# TRP-1 AS A MODEL TUMOR ANTIGEN FOR IMMUNOTHERAPY AND IMMUNE TOLERANCE IN THE THYMUS

Andrew G. Brandmaier

Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Doctor of Philosophy
In the Department of Microbiology and Immunology
Indiana University

June 2011

Accepted by the Faculty of Indiana University, in partial
Fulfillment of the requirements for the degree of Doctor of Philosophy

_____
Christopher Touloukian, M.D., Chair

_____
Janice Blum, Ph.D.

Doctoral Committee

_____
Randy Brutkiewicz, Ph.D.

June 25th, 2010

_____
Mark Kaplan, Ph.D.

_____
Jeff Travers, M.D./Ph.D.

ACKNOWLEDGEMENTS

I want to express gratitude to everyone who has contributed to my education and progress throughout the graduate program. I thank Dr. Chris Touloukian who provided me the foundation of an immune tolerance and tumor antigen model that is the backbone of this thesis. The field of tumor immunotherapy has captured my interest and inspired ideas for my future scientific efforts. I also thank Dr. Janice Blum who supported my progress, kept minutes on my committee, invited me to present at lab meetings and shared Western Blot reagents from her lab. I thank current and previous members of the Touloukian lab for their contributions and assistance on various techniques and experiments - Michael Ha, Garrett Kinnebrew and Nick Klemen. From the Broxmeyer lab, Giao Hangoc provided vital assistance with my mouse transplantation experiments. Also, I wish to acknowledge Victoria Crotzer from the Blum lab who has been a scientific resource and friend since my graduate rotations. I also want to recognize my committee members – Drs. Brutkiewicz, Kaplan, and Travers for their time and input along the way. Their thoughtful discussions on my project have generated useful suggestions and ideas throughout this process. Finally, I extend my gratitude to the M.D/Ph.D. program and its directors, Drs. Wade Clapp and Maureen Harrington. I have enjoyed the enriching seminars and retreats organized by them which offered insight into a wide variety of career opportunities in medical research. It has been a privilege to be a member of the Medical Scientist Training Program at Indiana. I extend sincere thanks to everyone mentioned herein for helping me along the way.

ABSTRACT

Andrew G. Brandmaier

TRP-1 AS A MODEL TUMOR ANTIGEN FOR IMMUNOTHERAPY AND IMMUNE TOLERANCE IN THE THYMUS

Tolerance mechanisms, which collectively work to prevent autoimmunity, play a key role in suppressing the adaptive immune response to tumor antigens. This phenomenon is attributed to the extensive overlap of tumor associated antigens with self peptides. We studied immune tolerance to tumor antigen TRP-1, a melanoma associated glycoprotein. Vaccination of Wild type (WT) and TRP-1 deficient (Bw) mice with TRP-1 antigen highlighted the substantial effect of tolerance on the T cell response: in the Bw population a log-fold differential was observed with greater clonal numbers and higher intensity of cytokine release from the antigen specific $CD4^+$ T cell population. Additionally, TRP-1-reactive T cells derived from Bw mice demonstrated significantly more efficacious tumor treatment ability than WT donor cells when adoptively transferred into recipients challenged with B16 melanoma. Furthermore, donor Bw T cells were so potent as to overcome suppression by endogenous $T_{regs}$ in mediating their effect. Probing for a tolerance mechanism, we isolated medullary thymic epithelial cells (mTECs) from WT mice and found that they promiscuously express TRP-1. Unexpectedly, TRP-1 expression in mTECs was found to occur independently of the prominent Autoimmune Regulator (Aire) transcription factor as well as the melanocyte specific transcription factor, mMitf. Our most recent data suggests that thymic dendritic cells may also express copies of the TRP-1 transcript.

Future transplant studies will test whether mTECs or thymic dendritic cells directly tolerize TRP-1 specific T cells. Overall, these findings highlight the relevance of central tolerance to cancer immunology and compel further investigation of its mechanistic impact on the development of tumor-reactive T cells.

Christopher E. Touloukian, M.D., Chair

TABLE OF CONTENTS

ABBREVIATIONS

| | |
|---|---|
| Ab | Antibody |
| ABS | Applied Biosystems |
| Aire | Autoimmune Regulator |
| APC | Antigen Presenting Cell |
| BL/6 | Mice on the C57BL/6 background |
| Bw | mice or cell samples with the $Tyrp1^{bw/bw}$ genotype |
| CCR | Chemokine receptor |
| CD (#) | Cluster of Differentiation marker on lymphoid cells |
| cTECs | Cortical Thymic Epithelial Cells |
| DCs | Dendritic Cells |
| DN | Double negative (thymocytes) |
| DP | Double positive (thymocytes) |
| DR4 Tg | DR4 transgenic mice |
| EBV-B | Epstein Barr virus transformed B cell line |
| EPCAM | Epithelial Cell Adhesion Molecule |
| HA | Hemagglutinin antigen |
| HLA | Human Leukocyte Antigen |
| IFN-γ | Interferon gamma |
| IL (#) | Interleukin (classification number) |
| i.v. | Intravenous |
| LN | Lymph nodes |
| MACS | Magnetic Assisted Cell Sorting (Miltenyi Corp) |

| | |
|---|---|
| MDA | Melanocyte Differentiation Antigen |
| MHC | Major Histocompatibility Complex |
| Mitf | Micropthalmia-associated transcription factor |
| mRNA | Messenger RNA |
| mTECs | Medullary Thymic Epithelial Cells |
| NK | Natural Killer |
| Nm | Nanomolar |
| qRT-PCR | Quantitative Real-time polymerase chain reaction |
| RAG | Recombinase activator gene |
| RBC | Red blood cell |
| SP | Single positive (thymocytes) |
| TCR | T cell receptor |
| $T_{eff}$ | T effector cell |
| Tg | Transgenic |
| Th (#) | T helper cell (classification number) |
| $T_{reg}$ | T regulatory cell |
| TRP-1 | Tyrosinase related protein |
| Tyrp1 | Tyrosinase related protein 1, gene symbol |
| $Tyrp1^{bw}$ | C57BL/6 mice of the $Tyrp1^{bw/bw}$ genotype |
| WT | C57BL/6 mice of the $Tyrp1^{+/bw}$ genotype |

# INTRODUCTION

## T cells

T cells play a central role in generating and modulating adaptive immune responses against peptide antigens. Classic αβ T cells have a range of functional profiles that are categorized by phenotype, but they share a common paradigm in that each expresses a unique surface molecule, the T cell receptor (TCR), which is a dimer of two globulin proteins, α and β. Individual TCRs are able to recognize specific antigenic peptides, known as epitopes, enabling immune detection of potentially dangerous challengers to the host such as microbes or tumor cells. The TCR variable region, a composite of the α and β chains, is the antigen binding end of the molecule. Each unique α and β chain is produced in a recombination step that entails random rearrangements of specific regions of the TCR gene (V + J for α and V + D + J for β) facilitated by the recombination activating proteins (Rag-1 and Rag-2). This process creates a diverse repertoire of TCRs which facilitates recognition of a massive spectrum of antigenic epitopes by the T cell population (1).

T cell recognition of an epitope occurs when its TCR binds with affinity to an antigenic peptide displayed by a molecular complex on an adjacent cell, the antigen presenting cell (APC). These display molecules are gene products of the major histocompatibility complex (MHC). Biochemically, they are globulin proteins that serve as vehicles for APCs to present peptide fragments on their surface, and the composite MHC:peptide molecules comprise one end of an

immunological synapse (2). Individual TCRs have uniquely arranged variable regions which allows them to recognize a specific cognate MHC:peptide complex; higher affinity binding generally leads to more potent stimulation. Studies have shown that synthetically tweaking natural peptide epitopes with truncations or minor amino acid substitutions to optimize affinity for a particular TCR can enhance this interaction (3). In general, individual T cells within the host repertoire are very precise in responding to a corresponding MHC:peptide complex. When recognition occurs and TCRs bind the complex with affinity, an immunological synapse is formed.

Formation of the immunological synapse is the key process that singles the T cell clone to mount an immune response. The αβ surface receptor transmits signals to other molecules associated with the TCR at the surface of the cell membrane, including the TCR zeta chain. Zeta contains immunoreceptor tyrosine-based activation motif domains (ITAMs) that are phosphorylated by the Src family kinase, Lck, when TCR binding occurs. The tyrosine kinase Zap70 is recruited to this domain and activated (4). Zap70 targets LAT (linker of T cell activation) for tyrosine phosphorylation, and LAT subsequently recruits an array of signaling molecules that mediate downstream signaling cascades leading to calcium release, Ras activation, actin polymerization and modulation of transcription (5,6). Ultimately, if this stimulation occurs in an inflammatory context with activation of costimulatory cell surface molecules, particularly the B7 family, the T cell will become activated (7). Depending on the cell's phenotype, it may

produce cytotoxic molecules or cytokines that coordinate an immune response to destroy the invading antigen. Moreover, the antigen-specific T cell clones will proliferate to magnify the number of cells responding to the invading antigen.

αβ T cells are classified based on phenotypic properties including their function and cytokine release profile. The two overarching phenotypes are divided according to whether they express the CD8 or CD4 surface marker and deemed $CD8^+$ or $CD4^+$, respectively. They are imprinted with this lineage during early development in the thymus (8). In host immunity, they perform diverse but complementary functions to maintain protection against a spectrum of antigenic challenges.

**$CD8^+$ T cells**

$CD8^+$ T cells respond to peptide epitopes presented by MHC class I complexes. These complexes consist of a three subunit alpha chain associated with the molecule β2 microglobulin (9). The pool of available class I antigens originates primarily originates from intracellular proteins that gain access to the cytosol where they are processed by the class I presentation pathway (10). MHC class I:peptide complexes are presented on the surface of cells of nearly every tissue type which facilitates global immune detection of the “danger” epitopes. This paradigm allows $CD8^+$ T cells to recognize and destroy infected cells throughout the body in a four phase process. First, the antigen specific T cells are activated by recognition of an MHC:peptide complex in conjunction with costimulation.

Next, the activated cell populations expand as much as 4 log fold over a period of 5-8 days. In the third phase, these populations contract by up to 95% over approximately a 1 week period. Finally, a subset of the remaining cells differentiates into a memory population that can re-expand vigorously on repeat exposure to the offending antigen (11). $CD8^+$ T cells directly mediate destruction of antigen carrying cells by secreting molecules such as granzyme and perforin that cause cytolysis (12). They also can promote target cell death by upregulating expression of CD95L which engages CD95 on adjacent cells and initiates an apoptotic signaling cascade (13). Additionally, they may release cytokines such IFN-γ and TNF-α to activate other effector immune cells including macrophages and neutrophils (14). Through these mechanisms, $CD8^+$ T cells are able to mediate elimination of virally infected cells and tumor cells.

**$CD4^+$ T cells**

$CD4^+$ T cells recognize MHC class II:peptide complexes which encompass a membrane-spanning heterodimer consisting of an alpha and beta chain, each with two subunits. most often presented by professional antigen presenting cells (APCs); the most prominent ones are dendritic cells and B cells, which can endocytose and process exogenous antigens for presentation. The class II epitopes include both extracellular and internally produced peptides that make it into the appropriate endosomal pathway. $CD4^+$ T cells are commonly referred to as $T_{helpers}$ for their role in releasing cytokines and chemokines that orchestrate other cells in the immune response, and they can adopt and sometimes

interchange between different subset phenotypes. The most prominent examples include IFN-γ secreted by $T_h1$ cells to promote cellular immunity, IL-4 from $T_h2$ cells which stimulates humoral immunity, and IL-17 from $T_h17$ cells which mediates pro-inflammatory responses. Depending on the context of their initial activation, $CD4^+$ T cells can differentiate along the different helper class lineages with the aforementioned cytokine profiles which allows a dynamic range of immune responses (15,16). Overall, $CD4^+$ T cells are a potent and critical component of the host's adaptive immune response to foreign antigens.

**Tumor immunity**

The phenomenon of tumor immunity has been observed for nearly a century with clinical case reports documenting resolution of tumor masses following associated infection or inflammation proximal to a malignancy. More specific confirmation of its existence was illustrated when tumor infiltrating lymphocytes (TIL) were identified in histological sections of malignant tissue. Further investigation of TIL has revealed that T cells are one of the central immune agents that recognize tumor antigens; they invade malignant sites and mediate the anti-tumor response. The first correlation between TIL and host survival was observed in melanoma patients where it was found that patients whose tumors had high levels of $CD8^+$ T cell infiltration enjoyed a more favorable prognosis (17,18). Since the discovery of a T cell response to tumors, investigators have attempted to utilize the immunogenicity of tumor-associated antigens for therapeutic purposes.

$CD8^+$ T cells specific for melanoma-associated antigens have been the primary focus of cell-based melanoma immunotherapy. Investigators have identified an extensive array of class I-restricted epitopes from melanoma antigens and also observed the significance of their cytotoxicity towards melanoma cells. For example, $CD8^+$ T cells specific for the melanoma antigen, MART-1, have been identified in infiltrates of the skin and retinal pigment epithelium of patients with autoimmune vitiligo - a cytotoxic immune response to melanocytes (19). Several studies have targeted MART-1 for melanoma immunotherapy including a recent gene therapy study that re-engineered T cells with a Mart-1 receptor and achieved tumor recognition and destruction (20). Another melanoma antigen, gp100, has been well characterized by Restifo and colleagues. They showed that immunization of C57BL/6 mice with a recombinant vaccinia virus expressing the gp100 gene elicited a cytotoxic response; *in vitro* co-cultures of the resulting T cells with various melanoma cell lines stimulated potent IFN-γ cytokine release. Also, adoptive transfer of an expanded gp100-specific $CD8^+$ T cell clone, derived from the vaccinated subjects, into mice challenged with B16 melanoma demonstrated superior tumor protection compared to a control group (21). The MART-1 and gp100 models are two prominent examples that have illustrated the potency of $CD8^+$ T cells bearing receptors specific for melanoma antigens.

**$CD4^+$ T cells in tumor immunity**

Despite the apparent potency of $CD8^+$ cytotoxic cells, increasing evidence points toward a central role for $CD4^+$ T cells in sustaining the type of comprehensive

immune response that may be needed for successful tumor immunotherapy. Various studies in mice have determined that $T_{helper}$ cells are essential for development and maintenance of $CD8^+$ memory T cells (22). More specifically, investigators have demonstrated that $CD4^+$ T cell help is required for optimal induction of cytotoxic T cells against class II-negative tumors (23). Additional studies have shown that $CD4^+$ T cells exert their effects through the induction and maintenance of B cells and $CD8^+$ T cells, and they are indispensable for the long-term maintenance of antigen-activated memory $CD8^+$ T cells (24,25). Several lines of evidence also suggest that $CD4^+$ T cells have other direct and indirect effects on tumor cells, including those deficient in major histocompatibility complex (MHC) class II, through the induction of a delayed type hypersensitivity-like reaction. This entails attracting inflammatory cells (macrophages, granulocytes, eosinophils, and natural killer (NK) cells) to the tumor microenvironment (26,27). The production of IFN-γ by the activated $CD4^+$ T cells has also been shown to have multiple effects, including direct tumor cytotoxicity, the up-regulation of MHC molecules which increases immune recognition, the enhancement of antigen processing, and the inhibition of tumor-induced angiogenesis (28-30).

One of the main mechanisms postulated to connect the $CD4^+$ and $CD8^+$ T cell responses in anti-tumor immunity is antigen cross-presentation. Dendritic cells endocytose, process, and present class II-restricted antigens to $CD4^+$ T cells, which respond by stimulating the DCs to increase surface expression of the co-

stimulatory molecules B7.1, B7.2, and OX-40 (31,32). The DCs then cross-present class I-restricted tumor epitopes to $CD8^+$ T cells and prime them by ligand binding of the co-stimulatory molecules so that the T cells are optimally activated to mount a potent and durable response to their target (33).

**Tumor immunotherapy**

Tumor immunotherapy involves clinical methods that are utilized to stimulate an immune response with associated tumor destruction. T cell immunotherapy, in particular, could entail vaccination with tumor antigens or adoptive transfer of reactive T cells specific for a tumor epitope to elicit a therapeutic anti-tumor response. Despite several clinical studies attempting T cell immunotherapy, there has yet to be shown a dependable, efficacious method of eliciting therapeutic immune responses to known tumor antigens. To date, several different modalities have been employed to target melanoma, but none have been overly successful. In addition to the previous examples, other attempts have included vaccination of patients with various melanoma differentiation antigens by injecting dendritic cells loaded with tumor lysates, which showed no appreciable efficacy (34). In a T cell adoptive transfer trial, patients were treated with their own tumor infiltrating lymphocytes, after expanding them *in vitro* (35). In a more sophisticated study, a group of 15 patients with advanced stage melanoma received gene therapy, whereby their PBMCs were transduced with a retrovirus containing the gene of a MART-1 specific, class I-restricted, TCR. Despite the potency of the MART-1 TCR, re-infusion of these transduced PBMCs

achieved long-term engraftment and clinical regression in only two of the patients (36). The multitude of unsuccessful attempts at immunotherapy has elicited interest in understanding the mechanistic barriers to its success. A central hypothesis in the field of cancer immunotherapy is that immune tolerance prevents or suppresses T cell responses to tumor antigens because these antigens are present in normal self tissue (37). The tolerance hypothesis is considered one of the main reasons for the shortcomings in melanoma treatment to date.

**TRP-1 and the melanosome**

Many of the target antigens for melanoma immunotherapy derive from protein substrates of melanocytes, the melanin producing cells present in skin and retinal pigment epithelium. Melanin pigments protect the skin and eyes by absorbing ultraviolet radiation (38). If they become malignant, melanocytes can develop into cancerous tissue (melanoma). Immunotherapy efforts directed at melanoma have been advanced by the identification of antigenic proteins, melanocyte differentiation antigens (MDAs); these are often structural or enzymatic molecules contained in the melanosome, the pigment-producing organelle of melanocytes (Figure 1). Studies have characterized the expression and MHC:peptide presentation of epitopes from several MDAs including MART-1, gp100 and tyrosinase (39,40). This project focused on the melanoma antigen TRP-1 (tyrosinase-related protein 1), which is a 75kDa molecule involved in melanin biosynthesis and the most abundant glycoprotein present in

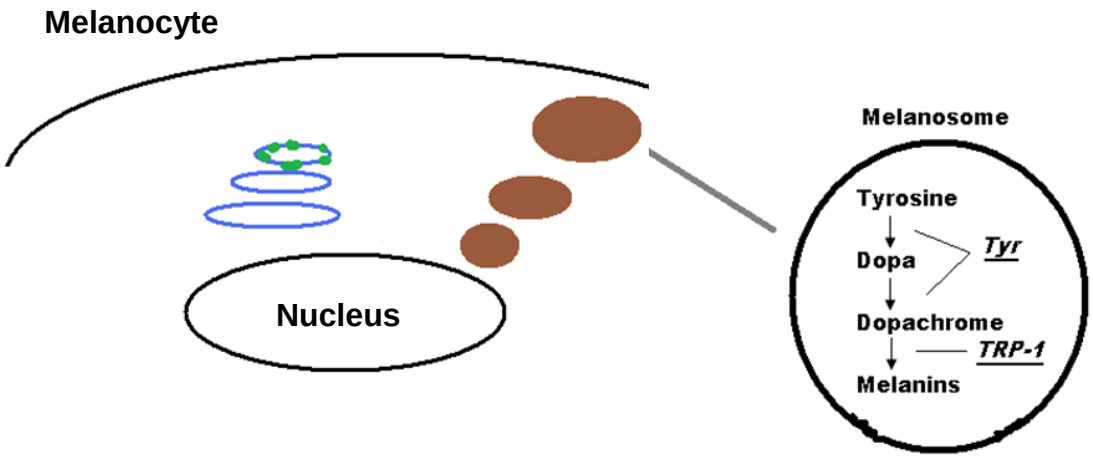


**Figure 1. Diagram of the melanocyte**. In melanocytes the characteristic organelle is the melanosome, a compartment that contains most of the melanocyte differentiation antigens. MDAs are often enzymes and structural proteins that support the synthesis of melanin, the protective pigment of skin, hair and retinal pigment epithelium.

melanocytes (41). Previous work has shown that when cohorts of C57BL/6 mice were immunized with a vaccinia virus construct expressing one of a panel of melanoma antigens (gp100, MART-1, tyrosinase, TRP-1, TRP-2), TRP-1 uniquely elicited autoimmune vitiligo. Additionally, T cells from the TRP-1 vaccinated mice were found to recognize and respond to a panel of melanoma cell lines and also provide protection against B16 melanoma in an MHC class-II dependent manner (42). Because of its antigenic potency and ability to stimulate a $CD4^+$ T cell response, we selected TRP-1 as an immunotherapy target.

**Mitf and TRP-1 transcription**

The micropthalmia transcription factor (Mitf) is an important upstream regulator of the program of pigment gene expression in melanocytes and melanomas. Several Mitf isoforms have been identified, all of which encode a basic/helix-loop-helix/leucine zipper molecule which transactivates expression by binding to a conserved promoter motif called the “E-box” (43). The M isoform (M-Mitf) is specific to neural crest derived melanocytes and is critical for activating expression of pigment proteins, melanocyte survival, and melanoma and melanocyte proliferation (Figure 2) (44). A conserved promoter element, the “M-box”, is an 11 base pair sequence shared by the tyrosinase and Tyrp1 gene promoters which Mitf binds to activate their transcription (45). Clinically, Mitf mutations such as Waardenburg syndrome type 2 and albinism-deafness illustrate the gene’s function (46-48). Waardenburg patients can manifest

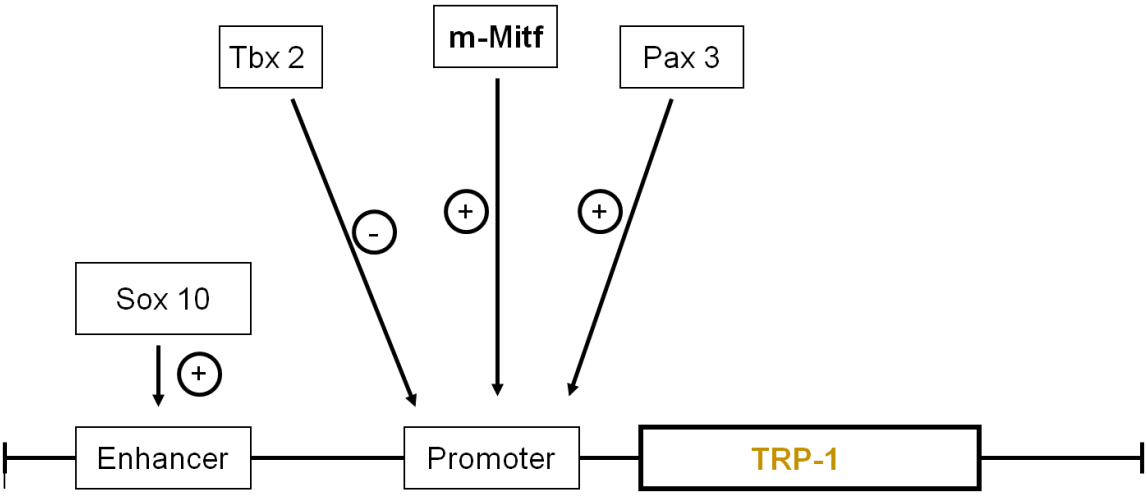


**Figure 2. Elements of the TRP-1 gene**. Transcription factors associating with the upstream promoter and enhancer elements individually upregulate TRP-1 expression. mMitf is the central controller of TRP-1, binding to a confined promoter region known as the “M box” which can individually induce TRP-1 expression.

different pigment-related defects including a white forelock and patches of white skin. Various mouse models of Mitf gene knockouts or mutants show ear, eye and coat color defects. All of these findings highlight the significance of Mitf as a gene regulator in melanocytes.

