TSC2 null lung lesions recruit myeloid derived suppressor cells indicating a multifaceted immunosuppressive milieu in LAM lung lesions (Krymsikaya et al., 2009).

LAM cells also have been shown to express activation inhibitors of T lymphocytes such as B7-H3 and B7-H1 (Boorjian et. al., 2009). B7-H1, also known as programmed death-ligand 1 (PD-L1), is a transmembrane protein that interacts with a surface receptor, PD1 (programed cell death protein 1) that is present on various immune cells, specifically T cells and induces their apoptosis. PD1 engagement causes an inhibition of T cell receptor signaling and production of
immunosuppressive cytokines by CD4+ T cells such as IL-10. PD-L1 expression is induced in
the presence of IFN-y on a myriad of cell types such as endothelial and epithelial cells. The
expression of PD-L1 by lesional cells render LAM an ideal target for adjuvants to adoptive T
lymphocyte transfer such as anti-PD1 blocking antibody pembrolizumab. This antibody blocks
PD1-PDL-1 interaction and prolongs INF-y and IL-2 release by T lymphocytes *in vitro* and may
serve as a key adjuvant to T cell therapy (Wang et al., 2014). Patients with the disease vitiligo
develop T cells that are reactive against pigment associated antigens (gp100) resulting in the
characteristic feature of vitiligo, depigmentation. Patient skin is an ideal source of melanosomal
antigen reactive T cells from which a T cell receptor was isolated and cloned (Oyarbide-Valencia
et al. 2006; Klarquist et al, 2016). Once expressed in primary human T cells and shown to
effectively and selectively recognize gp100 expressing targets, the vitiligo derived,
gp100reactive T cell receptor SILv44 (skin-infiltrating T cells from vitiligo, clone 44) can offer
great potential for the treatment of LAM (Klarquist et. al., 2009). LAM is more commonly
observed as a sporadic disease characterized by mutations in the *TSC2* gene, thus development
of an *in vitro* model of disease that realistically depicts the disease has proven difficult.
Surrogate models of LAM have included mouse embryonic fibroblasts (MEFs) which were
deficient in both TSC2 and Eker rat uterine leiomyoma Tsc2-deficient cells (ELT3)13.
While these models are deficient in Tsc2 similar to LAM, a cell line that has been derived from
an individual with LAM is of far greater utility in the development of new treatments.

**Melanoma Background**

While the primary pathologic hallmark of vitiligo is melanocyte death, the opposite is true in
the disease melanoma that results in the uncontrolled growth of transformed melanocytes. Skin
malignancies are the current most common malignancy across the world and are often classified as either non-melanoma skin cancer (NMSC) or melanoma skin cancer (American Cancer Society, 2012). NMSC, such as basal and squamous cell carcinomas which account for less mortality than melanoma, account for the highest percentage of skin cancer cases. Malignant melanoma accounts for only ~4% of all skin cancers but is the deadliest form of skin cancer (Gray-Schopfer, Wellbrock & Marais, 2013). Melanomas are tumors that arise from melanocytes which are found in the basal layer of the epidermis (American Cancer Society, 2012). These cancers can arise from various factors such as DNA damage due to ultraviolet radiation, and often result in immune suppression in the surrounding skin (Gordon, 2013). These factors contribute to the growth, and eventual takeover, of melanoma in patient skin. Melanoma is often treated surgically and has a good prognosis if the primary lesion can be removed. However, if the tumor has metastasized, the prognosis becomes much worse. The risk of metastasis of the primary lesion, as well as the presence of infiltrating T cells in melanoma lesions, has led to the development of immunotherapeutic options such as adoptive T cell transfer therapy in melanoma.

Vitiligo

Vitiligo is an autoimmune, T cell mediated skin disorder in which melanocytes are killed, resulting in depigmentation of the skin. In the diseased skin of vitiligo patients, melanocytes are destroyed by melanocyte-reactive T cells, as well as other immune or non-immune components (Das, van den Wijngaard, Wankowicz-Kalinska & Le Poole, 2001). Expanding vitiligo lesions have been shown to be infiltrated with T cells in the perilesional skin; these infiltrating T cells
are primarily of CD8+ cytotoxic T cells that are reactive to melanocyte-specific antigens (Wankowicz-Kalinska, et al., 2003).

Adoptive T Cell Transfer

It has long been known that adoptive transfer of autologous or syngeneic lymphocytes have the potential to effectively elicit anti-tumor response in mice (Andreini, Drasher, & Mitchison, 1955). However, the underlying mechanisms by which transferred lymphocytes enhance tumor immunity in a recipient animal have only recently begun to be understood. A key component in understanding the fundamental components of lymphocyte, and specifically T cell transfer therapy starts with a discussion of tolerance. Immunological tolerance is defined as “a state of unresponsiveness to self or foreign antigen” (Sprent & Kishimoto, 2001). This state of unresponsiveness is achieved through two separate, well-described mechanisms, namely central and peripheral selection. During these selection processes T lymphocytes are exposed to self-peptides presented in the context of host MHC; if host T cell interact with either too high or too low an avidity for MHC-peptide complex, the T cell is deleted. This poses a central problem to tumor immunity in that high affinity T cell clones to self-peptide are deleted as a protective mechanism for the host. As tumors arise from host tissue, they share expression of common antigens with non-malignant tissue. Host T cells have been pre-selected for low affinity interaction to these antigens. Thus, methods for introducing T cell receptors which possess high affinity and avidity to common self-antigens needed to be developed in order to augment lymphocyte transfer therapy. One such method is the isolation and subsequent expansion of high affinity T cells that have migrated into a tumor microenvironment coined TIL’s for Tumor Infiltrating Lymphocytes. The ability to isolate the rare, tumor specific T cells and perform ex-
vivo expansion of these clones has been a key advancement in immunotherapeutic of human cancer. Identifying key T cell growth factors such as IL-2 allowed this method to become a robust source of tumor reactive lymphocytes. However, this technology was still limited by the availability and access to expanded primary T cells. Advances in molecular biology allowed for the functional cloning of T cell receptor genes and gave researchers the ability to create tumor specific T cells in any population of patient derived T cells regardless of TIL abundance. By isolating and cloning T cell receptor genes, these genes may then be inserted into several different version of either gamma retrovirus such as MMLV (Maloney murine leukemia virus) or lentivirus such as HIV-1 (human immunodeficiency virus). The introduction of T cell receptor genes into viral vectors afforded the ability to introduce a specific reactive T cell receptor into any host via pseudo typing of viral particles with envelope proteins from viruses such as VSV-G and provided a way to generate cellular immunity to tumors in patients. Because these viruses utilize integrase, meaning they are incorporated into host genomes, they could be expanded and maintained in-vivo giving lasting, specific, tumor immunity. Viral transduction of T cell receptor genes remains the most widely used method in the creation of tumor specific T cells used in immunotherapetics.

**Hypothesis**

The use of adoptively transferred gp100 reactive transduced T lymphocytes in combination with anti-PD-1 antibody will effectively reduce the burden of LAM disease.

**Specific Aims**

**Aim I: Generate transient and stable expression of HLA-A2 in LAM10224 cells.** These experiments will allow for the generation and maintenance of an HLA-A2 positive LAM cell line
that will serve as a viable target for transduced, adoptively transferred T lymphocytes.

Development of a suitable, gp100 expressing, HLA-A2 cell line is essential in generating a relevant disease model in which to test T lymphocyte adoptive transfer therapy.

**Aim II: Generate gp100 reactive, HLA-A2 restricted T lymphocytes through SILv44 viral transduction and assess T cell functionality against HLAA2 expressing LAM10224 cells in vitro.** Primary human LAM10224 cells will be transfected with an HLA-A2 expression vector and will serve as a target for HLA-A2 restricted gp100 reactive transduced T lymphocyte. These T lymphocytes will be generated through transduction by a T cell receptor-encoding viral vector. T lymphocyte responses will be measured by ELISA for INF-γ. HLA-A2 expressing LAM10224 cells will be used as targets for SILv44 T cell receptor transduced cells, measuring secretion of IFN-γ and IL-17a.