**Thymocyte development**

To understand the T cell response in tumor immunity, it is important to consider early T cell development. The process begins in the bone marrow where hematopoietic stem cells reside and differentiate along various progenitor lineages including those that populate the thymus (49,50). Cells of the T progenitor lineage migrate from the bone marrow along a chemokine gradient – utilizing CCR7 and CCR9 – to reach the thymus (51,52). Upon arrival, T cell precursors (thymocytes) are in the pro-T cell stage and do not yet express any of the TCR genes or associated CD3 and zeta chains. They also lack expression of the CD4 and CD8 co-receptors and are thus classified as double negative (DN). The DN cells pass through a series of stages denoted DN1 through DN4, moving closer toward TCR development.

The DN3 stage is the first important step for mature TCR production. Recombination activating genes (Rag-1 and Rag-2) are expressed, and they mediate the rearrangement of V, D and J regions within the TCR β gene to produce a TCR with a random variable region, which corresponds to the antigen-detecting end. On a population scale, this gene rearrangement is responsible for

producing the substantial diversity within the TCR repertoire (53). Ultimately, each individual thymocyte expresses a uniquely rearranged TCR-β chain gene that, if viable, will associate with an invariant pre-α T chain and the CD3 signaling complex. Aggregation of a viable β chain in this pre-TCR complex autonomously signals the thymocyte in a ligand-independent manner (54). Resultant activation of downstream signaling cascades induces thymocyte proliferation and surface expression of CD4 and CD8 co-receptors which renders the thymocytes "double-positive" (DP) (55-57). Having already produced a functional globulin chain, the respective TCR-β gene of the thymocyte ceases to undergo further rearrangements. Subsequent steps then produce an α chain via recombination of V and J regions of the gene. During these early maturation steps, a large percentage of thymocytes undergo apoptosis after failing to produce a fully assembled αβ TCR. Cells that survive to the DP stage must undergo subsequent selection processes mediated by epithelial cells of the cortex and medulla. These steps, referred to as "thymic education", include positive and negative selection which are the mechanisms of central tolerance that ultimately sculpt a functional and safe T cell repertoire for the host.

**Positive selection**

Following synthesis of a viable αβ TCR, DP thymocytes scan their surrounding environment in the thymic cortex for ligand interactions. The histology of this region has been well described; it primarily consists of cortical thymic epithelial cells (cTECs) interspersed with fibroblasts. Studies have defined a characteristic

ensemble of surface markers for cTECs - $CD45^-$ $EPCAM^+$ $Ly51^+$ MHC class $II^+$ - a set of parameters that can be applied for cell sorting (58). cTECs present MHC class I and II peptide complexes as a means to engage thymocytes and participate in the first phase of thymic education. The thymocytes meander throughout the cortical microenvironment receiving feedback in the form of TCR engagement by MHC complexes on APCs. This step, known as “positive selection”, promotes survival of DP cells bearing functional TCRs. Confocal microscopy has illustrated the mobility of precursor thymocytes in real time, a pattern that has been described as “random walk migration” (52,59). Overall, positive selection enriches the thymocyte population with cells that possess TCRs capable of adhering to host MHC molecules with at least moderate affinity. Thymocytes that positively interact with cTECs survive with some even bearing receptors that are cross-reactive for peptide and MHC (60). Thymocytes that produce a TCR incapable of associating with MHC molecules on neighboring cTECs eventually undergo apoptosis due to lack of positive signaling. These clones, which manifestly lack functional utility in that they cannot respond to the host APCs, are eliminated from the repertoire. Ultimately, the positively selected thymocytes undergo a lineage commitment to either $CD4^+$ SP (when selected by MHC class II) or $CD8^+$ SP (when selected by MHC class I) based on differential intracellular signaling pathways via the tyrosine kinase and Lck molecules (61,62). Having passed this stage of thymic education, the SP thymocytes migrate to the thymic medulla under the guidance of CCR7 for the final stage of central tolerance (63).

**Negative selection and thymocyte elimination**

The thymic medulla is a unique tissue microenvironment that serves as a gatekeeper for SP thymocytes before they are released into the peripheral circulation. It primarily consists of stromal fibroblasts, dendritic cells and medullary thymic epithelial cells (mTECs). mTECs can be identified by the following surface marker expression profile: $CD45^-$ $EPCAM^+$ $Ly51^-$ MHC class $II^+$ (58). Having already undergone positive selection in the cortex, the SP thymocyte population migrating into the medulla has a self MHC-restricted T cell repertoire with wide-ranging antigen recognition ability. While this diversity of receptors offers broad protection against “danger” epitopes from invading pathogens, it also poses a potential hazard to the host from T cells that recognize peripheral self antigens.

Autoimmunity is the generation of inflammatory responses directed against host epitopes, and it can occur when self-reactive thymocytes are not adequately removed from the T cell repertoire. Thymic deletion is the core step of central tolerance whereby medullary thymic epithelial cells mediate apoptosis of thymocytes with TCRs specific for MHC:peptide complexes presented by the mTECs. Marrack and colleagues showed selective deletion of murine thymocytes expressing Vβ17a, which react strongly with the I-E protein, in mice expressing I-E (64). Additional studies have further characterized negative selection as a high-avidity process, in contrast to the lower baseline avidity required for positive selection. For example, fetal thymic organ cultures from

mice expressing a transgenic T cell receptor showed increased thymocyte deletion with addition of higher concentrations of the corresponding peptide epitope to the culture (65). Negative selection appears to require relatively extensive engagement of thymocyte TCRs with peptide:MHC complexes.

**Promiscuous gene expression by mTECs**

mTECs promiscuously express a spectrum of proteins from the host genome which creates a broad, representative pool of tissue-specific antigens, disease-associated autoantigens and cancer-germline genes available in the thymic environment (66). Epitopes from these antigens are then presented to developing thymocytes which mediates elimination of self-reactive cells (Figure 3). Bevan and colleagues have shown that mTECs directly present these self antigens to $CD8^+$ thymocytes whereas DCs appear to play the major role in engagement and elimination of self-reactive $CD4^+$ cells (67). Mitotic studies of mTECs have characterized them as a rapidly cyclic population, with apoptosis of the mature subset creating a veritable reservoir of self-antigens available to DCs for acquisition and presentation (68). This data is consistent with other reports that thymic DCs, particularly those with an activated phenotype, are constitutively provided with and present cytosolic as well as membrane-bound mTEC-derived peptides (69). Peripheral antigens can also be transported to the thymus. One study employing intravenous injection of peptide antigen into mice demonstrated that DCs acquire the peptide en route to the thymus where they mediate deletion

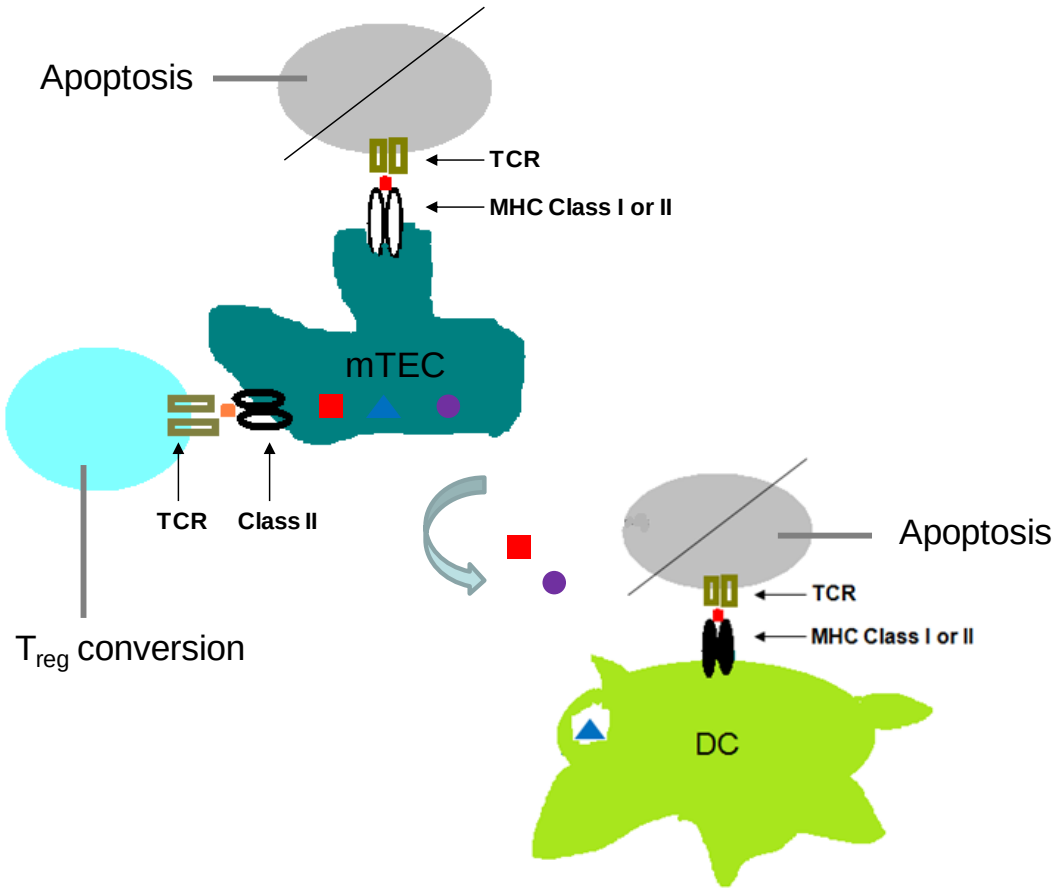


**Figure 3. mTEC mediated central tolerance.** Diagram illustrates the current paradigm of the medullary thymic epithelial cell in central tolerance. mTECs promiscuously express a representative ensemble of host antigens which are processed and presented in the form of MHC class I or II peptide complexes to developing thymocytes. Cells whose TCRs bind above an, as yet, undefined affinity threshold are induced to undergo apoptosis. Additionally, $CD4^+$ T cells whose receptors associate with a lesser affinity undergo conversion to the regulatory phenotype. Dendritic cells also have been shown to mediate central tolerance, particularly with thymocytes expressing class II-restricted TCRs. DCs are thought to either acquire host antigen from local mTECs or transport endocytosed antigens from the periphery.

of corresponding SP thymocytes specific for that peptide (70). Overall, the evidence shows there are several channels by which host antigens in the thymus are incorporated with the negative selection process.

Several studies have explored the patterns of mTEC gene expression and the transcriptional controls for this process. One of the first experiments characterized the ectopic antigen expression pattern by utilizing RT-PCR of mTEC RNA which detected transcripts representing a broad array of host tissue proteins. These included the melanoma associated antigens, gp100 and tyrosinase (71). More recent studies have attempted to determine the upstream transcription factors orchestrating the unique promiscuity of gene expression in mTECs. Aire, the autoimmune regulator gene, has been implicated as a critical transcription factor responsible for this phenomenon (72). Mathis and colleagues have linked defects in the Aire gene to autoimmune sequelae such as the APECED disease (autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy) (73). This was clearly demonstrated in their mouse model in which nude mice were transplanted with WT or $Aire^{-/-}$ thymuses. Only mice receiving a knockout thymus showed subsequent, spontaneous infiltration of immune cells and associated inflammatory pathology of several host tissues – salivary, stomach, liver, reproductive and retina (74). A separate study on the Aire knockout model reported absence of Mucin 6 expression in $Aire^{-/-}$ mTECs (normally expressed in WT) with a consequent, spontaneous autoimmune gastritis (75). Experiments investigating the transcriptional function of Aire have

actually shown that its role may be more complicated than simply promoter binding and transcriptional activation. It has more recently been found to have histone binding sites and demonstrable interactions with nucleosomes in addition to its DNA binding SAND domain (76). Furthermore, Aire is not the sole regulatory protein responsible for ectopic production of tissue antigens. Some mTEC gene expression levels are unchanged or even increased in the absence of Aire (77). Additionally, no mechanistic explanation has been elucidated for the various classes of tumor antigens expressed by mTECs. This includes transcripts of the aforementioned melanoma antigens described by Kyewski and more recent purified protein studies which revealed that MUC1 and CEA are expressed (78). Though additional pieces of the mTEC transcriptome continue to be revealed, the connection between the ectopic antigens and the factors regulating their expression remains relatively undefined.

## RESEARCH OUTLINE

We sought to determine the effect of host antigen deficiency on self-tolerance to tumor antigens by examining the TRP-1 antigen model. A previously discovered human class II DR4 restricted peptide epitope of TRP-1, 277-297, served as the target for our initial experiments (79). To model host antigen deficiency, we utilized Tyrp1 mutant mice, Bw, which were previously characterized by Oak Ridge National Laboratory as offspring from founders that had been exposed to gamma irradiation. The mice were noted to exhibit a cappuccino coat color phenotype which has been attributed to a recessive Tyrp1 gene defect characterized as an inversion of exon 1 (80). First, we attempted to examine in greater detail the antigen levels in the two mice. We determined that the paucity of TRP-1 transcript and protein in Bw melanocyte samples as well as the corresponding light brown coat color validated the Bw mouse as a credible antigen-deficient population relative to the WT background. WT and Bw mice were vaccinated with TRP-1, and their resulting T cell responses were compared on several parameters including numbers, cytokine potency and tumor treatment efficacy. These data provided a set of metrics for gauging the impact of tolerance on the TRP-1 antigen in the WT background.

The results from the vaccination and tumor treatment studies revealed a substantial difference in T cell phenotypes from the antigen-deficient Bw population in comparison to the WT cohort. Collectively, they highlighted the major impact of tolerance on the host's responding T cell repertoire. To

determine the source of these differences, we analyzed the potential effect of central tolerance, a major checkpoint of early T cell development in the thymus. These studies led to analyses of medullary thymic epithelial cells, which play a central role in deleting T cells specific for self antigens. Importantly, we found that mTECs promiscuously expression TRP-1 transcript and protein. Furthermore, we analyzed potential transcriptional controls for this phenomenon and found that neither Aire nor Mitf were necessary for activation of the TRP-1 gene. Most recently, we have observed detectable levels of TRP-1 transcript expressed in dendritic cells of WT mice. Additionally, ongoing studies are utilizing transgenic T cell receptor bone marrow – encoding an I-$A^b$ restricted TCR reactive against murine TRP-$1_{113\text{-}127}$ – for transplant experiments to probe deletion of TRP-1-specific thymocytes. These experiments are aimed at elucidating mTEC and/or DC contribution to deletion of the TRP-1 specific thymocytes.

## MATERIALS AND METHODS

**Experimental mice.** Tyrp1$^{bw/bw}$ mice, previously described (80), were fully backcrossed to C57BL/6 mice for 6 generations (81) and found to accept syngeneic tumor implants and skin grafts from C57BL/6 mice (data not shown). Murine class II-deficient, DR4-IE transgenic mice (DR4 Tg), (82) fully backcrossed onto C57BL/6, were purchased from Taconic Farms. To create a double-mutant strain expressing the Tyrp1$^{bw}$ mutation and the DR4-IE transgene and appropriate control littermates, Tyrp1$^{bw/bw}$ mice were bred with DR4 Tg mice. Mice were confirmed to have 2 copies of the Tyrp1$^{bw}$ mutation based on phenotype (cappuccino colored and confirmed to have at least 1 allelic copy of the DR4-IE chimeric transgene using polymerase chain reaction amplification of genomic DNA (oligonucleotide primers previously described). The F2 progeny yielded 2 experimental groups: Tyrp1$^{bw/bw}$//DR4$^{+}$ (Bw) and Tyrp1$^{+/bw}$//DR4$^{+}$ (WT) C57BL/6 (BL/6), OT-I, and OT-II transgenic (83,84) mice were purchased from Jackson Laboratories and used in an age range of 6-10 weeks.

**Cell lines.** Murine [B16.F10, MC-38, E.G7; American Type Culture Collection (ATCC), Manassas, VA], human tumor lines (397, 526, 624, 1088, 1102, and SK23 Mel, provided as a gift from S.A. Rosenberg, Surgery Branch, NCI/NIH) and human Epstein Barr virus (EBV)-B cell line (1088 EBV-B) were maintained in complete media (CM), as previously described (85). The 1088 Mel stably transduced with CIITA was prepared as previously described (79). Human leukocyte antigen (HLA)-DRB1* genotypes of tumor lines used in the following

experiments included 397 Mel (0404, 0408), 526 Mel (0401,1401), 624 Mel (0401,0701). 1088 Mel and EBV-B (0301,0401), as previously described (86). Murine tumors B16.F10 (B16) and MC38 were transduced with the retrovirus pLXSN (Clontech, Mountain View, CA) expressing the DR4 transgene (provided as a gift from P. Robbins, Surgery Branch, NCI/NIH).

**Immunizations.** Experimental mice [$Tyrp1^{bw/bw}$//$DR4^{+}$ (Bw) and $Tyrp1^{+/bw}$//$DR4^{+}$ (WT)] or control mice [OT-I or OT-II] were immunized with 50 µg of protein [mTRP-1, hTRP-1 or ovalbumin (OVA)]. Preparation of recombinant mTRP-1 and hTRP-1 has been previously described (79). Recombinant full-length OVA protein was purchased from Sigma; St Louis, MO. Proteins were emulsified in incomplete Freund's adjuvant (final volume 100 µL), divided equally and administered subcutaneously into the rear footpads. Fourteen days after primary vaccination, mice were boosted with the same amount of protein in incomplete Freund's adjuvant fluorescent antibody in the same location. Two weeks after the second immunization, inguinal lymph node (LN) cells were extracted and assessed for *ex vivo* reactivity or expanded in culture for subsequent experimental assays.

***Ex Vivo* assays.** Two weeks after the second immunization, LN cells were extracted and then assessed for phenotypic expression using fluorochrome-conjugated antibodies specific for murine CD4 (GK1.5, APC, BD Bioscience, San Diego, CA), CD8 (53-6.7, PE, BD Bioscience), CD3 (17A2, PE-Cy5, BD

Bioscience), CD25 (7D4, FITC, BD Bioscience), and Foxp3 (FJK-16s, Pacific Blue, eBioscience, San Diego, CA). ELISPOT *ex vivo* assays were performed in the following manner: M200 plates (CTL, Shaker Heights, OH) were precoated with IFN-γ antibody (R46A2; BD Biosciences) at 4 μg/mL and incubated overnight; LN cells ($4\times10^5$/well) and targets (pulsed $1\times10^5$ 1088 EBV-B cells) were added to wells in triplicate in 200 μL of HL-1 CM (HL-1 Media, Biowhittaker, Walkersville, MD, L-glutamine 200 mM; and gentamycin sulfate 50 mg/mL); EBV-B cells were pulsed the day before (from 12 to 18 h) with peptide (50 μM), protein (50 μg/mL) or lysate ($10^5$ cell equivalents/well; and prepared as previously described, (79). After a 24-hour coculture, plates were incubated with biotinylated IFN-γ antibody (XMG1.2; BD Biosciences) at 2 μg/mL. The next day plates were incubated with streptavidin-alkaline phosphatase (Southern Biotechnology, Birmingham, AL) at a 1:1000 dilution for 2 hours, after the addition of p-nitroblue tetrazolium chloride-5-bromo-4-chloro-3-indolyl phosphate (Thermo Scientific, Rockford, IL); the resulting spots were counted on a computer-assisted ELISPOT image analyzer (Immunospot Series I Analyzer, CTL). Monoclonal antibodies (mAbs) were used (at 50 μg/mL) to inhibit the recognition by T cells included HB55 (against HLA-DR; $IgG_{2a}$; ATCC) and W6/32 (against HLA-A, HLA-B, HLA-C; $IgG_{2a}$; ATCC).

**Generation of established $CD4^+$ T cell lines.** Extracted LN cells from immunized mice were cultured in 24-well plates at $5\times10^6$ cells per well with peptide hTRP-$1_{277–297}$ at 50 μM (or control class I $OVA_{257–264}$ at 10 μM or control

class II $OVA_{323–339}$ 50 μM). Twelve days after the first *ex vivo* stimulation, both lines were restimulated with peptide-pulsed, irradiated, syngeneic DR4 Tg splenocytes. Splenocytes were pulsed with hTRP-$1_{277–297}$ at 50 μM for 3 hours at 37°C, washed, irradiated with 3000 rads, then added to each T cell culture at a 10:1 ratio ($5\times10^6$ APCs per well). CM containing interleukin (IL)-2 (Chiron, Emeryville, CA) at 7.5 cetus units/mL was added 2 days after the stimulation (day 14). Multicolor flow cytometry for CD4, CD8, CD25, and Foxp3 T cell expression was performed using the mAbs described above. Lines were stimulated and maintained using the same methods every from 10 to 15 days. To evaluate specific T cell reactivity, splenocytes or 1088 EBV-B cells were pulsed for 3 hours with peptide (at various concentrations) or with recombinant protein overnight. Whole tumor cells (pretreated with IFN-γ × 48h at 200U/mL) or pulsed APCs ($10^5$ cells per well) were then cocultured with $10^5$ T cells per well in U-bottom 96-well plates for 24 hours. The mAbs HB55 and W6/32 were again used to block T cell interactions. Culture supernatants were assayed for IFN-γ, IL-4, or IL-17 using commercially available enzyme-linked immunosorbent assay (ELISA) kits (BD Bioscience).

**Tumor treatment and antibody depletion assays.** Six to eight week-old female C57BL/6 and DR4 Tg mice were injected intravenously through the tail vein with $5\times10^5$ B16 melanoma cells, MC-38 or E.G7 tumor cells at 5 animals per group (day -4). Four days later, mice were injected intravenously (day 0) with various quantities of T cells generated from immunized OT-1, OT-II,

TRP1$^{bw/bw}$//DR4$^{+}$, and TRP1$^{+/bw}$//DR4$^{+}$ mice. Twelve days later (day $^{+}$12), lungs were removed, countered stained with India ink and metastases enumerated in a blinded fashion (87). For tumor treatment experiments involving antibody depletion, mAbs to murine CD4 (GK1.5), CD8 (53-6.7), and NK and natural killer T cell (NKT) (PK136) were purified from hybridoma culture supernatants (ATCC), and injected (100 µg) intraperitoneally into host mice at 5 animals per group every 3 days beginning 9 days (day -13) before tumor injection. $T_{reg}$ depletion was performed with a single intraperitoneal injection of 400 µg (anti-CD25 PC61 or control rat IgG) 4 days before tumor implantation (day -8) into 5 experimental mice per group. Depletion analysis by fluorescence-activated cell sorting was performed on peripheral blood on the day of adoptive transfer (day 0). Treatment results are shown as standard error of the mean and statistical significance between groups was determined by Student t test.

**Thymic digestion for mTEC isolation.** Thymuses (5-20) were removed from experimental mice (optimal age: 6-10 weeks) and placed into ice cold RPMI. Fat and connective tissue was pulled off the lobes with forceps. All of the lobes were separated, nicked with scissors, and placed into a round bottom tube with a magnetic stir bar. 10mL of RPMI was added to the tube, and the thymuses were agitated with stirring for 10 minutes. Supernatant was poured off, and then enzyme solution 1 was added to the tube (RPMI, Collagenase D [Roche] .125% w/v, DNase I [Biomatik] .1% w/v). The thymus tissue was stirred for 15 minutes on a hot plate at 40°C and supernatant was collected and kept on ice. This

digestion step was repeated 3 more times. Next, the thymus tissue was incubated with enzyme solution 2 (RPMI, Collagenase/Disapse [Roche] .125% w/v, DNase I .1% w/v) for 15 minutes – or until satisfactorily disaggregated – and the supernatant was collected. The pooled supernatant sample from all digests was centrifuged at 470xg for 5 minutes, resuspended in EDTA/FACS buffer (1x PBS, 5mM EDTA, 2% FCS, $NaN_3$ .02% w/v) and incubated at 4°C for 10 minutes. Next, the cell suspension was poured over a 40µm filter and counted with a hemocytometer. The sample was centrifuged at 470xg for 5 minutes and resuspended in MACS buffer (Miltenyi formulation) at 95µL/$10^7$ cells. Magnetic anti-CD45 microbeads (Miltenyi) were added to the suspension at 5µL/$10^7$ cells. From this point onward, the directions for the Miltenyi CD45 kit were followed to deplete the sample of $CD45^+$ cells using the appropriate number of LS columns with a MACs magnet. The $CD45^-$ fraction was washed and prepared for mTEC cell sorting.

**mTEC cell sorting.** Cells were acquired on a FACS Vantage or FACS Aria sorter using either Cell Quest or FACSDiva software, respectively. The live population was gated based on forward and side scatter parameters. Cells were then sorted on the following gated parameters: $CD45^-$ (30-F11, PercpCy5.5, BD Biosciences), $Ly51^-$ (BP-1, PE, BD Biosciences), $Epcam^+$ (G8.8, APC, eBioscience), MHC class $II^+$ (M5/114.15.2, FITC, eBioscience).

**Confocal microscopy and immunofluorescence.** 8-well chamber slides (Biomatik) were coated with 1% gelatin for 15 minutes at room temperature. Cells were then plated overnight in individual wells with their customary media (mTECs were plated in RPMI complete media supplemented with L-glutamine, 5%). The following day, culture media was removed, and all samples were fixed with 3.7% paraformaldehyde for 15 minutes at room temperature. After fixing, paraformadehyde was pipetted off, and the wells were gently washed three times by adding 1x PBS for consecutive 5 minute incubations. Next, the cells were permeabilized by adding methanol for a 3 minute incubation after which cells were washed 3 times with 1x PBS. After washing, each well was coated with blocking solution (5% Bovine Serum Albumin & 1% goat serum in 1x PBS) for a 30 minute incubation. The blocking solution was removed and cells were washed with 1x PBS for five minutes. 1µg anti-TRP-1 Ab (Mel-5, Covance) was conjugated via the Alexa Fluor 488 Antibody Labeling Kit (Invitrogen). Sample wells were labeled with 1µg of the conjugated Mel-5 Ab or conjugated Mouse IgG2a isotype control Ab and left for 1 hour at room temperature. All wells were washed 3 times with 1x PBS, and cells were left with a residual layer of buffer after the final wash. Finally, immunofluorescent images of the samples were acquired on an Olympus FV1000-MPE Confocal/Multiphoton Microscope.

**RNA isolation.** Cell samples were transferred to RNase-free tubes and centrifuged to pellets. Samples were lysed followed by RNA spin-column isolation using either the Qiagen RNeasy Mini kit (when >2x$10^5$ cell sample) or

RNeasy Micro kit (when <$2x10^5$ cell sample). RNA was either used fresh or stored at -80°C for later experimentation.

**mRNA amplification.** All mTEC RNA samples were harvested with the RNeasy Micro kit (Qiagen) and then linearly amplified with the MessageAmp III RNA Amplification Kit (Ambion). For amplification, 5µL of sample RNA was incorporated into a sequence of PCRs according to the manufacturer's protocol. Afterwards, the amplified samples were conjugated with RNA binding beads, washed, and then eluted.

**Quantitative real time PCR gene expression assays.** Reverse transcriptase (Applied Biosystems, ABS) was utilized according to the manufacturer's protocol for synthesis of cDNA from mRNA samples. The cDNA samples were then incorporated into quantitative real-time PCR reactions to probe for transcript levels of the gene of interest with the following reaction setup: 10µL PCR Mastermix (ABS), 1µL gene specific FAM probe (ABS), 5µL cDNA sample, 4µL $H_2O$. Samples were run on a StepOnePlus Real-Time PCR System (ABS) using the StepOne v2.1 software in "relative" format with the housekeeping gene β-actin used as an internal standard for normalization. Sample results were reported as "RQ" values, representing the ratio of transcript with respect to the experimental sample defined as '1'. Reactions were performed in triplicate and standard error was calculated by the software ($p < .05$).

**Bone marrow transplant.** Mice with three distinct genotypes – C57BL/6, DR4Tg, and Tyrp1$^{bw/bw}$ – were utilized as recipient cohorts with 5 mice per cohort. All subjects were given doxycycline feed on d -3 to reduce bacterial load (prior to the subsequent neutropenia from irradiation). Mice were lethally irradiated on d -1 with 900Gy. On d 0, 7 Trp-1 TCR Tg mice were euthanized; femurs were extracted and flushed with 1x PBS to obtain donor bone marrow cells. The donor bone marrow was treated with RBC lysis buffer (Qiagen) to remove RBCs and then washed and counted with a hemocytometer. Cells were conjugated with the following 3 biotinylated, monoclonal Abs: anti-CD3, anti-NK1.1 and anti-CD19 (eBioscience), corresponding to markers on mature T, NK, and B cells, respectively. Strepavidin magnetic beads (Miltenyi) were added to the cells to bind to the antibody-coated cells. The samples were then washed and run over a magnetized LS column (Miltenyi) to remove the aforementioned mature lymphocyte populations from the donor bone marrow. The sample was resuspended in 1x PBS at 7x10$^6$ cells/400µL. Each recipient mouse received a tail vein injection of 400µL of the donor cell suspension.

**Transplant analysis.** At 8 weeks, transplanted mice were euthanized. Their thymuses and spleens were removed, homogenized, washed and filtered. Samples were Fc blocked and then stained for analysis. To analyze thymocytes, samples were stained with mAbs specific for the TRP-1 transgenic beta chain, Vβ14 (14-2, FITC, BD Bioscience) as well as CD4 (APC), CD8 (PercpCy5.5), CD25 (PE), Foxp3 (Pacific Blue) (clones previously catalogued in earlier

Methods). Additionally, samples were stained with Annexin V (PE, eBioscience) and mAbs specific for Vβ14, CD4 and CD8 to analyze apoptosis. Splenocytes were stained with mAbs specific for Vβ14, CD4 and CD8 to analyze the presence of transgenic T cells in the periphery.

# RESULTS

## Assessing the T cell immune response to TRP-$1_{277-297}$ in TRP-$1^{bw}$ versus WT mice *ex vivo*

TRP-$1^{bw}$ breeder mice were crossed with DR4 Tg mates (both on a C57BL/6 background). Offspring were crossed to produce an $F_2$ generation comprised of Tyrp$1^{bw/bw}$//DR$4^+$ (Bw) and Tyrp$1^{+/bw}$ //DR$4^+$ (WT). Bw and WT mice were vaccinated with recombinant hTRP-1 protein and given a booster vaccination two weeks later. Two weeks following the booster, inguinal lymph nodes were removed from each cohort and analyzed by flow cytometry. Both WT and Bw populations were similar in their overall population of $CD4^+$ T cells, 74% vs 83% respectively, and $T_{reg}$ cells ($CD4^+$/$CD25^+$/$Foxp3^+$), 2.2% versus 3.6% respectively (Figure 4). The lymph node cells were also tested for ex-vivo reactivity by stimulation with TRP-1 in the form of peptide (100µM), protein (50µg/mL) or cell lysate material ($10^5$ cell equivalents) pulsed onto DR$4^+$ EBV-B cells. An ELISPOT assay for IFN-γ production was performed to determine the number of responding T cells from both the WT and Bw populations in response to these various stimuli (Figure 5). The Bw T cells demonstrated a greater magnitude of clonal reactivity to mTRP-1 peptide, human and murine TRP-1 protein, and human and murine melanoma cell lysates – B16 and SK23. Blocking the HLA-DR molecule with mAb HB55 effectively diminished the number of detectable T cells, whereas the HLA class I-specific mAb W6/32 had no effect; this illustrated the HLA-class II specificity of the cytokine response.

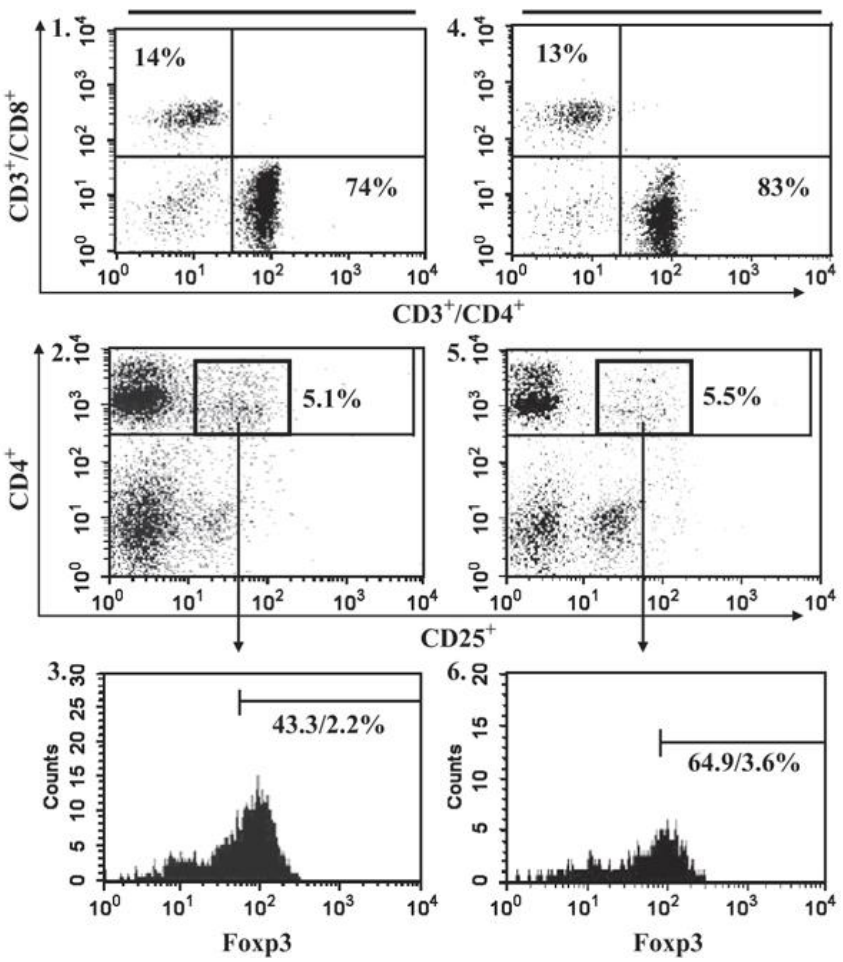


**Figure 4. Prevalence of $T_{regs}$ in immunized WT and Bw.** LN populations obtained after vaccination are comparable in $T_{reg}$ prevalence. TRP1$^{bw}$//DR4$^{+}$ (Bw) and WT//DR4$^{+}$ (WT) littermates were immunized twice with recombinant hTRP-1. Fourteen days after the second immunization, LN cells were harvested and stained with antibodies specific for murine CD4, CD8, CD3, CD25, and Foxp3. The panels are representative of 3 distinct experiments.

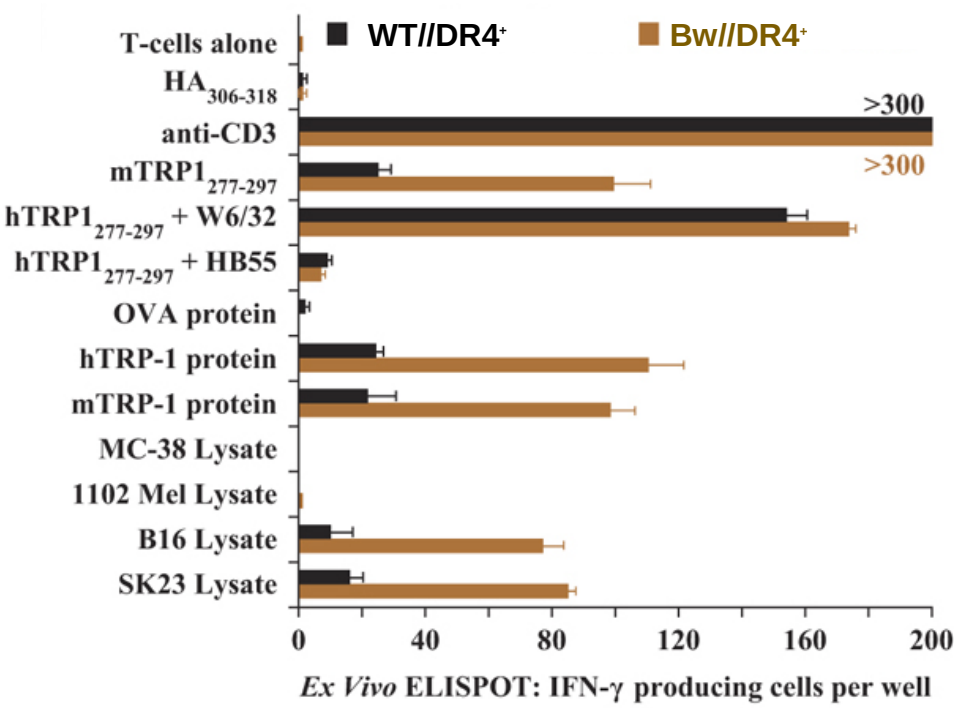


**Figure 5. ELISPOT Assay probing response to peptide and tumor.** LN cells obtained from TRP-1 KO mice are more potent than WT controls. After immunization, LN cells ($4x10^5$ per well) were stimulated ex vivo in ELISPOT plates with soluble anti-CD3 (2 µg/mL) or $DR4^+$ EBV-B cells ($1x10^5$ per well; 1088 EBV-B) pulsed with peptide (100 µM) or protein (50 µg/mL) or lysate ($10^5$ cell equivalents). LN cells from both groups produce interferon-γ to anti-CD3 and EBV-B cells pulsed with mTRP-$1_{277–297}$ or hTRP-$1_{277–297}$ peptide, hTRP-1 or mTRP-1 protein, and to B16 and SK23 Mel lysate. Specific reactivity from both groups was blocked with anti-DR antibody, HB55, but not with anti-MHC class I antibody, W6/32. No reactivity was observed to either the control peptide ($HA_{306–318}$), protein (ovalbumin) or the lysates (MC-38, or 1102 Mel, both negative for TRP-1).

Interestingly, both cohorts demonstrated a similar number of responding cells to hTRP-$1_{277-297}$ peptide at the 100µM concentration. However, differences became more apparent when we probed with titrating amounts of human peptide to assess the sensitivity of the two populations. The aforementioned ELISPOT was repeated but with a diminishing, log-fold range of peptide concentrations. Notably, a 1.5 log-fold greater magnitude of responding T cells was observed at half maximal peptide concentration (1µM) for the Bw group compared to the WT group (Figure 6). As before, similar numbers of responding cells were observed in both populations at 100µM of TRP-1 peptide, suggesting that the presence of self antigen may exert its effects primarily on the higher sensitivity T cells specific for that antigen, while not impacting the less sensitive clones.

**Phenotypic differences persist between WT and BW TRP-$1_{277-297}$ specific T cells after *in vitro* expansion**

Having shown a significant difference in the *ex-vivo* antigen response of $CD4^+$ T cells from Bw mice compared to WT, we explored whether cells that were expanded from both populations *in vitro* would demonstrate similar results after manipulation in culture. To address this question, LNs were harvested from immunized mice, as in the previous experiments. After homogenizing the LNs and collecting all cells, the populations were stimulated in culture conditions with TRP-$1_{277-297}$ peptide and IL-2; two rounds of stimulation were performed. Following the expansion stage, we analyzed the cells by flow cytometry and

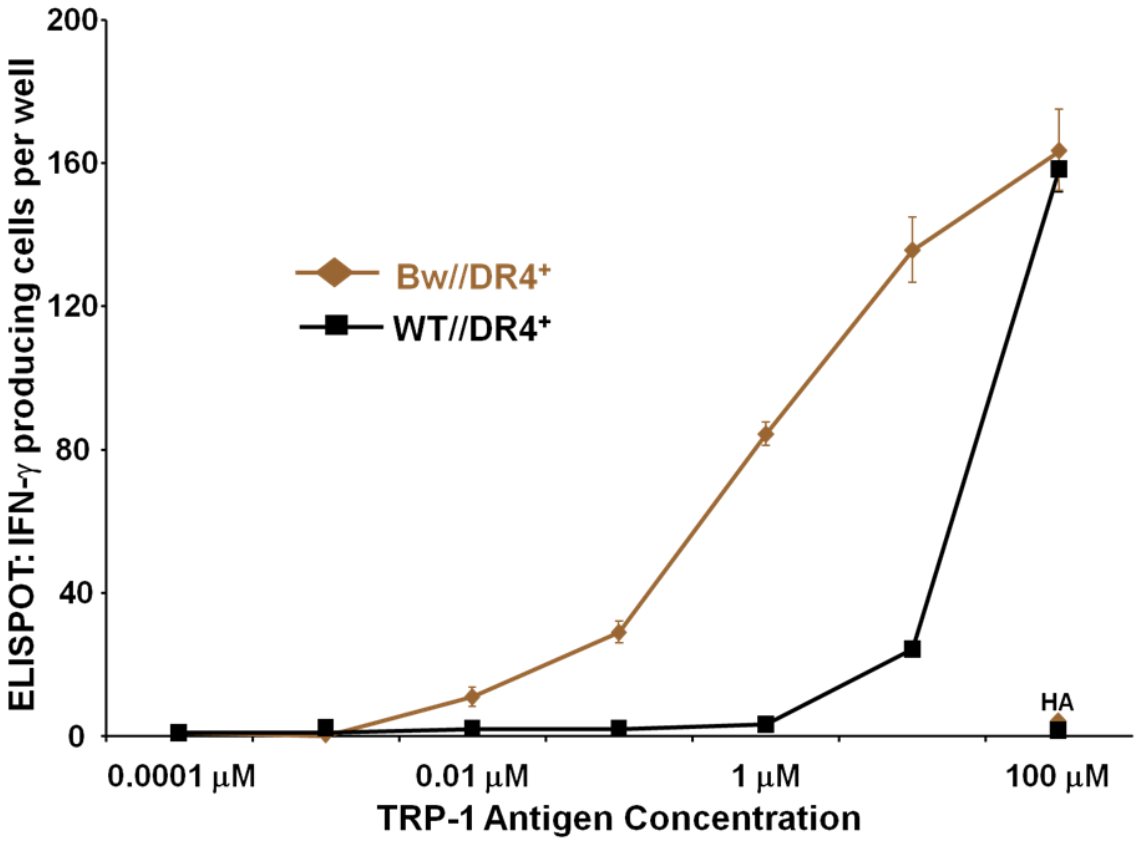


**Figure 6. *Ex Vivo* ELISPOT titration assay. hTRP-$1_{277–297}$-specific T cells from Bw//DR4$^+$ mice are present at a higher frequency.** Bw and WT littermates were immunized with hTRP-1 protein as described in Figure 4. LN cells were harvested and stimulated ($4x10^5$ per well in triplicates) *ex vivo* with hTRP-$1_{277–297}$ pulsed onto DR4$^+$ EBV-B cells ($1x10^5$ per well; 1088 EBV-B) at titrating (100 to 0.0001µM) peptide concentrations. All experiments were performed 2 to 3 times with similar results.

found that, in both groups, greater than 90% were $CD4^+/CD25^+/Foxp3^-$ indicating a high level of $T_{effector}$ cells and a relatively low level of $T_{regs}$ (Figure 7). We then gauged the response of these populations to TRP-1 by co-culturing them with EBV-B cells (APCs) pulsed with titrating levels of TRP-$1_{277-297}$ peptide. Supernatant was collected from the co-cultures and assayed for quantity of IFN-γ release by ELISA. Similar to the previously described *ex vivo* assay, Bw T cells exhibited a more sensitive cytokine response than WT. This phenomenon became most apparent at diminishing levels of antigen where at half maximal concentration (100nM), the Bw:WT IFN-γ secretion ratio was 15:1; at 1nM, the observed ratio was 25:1 (Figure 8).

**$CD4^+$ T cells derived from Bw mice and expanded *in vitro* respond to melanoma cells with greater INF-γ production than those derived from WT mice**

After finding that TRP-1 specific $CD4^+$ T cells derived from the antigen-deficient Bw population possessed greater sensitivity and potency to their cognate peptide epitope verus the WT cells, we next explored their functionality in a more clinical context. Both T cell populations were co-cultured with either recombinant TRP-1 protein or various melanoma cell lines, and their respective responses were assessed with an ELISA that probed IFN-γ secretion (Figure 9). This design allowed for comparing the samples in a setting where antigen processing and presentation mechanisms were necessary and also where actual tumors with

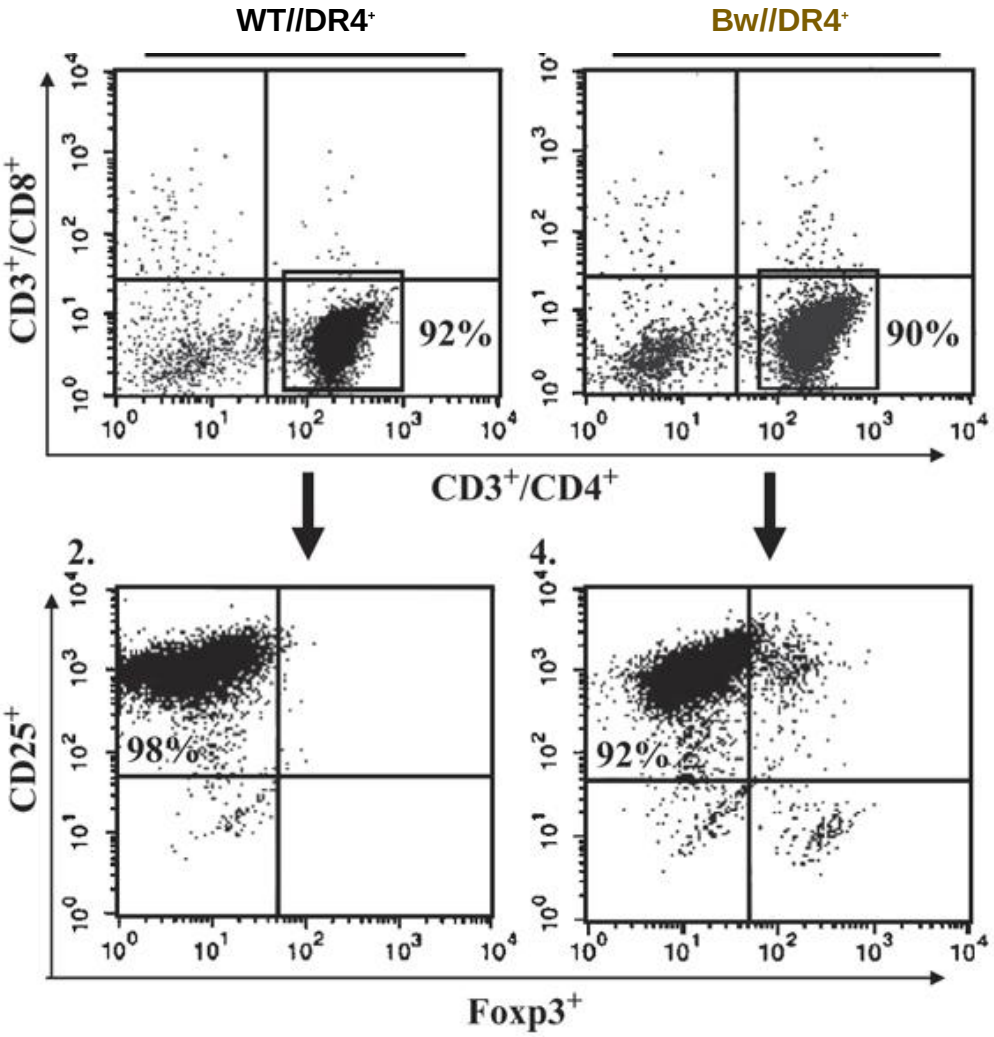


**Figure 7. $T_{regs}$ in ex vivo expanded cells.** TRP1$^{bw/bw}$//DR4$^{+}$ and WT//DR4$^{+}$ Tg littermates were immunized with hTRP-1 protein and expanded *in vitro*. A, Flow cytometric analysis of experimental T cell populations. Both groups of *in vitro* IL-2 expanded WT and KO T cells are equivalently CD4$^{high}$/CD8$^{dim}$ (panels 1 and 3) and $T_{reg}$ negative CD4$^{high}$/CD25$^{high}$/Foxp3$^{dim}$ (panel 2 and 4).