**Aim III: Identify a role for anti-PD1 and TCR Transduced T cells in containing LAM lesions in mice.** Mice challenged intravenously with LAM10224A2 cells will be injected with T cell receptor transgenic T cells. LAM disease severity will be monitored via lesion size over time and lesion area in lung tissue. These experiments will test the efficacy of HLA-A2 restricted, gp100 reactive transduced T lymphocytes in reducing the tumor burden of LAM10224 challenged, immunocompromised mice. Additional LAM10224-A2 challenged mice that have been treated with HLA-A2 restricted, gp100 reactive T lymphocytes will also be treated with anti-PD1 antibody to examine effects of checkpoint blockade on T cell response to LAM.
CHAPTER 2

AIM 1

AIM 1 – Generate Transient and Stable Expression of HLA-A2 in LAM10224 Cells

Experimental Approach.

Generation of a HLA-A2+ LAM cell line. LAM10224 is a cell line isolated from a lung biopsy of a patient who was subsequently diagnosed with lymphangioleiomyomatosis (LAM). LAM10224 cells were isolated and cultured from the lung biopsy of a premenopausal female patient who first exhibited dyspnea and cystic lung disease 3 months prior to the biopsy. LAM diagnosis was confirmed by indirect HMB45 immunostaining (Dako, Glostrup, Denmark). The sample was obtained with patient informed consent according to approval from Loyola’s Institutional Review Board (IRB) in accordance with the Declaration of Helsinki. Cells were isolated by overnight incubation with collagenase type IV (Sigma-Aldrich, St. Louis, MO), thermolysin (Sigma-Aldrich), trypsin (Invitrogen, Carlsbad, CA), and DNaseI (Roche, Madison, WI). Cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Cellgro, Manassas, VA) containing 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and antibiotics (penicillin, streptomycin, and amphotericin; Invitrogen) at 37°C in the presence of 5% CO₂. Incredibly, these cells became pigmented after two passages in culture and are unique in their uniform expression of the associated enzyme tyrosinase. Furthermore,
LAM10224 cells exhibit anchorage independent cell growth as well as the ability to form lesions on mouse lungs when injected intravenously (not shown).

However, these cells were isolated from an individual who does not express HLA-A2. This necessitates introduction of HLA-A2 expression so that they may serve as a viable target for our transduced T cell receptor. T lymphocyte responses do not merely depend on the specificity of the T cell receptor for a given peptide, but also require recognition of the cognate peptide in the context of the correct MHC molecule. HLA matching of a T cell and target cell must be considered when developing T cell mediated therapies. The vitiligo lesion isolated, gp100-reactive T cell receptor SILv44, is an HLA-A*0201 restricted T cell receptor, meaning that it will only recognize its cognate peptide gp100 in the context of the MHC class I molecule HLA-A2. This interaction is necessary for these T cells to respond to target cells.

HLA-A2 expression in LAM10224 was established by transfection of an HLA-A2 plasmid bearing an antibiotic selection marker for blasticidin or an HLA-A2-GFP fusion plasmid. LAM10224 cells were transfected with HLA-A2 expression plasmid pEF6/V5-His-TOPO and the resulting cells (Figure 1) were selected using 20 micrograms/ml of blasticidin until all control, non-transfected cells were eliminated. As an alternative approach, the use of an additional plasmid bearing an HLA-A2 and an N-terminal GFP fusion cassette were used to monitor HLA-A2 expression via fluorescence microscopy during in vitro assays.

For flow cytometry analysis, a single cell suspension of LAM10224 transfected with HLA-A2 in pEF6/V5-His-TOPO expression vector or HLA-A2-GFP fusion expression plasmid (Tone Fredsvik Addgene plasmid # 85162) was stained extracellularly prior to running the samples on the cytometer. Cells were separately stained with antibodies against HLA-A2 (BB7.1) (BD Biosciences) and HMB45 to gp100 (Dako). Briefly, cells were incubated with
primary antibodies for 30 minutes at 4°C, washed, and then incubated with species specific, fluorescently labeled secondary antibodies for 30 minutes at 4°C. Fluorescence was detected and quantified with FACS Canto II and Fortessa LTR II instrumentation (BD Biosciences, San Jose, CA). Data were analyzed with FlowJo software (TreeStar Inc. Ashland, OR).

Results.

LAM10224 cells transfected with pEF6/V5-His-TOPO HLA-A2 containing expression vector were assayed for HLA-A2 expression via flow cytometry after a 24-hour incubation period. Both transfected and non-transfected cells were placed under blasticidin selection at a concentration of 20 micrograms/ml of blasticidin (Figure 1). The transfected LAM10224 cell population growth was uninhibited by the blasticidin (Figure 1A) whereas the non-transfected LAM10224 cells were eliminated (Figure 1B). LAM10224 cells were successfully transfected to express both the GFP (Figure 3) and antibiotic resistance containing HLA-A2 plasmids (Figure 3) through the use of liposome based transfection systems. These cells were able to functionally present gp100 to several HLA-A2 restricted T cell receptors, including SILv44, demonstrating that the HLA-A2 transfections were successful. Both the HLA-A2 GFP fusion plasmid and the pEF6/V5-His-TOPO HLA-A2 plasmid could elicit IFN-γ and CD107a release from CD8+, SILv44+ T cells in vitro (Figure 6B).

For the experiments discussed, transfection of an HLA-A2 expression vector was used to generate LAM10224-HLA-A2+ target cells. The transfected expression vectors utilize DNA incorporated into liposomes with a lipid based transfection reagent, Purefection (SBI). This reagent allows the DNA/lipid complexes to pass through cell membranes, and the autonomously
replicating plasmid is then translated and expressed. Because these plasmids do not efficiently integrate into host genomes, expression is transient in nature. However, this method of protein expression is both rapid and highly efficient (Figure 3). Greater than 50% of cells in these assays express the protein of interest (Figure 3B), and this protein expression is maintained in vivo for as long as 14 days (Figure 3C), indicating that transfection based MHC expression is sufficient to elicit and measure responses to treatment in an in vivo model system.

Figure 1. HLA-A2(+) LAM10224 cells can be generated through transfection of an HLA-A2 expression plasmid
A.) LAM10224 cells transfected with PEF6-V5his TOPO HLA-A2 containing vector. Cells were transfected using LTX transfection reagent and placed under 20 micrograms/ml blasticidin for 4 days and examined for presence of live cells B.) Untransfected LAM10224 cells treated with 20 µg/ml of blasticidin after 4 days to ensure that the concentration of blasticidin was sufficient for selection of transfected cells.
Figure 2. LAM10224 shows similar melanocyte associated antigen expression when compared to 888 melanoma cells.

A.) Electron micrograph depicting melanosomes present in LAM10224 P5 cell cytoplasm

B.) Flow cytometry analysis depicting tyrosinase and gp100 expression by LAM10224 P11 cells in comparison to 888 melanoma cells. LAM10224 cells were used as the unstained control
Figure 3. HLA-A2 expression in transfected LAM10224 cells can be maintained in vivo.
A.) Flow cytometry performed on untransfected LAM10224 P85 cells as a control for HLA-A2 transfection B.) Surface HLA-A2 expression on HLA-A2 transfected LAM10224 P85 cells C.) Flow cytometry performed on LAM10224-A2 P85 challenged SCID/Beige mouse lung depicting surface HLA-A2 expression of 60% in vivo

Discussion.