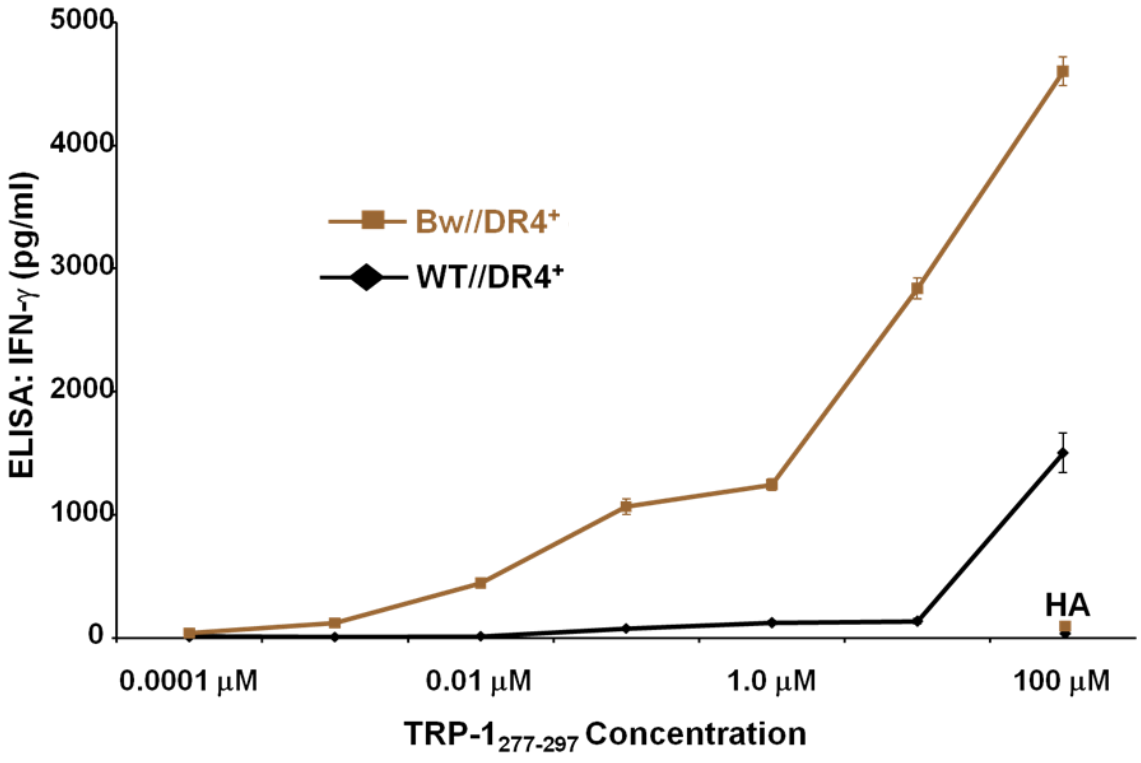


**Figure 8. ELISA comparing Bw and WT T cells at diminishing peptide concentrations.** TRP-1-specific T cells from WT and Bw mice were expanded *in vitro* by peptide pulsing (TRP-$1_{277-297}$) upon LN removal, pulsing again on d 12, and stimulating with IL-2 on d 14. Cell lines were stimulated and maintained using the same methods every from 10 to 15 days. The expanded populations were co-cultured with $DR4^+$ EBV-B cells (1088 EBV-B) pulsed with titering concentrations of hTRP-$1_{277–297}$. Significant differences in potency of IFN-γ release were observed between the groups with consistently higher concentrations secreted by Bw T cells over a range of peptide concentrations.

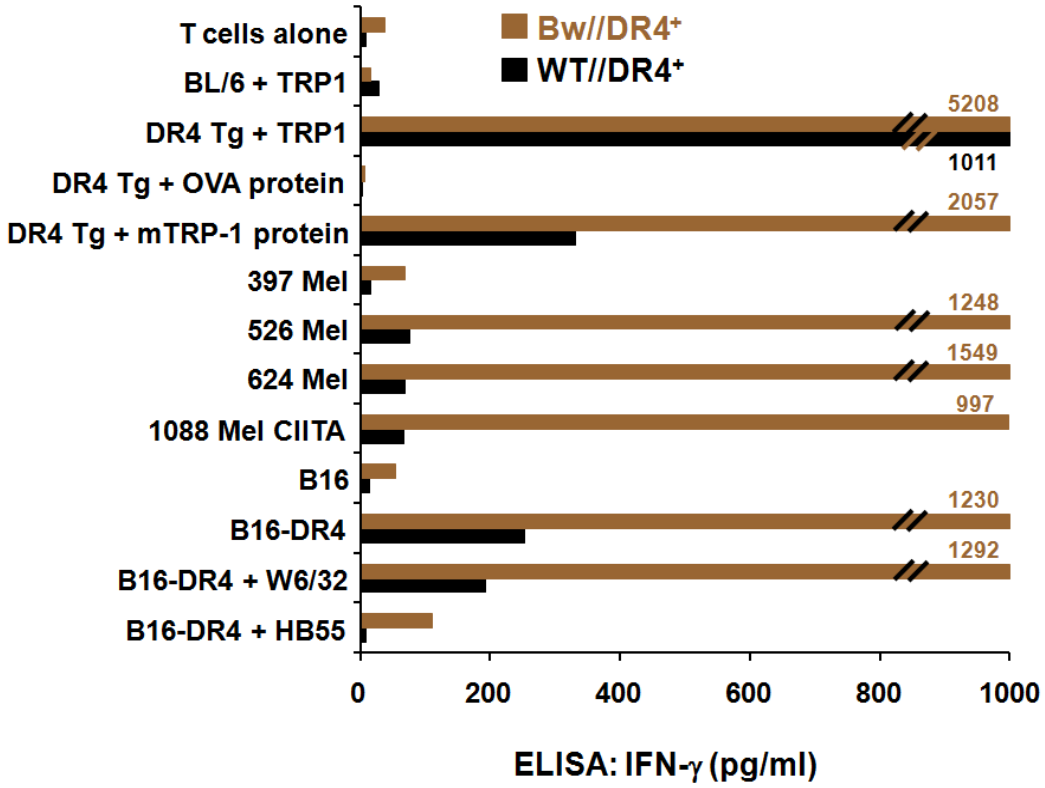


**Figure 9. ELISA demonstrating IFN-γ release in response to antigen and tumor.** $CD4^+$ T-cells derived from TRP-1-deficient hosts (Bw) release high levels of cytokine in response to intact melanoma. $CD4^+$ T cells from WT and Bw react differentially react to both the TRP-1 peptide and the recombinant protein when pulsed on $DR4^+$ targets (DR4 Tg splenocytes), but not when pulsed onto $DR4^-$ antigen presenting cells (C57BL/6 splenocytes, $I\text{-}A^{b+}$). No reactivity was observed to control protein ovalbumin. Specific reactivity was also observed to both HLA-$DR4^+$ and $TRP\text{-}1^+$ human melanomas (526 and 624 Mel pretreated with IFN-γ; and 1088 Mel stably transduced with CIITA) and to the murine melanoma B16-DR4 (B16 stably transduced with HLA-DRB1*0401). No reactivity was observed against control tumor B16 ($DR4^-$). Recognition of B16-DR4 was blocked with monoclonal antibody HB55 (anti-class II), but not with W6/32 (anti-class I).

endogenous antigen levels could be tested against the reactive cells. When pulsing recombinant TRP-1 protein onto $DR4^+$ Tg splenocytes, both T cell populations demonstrated reactivity but with a clear differential in quantity of cytokine released. Additionally, pulsing of BL/6 (DR4-negative) splenocytes elicited no reactivity in either T cell population, illustrating the specificity of the 277-297 epitope for the DR4 restriction element. To assess the response against tumor cells, the TRP-1 positive human melanomas, 526 Mel and 624 Mel, as well as murine B16 melanoma transduced with the DR4 gene were all included in separate co-cultures with the T cell populations. Bw-derived cells exhibited a more than 20-fold greater potency of IFN-γ production in response to the two human tumor lines versus WT-derived cells as well as a greater response to the B16-DR4 line expressing murine TRP-1. Neither T cell population secreted significant cytokine against 397 Mel, a DR4 negative melanoma line. Moreover, inclusion of HB55, an anti-class II Ab, abrogated the response to B16 DR4 melanoma, again demonstrating class II specificity for the 277-297 epitope (Figure 9).

**Cytokine analysis of cells derived from TRP-1 vaccinated Bw and WT mice reveals a polarized $T_h1$ profile**

The Trp-1-specific T cells from Bw and WT vaccinated mice were cultured for 3 weeks *in vitro* to expand their numbers for assays and immunotherapy experiments. Given the possibility of cells undergoing phenotypic changes while in culture, it was important to characterize the cytokine secretion profile of the

populations following the artificial expansion. The T cells were co-cultured with TRP-$1_{277-297}$ peptide-pulsed APCs (EBV-B cells) as well as antigen positive B16-DR4 cells. Additional negative control co-cultures included HA peptide pulsed APCs as well as MC38-DR4, a TRP-1 negative carcinoma line. Supernatants were collected from the co-cultures and characterized by ELISA for levels of IFN-γ, IL-4 and IL-17. The levels of IL-4 and IL-17 did not rise above background for any of the co-culture conditions suggesting little to no presence of $T_h2$ or $T_h17$ cells. However, IFN- γ was released in substantial amounts (and at higher levels by the Bw mice) in the peptide pulsed and antigen positive co-cultures. This finding characterizes the T cell response to TRP-1 as consistently polarized towards T helper type 1 (Figure 10).

**High-avidity TRP-1 specific $CD4^+$ T cells derived from Bw hosts mediate tumor destruction**

Given that Bw mice immunized with TRP-1 produced a greater magnitude of $CD4^+$ T-cells which secreted higher concentrations of cytokine per cell than WT in response to antigen, we sought to determine if the Bw T cells were also more effective at eradicating tumor *in vivo*. Additionally, we assessed whether the treatment effect was antigen-specific, DR4-restricted and also whether it was mediated by direct or indirect recognition of tumor. To test these questions, we took advantage of the unique genetic differences in MHC expression on both the tumor cells ($DR4^-$) and host APCs ($DR4^+$), as well as the restriction elements that

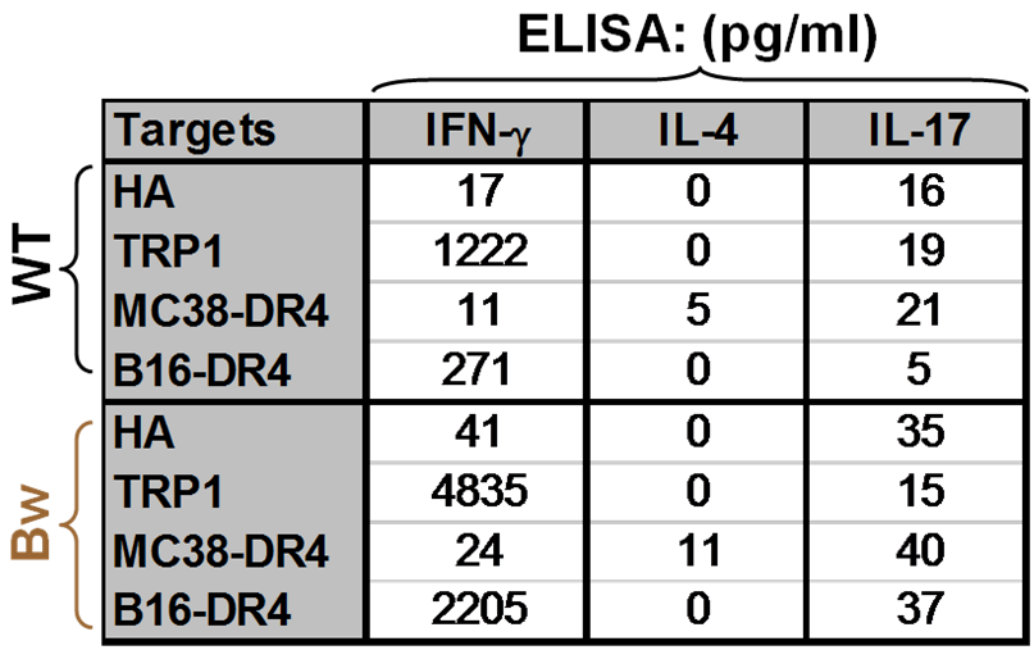


**Figure 10. Cytokine release profile of TRP-1 specific T cells.** $CD4^+$ T cells from both groups exhibit a $T_h1$ cytokine profile (IFN-γ production, but none to IL-4 or IL-17A by enzyme-linked immunosorbent assay) in response to specific peptide and tumor (B16-DR4, but not MC-38-DR4). All experiments were performed 2 to 3 times with similar results. EBV-B indicates Epstein Barr virus-B; IL, isoleucine; TRP, tyrosinase-related protein.

mediate the reactivity of both host $CD8^+$ T cells ($K^b/D^{b+}$) and the adoptively transferred $CD4^+$ T cell treatment population ($DR4^+$). A general schematic for all tumor treatment experiments is shown in Figure 11.

We initially performed a 4-day lung metastasis treatment experiment using 3 different tumor-bearing hosts: (1) B16 ($TRP\text{-}1^+$) lung metastases in DR4 Tg ($DR4^+$); (2) B16 lung metastases in C57BL/6 ($DR4^-$); and (3) MC38 ($TRP\text{-}1^-$) lung metastases in DR4 Tg. Mice received intravenous injection of B16 melanoma (or control MC38 tumor) which metastasizes to the lungs, engrafting as quantifiable tumor nodules. T cells were obtained from the lymph nodes of WT and Bw mice after immunization then expanded *in vitro* and adoptively transferred ($10^7$ cells per mouse) into tumor-bearing recipients. Importantly, the donor populations from WT and Bw had statistically similar percentages of $CD4^+$ T cells including a small subpopulation of $T_{reg}$ cells. A third cohort of tumor bearing mice received control OVA-specific $CD4^+$ T cells which were obtained from OT-II mice following an analogous immunization and *in vitro* expansion process with the $OVA_{323–339}$ epitope (83). We observed a significant difference in treatment effect using T cells obtained from Bw mice (mean=5 lung metastases; P=0.0006 using a 2-tailed Student t-test) versus WT littermates (mean=68 lung metastases) versus control OVA specific CD4s (mean=156 lung metastases) in DR4 Tg recipients bearing B16 lung metastases (Figure 12). This result was contrasted with the complete loss of any treatment effect (mean ≥ 150 lung metastases for all

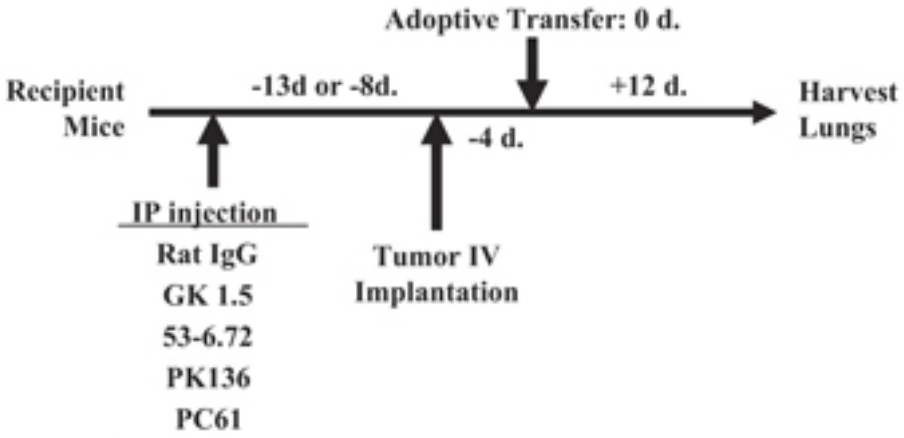


**Figure 11. Schema for adoptive transfer of TRP-1 specific T cells.** Recipient mice were depleted of specific immune cell populations with monoclonal Abs. On d –4, the mice received i.v. injection of B16 melanoma. At d 0, $10^7$ T cells from each experimental population was adoptively transferred into its respective tumor-bearing mouse cohort. 12 days later, lungs were removed from all the tumor-bearing mice, and metastases were counted in a double-blind fashion.

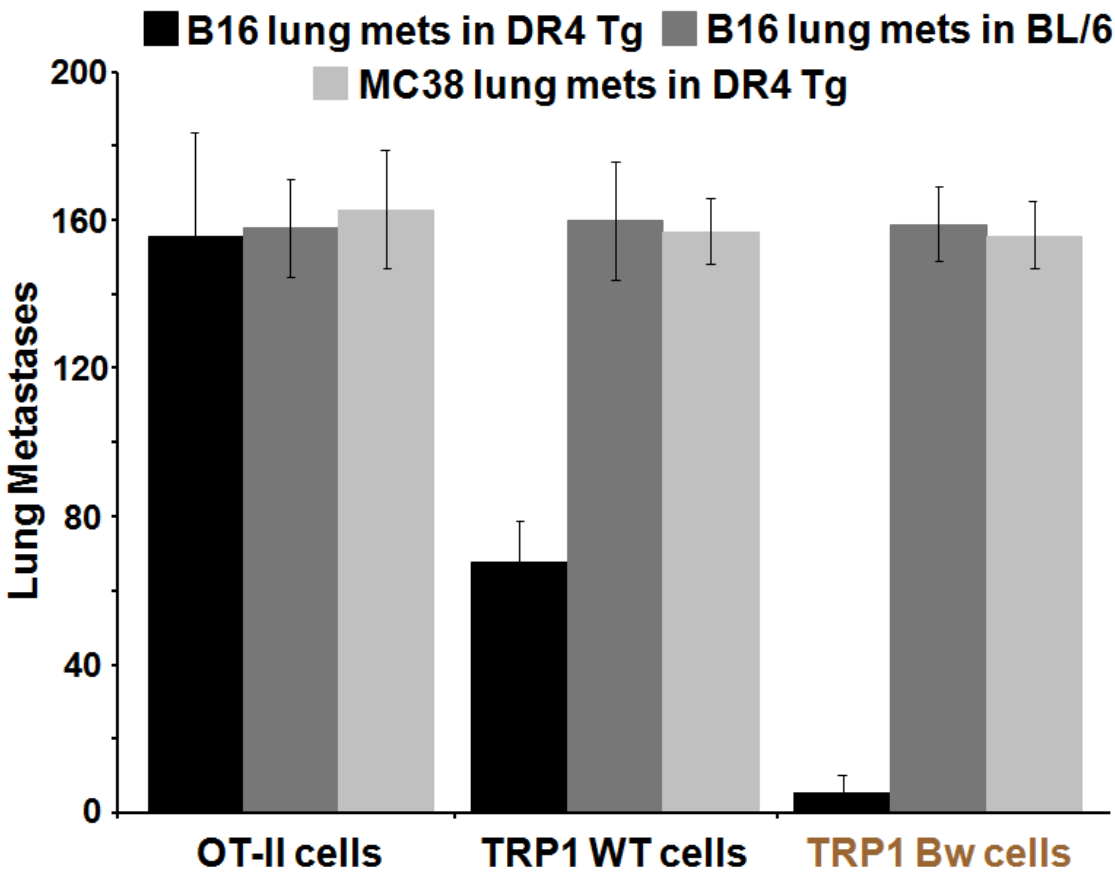


**Figure 12. Melanoma treatment is antigen-specific and DR4 restricted.** Expanded T cells were adoptively transferred ($10^7$ per mouse) into three different tumor-bearing hosts at 5 mice per group: (1) B16 (TRP-$1^+$) lung metastases in DR4 transgenics (DR$4^+$); (2) B16 lung metastases in C57BL/6 mice (DR$4^-$); (3) MC38 (TRP-$1^-$) lung metastases in DR4 transgenics. Each group had been previously intravenous tail vein inoculated with tumor. $CD4^+$ T cells derived from the TRP-$1^{Bw}$//DR$4^+$ mice nearly fully eradicate the 4-day lung metastases (mean = 5; P = 0.0006 using a 2-tailed Student t test) when compared with littermate (WT) controls (mean = 68) and control OVA-specific $CD4^+$ T cells (mean: 156).

treatment groups) in mice bearing our control, antigen-deficient tumor cell line (MC38) or in recipient mice lacking the specific restriction element (C57BL/6 mice; $DR4^-$). Unlike other models of adoptive transfer (88,89), exogenous IL-2 was not administered, and recipient animals were neither vaccinated nor irradiated for these experiments. These results confirmed that the treatment effect was both melanoma-specific and restricted by host DR4 expression.

**The TRP-1-Specific $CD4^+$ Donor T Cells Mediate Tumor Treatment Via Host $CD8^+$ T Cells**

To more specifically determine if the treatment effect was mediated by individual T cell subset populations (from either the host or those transferred), we performed a similar adoptive transfer experiment using $CD4^+$ T-cells derived only from Bw mice. The B16 tumor-bearing recipient mice were first depleted of individual effector cell subsets (CD4, CD8, NK, and NKT) before adoptive transfer. Elimination of >95% of each population was confirmed by flow cytometry. The treatment effect was entirely abrogated after host $CD8^+$ T cell depletion with no significant difference between the CD8 depletion group and the OVA control group (mean=121 vs. 127 lung metastases; P=0.106). Moreover, we excluded the contribution of other host components by observing a nearly complete and equivalent treatment effect after host CD4 and NK/NKT depletion (mean=7 vs. 4 lung metastases; P=0.128) or with control rat IgG administration (Figure 13). These results identified endogenous $CD8^+$ cells as the major mediators of tumor treatment.

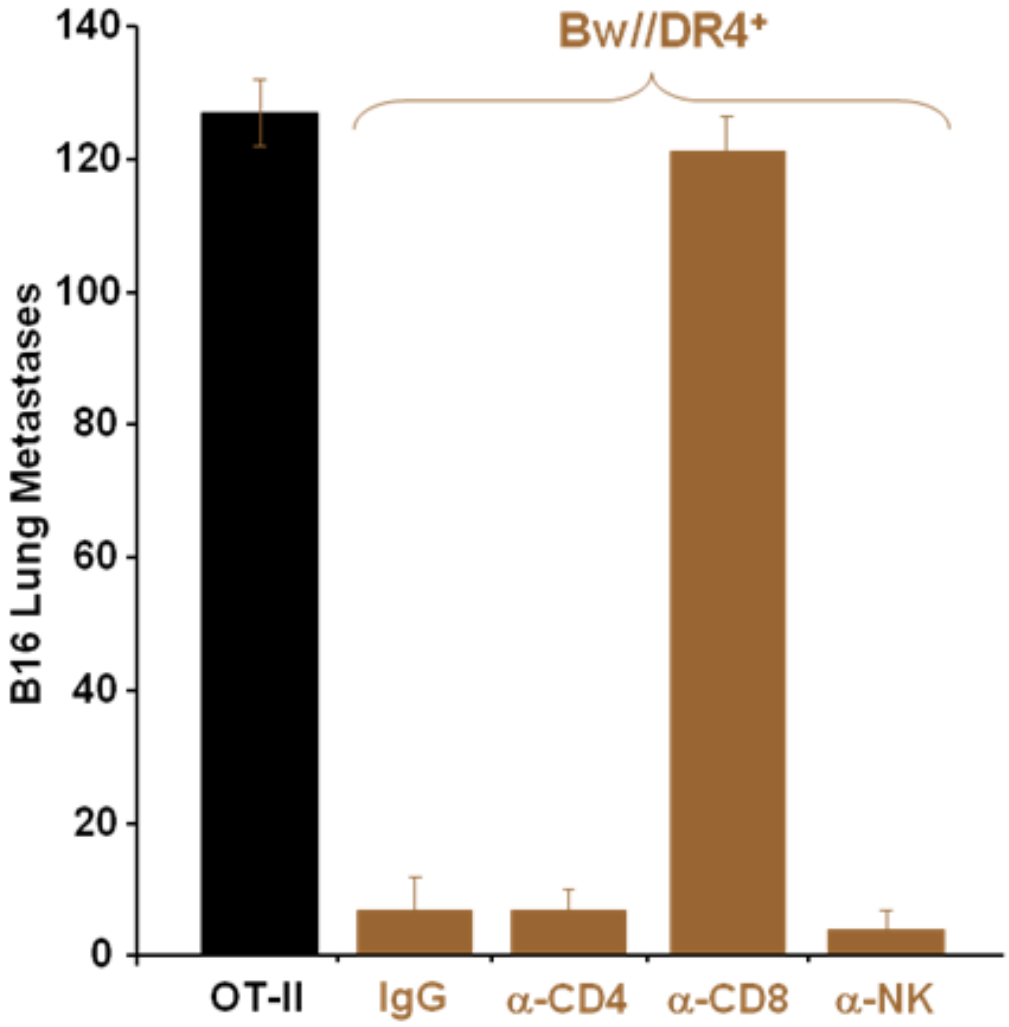


**Figure 13. Role of endogenous CD8⁺ T cells in immunotherapy.** Treatment of B16 lung metastases is dependent upon host CD8⁺ T cells. Tumor-bearing (B16 only) DR4 Tg mice were depleted of individual effector-cell subsets (CD4, CD8, natural killer and natural killer T cell) after IP injection of monoclonal antibodies before adoptive transfer of CD4⁺ T cells derived from TRP1$^{Bw}$//DR4⁺ mice. Control OVA-specific CD4⁺ T cells were used to measure the nonspecific effects of adoptive transfer. Each experimental arm involved 5 mice per group.

**High-avidity $CD4^+$ T cells derived from Bw mice overpower host $T_{regs}$ in effecting tumor destruction**

A preponderance of animal and clinical studies have demonstrated that the elimination of $T_{regs}$ can break self-tolerance, enhance antitumor immunity, and improve the antitumor effects of both cancer vaccines and adoptively transferred cells (90,91). We hypothesized, therefore, that depletion of host $T_{regs}$ would improve the treatment effect of adoptively transferred $CD4^+$ T cells. To address this question, we incorporated an established protocol (92) using an anti-CD25 mAb (PC61) capable of depleting $CD4^+/CD25^+/Foxp3^+$ $T_{regs}$. The extent of depletion was determined by flow cytometry on the day of adoptive transfer from peripheral blood samples. This protocol has shown no effect on host $CD25^-$ cells (both $CD8^+$ and $NK^+$), nor any suppressive effect on members of the IL-2 cytokine family including IL-21 (93). To more precisely measure the depletion impact on treatment, we adoptively transferred titered numbers of $CD4^+$ T cells derived from Bw mice in a side-by-side comparison with those from control OVA-specific cells ($CD4^+/CD25^+$, as determined by flow cytometry, Figure 14). Although we found a decreasing treatment effect with diminishing numbers ($10^7$, $10^6$, and $10^5$) of infused T cells, we also found that pretreatment with PC61 had no demonstrable impact on the overall efficacy when compared with the control IgG administration. This result was contrasted by a demonstrable treatment effect that favored $T_{reg}$ elimination before adoptive transfer of the lower avidity WT TRP-1 specific T cells ($10^7$ cells only). As in the prior experiment, we found

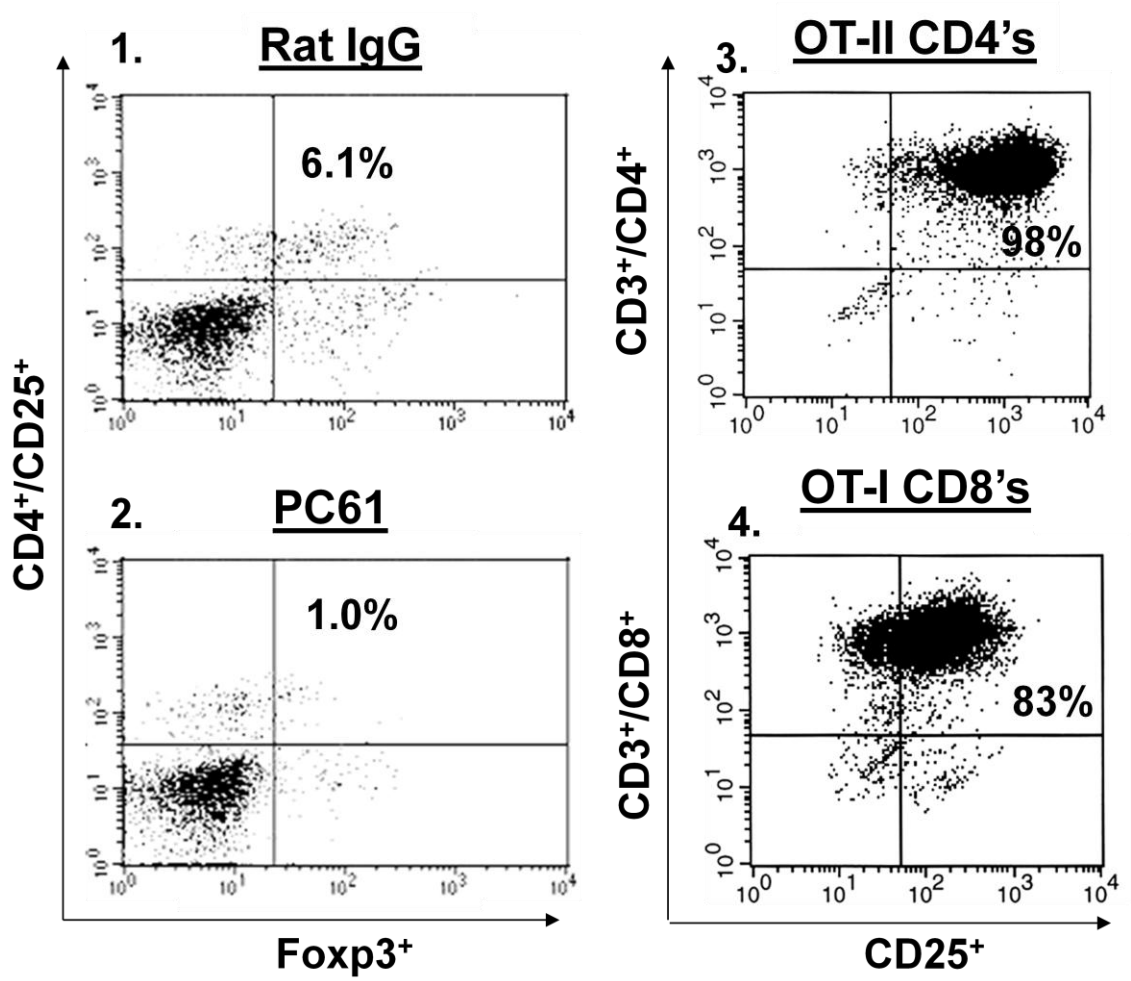


**Figure 14. $T_{reg}$ status of transplanted mice.** Flow cytometric analysis obtained from peripheral blood samples on the day of adoptive transfer of host $T_{reg}$ status ($CD4^+/CD25^+/Foxp3^+$) after depletion with control rat IgG (panel 1) or with PC61 (panel 2), respectively. Flow cytometric analysis of control in vitro expanded OT-II and OT-I cells are $CD4^+//CD25^{high}$ (panel 3) and $CD8^{high}/CD25^{high}$ (panel 4), respectively; the panels are representative of three distinct experiments.

no treatment effect with the control OVA-specific $CD4^+/CD25^+$ T cell transfer (Figure 15).

Although neutralizing levels of PC61 appeared to have no functional inhibitory effect on the subsequently infused $CD4^+/CD25^+$ T cell population, a finding noted by other investigators (94-96), that issue still remained unclear for our studies. To control for the confounding effects of residual circulating CD25 antibodies on the adoptive transfer cell population and gauge the relative impact of $T_{reg}$ depletion on antitumor responses, we infused various titers of $CD8^+/CD25^+$ $K^b$-restricted OVA-specific T cells (generated with 3 rounds of *in vitro* stimulation using peptide and IL-2) into C57BL/6 mice implanted with the OVA-expressing thymoma cell line E.G7. Unlike high avidity $CD4^+$ T cells, OVA-specific $CD8^+$ T cells had statistically greater ($P=0.0002$ at $10^6$ and $P=0.0001$ at $10^5$ cells infused) therapeutic efficacy when host animals were depleted of $T_{regs}$ before adoptive transfer when compared with control depletion (rat IgG) or control cells (TRP-1-specific CD4s) (Figure 15). These results show that high avidity TRP-1-specific $CD4^+$ T cells were maximally effective against established tumor regardless of host $T_{reg}$ status.

**Histology of Bw skin compared to WT and $Mitf^{-/-}$ samples**

The previously published findings on the Bw mouse were suggestive of TRP-1 antigen deficiency, though not conclusive. The distinct cappuccino coat color

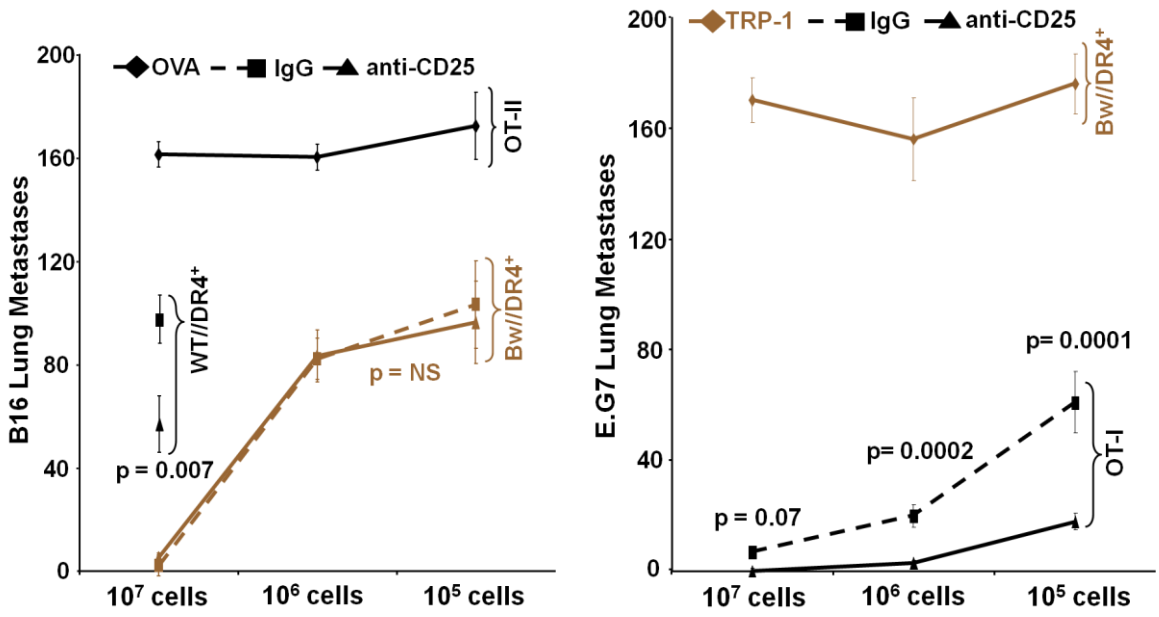


**Figure 15. The effect of $T_{reg}$ depletion on tumor treatment efficacy.** Treatment of B16 lung metastases is unaffected by $T_{reg}$ depletion. Tumor-bearing (B16 only) DR4 Tg mice were depleted of $T_{regs}$ after IP injection of monoclonal antibody PC61 before adoptive transfer. $CD4^+$ T cells derived from KO mice and control OT-II mice were transferred at titering concentrations ($10^7$, $10^6$, and $10^5$). $CD4^+$ T cells derived from TRP-1 WT mice were transferred at $10^7$ cells only. Pretreatment with PC61 has no impact on treatment when compared with control rat IgG administration using KO cells when compared with WT cells. Also, the cohort adoptively transferred with OVA-specific $CD4^+$ T cells gained no treatment effect. C, OVA-specific $CD8^+/CD25^+$ T cells more effectively eradicate established tumor in hosts depleted of $T_{regs}$. Tumor-bearing (E.G7 only) C57BL/6 mice were similarly depleted of $T_{regs}$ before adoptive transfer. $CD8^+$ T cells derived from OT-I mice and control TRP-1-specific CD4's were adoptively transferred at titering concentrations. Pretreatment with PC61 significantly

improved the overall treatment effect at both $10^6$ and $10^5$ cells infused when compared with control rat IgG. No significant treatment effect was observed using control TRP-1- specific $CD4^+$ T cells. Each experimental arm involved 5 mice per group, and all experiments were performed at least 2 to 3 times with similar results. Ig indicates immunoglobulin; OVA ovalbumin; TRP, tyrosinase-related protein.

phenotype of these mice points to a defect in melanin production. Furthermore, molecular analysis of whole skin homogenates (which are dominated by keratinocytes, not melanocytes) via RT-PCR failed to amplify a detectable level of TRP-1 RNA transcript (97). Given that TRP-1 expression is primarily confined to the melanocyte population, a cell that is relatively sparse in whole skin, these results were limited in their ability to precisely define TRP-1 expression in the Bw host. A primary objective of this project was to probe the role of tolerance in manifesting the differential immune response between the BL/6 and TPR-$1^{bw}$ cohorts. Therefore, it was necessary to more thoroughly analyze skin histology and melanocyte characteristics of the TRP-1 antigen in the Bw mouse. First, skin samples were taken in sections from the neck and upper back of WT, Bw, and $Mitf^{-/-}$ mice and sliced for immunohistochemistry. The slices were stained with S100 Ab, a probe for cells of neural crest origin including melanocytes. The result from our WT sample illustrated a classic histological pattern of melanocytes in the skin with cell clusters staining positive, particularly around the epithelium of the hair follicle (Figure 16). Conversely, the $Mitf^{-/-}$ skin sample was devoid of melanocytes with no detectable S100 staining anywhere around the hair follicles or throughout the epithelium. This result was consistent with findings in the literature that show mice without functional Mitf lack viable melanocyte precursors known as melanoblasts (44). Notably, the Bw sample was sparsely stained by S100 with more isolated, punctuate areas reading positive. The cappuccino coat color of Bw mice is consistent with diminished or impaired

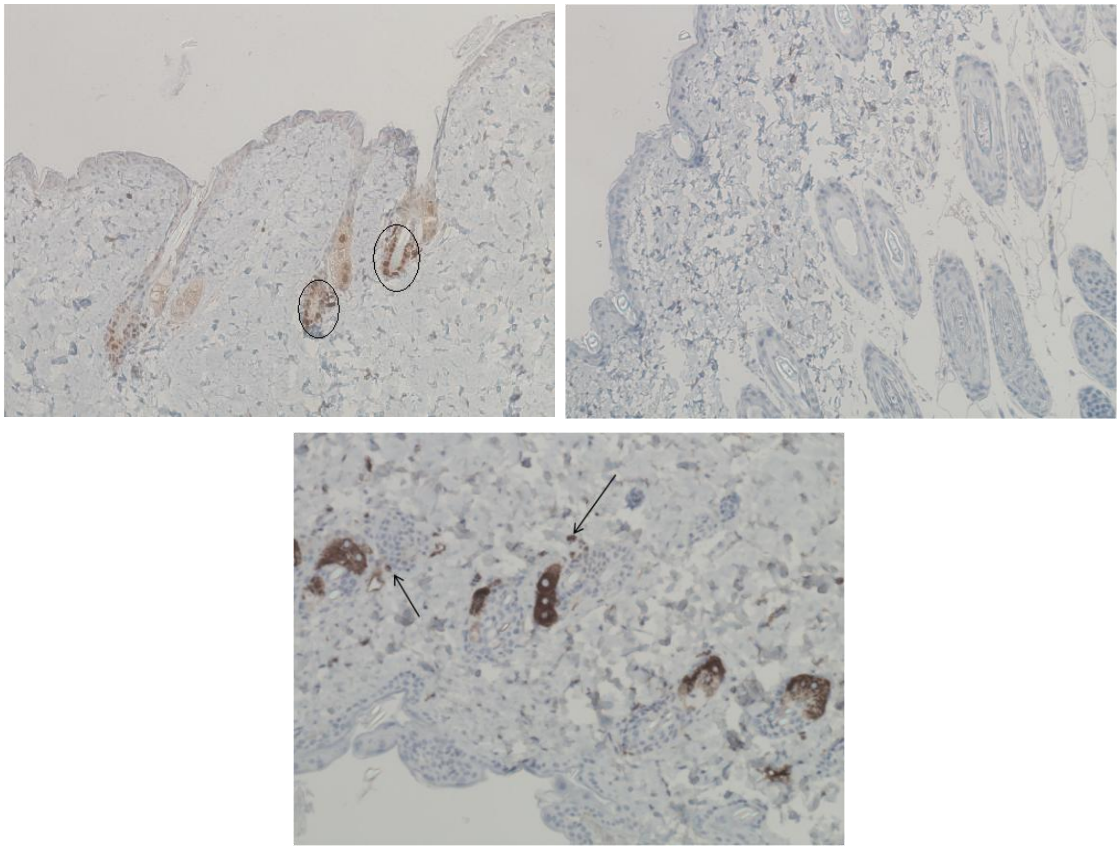

**Figure 16. Immunohistochemistry of skin sections from WT, Mitf$^{-/-}$ and Bw.** Skin was collected from each respective mouse and paraffin embedded. Samples were stained with S100 Ab and immunohistochemistry was performed. A. WT skin sample - ovals encompass the brown-staining clusters surrounding hair follicles in a typical pattern for melanocytes. B. Mitf$^{-/-}$ skin sample - No staining is evident as these mice lack viable melanocytes. C. Bw skin sample - Individual melanocytes are indicated by arrows. Bw mice apparently have detectable melanocytes in the vicinity of hair follicles but display drastically reduced density compared to the WT. (The smudge staining is consistent with sebaceous glands.)

melanin production which may correspond to a defect at the gene level resulting in TRP-1 deficiency; based on S100 levels, a defect at the tissue level (fewer melanocytes) may also explain this.

**Analysis of TRP-1 expression in Bw and WT melanocytes**

A more thorough analysis was conducted to characterize the tissue specific TRP-1 levels in Bw mice and gauge the relative levels with respect to WT mice. To do this, melanocytes were cultivated from neonatal Bw offspring for gene and protein expression assays. Whole skin was collected from a neonatal cohort and disaggregated in media. The epidermis was removed from the dermal layer then homogenized and cultured with growth factors to promote melanocyte differentiation and proliferation. We performed confocal microscopy to probe for protein level expression of gp100 and TRP-1 in the Melan-a and 5-2 lines. Cells were cultured in chamber slide wells and then incubated with either hPEP7 (anti-gp100) or MEL5 (anti-TRP-1) primary Abs followed by FITC-labeled secondary Abs. Immunofluorescence imaging revealed detectable gp100 levels dispersed in a similar pattern within both the Melan-a and 5-2 populations (Figure 17). Importantly, the MEL5 staining showed substantial levels of TRP-1 in the Melan-a cells but no detectable protein within the 5-2 population, further validating the gene expression results and solidly confirming TRP-1 protein deficiency in Bw mice (Figure 17).

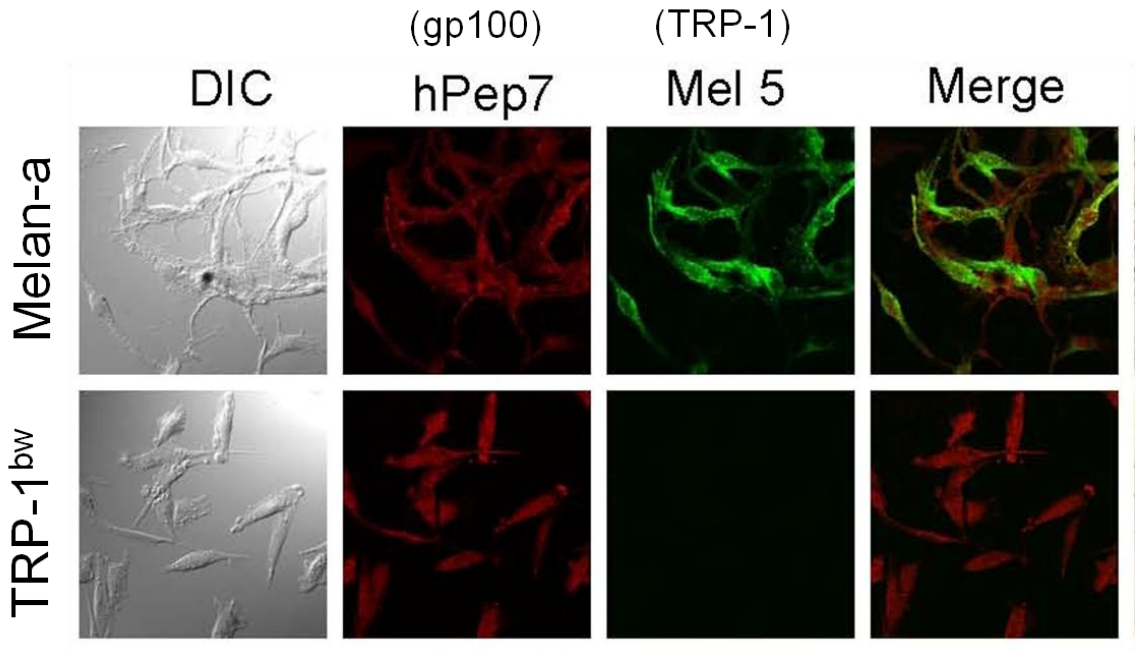


**Figure 17. Confocal microscopy of melanocytes.** Immunofluorescence staining of a C57BL/6 melanocyte line (Melan-a) and a TRP-1$^{bw}$ melanocyte line demonstrates the presence of a characteristic MDA, gp100, and the absence of detectable TRP-1 in the Bw population.

**Medullary thymic epithelial cells of WT mice express detectable levels of TRP-1**

Mouse studies have illustrated the significance of peripheral antigen expression in medullary thymic epithelial cells for mediating central T cell tolerance to self antigens. As previously described, artificial models with OVA and HA as well as endocrine related antigens, such as insulin, have all agreed on this concept. In consideration of the potential role of central tolerance in the context of the TRP-1 antigen, we isolated murine medullary thymic epithelial cells (mTECs) for gene expression analysis. Sample thymuses were harvested and digested, depleted of CD45 cells via MACS and sorted for the $CD45^-$ $Ly51^-$ MHC Class $II^+$ $EPCAM^+$ fraction. The yield from this protocol over multiple mTEC collections was between 10-20% of the input CD45 depleted population (Figure 18). Additionally, mTECs were isolated for analysis by confocal microscopy. A sorted cell suspension was plated in a chamber slide side-by-side with B16 melanoma (TRP-1 positive) and E89 melanoma (TRP-1 negative). All cells were stained with MEL-5 Ab, labeled with AlexaFluor 488 and then imaged. Microscopy revealed substantial levels of TRP-1 in the cytoplasm of the B16 cells, whereas the E89 population showed only weak background staining. Most importantly, a subset within the mTEC population stained at a detectable level pointing to a baseline level of TRP-1 protein expression within the thymus (Figure 19).