The creation of HLA-A2+ cell lines to serve as targets for pre-clinical research from cell lines not initially expressing that haplotype is uncommon in the current literature. The most common methods of obtaining HLA-A2+ tumor tissue are either naturally occurring tumors in patients who express HLA-A2 or transgenic HLA-A2 expressing mice which are aged until cancer develops (Kaplan, et al., 2005). In the literature transfection of permissive cell lines appears to be the most common method to achieve HLA-A2 expression and the methods behind this strategy are sparsely discussed (Ding, et al., 1998; Mentzer et al. 1986). The method utilized in my experiments was described by (Kawakami, Zakut, Topalian, Stotter & Rosenberg, 1992)
This method was used to generate several HLA-A2 melanoma cell lines that can now be purchased for T cell recognition experiments. Because these experiments involve primary LAM cells, in which we possess the only human cell line, modification of these cells to express HLA-A2 was required. Although evidence exists to support smooth muscle origin of LAM cells, the originating cell type has yet to be identified. The use of a surrogate, HLA-A2, gp100 expressing cell line should not be a strategy because of the uniqueness of LAM and the effect these differences might have on the outcome of T cell based immunotherapy. Primary LAM10224 cells are unique in their ability to maintain their pigment over 107 passages, something not seen in melanoma cell lines that will begin to lose their dark appearance after serial passage.

LAM10224 also express the immunosuppressive molecules PD-L1 and PD-L2 to a greater degree than melanoma, thus making gp100 expressing melanoma cell lines unsuitable candidates for representative in vitro assays. The establishment of HLA-A2 expressing LAM10224 cells has been a difficult task; LAM10224 has shown nearly complete resistant to G418 selection and initial attempts to create a stable line using the vector pcDNA3 bearing G418 resistance gene failed because the untransfected cells were not killed by G418 using concentrations up to 2000μg/ml. Because of this finding, another HLA-A2 expression vector bearing a resistance gene for blasticidin, pEF6/V5-His TOPO. LAM10224 cells were susceptible to this antibiotic and pure populations of HLA-A2 expressing LAM10224 cells could be established for use in in vitro assays. However, these cells, even if left under antibiotic selection would lose their surface expression of HLA-A2 after approximately 4 days, making them unsuitable for future use in in vivo assays. In an effort to establish stable HLA-A2 expression, we sought to use viral transduction in order to achieve stable integration of HLA-A2 into the genome of LAM10224
cells. However, there was only 1 HLA-A2 expressing viral vector available to the lab, and although our lab has been able to successfully produce infectious lentivirus, we were unable to make infectious retrovirus with this HLAA2 retroviral vector. We thus turned to our final HLA-A2 expression plasmid pClpA102-G-HLA-A2_GFP which had been purchased on addgene (Tone Fredsvik Gregers & Sébastien Walchli (Addgene plasmid # 85162). This plasmid allowed for faster analysis of transfection efficiency via microscopy as well as provided an additional measure for monitoring tumors in vivo. This plasmid was able to transfect LAM10224 cells at high efficiencies with a mean transfection of 45% over 5 transfection attempts. These transfected cells were also able to maintain expression in vivo for 14 days in a pilot experiment (Figure 3C) thus making it suitable for examining therapeutic effects of HLA-A2 restricted transgenic T cells on LAM10224 challenged mice.
AIM II: Generate gp100 Reactive, HLA-A2 Restricted T Lymphocytes Through SILv44 Viral Transduction and Assess T Cell Functionality Against HLA-A2 Expressing LAM10224 Cells in vitro

For an immunotherapeutic approach to be successful, reliable and functional transduction of T lymphocytes must be achieved. Using a lenti-viral transduction system, the desired DNA encoding for the T cell receptor of interest (in our case SILv44) can be packaged into viral particles that can bind and enter host lymphocytes. Lentivirus systems make use of the viral enzyme integrase. This enzyme facilitates integration into the genome allowing progeny of a successfully transduced cell to be expanded and to maintain expression of the desired receptor. This is especially important when working with primary lymphocytes where only limited numbers of cells can be isolated from patients. The ability to rapidly and effectively expand successfully transduced cells allows for treatment even when only small quantities of cells are available. An additional benefit to lentiviral based transduction systems is the ability to transduce both actively dividing and nondividing cells allowing for more versatile transduction conditions. Our SILv44 containing lentiviral vector also contains GFP in order to more easily identify both transfected viral producing cells and eventually transduced T cells. GFP expressing vectors allow for the ARIA cell sorting system to sort successfully transduced cells to ensure a pure population
of transduced T cells while avoiding staining for markers such as CD3 that could potentially activate and exhaust T cells before transfer into subjects.

**Experimental Approach.**

**Cloning the TCR into a viral plasmid.** Existing SILv44 T cell receptor expressing retroviral construct pQCXIN was digested using the restriction enzymes PacI and NotI (Thermo Fisher, Waltham, MA) for 1 hour at 37 °C. Digested vector DNA was then loaded directly into a 1% agarose gel run at 100V for 1 hour and the desired fragment was isolated with a DNA gel extraction kit (Thermo Fisher, Waltham, MA). The resulting overhangs generated by PacI and NotI digestion directly flank the T cell receptor insert, allowing for subcloning of the T cell receptor genes and the viral slippage P2A sequence into the lentiviral vector. The pLVXIRESZsGreen lentiviral transfer vector was obtained from Clontech Laboratories (Mountain View, CA). This transfer vector was chosen because the presence of the ZsGreen fluorescent moiety aids in verification of viral production and because homologous restriction sites in the multiple cloning site of the vector facilitate efficient transfer of the existing excised T cell receptor α and β genes. The pLVXIRESZsGreen vector was digested with PacI and NotI as described above and the T cell receptor gene fragment and empty pLVX-IRES-ZsGreen vector were subsequently incubated in the presence of T4 DNA ligase for 1 hour at 37°C. The ligation mixture was then used to transform heat shocked chemically competent OneShotTOP10 E. coli cells and plated on ampicillin containing LB-agar plates. Colonies were collected and expanded in liquid LB-medium with ampicillin. DNA was isolated from the transformed E. coli cells using a DNA isolation kit (Thermo scientific), and the resultant DNA was confirmed to contain the T cell
receptor genes via restriction digest with PacI and NotI to ensure TCR gene insertion by way of gel electrophoresis.

**Lentiviral vector construction and transduction.** Lentiviral vector pLVX-IRES-ZsGreen (Clontech) served as a backbone for all transductions. TCR α and β chains for T cell receptor SILv44 were joined via a viral 2A self-cleavage sequence and inserted at NotI and PacI restriction sites on pLVX-IRES-ZsGreen. pLVX-IRES-ZsGreen encoding SILv44 was transfected into 293TN viral producing cells via Purefection transfection reagent (SBI). Infectious virus was generated following transfection with envelope gene from vesicular stomatitis virus and the viral gag-pol plasmid psPAX2. Virus production was confirmed by transduction of titering cell line HT1080. For CD8+ Jurkat cell transduction, $10^6$ CD8+ Jurkat cells/mL were added to washed plates and spun at 1000g for 30 minutes in virus containing media. CD8 expression was maintained in these cells using 2 mg/ml G418 (constant exposure). For CD8 T cell transduction, CD8+ T cells were activated in culture medium for 3 days using OKT3 and IL-2 prior to introduction to the virus. Non-adherent cells were negatively sorted for CD8 (Stem Cell Technologies, Vancouver, Canada) and transduced using the same method as described previously.

**Fluorocytometric analysis.** T cells were stained with fluorochrome conjugated antiVβ17-PE (Beckman Coulter) and CD107a-Alexa Fluor 700 (BD Biosciences). Intracellular cytokine staining was performed with anti-IFN-γ-Bv711 (BioLegend, San Diego, CA) after transduced T cell cultures were stimulated in presence of Brefeldin A (BioLegend) with equal numbers of T2 cells loaded with HLA-A2 restricted peptide 209-217 (0.5 ng-5 µg) of gp100. As indicated, gp100209-217 2M peptide IMDQVPFSV was used to match the peptide used to vaccinate melanoma patients. After stimulation, cells were stained for surface antigens prior to permeabilization with
0.3% saponin. Cells stained for intracellular antigens were postfixed with 2% paraformaldehyde before analysis using Fortessa LSR II instrumentation (BD Biosciences).

**Cytokine release assays.** In cytokine release assays, effector T cells and target cells were combined 2:1 in flat-bottom 96-well plates at 50,000 effector cells/well. For PMA/ionomycin stimulation, 10 ng/mL of PMA was added and 1 µg/mL ionomycin was used for positive control samples (Life Technologies). T2 target cells were peptide-pulsed for two hours before adding effectors. Cocultures were incubated for 48 hrs. before measuring cytokine release via ELISAPRO kits for IFN-γ, IL-2 (Mabtech) or IL-17A ELISA (Mabtech, Cincinnati, OH) per manufacturer’s instructions.

**Sequence verification.** The T cell receptor containing viral vector pLVXIRES-ZsGreen purified plasmid DNA was sent to ACGT (Wheeling, IL) for sequence verification by Sanger sequencing. The correct sequence was confirmed.

**Transfecting a producer line.** The viral production cell line used for these experiments was the Human embryonic kidney cell line 293TN (SBI, Palo Alto California). These cells were first expanded to the desired number using 10 cm culture plates for the given experiment. Producer cells were transfected at approximately 80% confluency. Transfection was carried out by two different reagents, LTX and Purefection, both of which are liposomal based systems. Plasmids psPAX2, which contains DNA encoding the HIV gag structural protein, viral polymerase/tat transcriptional enhancer protein PMD2.G (a vesicular stomatitis virus glycoprotein encoding plasmid) and the pLVX-IRESZsGreen lentiviral transfer vector encoding the T cell receptor SILv44 were mixed in serum-free DMEM or OPTIMEM (Thermo Fisher,
Waltham, MA) at a ratio of 2:1:1 and incubated with either Purefection or LTX transfection reagent for 15 minutes at room temperature. The resultant mixture was applied to the 293TN producer cells and incubated for 18 hours (Figure 7A). After 18 hours, transfection media was removed and viral collection media was applied to viral producing cells for 24 hrs. After 24 hours media was collected into a single ultracentrifugation compatible tube (38 ml SW28). The viral supernatant was then centrifuged at 1x10^5 g for 2 hours at 4°C, resuspended and stored at -80°C. Virus was titered after thaw to account for loss of infectivity (Figure 7B).

**Isolating T cells.** Human T cells are isolated from buffy coats or whole patient blood by Ficoll gradient separation. The resulting 4-layer suspension contains red blood cells (bottom fraction), Ficoll (second to bottom fraction), peripheral blood mononuclear cells (second to top fraction) and plasma (top fraction). The PBMC layer is placed in a new tube. These cells are then centrifuged and washed. Once purified, the PBMCs are subjected to a CD8 negative separation kit to avoid premature stimulation of CD8+ T cells.

**Transducing human T cells.** Human T cells are isolated from buffy coats by Ficoll gradient centrifugation. CD8+ cells are magnetically, negatively separated to avoid premature stimulation. After isolation, T lymphocytes are activated with a combination of anti-CD3 (OKT3), IL-2, and IL-15 for 72 hours. T cells are then placed in a 96 well plate coated with retronectin at ~3x10^5 cells per well to be transduced by lentiviral vector pLVX-IRES-ZsGreen containing the T cell receptor SILv44. The cells are then spun in a table top centrifuge for 1 hour at 1000 g in the presence of 8 µg/ml polybrene and subsequently placed in a 37°C incubator for 8 hours post spinoculation. The transduction media is then removed and media containing fresh 100 international units of IL-2 per milliliter is added. The cells are assayed for transgene
expression at 48 hours. These cells can then be subjected to a rapid expansion procedure in the presence of irradiated feeder cells for 5 days. Briefly, this process entails irradiating PBMCs isolated from patient blood so that these irradiated cells may act to produce growth signals as well as cytokines for the expansion of the selected T lymphocyte population (Roy et al). The cells, however, are often used directly to avoid possible exhaustion.

**Surface CD107a and intracellular cytokine staining.** Human CD8+ T lymphocytes were transduced with SILv44 containing lentiviral vector pLVX-IRES-ZsGreen and allowed to incubate in AIMV media containing 100 international units/ml of IL-2 for 24 hours post transduction. T cells were added to 888-A2 melanoma cells, LAM10224 HLA-A2 transfected cells, or gp100 peptide pulsed T2 cells at a ratio of 2:1 effector to target. AIMV media containing 1x Brefeldin A and 1x Monensin was used to resuspend T cells. T cells and target cells were incubated for 6 hours before FACS analysis. Cells were collected and stained with antibodies against surface CD107a labeled with Alexa Fluor 700 at 4 °C. Cells were washed and resuspended in permeabilization buffer. Primary antibodies against cytokines IFN-γ and IL17a were added, labeled with BV711 and APC, respectively. Fluorescence was measured on Fortessa LSR II (BD Biosciences San Jose, CA) the same day and analyzed.

**Results.**

Transgenic T cells conferred specific reactivity to HLA-A2+, gp100+ cells including LAM10224 HLAA2 transfected cells *in vitro* (Figure 4A). Cytotoxicity was measured by CD107a expression (Figure 5B, C). SILv44 transduced T cells were measured for reactivity towards relevant targets. Responses were measured by ELISAs detecting IFN-γ, IL-5, and IL13 after 48 hrs. CD8 transfected Jurkat T cells were also examined for IL-2 secretion and T cell
receptor expression (Figure 6,7). The SILv44 T cell receptor transgenic T cells demonstrated specific IFN-γ release in response to gp100, HLAA2+ LAM10224 and melanoma cells, as well as comparable CD107a expression to that of PMA/ionomycin stimulated cells. When compared to two other melanoma reactive T cell receptors T4H2 (Figure 4E), R6C12 (Figure 4F), SILv44 (Figure 4D) displayed greater IFN-y release in response to physiological concentrations (0.5nM) of gp100\textsubscript{209-217} presented by T2 cells. The mean fluorescent intensity for each T cell receptor variable beta subunit condition was as follows for the peptide conditions, SILv44-175, T4H2-154, R6C12-167 and untransduced-101. The MFI of the variable beta subunits in the null peptide condition were, SILv44- 85.1, T4H2-84.5, R6C12-101.5 and untransduced-72. SILv44 mediated comparable CD107a, IFN-y (+) cells, with a mean fluorescence intensity of 2039, as compared to T4H2 1533 and R6C12 2353. The null peptide conditions resulted in MFI’s of SILv44-934, T4H2-654 and R6C12-659. The PMA/ionomycin condition had an MFI of 3379 (Figure 5A).