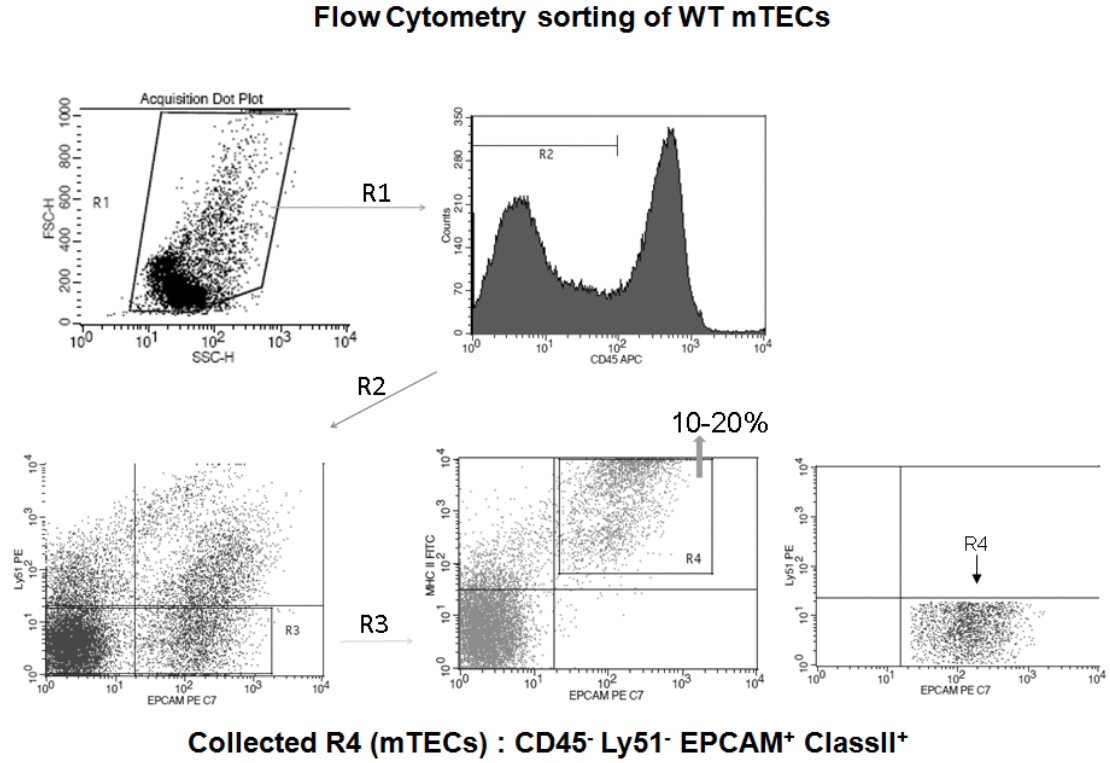


**Figure 18. Flow sorting schema for collection of mTECs.** mTECs were harvested by digesting thymic tissue into homogenates, then depleting samples of $CD45^{+}$ cells with anti-CD45 Miltenyi MACS beads. The typical yield of WT mTECs from total cells placed into the sorter was 10-20%.

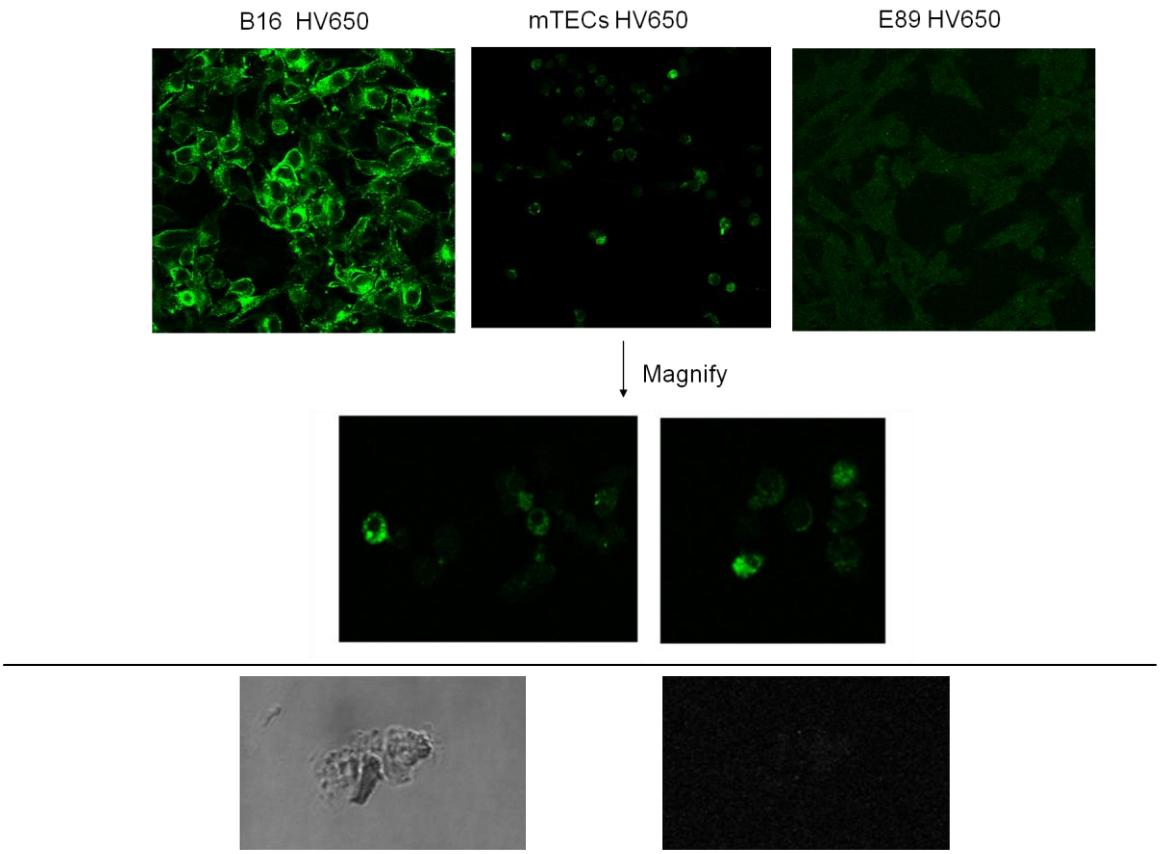


**Figure 19. Confocal microscopy of mTECs.** Cells were plated in 8-well chamber slides overnight. Each sample was fixed with 4% paraformaldehyde and permeabilized with methanol. A. Wells were stained with Mel-5 Ab conjugated with AlexaFluor 488 fluorophore. B16 melanoma stained positive, and E89 was negative, as expected. The mTEC sample was interspersed with dim positive staining cells. B. (Separate experiment from A) Top Panels: mTECs stained with isotype conjugated with AlexaFluor 488 (Mouse IgG2a). Left side is light microscopy view, and right side is immmunofluorescent view. No apparent isotype staining was detected. Bottom Panels: mTECs stained with Mel-5 Ab conjugated with AlexaFluor 488.

**Transcription factor expression in mTECs (Aire and Mitf)**

Clarifying the transcriptional control of promiscuous antigen expression in mTECs has remained elusive. The previously described studies in the literature examined various transcription factors, cell signaling molecules and chromatin modifiers as contributing factors. While certain causal relationships have been established (particularly Aire promotion of endocrine gene expression), so far no unifying mechanism has emerged. Having already discovered that TRP-1 is expressed in mTECs, we explored the question of what promotes the Tyrp1 gene in the mTEC population. One target of interest was the Aire molecule, which has been associated with mTEC development and promiscuous expression of insulin. The second target of interest was mMitf, which is a core transcription factor employed by melanocytes to promote expression of MDAs, including TRP-1. To test these questions, we acquired $Aire^{-/-}$ and $Mitf^{-/-}$ mice (Figure 20), and isolated their mTECs for analysis (Figure 21). Before proceeding, we confirmed the knockout genotype of the $Aire^{-/-}$ mice received from Jackson Labs; $Aire^{-/-}$ mTEC RNA as well as WT mTEC RNA was amplified in an RT-PCR reaction with primers flanking the region as done by Anderson et al. (98). The PCR products were run on a 1% agarose gel with a 1kb ladder to confirm the band sizes. In the lanes containing the Aire product, the WT sample showed up in a size-appropriate position, and the $Aire^{-/-}$ sample appeared in the appropriate smaller size position on the gel, corresponding to the knockout size (Figure 22). Next, we performed a qRT-PCR with all 4 mTEC samples (WT, Bw, $Aire^{-/-}$, $Mitf^{-/-}$) to assess TRP-1 expression.

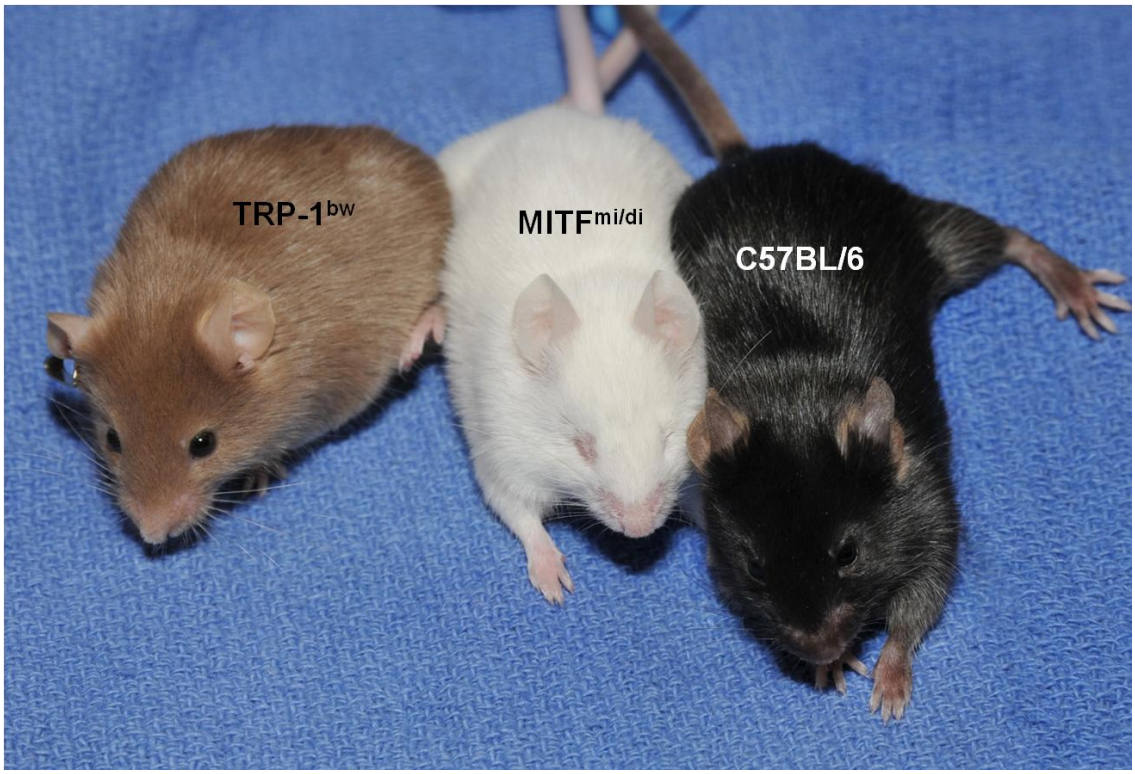


**Figure 20. Illustration of coat color phenotypes**. Three populations of mice were utilized for skin histology, mTEC preparations and gene analysis: WT, Bw, and MITF$^{-/-}$. WT and Aire$^{-/-}$ mice have a traditional black coat color whereas Bw mice demonstrate a lighter cappuccino phenotype. Mitf$^{-/-}$ mice, lacking viable melanoblasts early in development, have no melanocytes and are consequently white.

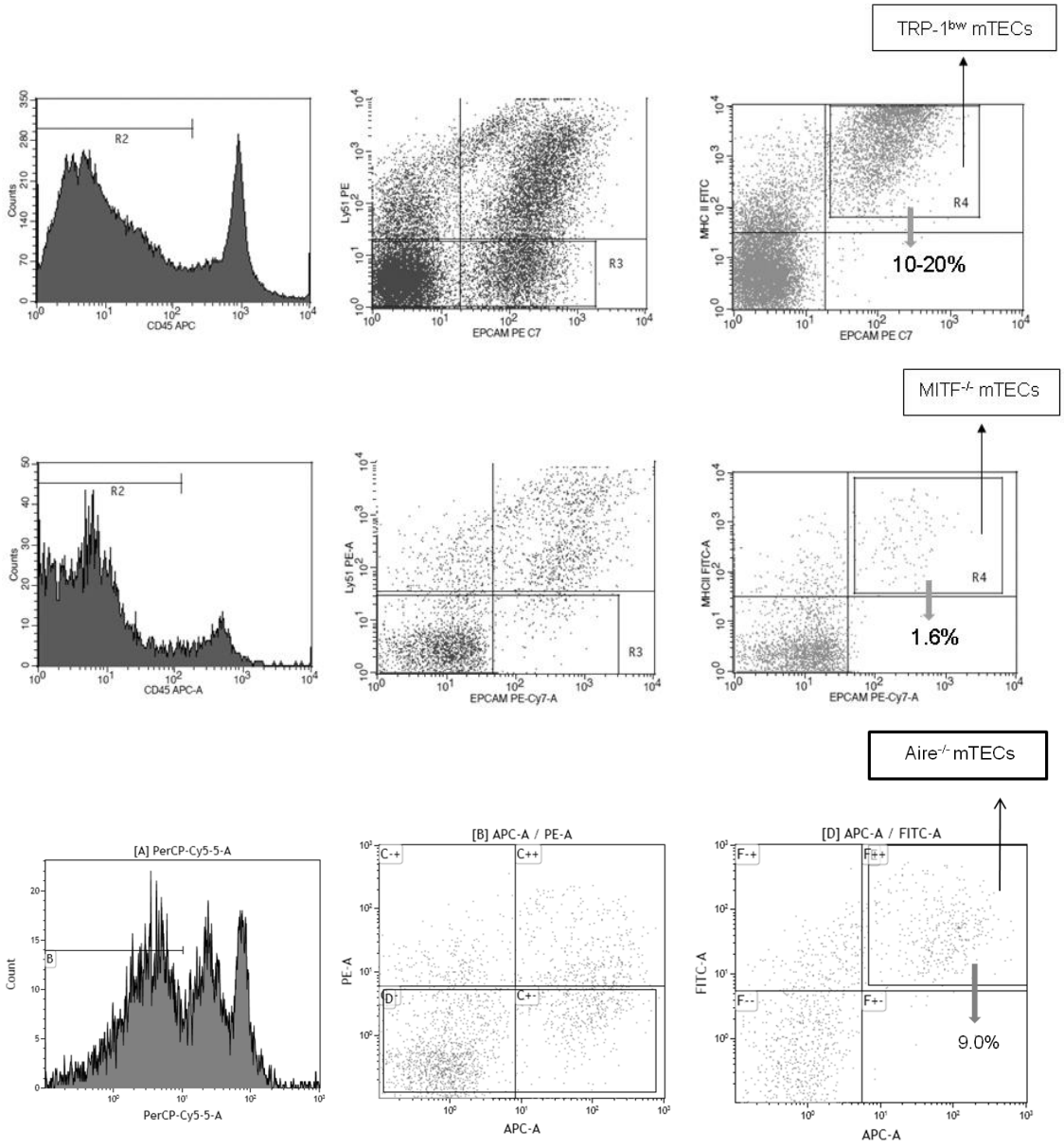


**Figure 21. FACS sorting of mTECs from Bw, Mitf$^{-/-}$ and Aire$^{-/-}$ mice.** Sorting schemas and final yields are noted for each respective sample. Mitf$^{-/-}$ sample prep yielded lower percentage of mTECs from the population compared to WT, Bw and Aire$^{-/-}$.

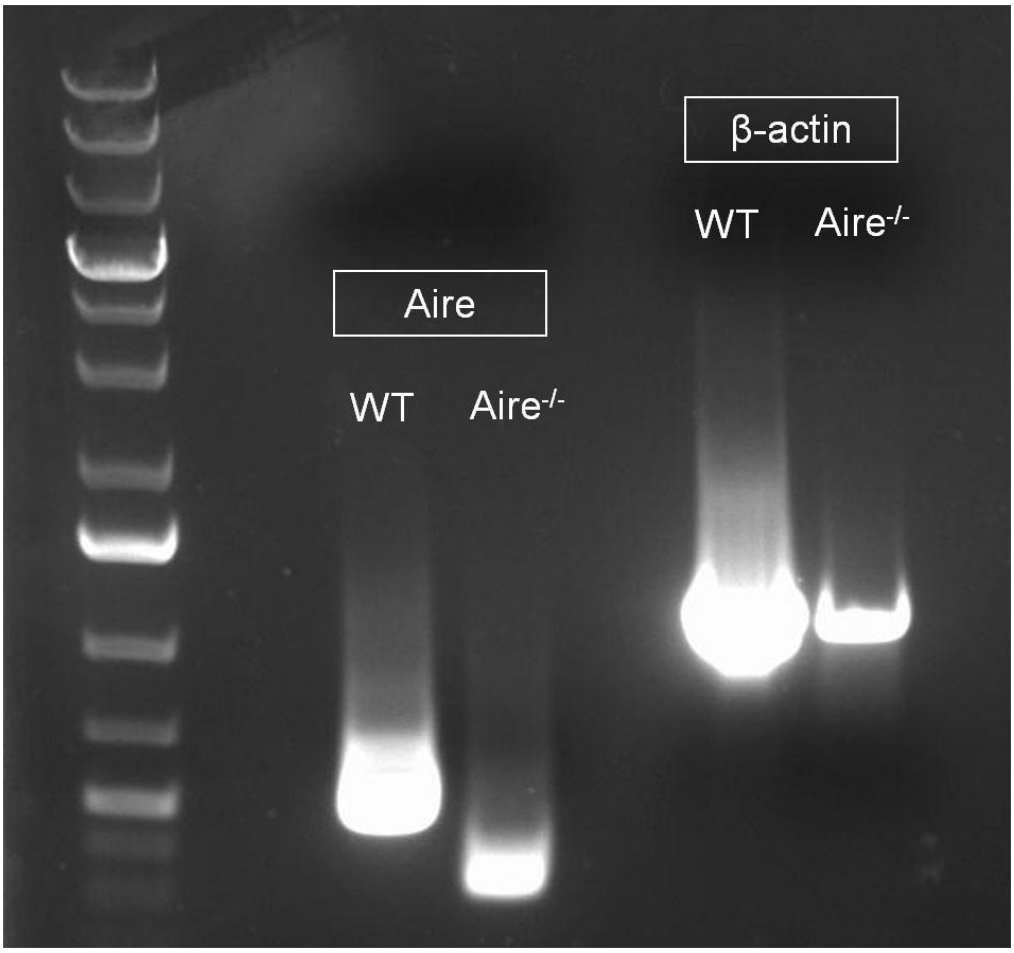


**Figure 22. RT-PCR analysis for Aire expression.** mTEC RNA samples from WT and Aire$^{-/-}$ were amplified to confirm the size differential expected between the two samples. Aire$^{-/-}$ were generated by a truncation between exon 2 and 3. Samples were amplified with primers flanking the proximal upstream and downstream region of the exon2/3 truncation and the products were run on a 1% agarose gel.

Interestingly, the Aire$^{-/-}$ background showed elevated levels of TRP-1 transcript compared to WT by a 7-fold magnitude (Figure 23). Also, we found a concomitant elevation of Mitf expression in the Aire$^{-/-}$ mTECs, with substantially higher transcript levels – a 450-fold increase compared to the WT (Figure 24). The absence of functional Aire in the Aire$^{-/-}$ mice possibly de-repressed one or both genes resulting in their noticeable increase.

The data derived from the Mitf$^{-/-}$ sample was also noteworthy. First, when performing the mTEC isolation procedure, we observed a substantially diminished cell yield as a percentage of the total. Whereas an mTEC harvest from WT and the other murine populations yielded in the range of 10-20% of the total cell population fed into the sort, the mTEC yield from Mitf$^{-/-}$ mice was closer to 1%, an apparent log-fold decrease compared to WT (Figure 25). Mitf has a broad, diverse role as a developmental transcription factor with a multitude of isoforms distributed amongst various tissue types. These results may imply a role for Mitf in mTEC development. The 4-sample qRT-PCR data was also suggestive in this regard. We noted a log-fold reduction in Aire transcript levels expressed in the Mitf$^{-/-}$ samples compared to the WT population (Figure 26). As Aire is an important transcription factor in mTEC differentiation, there could be a causative link between these findings. Finally, we found that TRP-1 transcript levels were not diminished in the Mitf$^{-/-}$ sample compared to the WT sample, suggesting that, in mTECs, TRP-1 expression occurs independently of the Mitf transcription factor (Figure 23).

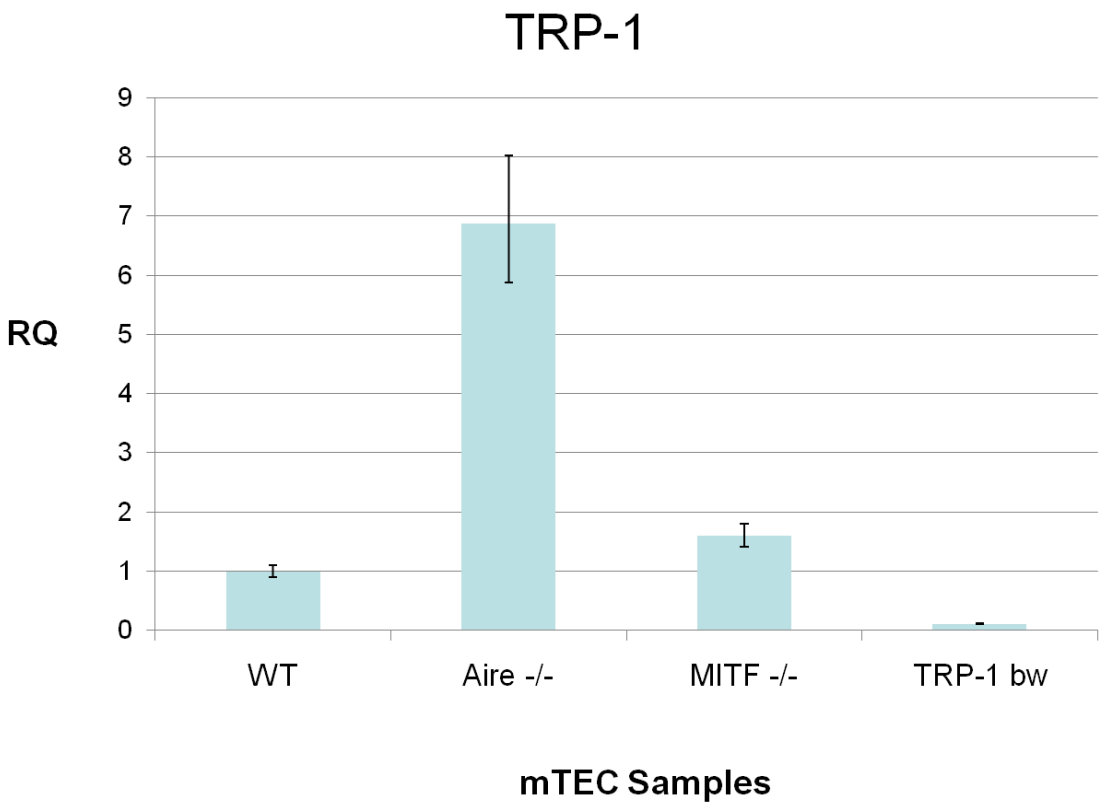


**Figure 23. Gene expression analysis of TRP-1 by mTECs.** qRT-PCR was peformed comparing the mTEC RNA samples from WT, Bw, Aire$^{-/-}$ and Mitf$^{-/-}$ mice. Samples were probed for Insulin expression and results were normalized to β-actin expression. The results showed the expected loss of Insulin expression in Aire$^{-/-}$ mice. Additionally mTECs from MITF$^{-/-}$ mice express log-fold lower fold lower levels of Insulin compared to WT sample.

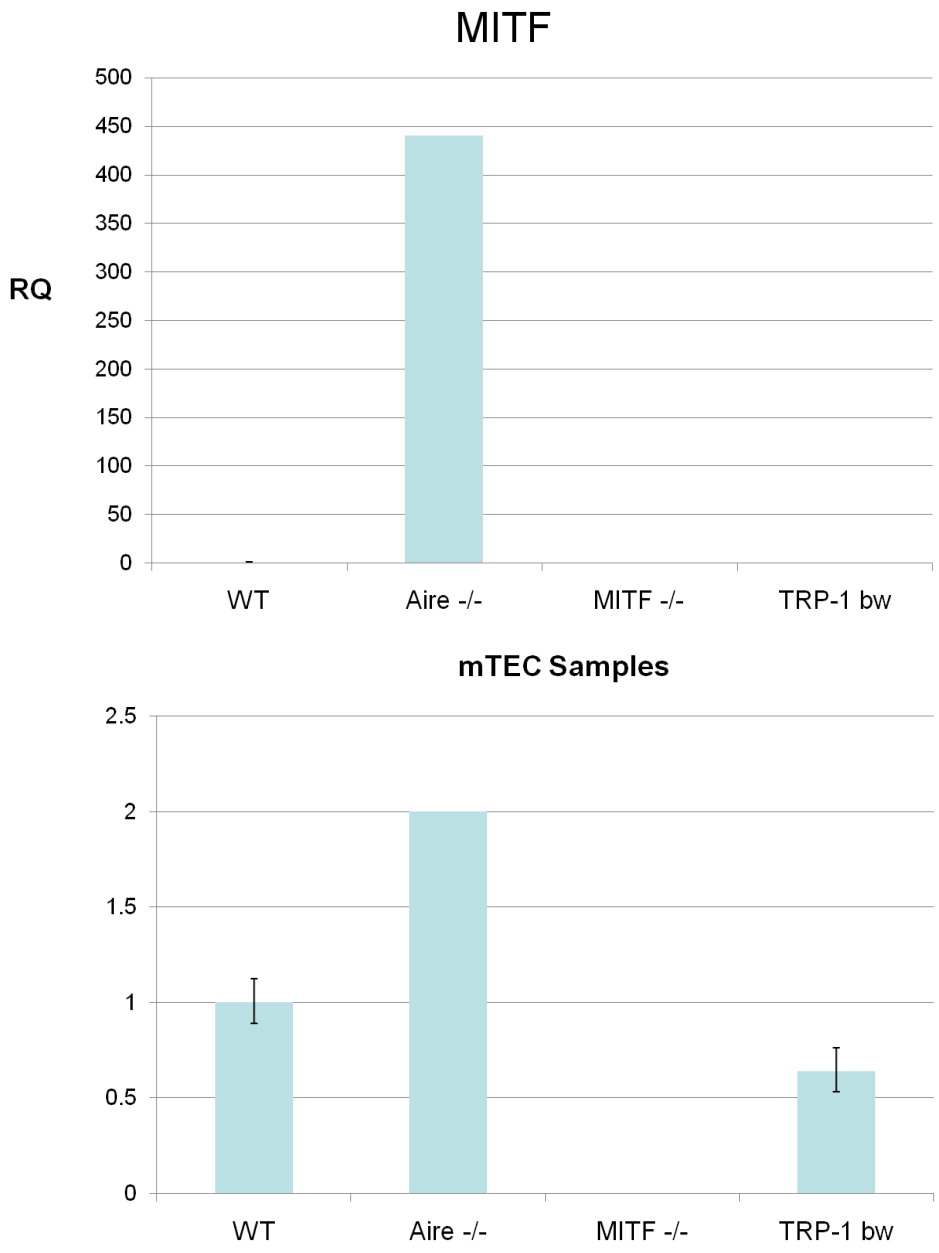


**Figure 24. qRT-PCR of mTECs for Mitf expression.** qRT-PCR was peformed comparing the mTEC RNA samples from WT, Bw, Aire$^{-/-}$ and Mitf$^{-/-}$ mice. Samples were probed for MITF expression and results were normalized to β-actin expression. The results showed a substantial elevation in Mitf expression in the Aire$^{-/-}$ sample.

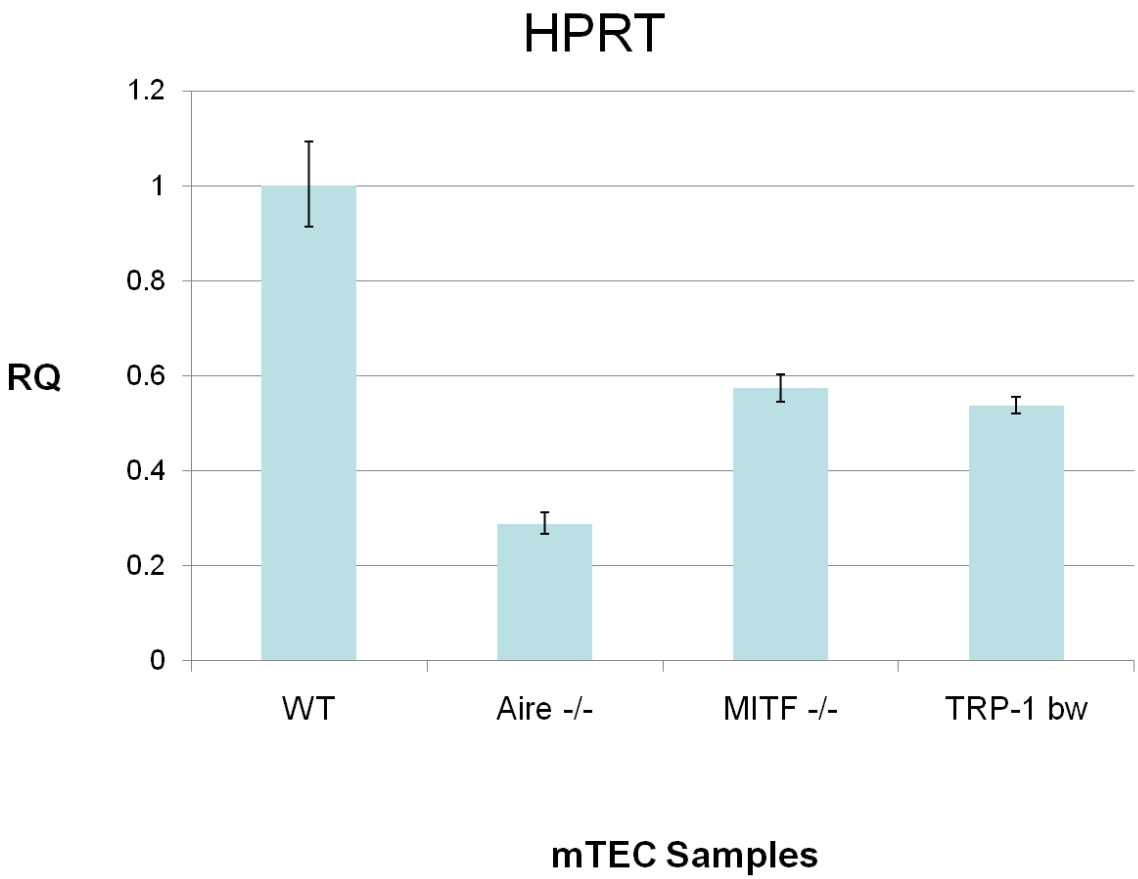


**Figure 25. Gene expression analysis of HPRT by mTECs.** qRT-PCR was peformed comparing the mTEC samples from WT, Bw, Aire$^{-/-}$ and Mitf$^{-/-}$ mice. HPRT, considered an endogenous housekeeping gene, was probed to assess internal variation among the four samples. Samples were normalized to β-actin expression.

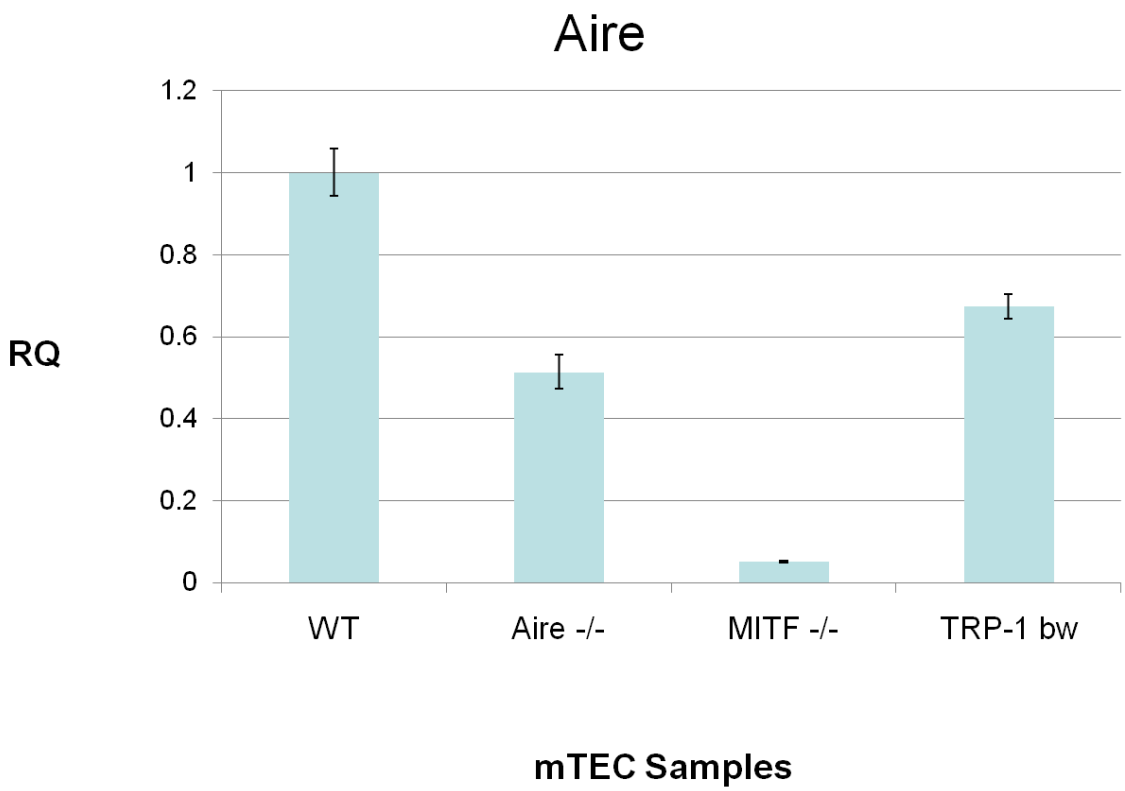


**Figure 26. Gene expression analysis of Aire.** qRT-PCR was peformed comparing the mTEC RNA samples from WT, Bw, Aire$^{-/-}$ and Mitf$^{-/-}$ mice. Samples were probed for Aire expression and results were normalized to β-actin expression. The results revealed that mTECs from MITF$^{-/-}$ mice express log-fold lower levels of Aire compared to WT sample.

**Thymocyte Analysis of WT, Bw, and DR4$^+$ Tg mice transplanted with TRP-1 TCR Tg bone marrow**

To test the impact of central tolerance on the TRP-1-specific T cell repertoire, we performed bone marrow transplants into WT and Bw mice - antigen high and antigen-deficient backgrounds, respectively. A third cohort of DR4$^+$ Tg mice (murine MHC class II$^-$, HLA DR4$^+$) was incorporated to control for the MHC class II restriction element. A TRP-1-specific TCR transgenic mouse, previously generated by Restifo and colleagues on a C57BL/6 background, was utilized as the donor bone marrow population (88,99). The transgene encodes an I-A$^b$ restricted TCR that recognizes the murine TRP-1$_{113\text{-}127}$ epitope. Following lethal irradiation, the WT and Bw cohorts were i.v. injected with TCR Tg donor bone marrow that was previously depleted of mature lymphoid populations. The recipient mice were then given 6 weeks for engraftment and hematopoietic reconstitution. Thereafter, we analyzed the thymocyte populations of the two cohorts to probe for any tolerizing pressure on the TCR Tg population as assessed by cell prevalence or apoptosis. Samples were stained with anti-Vβ14 Ab to identify the cells expressing the TRP-1 specific TCR (Figure 27). Looking at single positive thymocytes, we found a similar distribution of total SP CD4$^+$ cells in the WT, Bw, and DR4 recipients between 10-12% of total thymocytes. Within just the SP subpopulation, we calculated the percentage of Vβ14$^+$ thymocytes. In that category, there was no statistical difference between the three recipient populations with all showing transgene expression in about 42-

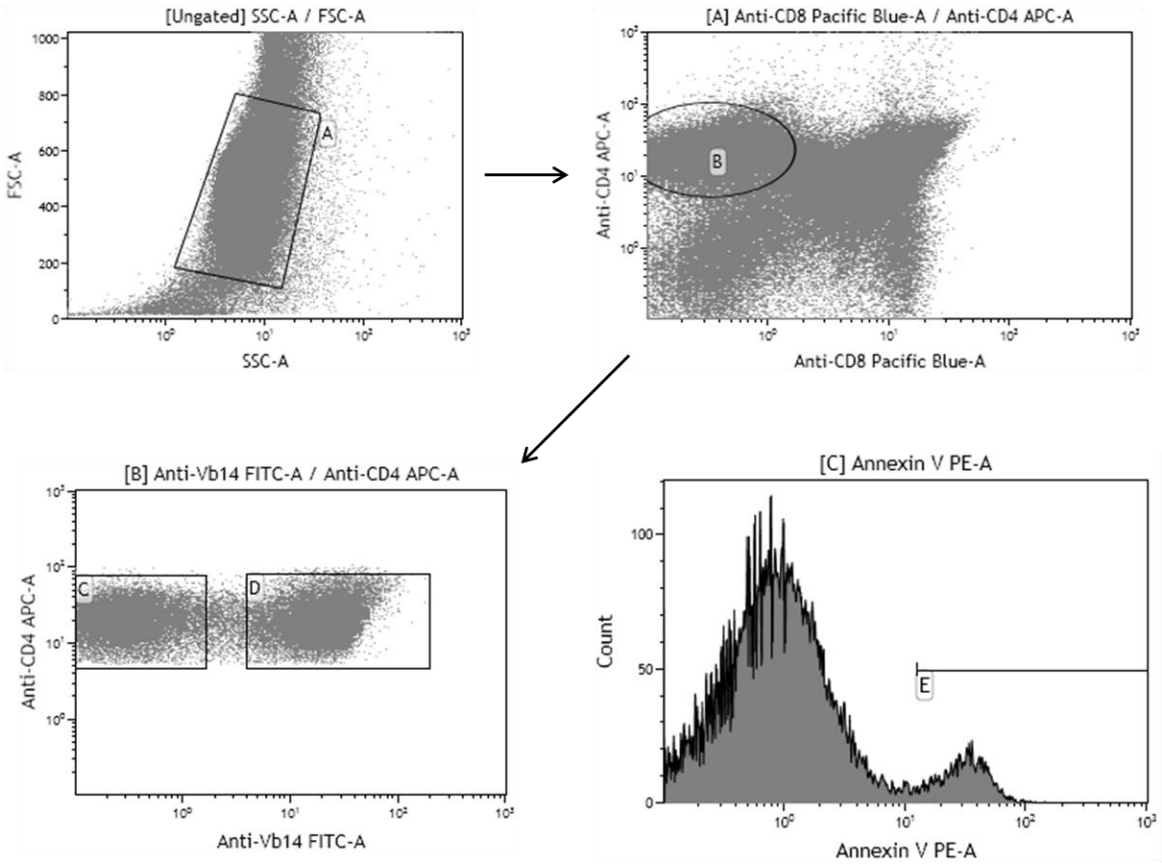


**Figure 27. Thymocyte analysis of TRP-1 TCR Tg bone marrow transplants.** WT and Bw mice were lethally irradiated then transplanted with $6x10^6$ BM cells from TRP-1 TCR Tg mice. Following engraftment, thymuses were harvested from recipient mice. Samples were stained with anti-CD8, anti-CD4, Anti-Vβ14 and Annexin V and data acquired by FACS. Samples were analyzed for the $CD4^+$ SP compartment, the Vβ14 positive and negative subsets, as well as the percentage cells positive for an apoptosis marker.

44% of $CD4^+$ SP cells (Figure 28). Finally, we assessed apoptosis statistics for the SP $CD4^+$ thymocytes (both $V\beta14^+$ and $V\beta14^-$) based on percentage positive for Annexin V (Figure 29). In all 3 recipient populations the percentage of cells staining for Annexin V ranged from 6-7% for both $V\beta14^+$ and $V\beta14^-$ with no statistical differences noted between transgene expressing versus transgene non-expressing thymocytes or across the three murine populations (Figure 29).

**Splenocyte analysis of mice transplanted with TRP-1 TCR Tg bone marrow**

In addition to monitoring thymocyte development of TRP-1 specific T cells, we also compared the peripheral compartment by analyzing splenocytes of the WT, Bw, and DR4 recipients. Cells were gated on $CD4^+$ and then either $V\beta14^+$ and $V\beta14^-$ to analyze the prevalence of transgene expressing and non transgene expressing peripheral T cells (Figure 30) The prevalence of $V\beta14^+$ expressors in the $CD4^+$ population was 44% in the WT, 50% in the Bw, and 51% in the DR4 population. The differences among mouse populations were not statistically distinguishable.

**Gene expression analysis of DCs from WT, Bw, and $Mitf^{-/-}$ mice compared to WT mTECs**

In addition to cTECs and mTECs, dendritic cells also contribute to the thymic education process. They present MHC class II complexes which engage and positively select thymocytes. Also, data reported by Bevan with MHC mismatch

| | SP CD4+ | SP CD4+ Vβ14+ | SP Vβ14- AV + | SP Vβ14- AV + |
|---|---|---|---|---|
| **WT** | 10.5 | 43.5 | 6.4 | 5.5 |
| | 10.3 | 40.5 | 5.7 | 5.5 |
| | 7.5 | 33.8 | 7.7 | 6.7 |
| | 10.1 | 43.4 | 8.8 | 7.2 |
| | 9.3 | 47.6 | 9.1 | 8.4 |
| | **9.5%** | **41.8%** | **7.5%** | **6.7%** |
| **TRP-1$^{bw}$** | 10.2 | 46.8 | 7.6 | 5.2 |
| | 18.4 | 46.2 | 6.9 | 7 |
| | 9.3 | 44.6 | 7 | 5 |
| | 15.4 | 41.1 | 8.3 | 7.3 |
| | **13.3%** | **44.7%** | **7.5%** | **6.1%** |
| **DR4** | 12.1 | 33.3 | 5 | 5.3 |
| | 13.3 | 43.9 | 7.4 | 7.3 |
| | 10.1 | 40.2 | 6.5 | 6.5 |
| | 12.8 | 42.6 | 4.9 | 5.1 |
| | 9.6 | 58.6 | 9 | 8.6 |
| | **11.6%** | **43.7%** | **6.60%** | **6.60%** |
| **Untx WT** | **4.50%** | **7.50%** | **8.20%** | **5.90%** |

**Figure 28. Summary of thymocyte populations in transplant recipients.** Column 1: Percentage of total thymocytes that were SP CD4$^+$. Column 2: Percentage of SP CD4$^+$ thymocytes that were V14$^+$. Column 3: Percentage of SP CD4$^+$ Vβ14$^-$ thymocytes positive for Annexin V. Column 4: Percentage of SP CD4$^+$ Vβ14$^+$. Data represents averages of at least 4 mice/group.

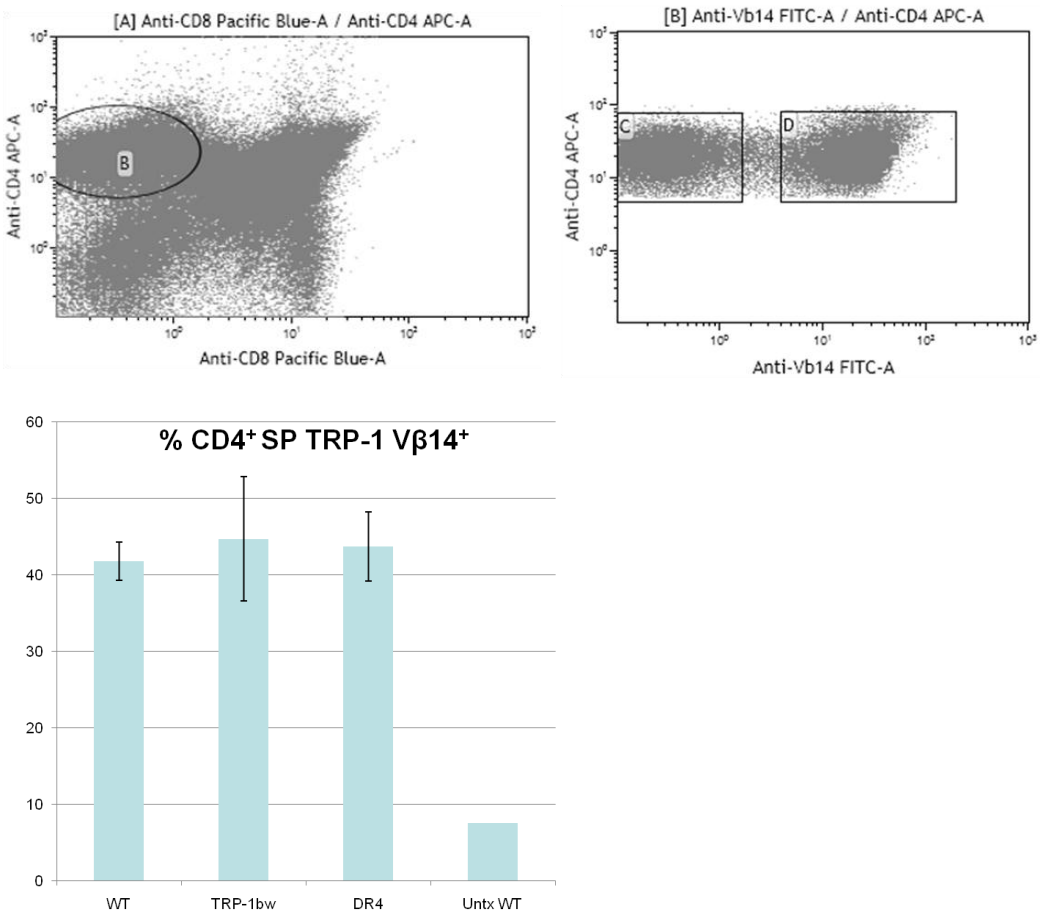


**Figure 29. Splenocyte analysis of TRP-1 TCR transplant recipients.** Top panel: Peripheral T cells were analyzed on the $CD4^+$ cells and determiningd the percentage $V\beta14^-$ versus $V\beta14^+$. Bottom panel: WT recipients had approximately 44% transgene positive T cells compared to 50% and 51% in Bw and DR4, respetively; however, the differences among the 3 groups were not statistically significant.