CD107a, IL-17(+) cells were also measured the resulting MFI for the peptide containing conditions were as follows SILV44-1945, T4H2-1221, R6C12-2009 and untransduced-1200. Peptide null T2 cells were used as control; their MFI were SILv44-705, T4H2-1557, R6C12-2165, untransduced and PMA/ionomycin 698, 7904 respectively (Figure 5B). MFI for the specific introduced variable beta subunit were also measured for the null peptide conditions and were as follows SILv44-85.1, T4H2-84.5, and R6C12-101, untransduced-26.2 these MFI increased to SILv44- 237, T4H2- 170, R6C12-180, untransduced-104 when gp100 containing wells were measured. (Figure 5D)

Illustration of the gating strategy used to determine these data. (Figure 6B) Those in Figure 5 were also measured for secretion of IL-5 and IL-13. The results of this ELISA were as follows
for the peptide containing conditions for IL-5 secretion SILv44-21.58, T4H2-394.84, R6C12-362.06 and untransduced-337. For the no-peptide controls SILv44-39.66, T4H2-172.04 and R6C12-90.66 illustrating SILv44 secretes significantly less IL-5 than the two to other gp100 reactive T cell receptors T4H2 (p=<0.0001), R6C12 (p=0.0011) and untransduced cells (p=0.0251) calculated using Welch’s corrected T test. (Figure 6C)

For the IL-13 ELISA the results were as follows for the peptide containing conditions, SILv44-85.28, T4H2 699.64, R6C12-554.46 and untransduced-384.97. The nopeptide controls SILv44-95.76, T4H2-227.72 and R6C12-394.97. These data indicate that SILv44 produced significantly less IL-13 than the other two gp100 reactive T cell receptors T4H2 (p=0.0009), R6C12 (p=<0.0001) and untransduced cells (p=0.0397) these data were analyzed using Welch's corrected T test.

A representative example of 38.9% SILv44 surface expressing CD8+ T cells is shown in (Figure 7A). These results were recapitulated for SILv44 in ELISA assays for IFN-y in response to HLA-A2 expressing LAM10224 cells (Figure 7B). CD8 transfected Jurkat T cells were transfected with SILv44, T4H2 and R6C12 and reacted 2:1 with 0.5 nM gp100; results were measured via ELISA for IL-2. SILv44, T4H2 and R6C12 were each able to mediate IL-2 release from CD8 transfected Jurkat T cells. The MFI was measured for each of these TCR transduced Jurkat populations and the results were SILv44-1277, R6C12-599, T4H2-325 and empty vector-50.2. (Figure 7 D, E). SILv44 CD8+ T cells show specific CD107a expression when cocultured with LAM10224-A2+ cells: 37% CD107a-IFN-y positive cells (Figure 8) in comparison to only 7% CD107a-IFN-y positive cells in the non-transduced control and 2.25% in the non-HLA-A2 expressing LAM10224 control (Figure 8).
Figure 4. SILv44 expressing CD8 T cells show increased IFN-γ production when compared to other gp100 reactive T cell receptor expressing CD8 T cells.

A.) LAM10224-A2 P83 cells are under attack by SILv44 transduced CD8+ T cells B.) Representative example of SILv44 surface T cell receptor expression 48 hours after viral transduction (C, D, E, F) depiction of IFN-γ intracellular cytokine staining performed with SILv44 and two melanoma reactive T cell receptors T4H2 and R6C12. The flow cytometry graph directly to the right of each letter is the corresponding no peptide control for that T cell receptor. T cells were incubated with gp100 pulsed T2 cells at a ratio of 2:1, E:T, peptide concentration was 0.5nm C.) untransduced CD8 T cells D.) SILv44 transgenic CD8+ T cells E.) T4H2 transgenic CD8+ T cells F.) R6C12 transgenic CD8+ T cells. SILv44 expression in CD8 T cells imparts a Tc17 phenotype gp100 pulsed T2 cells at a ratio of 2:1, E:T, peptide concentration was 0.5nm
Figure 5. SILv44 expression in CD8 T cells imparts a Tc17 phenotype.
A.) CD107a surface expression as a function of IFN-γ among T cell receptor positive CD8+ T cells. SILv44, T4H2 and R6C12 transgenic CD8+ T cells incubated with 0.5nm gp100209-217 pulsed T2 cells. B.) CD107a surface expression as a function of IL17a among T cell receptor positive CD8+ T cells C.) Variable beta subunit staining MFI for each transduced TCR D.) gating strategy used to determine mean fluorescence represented in each bar graph
Figure 6. SILv44 expression in CD8 T cells imparts differential cytokine secretion.
A.) Variable beta subunit staining for the cells used to generate the data for graph’s B, C.  B.) SILv44 shows significantly reduced secretion of IL-5 when compared to T4H2, R6C12 and untransduced T cells C.) SILv44 shows reduced IL-13 secretion when compared to T4H2, R6C12 and untransduced CD8+ T cells.
Figure 7. SILv44 expression in CD8 T cells generates specific IFN-γ release when reacted with HLA-A2(+) LAM10224 cells.

A.) representative SILv44 Vb17 surface expression 48 hours after viral transduction by the CD8+ T cells  
B.) Transduced CD8+ primary T cells were reacted with LAM10224-A2 cells at effector:target ratios of 2:1, demonstrating INF-γ release mediated by SILv44  
C.) Gating strategy used to determine T cell receptor presence and MFI on CD8 transfected Jurkat T cells  
D,E.) Preliminary assay’s illustrating that SILv44,R6C12, and T4H2 could be virally transduced into Jurkat T cells and respond to cognate antigen.
Figure 8. SILv44 expressing CD8 T cells show similar CD107a expression to stimulation condition when reacted with HLA-A2 expressing LAM10224 cells.

A.) CD107a-IFN-γ co-expression among SILv44 T cell receptor positive CD8+ T cells incubated 2:1 with HLA-A2 transfected LAM10224 P83 cells. Lack of CD107a expression among untransduced T cells incubated with HLA-A2 expressing LAM10224 P83 cells

Figure 9. Lentiviral transduction of the HT1080 cell line allows for titering of infectious TCR containing lentiviral particles,

A.) Lenti-X 293T viral producing cells transfected using LTX transfection reagent containing the SILv44 lentiviral expression vector pLVX-IRES-ZsGreen B.) Transduction of HT1080 viral titer cell line using the transduction protocol outlined in experimental approach in AIM 2 infectious viral titers determined to 5.6 x 10^6/ml
Discussion.

The first use of a lentiviral vector system was in 1989, when Terwilliger, Godin, Sodroski and Haseltine (1989) replaced the nef gene from replication competent HIV-1 with Chloramphenicol acetyl transferase and infected Jurkat T cells. Since that time the use of lentiviral vector systems has become a forefront in genetic engineering. Lentiviral vectors have been generated to both induce expression of desirable genes such as T cell receptors and to silence undesirable genes as in the case of RNAi lentiviral vectors (Stewart et al., 2003). The original replication competent viruses were both a significant biosafety risk and also were restricted in their tropism in that they could only infect the same host cell range as that of the pathogenic virus (Sakuma, Barry & Ikeda, 2012). Modern lentiviral vector systems employ several strategies to both increase safety and efficacy of gene delivery by these viruses. First, viral proteins essential for replication are put on separate plasmid so that only one round of replication may occur, thus increasing safety for both the researcher and eventually patient. Second the pseudo-typing of HIV-1 virus with pan-tropic envelope proteins such as vesicular stomatitis virus glycoprotein has allowed for the expansion of this method to virtually any cell type, including those important in our data, CD8 T lymphocytes. One of the first examples of redirected specificity by viral introduction of a specific T cell receptor came when (Cooper et al, 2000) used viral transduction to engineer CD8 T cells against an HIV-1 epitope. These data demonstrated that viral transduction could both alter the specificity of isolated T cells by introduction of T cell receptor genes as well as sustain the expression of the desired receptor as the expression data collected for this manuscript was collected 21 weeks after transduction of the T cells. Although lentiviral biology has been intensely studied since it was first used to generate
cellular immunity in T cells, much of the methodology remains similar. We have utilized a similar vector system as described above however taken advantage of the more recent incorporation of fluorescent moieties such as GFP in our case to further aid in characterization of our transduced cells. The classical determinant of a T lymphocytes cytokine expression is defined by the transcription factors that are active within a cell for example, TH1 and TH2 cells that are characterized by their expression of T-Bet and GATA3 respectively. However, in the data presented here, we should that the phenotype of CD8+ T cells can be altered by the introduction of a nonendogenous T cell receptor. The differential secretion of IL-5, IL-13 and IL-17 shown in Figures 5 and 6 demonstrate both differential cytokine secretion of SILv44 compared to two other gp100 reactive T cell receptors as well as untransduced CD8+ T cells from the same donor. These data provide evidence that the T cell receptor may play a role in the phenotype of a given T cell, and that introduction of a foreign T cell receptor may alter the previously-determined cytokine profile of that T cell. Before this conclusion can be made, additional experiments are required. Namely, SILv44 must be tested across several donors under the same experimental conditions, and across several target cell populations, such as T2, melanoma and LAM10224 cells to ensure that the T cell receptor is truly mediating a change in T cell phenotype. In the data presented in this text, the presence of the introduced T cell receptor was determined by staining for the variable beta subunit of the introduced T cell receptor as well as the presence of a fluorescent moiety housed within the viral transfer vector. While this method is effective in differentiating transduced, from non-transduced cell populations, it does not definitely give detailed information about the T cell receptor, such as the amount of correctly paired introduced T cell receptors on the cell surface. Additional work using either anti-idiotype,
or MHC-tetramer flow cytometry staining must be done to elucidate whether the differences in cytokine secretion seen in these data are due to expression levels, TCR stability at the surface or mispairing with endogenous T cell receptors.
LAM10224-A2 challenged mice are treated with HLA-A2 restricted, gp100 reactive T lymphocytes, with and without anti-PD-1 antibody will be administered and antitumor responses will be measured. Severe combined immunodeficiency (SCID) mice possess a bi-allelic recessive mutation on chromosome 16 within the gene that encodes the enzyme protein kinase, DNA activated, catalytic polypeptide (Prkdc). Leaving the cellular immune system unable to perform VD(J) recombination severely impacts the ability to produce a function T and B lymphocyte repertoire. Because of the lack of adaptive immunity these mice are prime candidates to accept allogeneic cell types such as LAM10224, as a model for studying the effectiveness of adoptively transferred T cells. Because the adoptively transferred T cells would provide the only adaptive immune responses within the mouse, their activity can easily be correlated with disease outcome. Mice will be intravenously and subQ challenged with LAM10224-A2 cells as well as subcutaneously as a monitor for lung lesions, and animals will be treated with untransduced or SILv44 transduced human T cells with and without anti-34PD1 antibody when tumors arise. Subcutaneous tumors are measured via calipers daily, for changes in
tumor growth. PD-1 receptor activation inhibits T cell receptor mediated effector functions and increases T cell migration within tissues, reducing the time that a T cell has to scan the surface of engaging cells for the presence of cognate peptide-MHC complexes. By reducing this interaction time, T cells may not recognize target cells expressing lower levels of peptide-MHC complexes (Honda et al., 2014; Topalian et al., 2012). PD-1 ligands PDL-1 and 2 are overexpressed by some tumor cells, including LAM10224 (Figure 15). During antigen specific T cell engagement of a target cell, surface expression of PD-1 is increased upon stimulation of the T cell receptor (Topalian et al., 2012). In consequence, T cells engaging in persistent infection or tumor challenge in our case can exhibit “exhaustion” which may lead to expression and engagement of these death receptors and subsequent loss of T cell populations combating these malignancies.

**Experimental Approach.**

**Injection of mice and description of experiment.** LAM10224 cells were transfected with HLA-A2-GFP encoding plasmid and analyzed in part for expression of HLA-A2 via flow cytometry. After determination of transfection efficiency (Figure 3B), Each group of SCID beige mouse (N=2) received 10^5 HLA-A2 expressing LAM10224 cells via tail vein injection and 10^6 HLA-A2 expressing LAM10224 cells subcutaneously. Subcutaneous tumors were monitored daily. When a palpable mass could be measured.2x10^6 SILv44 transduced CD8+ T cells measured to be ~26% T cell receptor (TCR) positive (thus, 5.20x10^5 TCR transgenic T cells were injected via retro orbital injection under isoflurane anesthesia (Figure 12). Daily subcutaneous tumor measurements were carried out until the end of the experiment. Measurements were averaged for each group and graphed.
Results.

The transgenic T cell treated mice that received pembrolizumab both transgenic T cell treated groups showed less average tumor volume than mice treated with untransduced T cells (Figure 11). Mean tumor volume for untransduced treated group was 180.25 mm$^3$, SILv44 treated group was 150mm$^3$, and SILv44 + pembrolizumab was 42mm$^3$. SILv44; pembrolizumab was also the only group in which SubQ tumor showed recession during the experiment. More importantly, this experiment allowed for the determination that the tumor challenge and treatment procedure could successfully be completed using the methods outlined previously in this text. The purpose of this experiment was to develop a strategy to complete larger, more complex experiments from which data could be collected.

Tumor growth in these mice matched earlier data collected after challenge with lower passage LAM10224 P11 cells (Figure 10). Subcutaneous tumors, lungs, kidneys and spleens of each mouse were harvested at the end of the experiment and either processed for HLA-A2 expression in tumor tissue via flow cytometry (Figure 3C) or frozen in OCT blocks for immunohistochemical staining. Lungs were imaged immediately after excision (Figure 11). Lung images were used to quantify lesion number. SILv44 pembrolizumab treated group had a total of 1 lung lesion, the untransduced treated group had a total of 3 lung lesions and the SILv44 only group had 0. A repeat experiment using the same methods as those described previously was done using three groups, N=6 SCID beige mice. The mean tumor volume at end point for the groups are as follows: untransduced T cell treated 214.6mm$^3$, SILv44 treated 103mm$^3$, SILv44+pembrolizumab 166mm$^3$ (Figure 14 A). (Figure 14 B & C) Flowcytometry was performed on spleen homogenate showing the greatest amount of CD3,CD8(+) cells present in
the SILv44+anti PD-1 treatment group with an average of 6.57%, followed by 1.8% in the SILv44 treated group and finally 1.11% in the untransduced treated group. Similarly, spleen homogenate was also stained for Vb17 and CD8, Vb17 (+). The SILv44+ anti-PD1 treatment group showed 3.48%, SILv44 only 0.82% and the untransduced group 0.54% double positive cells in spleen homogenate (Figure 14 C). Figure 14 D illustrates the gating strategy used to generate bar graphs for Figure 14 B and 14 C. Using linear regression analysis the mean values for tumor growth were plotted as function of time and the slopes were determine to be significantly different (p=0.0128). The slope of each line was calculated to be, antiPD1+SILv44-19.22, SILv44 only-13.15, and untransduced- 32.81 with R² values of 0.6696, 0.8217, 0.9108 respectively.

Figure 10. Primary LAM10224 cells show similar growth kinetics in vivo when compared to E6, E7 immortalized LAM10224 cells. Figure 10) Previous experiment done by our group providing evidence of similarity between immortalized and non-immortalized LAM10224 P11 and P11 cells
Figure 11. SILv44 alone and in combination with pembrolizumab treated mice show decreased subcutaneous tumor volume.  
Figure 11.) Growth of LAM10224-A2 P85 cells SubQ tumor challenged SCID/Beige mice. Three groups of SCID/Beige mice were challenged both with 1x10^5 LAM10224-A2 cells via tail vein injection and with 1x10^6 LAM10224-A2 cells subcutaneously. Two groups were then treated with 2x10^6 SILv44 transduced CD8 T cells, with and without anti-PD1 antibody pembrolizumab. The third group was treated with untransduced human CD8+ T cells.

A

B

Figure 12. SILv44 in combination with pembrolizumab showed no presence of LAM10224 lung lesions in challenged SCID mice. 
A.) Representative example of untransduced T cell treated along with excised lung from that group.  B.) Representative mice from the SILv44+anti-PD1 along with excised lung from that group.
Figure 13. Vb17 surface expression on CD8 T cells injected into SCID mice challenged with LAM10224-A2 cells.
Surface TCR Vβ17 staining performed on SILv44 transduced CD8+ T cells to confirm surface expression of the TCR before injection into SCID/Beige mice, showing 26% transduction. A.) untransduced control B.) 26.5% surface expression of SILv44 T cell receptor 24 hours post transduction.
A. Tumor Volume (mm$^3$) over Days post treatment:
- RED: SILv44
- BLUE: anti-PD1 + SILv44
- BLACK: Untransduced

B. % CD8/CD17 (+) cells:
- SILv44 + anti-PD1
- SILv44 only
- Untransduced

C. % CD3/CD8 (+) cells:
- SILv44 + anti-PD1
- SILv44 only
- Untransduced

D. Flow cytometry plots:
- Single cells: 64.5
- Lymphocytes: 93.1
- CD3/CD8 (+) measured
Figure 14. SILv44 in combination with pembrolizumab shows increased persistence of both CD3, CD8 expressing T cells as well as CD8,Vb17 expressing T cells in vivo.

Figure 14) A.) Subcutaneous LAM10224-A2 tumor volume across 3 treatment groups, N=6 for each group. Both treatment groups were given 1 million transduced T cells, as well as 20,000 units of IL2 for all groups every other day. The pembrolizumab group was given 250 μg pembrolizumab IP every other day until termination. The untransduced group was given 2 million untransduced T cells B.) CD8, Vb17 (+) cells recovered from SCID mice spleen at experimental end point C.) CD3, CD8(+) cells recovered from SCID mice spleen at experimental end point D.) gating strategy used to determine cell populations shown in B, C

Figure 15 SILv44 in combination with pembrolizumab shows increased persistence of both, CD8, IFN-γ expressing T cells as well as CD8,IL-17 expressing T cells in vivo  A ,B) The SILv44+ anti-PD1 mice show higher % CD8, IL-17 and CD8, IFN-γ expressing cells in the spleen at end point. C.) gating strategy used to generate bar graphs for A, B
Figure 16. LAM10224 cells show increased PD1 ligand expression when compared to 888A2 melanoma cells in vitro.

Surface programed death ligand 1 and 2 expression in 888-A2 melanoma cells (left) and LAM10224 cells (right)

Discussion.

The use of human tumor xenografts into immunocompromised mice has been utilized in the modeling of human disease since the late 1960's (Rygaard, 1969). These methods involve the isolation of either human tumor or tumor cells that may then be transferred to mice that lack the ability to reject the graft. As one would surmise, this allows the researcher to study in vivo growth of human tumors and subsequently ways in which to impede such growth. Since its development the human tumor xenograft has resulted in mouse models of many human malignancies, Charles Rivers lists 23 histotypes of human xenograft cancers in nude mice. One human malignancy not listed here nor anywhere else in the literature is lymphangioleiomyomatosis. There are currently no available in vivo models of LAM disease.
using human allogeneic cell challenge. Currently, the existing model of LAM disease in mice utilizes TSC2(-,-) mouse kidney cells that demonstrate the ability to form lung lesions in nude mice (Krymskaya, 2012). Although this particular model has been useful in examining the effects of pharmacologic based treatments for LAM disease, this model can be improved upon to test approaches such as immunotherapy. In order mimic the clinical disease and effective treatment it is most useful to maintain as much similarity to the clinical scenario as possible. Our group has done so by utilizing not only the first human LAM cell xenograft into SCID mice, but also through the use of human transgenic T lymphocytes and checkpoint inhibition directed at human PD-1. This allows for the most interaction between human T cells and human tumor as it would occur in patients. Although the LAM10224 xenograft is currently the most clinically similar model of LAM disease for in vivo therapeutic use, our model may still be improved upon.

The first example of clinical success using adoptive T cell transfer of genetically modified T cells was described in 2006, when patients were given T cells reactive to the melanoma peptide MART-1 (Morgan et. al., 2006). This trial demonstrated that solid tumors could be treated by conferring antigen specificity via a T cell receptor in patient derived lymphocytes. Since this initial study, there have been many recapitulations of this trial using different melanoma antigens such as eMAGE-A3 and gp100 (Sharpe & Mount, 2015). During these trials, 30% of patients were shown to exhibit tumor regression as defined by monthly CT scan (Johnson et. al., 2009). However, on target, off tumor reactivity was observed. It is generally understood that although peptide selection for TCR transgenic T cell receptors is designed to minimize the presence of the target peptide in off target tissue, this has not been completely possible. The current clinical approaches aim to select markers that are present in non-essential cells such as melanocytes so
that on target, off tumor reactivity is not lethal. Although depigmentation as a result of adoptive T cell transfer therapy is non-lethal, it can result in social consequences for patients and thus must be seriously considered when proposing melanocytic antigen reactive T cell therapy. Previous use and clinical success of adoptive transfer of genetically modified T cells lends support to the utilization of this treatment method for other diseases such as LAM where links can be made to existing therapies. Although these data share similarities to existing therapies, we are the first group to propose a combination therapy of transgenic T cell adoptive transfer and anti-PD-1 antibody treatment. There are currently multiple anti-PD1 antibodies in clinical use. The antibody used in these studies, pembrolizumab was FDA approved in 2014 for use in melanoma after it was shown to reduce tumor size in 34% of patients (Schachter et al., 2015). The FDA approval benefits our approaches because the safety of this treatment has already been evaluated and deemed acceptable for clinical use. Immune checkpoint blockade is quickly becoming utilized in other malignancies as well; the FDA recently approved another PD1 blocking antibody for use in bladder cancer after a mean tumor regression of 19.4% was demonstrated (Sharma et al. 2015). In our own hands, pembrolizumab in combination with SILv44 transgenic T cells has both demonstrated the ability to reduce tumor volume in LAM10224 challenged SCID/Beige mice when compared to untransduced T cell treated mice, as well as increase persistence of SILv44 transgenic T cells in-vivo. At this time, these data require further validation by additional experimental parameters that were not present in this work. During this work, there were a number of factors that limited our ability to perform certain controls, such as subject number. The sample size (N=2 and N=6) respectively are relatively small and make statistical analysis more challenging. With a limited number of mice, additional
controls must also be omitted in order to collect useable data. Two such control groups that were not present in these data were a group using anti-PD1 antibody in combination with untransduced T cell transfer to examine the effect of checkpoint inhibition on allogeneic response. An additional control that must be added is the complete absence of any introduced human T cells to examine how tumors will progress in the absence of any cells capable of inhibiting their growth via specific or non-specific mechanisms. As a personal interest, our lab would also like to examine the dose response of SILv44 transgenic T cells by using increased numbers of transgenic T cells, as well as additional treatments to examine whether greater amounts of cells provide additional benefit. When LAM10224 challenged mice are sacrificed and spleen homogenate is collected, the mice treated with pembrolizumab show both higher CD3, CD8(+) cells as well as higher CD8, Vb17(+) when compared to mice treated with SILv44 or untransduced T cells alone, indicating that pembrolizumab is allowing these cells to persist in-vivo. Taken together the current literature and clinical history of both transgenic T cell therapy as well as immune checkpoint blockade lend strong support to the clinical feasibility of our treatment for LAM.
CHAPTER 5

CONCLUSION

Discussion

Taken together, these data illustrate that LAM10224 may be reliably and efficiently transfected to express the MHC class I peptide HLA-A2 and functionally present antigen to antigen specific, transgenic CD8+ T lymphocytes. The ability of LAM10224 to be modified to express nonendogenous MHC haplotypes lends support to its ability to serve as a reliable *in vitro* and *in vivo* target for immunotherapeutics. This is because the HLA-A2 haplotype is common haplotype across most ethnic groups with an average total prevalence of 47.6% across ethnicities (Ellis, et al., 2000). This allows for the broadest spectrum of patients who may benefit from this treatment. With LAM disease exhibiting a known prevalence of only 3.35 cases per million women in the United States (Harknett et al., 2011) it is essential to develop a therapy that targets the largest group of patients. There have been no studies that have looked at HLA haplotype presence in LAM exclusively, so one must apply the general population statistics to this particular patient population. Because these cells may be transfected to express exogenous MHC molecules at efficiencies greater than 50% as well as to maintain that expression *in vivo* for as long as 14 days post injection they offer substantial, superior benefits over existing models such as TSC (-) MEF cells in the context of testing immunotherapeutic approaches to LAM. The most important of which is that it employs the use of human LAM cells and human T cells which most closely resembles the disease and treatment approach as they would be utilized in patients.
LAM10224 demonstrates the ability to form lung lesions, in mice, similar to what is observed in patients. These tumors also progress slowly in these mice. Sub cutaneous injections of $10^6$ cells reach only 300 mm$^3$ in untreated mice; this can be correlated to natural disease progress of LAM, giving opportunities for interventions such as those we propose with these data. Utilizing autoreactive T cell receptors from vitiligo patients to treat pigmented malignancies offers a novel concept, combining expertise from two highly studied areas of immunology, autoimmunity and immune evasion by cancer. The SILv44 vitiligo derived T cell receptor is a novel and unique receptor in that it imparts different effector function on CD8+ T cells when compared to T cell receptors reactive toward the same antigen (Figure 5). Melanoma is a highly studied cancer in the field of immunotherapy; one might suggest that because gp100 is the diagnostic marker for LAM disease, that a preexisting melanoma isolated, gp100 reactive T cell receptor would be the best therapeutic option. However, when compared with other gp100 reactive T cell receptors such as T4H2 and R6C12, SILv44 has the ability to induce greater IL-17a release than the other two T cell receptors even though all 3 share similar ability to produce IFN-y in response to TCR stimulation. These findings offer new insight into the functions defined by T cell receptors, as it appears they define not only the quantity, but also the quality of the response. (the criteria in which immunotherapeutic T cell receptors are defined and chosen). IL-17 has been to have diverse roles in tumor immunity and seems to be variable depending on the tumor microenvironment. Recently, however, IL17 has been associated with the reduction of T regulatory cells present in tumors, thus augmenting immune response against the tumor (Majchrzak et al., 2016). Th17 cells have also been shown to activate CD8+ T cells in melanoma models and improve overall outcome (Martin-Orozco, 2009). Utilization of a CD8+, antigen
specific, IL17 secreting T cell receptor, thus offers great promise in unexplored malignancies such as LAM. Adoptive T cell transfer might offer greater therapeutic benefits for LAM patients than existing therapeutics. T cell-based therapeutics are cytotoxic towards LAM, thus eliminating LAM cells from the lungs rather than merely inhibiting tumor progression. The only current available therapy for patients with LAM disease is the immunosuppressive drug Rapamycin. Rapamycin was FDA approved for use in LAM after the MILES trial demonstrated stabilized lung function in patients responding to treatment (McCormack et al., 2011). Stabilization was the best achievable outcome for these patients. The cytotoxic effector function of gp100-reactive, CD8+ T cells may instead allow for the elimination of LAM cells in patient lungs possibly allowing them to regain lung function.

Although transient expression of HLA-A2 has been sufficient for \textit{in vitro} and \textit{in vivo} experiments in our current system it must be acknowledged that stable expression of HLA-A2 in LAM10224 cells would be ideal moving forward. Our lab was forced to make use of transient transfection methods due to lack of access to a reliable HLA-A2 viral vector that could be used to produce high quality, infectious virus. Multiple methods were used to produce HLA-A2 containing retrovirus with the vectors available including utilization of several different viral producing cell lines and viral packaging plasmid combinations, however, these were unsuccessful. In future experiments, \textit{de novo} synthesis of an HLA-A2 expressing viral vector that may reliably allow for transduction of LAM10224 and creation of long term, stable expression will be ideal for furthering this model. LAM10224 offers promising ability to provide a clinically relevant model of human LAM disease and gives researchers the ability to develop innovative new therapies such as those discussed within this text.
Materials and Methods

Cell lines. T2 and Jurkat RT3-T3.5 (J76) were obtained from the American Type Culture Collection (Manassas, Virginia), (ATCC/CRL-1992 and ATCC/TIB-153, respectively). T2 and J76 cells were maintained in RPMI 1640 medium (Mediatech, Manassas, VA) with 10% heat inactivated fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 250 ng/mL Amphotericin B, 100U/mL Penicillin, 100 μg/mL Streptomycin (Invitrogen, Carlsbad, CA), and 2 mM L-glutamine (Invitrogen). J76 cells expressing CD8 were described elsewhere (57). Cells were grown at 37° C and 5% CO₂. 293 viral producing cells, lentiviral vector pLVX-IRESZsGreen, PMD2.G (VSV-G), and PSPAX2 were obtained from Clontech Laboratories, Inc (Mountain View, CA), and maintained in Dulbecco’s Modified Eagle Medium (Mediatech) with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals), 250 ng/mL Amphotericin B, 100U/mL Penicillin, 100 μg/mL Streptomycin (Invitrogen), 4.5 g/L L-glutamine, 4.5 g/L glucose, and 4.61 g/L sodium pyruvate (Mediatech). Transfected CD8⁺ J76 cells were maintained in presence of 2 mg/mL G418 sulfate (Mediatech).

LAM10224 cells were derived from the lung biopsy of a female patient who had clinical presentation consistent with LAM disease. LAM diagnosis was confirmed by indirect HMB45 immunostaining (Dako, Glostrup, Denmark). Sample was obtained with patient’s informed consent according to approval from Loyola’s Institutional Review Board (IRB) in accordance with the Declaration of Helsinki. Cells were isolated by tissue disassociation with collagenase type IV (Sigma-Aldrich, St. Louis, MO), thermolysin (Sigma-Aldrich), trypsin (Invitrogen, Carlsbad, CA), and DNaseI (Roche, Madison, WI). Cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; (Cellgro, Manassas, VA), containing 10% heat-inactivated fetal
bovine serum (Atlanta Biologicals, Lawrenceville, GA) and antibiotics (penicillin, streptomycin, and amphotericin; Invitrogen), at 37°C in the presence of 5% CO₂. Cells were defined as LAM cells by staining for gp100 and smooth muscle action. CD8+ primary T cells were maintained in AIMV medium (Invitrogen) supplemented with 5% heat-inactivated pooled human AB serum (Valley Biomedical, Winchester, VA), 250 ng/mL Amphotericin B, 100 U/mL Penicillin, 100 μg/mL Streptomycin (Invitrogen), 300 IU/mL recombinant human IL-2 (R&D Systems, Minneapolis, MN), 100 ng/mL recombinant IL-15 (R&D Systems), and 50 ng/mL OKT3 (eBioscience, San Diego, CA). PBMCs from hemochromatosis apheresis patients were isolated by Ficoll gradient centrifugation unless otherwise noted.

Human melanoma cell line 888-A2⁺, transfected to express HLA-A2 (59, 60, and 61). Cells were maintained in RPMI 1640 medium (Mediatech, Manassas, VA) with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 250 ng/mL Amphotericin B, 100 U/mL Penicillin, 100 μg/mL Streptomycin (Invitrogen, Carlsbad, CA), and 2 mM L-glutamine (Invitrogen).
REFERENCES


VITA

Christian Ankney was born in Franklin, IN. He attended University of Indianapolis where he received a Bachelor’s of Science in Kinesiology in 2015. While attending University of Indianapolis he worked as a teaching assistant in the physiology department. Christian became interested in immunology after taking a senior capstone course on the subject. His thesis work is on using gp100 reactive T cell receptors to treat lymphangioleiomyomatosis in the lab of Caroline Le Poole.

After completion of his Master’s degree in Infectious Disease and Immunology at Loyola University Chicago he plans to attend medical school.