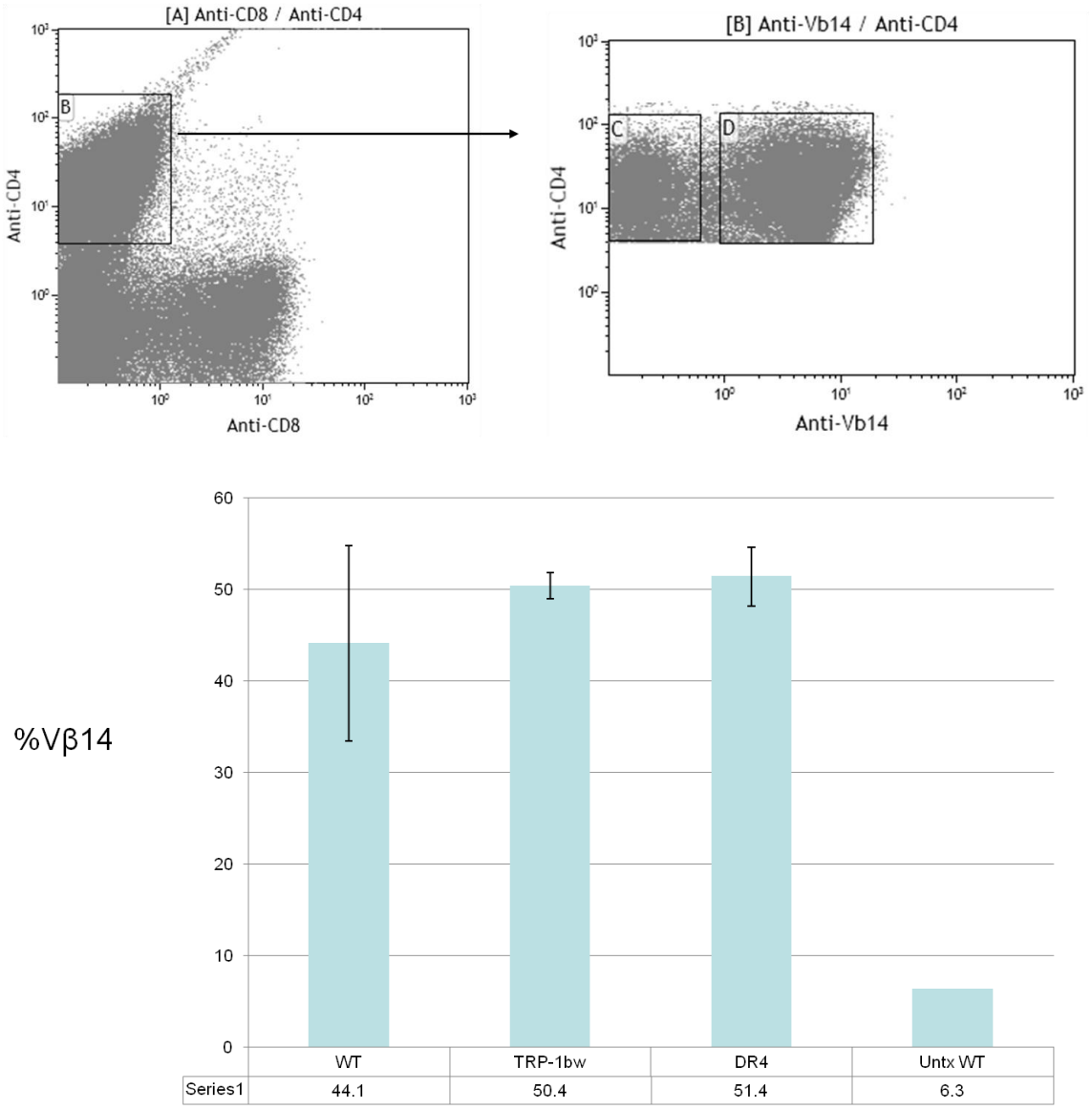


**Figure 30. Splenocyte analysis of TRP-1 TCR transplant recipients.** Top panel: Peripheral T cells were analyzed on the $CD4^+$ cells and determining the percentage $V\beta14^-$ versus $V\beta14^+$. Bottom panel: WT recipients had approximately 44% transgene positive T cells compared to 50% and 51% in Bw and DR4, respetively; however, the differences among the 3 groups were not statistically significant.

experiments on the OT-II and Ova antigen transgenic model suggests that DCs play a definitive role in negative selection of SP $CD4^+$ thymocytes (100). We performed a pilot experiment with isolates of thymic, splenic and lymph node DCs from mice on a C57BL/6 background to analyze TRP-1 gene expression in these populations (Figure 31).

The data generated is from a preliminary experiment with samples that were not derived from a uniform mouse population (with thymic tissue from a DR4 Tg C57BL/6 background). DCs were sorted by staining the cell homogenates with anti-MHC class II and anti-CD11c Abs and collecting the double positive population. RNA was then isolated from the sorted cells and reverse-transcribed into cDNA for qRT-PCR. The assay analyzed 3 DC samples and 1 WT mTEC sample to probe for relative gene expression of TRP-1. Notably, the results showed that DCs expressed TRP-1 in the range of the WT mTEC sample (Figure 32). Given the interest in the contribution of DCs to thymocyte selection, these findings expand the inquiry into the source of promiscuous antigens in the thymus. The current paradigm in the literature is that DCs acquire exogenous antigens in their vicinity, and in the thymus, mTECs are the local source of antigens.

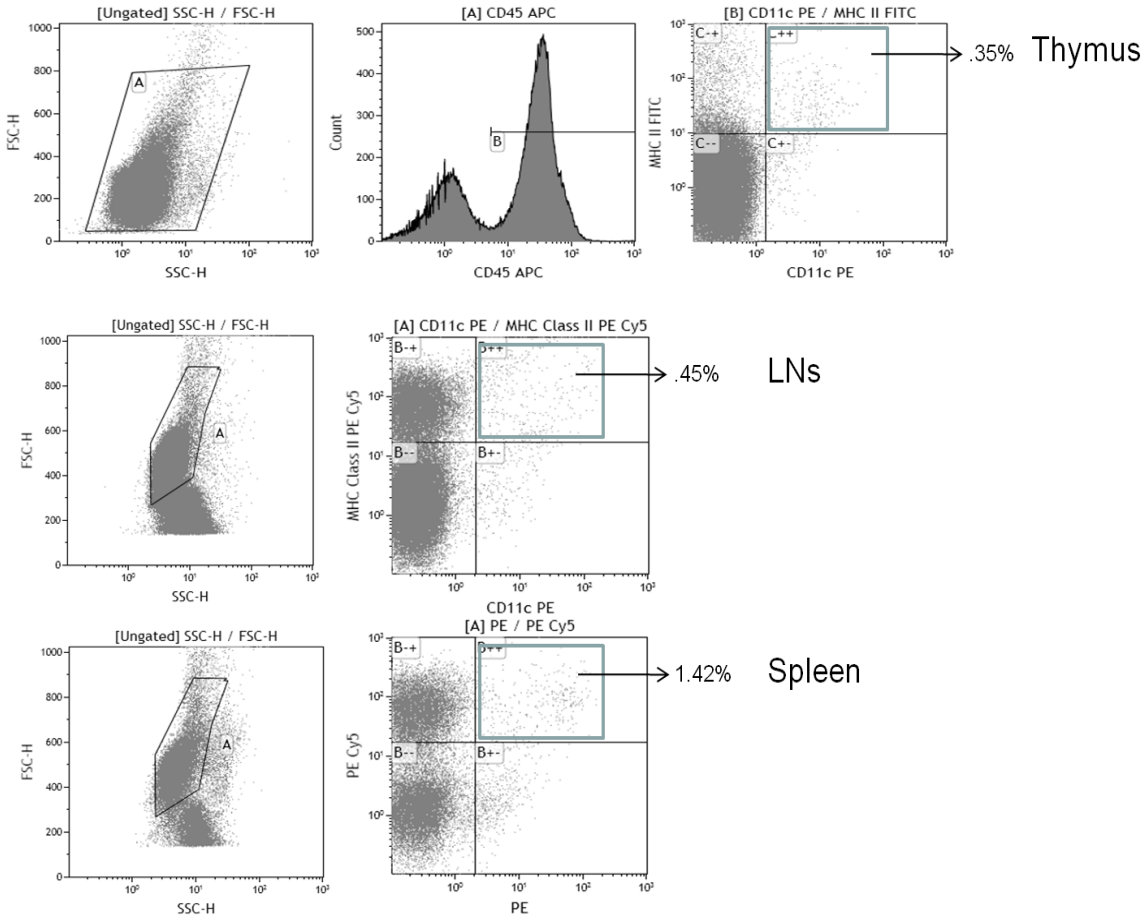


**Figure 31. Dendritic cells sorted from Thymus, Lymph nodes, and Spleen.** Respective tissues were homogenized and stained with anti-MHC class II and anti-cd11c Abs. The double positive DC population was collected from each sample with a yield between 0.4-1.4% of total cells.

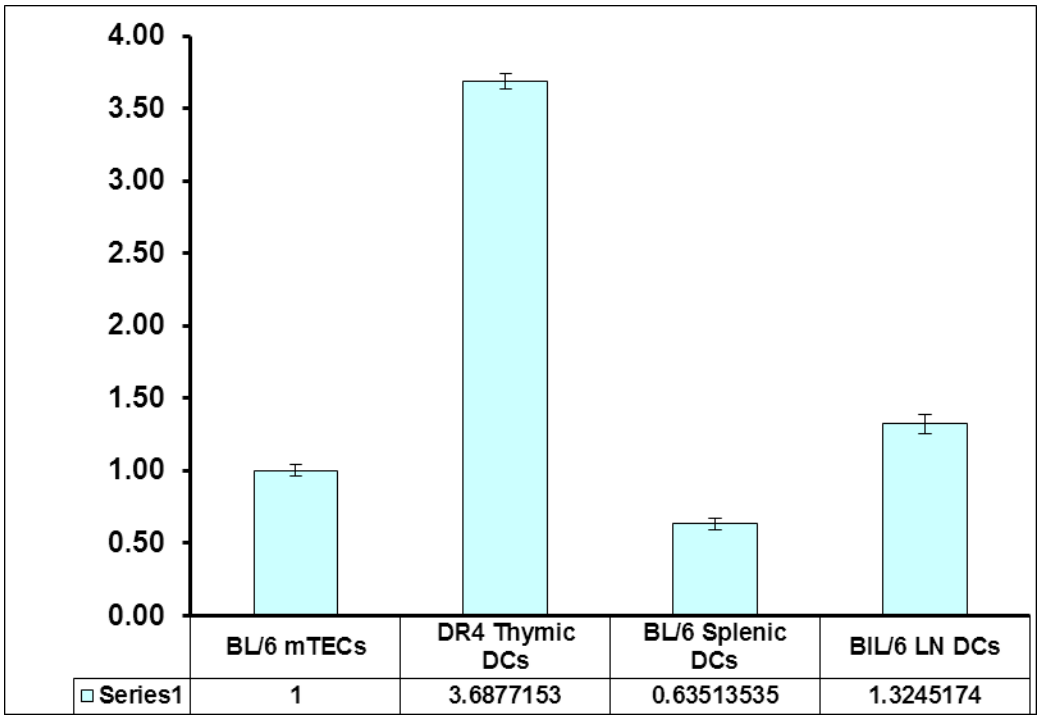


**Figure 32. qRT-PCR assessing TRP-1 expression in DCs.** RNA from the DC samples described in Figure 29 as well as WT mTECs analyzed in a quantitative assay to compare relative TRP-1 expression (normalized to β-actin). Interestingly, all 3 DC populations showed comparable levels of TRP-1 to the WT mTEC population.

# DISCUSSION

## Chapter 1

The first aim of this project focused on optimizing a novel T cell based immunotherapeutic approach to melanoma in a mouse model. Over the past 20 years, several such immunotherapy studies have relied on the unilateral activation or delivery of $CD8^+$ T cells specific for tumor antigens. Although these efforts have clarified basic principles of cancer immunotherapy, the clinical outcomes have generally been disappointing. The rationale behind studies has been based several factors including: the availability of known immunogenic MHC class I epitopes; the high frequency of 1 particular class I allele (HLA-A*0201) in melanoma patients (50%); the straightforward assays used to measure class I-restricted immune responses; the relative ease in cultivating $CD8^+$ T cells compared with their $CD4^+$ T cell counterparts; the widespread MHC class I expression on the tumor surface; and the counterproductive actions of regulatory T cell activation within the $CD4^+$ population (101). Given the many practical reasons for utilizing $CD8^+$ T cells, we questioned the fundamental T cell response itself and also expanded the focus to the $CD4^+$ population as a potential role player in immunotherapy applications.

Our initial experiments set out to characterize the immunogenicity of the melanoma antigen, TRP-1, as both a self and foreign moiety by using WT and antigen deficient (Bw) hosts. To test this question, vaccination experiments with TRP-1 protein were performed to assess immune targeting of a previously

identified HLA-DR4-restricted epitope, TRP-$1_{277-297}$. We focused on comparing and contrasting the intensity of the T cell response to this antigen within both hosts. Using an ELISPOT assay, we tested reactivity to exogenously loaded antigen over a log-fold range of peptide concentrations. At the highest concentration of peptide, no difference in T cell frequency was observed. However, at diminishing levels, we found a significantly more abundant population of responding T cells from the antigen-deficient host, Bw. Whereas the number of WT responders sharply tapered off over the first two logs, the Bw population persistently responded with cytokine secretion, even at a three-fold log decrease in peptide concentration. Overall, our findings demonstrated that systemic expression of TRP-1 (WT) markedly alters the functionality of the corresponding antigen specific T cell repertoire. These results suggested that tolerance to TRP-1 does not result in the complete loss of autoreactive T cells, but does significantly dampen the overall potency of the host's antigen-specific population manifesting in fewer and lower sensitivity clones as seen in the WT population. This same trend was observed after expanding the TRP-1 specific T cells *in vitro* and assaying both populations.

Despite the emphasis on $CD8^+$ T cell responses by previous immunotherapy clinical trials, there has been mounting data to support the critical role for $CD4^+$ T-helper cells in autoimmunity, antitumor immunity, and long-term immunity (102,103). There is also accumulating evidence that the combined application of class I and class II epitopes derived from the same tumor antigen can potentiate

durable antitumor effector function (104-106). We explored this potential with our TRP-1 model. After elucidating the substantial difference in the TRP-1 T cell immune response mounted by WT and Bw cohorts, we proceeded to assess the antitumor efficacy of both respective $CD4^+$ T cell populations, when adoptively transferred into melanoma challenged mice.

Using genetically MHC-mismatched combinations of tumors and tumor-bearing hosts, we also showed that fully polarized high IFN-γ producing $T_h1$ $CD4^+$ T cells eradicate established lung metastases in a manner that is both melanoma-specific and DR4-restricted. More importantly, we showed that this antitumor effect was more efficacious when mediated by $CD4^+$ T cells-derived from antigen-deficient donors. These results were, therefore, consistent with the aforementioned differences in T cell sensitivity. Although cells from WT and Bw mice were infused into TRP-1 "self"-expressing DR4 Tg mice, systemic expression of TRP-1 seemed to have no apparent deleterious effect on the Bw cells' ability to outperform their WT counterparts in tumor destruction.

By depleting individual individual lymphocyte populations with monoclonal antibodies, we then demonstrated that the antitumor effect is entirely dependent on host $CD8^+$ T cells. Although a $K^b$-restricted epitope from TRP-1 has been previously described (107), it is still unknown what specific antigen or restriction element is used by the cytotoxic T cells (CTLs). We had found that MCA-205 stably transfected with mTRP-1 was rejected after adoptive transfer but not a

control GFP transfectant (data not shown, C. Touloukian) confirming that antitumor responses were indeed TRP-1-specific. The results, however, still do not exclude the contribution of other antigens to the overall effector $CD8^+$ response. Regardless of the $CD8^+$ T cell clone or clones in question, it is clear that they require expression of HLA-DR4, presumably on host APCs. The ability of activated, adoptively transferred $CD4^+$ T cells to treat established tumor suggested that endogenous APCs are unable, on their own, to activate $CD8^+$ T cells, but become de novo-induced after adoptive transfer. It is also important to note that specific $CD4^+$ T cells were incapable of recognizing the TRP-$1_{277–297}$ epitope when presented on murine class I or II (present on C57BL/6 splenocytes), thus excluding a false positive interaction between the effector population and tumor.

In the final immunotherapy experiment, we demonstrated, unexpectedly, that the tumor treatment effect was unimproved (with increased numbers of infused cells) after the depletion of host $T_{regs}$. WT TRP-1-specific CD4 T cells were more effective against tumor when host $T_{regs}$ were depleted, whereas the cells derived from the Bw mice were maximally active against tumor and thus capable of overcoming the inhibitory effects of $T_{regs}$. In contrast, despite their high avidity and action against a highly immunogenic foreign antigen, OT-I $CD8^+$ T cells were relatively less effective in $T_{reg}$-replete hosts than the autoreactive Bw-derived T cell population. So far, there is a dearth of evidence that the host tumor-bearing state or the relatively naïve unprimed state of the endogenous APC pool has any

negative effects on the ability of activated, high-avidity $CD4^+$ T cells to provide help to antitumor $CD8^+$ T cells.

Although other investigators have used either ubiquitous or foreign antigen models or used TCR transgenic T cells, our immunotherapy data demonstrated that the adoptive transfer of nontransgenic $CD4^+$ T cells specific for a normally expressed endogenous self/tumor antigen could directly cross-prime host-derived endogenous $CD8^+$ T cells. Reports in the literature indicate that $CD4^+$ T cells recognize antigen/MHC complexes either on classical APCs or on class II-expressing endothelial cells. $CD4^+$ T cells in turn activate or condition dendritic cells (DCs), principally through the interaction of CD40 with its ligand, resulting in the up-regulation of B7-1, B7-2, and OX40 costimulatory molecules (31,32,108). Conditioned DCs can then cross-present antigen to cognate $CD8^+$ T cells at the tumor site or in remote locations (109). We hypothesized that the antitumor effects of the transferred $CD4^+$ T cells could occur by several different mechanisms of action, including: the release of cytokines that directly kill tumor or the supporting stroma; the activation of APCs that in turn activate NK/NKT cells or macrophages that in turn kill stromal cells; or through the activation of APCs which in turn cross-prime and activate $CD8^+$ T cells on the same APC. It is also plausible that IFN-γ production by $CD4^+$ T cells potentiates or enables the action of CTLs either by up-regulating tumor MHC class I and/or by enhancing the antigen processing. In the end, our pre-conditioning studies revealed that host $CD8^+$ T cells were required for tumor elimination.

Despite substantial evidence that $T_{regs}$ are crucial in maintaining self-tolerance and blunting the onset of autoimmunity and tumor immunity, the most surprising result was the demonstration that the suppressive actions of $T_{regs}$ are not absolute. In particular, we discovered that the actions of high-avidity autoreactive $CD4^+$ T cells can overcome $T_{regs}$ and mediate tumor immunity. High avidity $CD8^+$ T cells have been shown to be susceptible to regulatory suppression in similar adoptive transfer and tumor treatment models (110,111), including suppression generated within the immediate tumor microenvironment (112). In our hands, high avidity OT-I $CD8^+$ T cells were in fact susceptible to suppression. $T_{regs}$ are thought to oppose the sustained activation and expansion of $CD4^+$ and $CD8^+$ T cells through the secretion of suppressive cytokines (IL-10 and TGF-β) the engagement of CTLA-4 or PD-1, the down-regulation of DC antigen presentation function, or through the direct elimination of autoreactive $CD4^+$ T cells (113-116). However, the adoptive transfer of high avidity $CD4^+$ TRP-1-specific T cells may override these constraints of regulatory suppression. For example, an overwhelmingly strong avidity interaction between TCR and the antigen/MHC complex may counterbalance the actions of CTLA-4 or PD-1. This would explain the observed potent suppression of WT (tolerized) TRP-1-specific $CD4^+$ T cells as opposed to weak or nonexistent suppression of Bw (non-tolerized) T cells. A second explanation for this trend could be based on the release of cytokines (perhaps the high level release of IFN-γ alone by Bw T cells) which counterbalances the suppressor cytokines released by $T_{regs}$.

The TRP-1 vaccination and immunotherapy results highlight the importance of isolating high-avidity T-helper cells. The antitumor potency of $CD4^+$ T cells is still largely untested, and the exploitation of T cells derived from antigen-deficient hosts, as demonstrated in the tumor treatment studies, magnifies their potential experimental and clinical utility. High avidity $CD4^+$ T cells have the capacity to disrupt homeostatic balance and overcome the inhibitory effects of $T_{regs}$. A further advantage of this model system is that it relies on a normally expressed self-antigen, presented by the most common class II allele (DR4) in patients with metastatic melanoma (117,118). The binding of the hTRP1 peptide to HLA-DR1, DR7, and DR11 has also been found to be very high (data not shown), thus expanding the potential scope of its clinical application to more than 50% of patients with melanoma (119).

Unlike other models, our system also did not involve the exogenous administration of IL-2 or vaccination or require myelodepletion to facilitate the antitumor effects. These results further emphasize the self-sufficiency of high-avidity T cells introduced into transplant recipients. We believe these overall findings have tremendous therapeutic implications if TCR genes from $CD4^+$ T cells can be isolated from antigen-deficient hosts and transferred either to peripheral T cells or hematopoietic stem cells. Doing so will maximize the therapeutic efficacy that can be achieved with adoptive transfer of $CD4^+$ T cells.

Chapter 2

The results in Chapter 1 revealed the magnitude of immune tolerance to the self-antigen, TRP-1. Systemic expression of this antigen effectively blunts the sensitivity and cytokine potency of $CD4^+$ T cells specific for its 277-297 epitope significantly inhibiting their ability to recognize and eliminate a challenge with antigen-positive melanoma cells. Given the significance of these findings, we decided to query a specific mechanism of tolerance that could be responsible. Globally, immune suppressive mechanisms include deletion, anergy, regulation, activated-induced cell death and ignorance. Each of these blunts autoimmune responses to self-antigens. From a developmental standpoint, promiscuous expression of tissue-specific genes within medullary thymic epithelial cells (mTECs) has emerged as a powerful mechanism of central tolerance. Deletion of immature thymocytes serves as an initial gatekeeping stage to prune the entire T cell repertoire before it can even reach the systemic circulation. Although RNA transcripts of the MDAs gp100 and tyrosinase have been detected in mTEC RNA by polymerase chain reaction, no causal relationship to thymic selection has been demonstrated. To date, no studies in the literature have confirmed that TRP-1 is expressed within mTECs. However, it has been shown that TRP-1 is the most abundant glycoprotein in melanocytic cells, and a secreted fraction of the protein has been found to contain the 277 to 297 epitope (120). Given these circumstantial findings, we hypothesized that negative selection of the TRP-1 specific T cell repertoire could be mediated by ectopically presented antigen from mTECs or thymic DCs.

The broad framework for our interest in central tolerance was the modest results of past clinical studies that employed immunotherapy modalities for melanoma. Vaccine trials have attempted to deliver highly potent stimuli for T cells specific for tumor antigens. Additionally, adoptive transfer studies have aimed to introduce into patients T cells transduced with a functional TCR, often derived from a T cell clone previously cultivated from TIL. Attempting to engage the host T cell response by immunizing or utilizing gene therapy with TCRs poses a paradoxical challenge if, in fact, our hypothesis that central tolerance eliminates the most potent tumor reactive thymocytes is valid. Thymic deletion of tumor reactive T cells, such as those specific for TRP-1, preempts targeting of said cells by these modalities. Even when priming with the most optimal vaccine regimen or cloning the most highly-avid TCR for gene therapy, these methods are ultimately drawing from a population of TCRs that has been pruned by thymic selection. Therefore, they manifestly fail to achieve the true immune potency that would be directed at a foreign antigen.

Our first objective was to clarify the expression status of the TRP-1 antigen in the Bw mouse population. This information would help classify its status as a foreign or self protein with respect to the immune system. Data on the Bw mouse in the literature was suggestive of TRP-1 deficiency: the cappuccino coat color phenotype (as opposed to the normally pigmented black), the defined gene inversion of exon 1 within the Tyrp1 locus as well as RT-PCR studies of whole skin homogenates (121). We grossly compared Bw to WT mice by first acquiring

skin samples for histology and staining. Sections from both populations were stained with S100 Ab, specific for cells of neural crest origin, and thus a marker for melanocytes in the epidermis. Interestingly, while Bw cells did have some melanocytes in proximity to the hair follicle, they were extremely sparse in comparison to the WT skin section. TRP-1 is an important cofactor in melanin biosynthesis, but it has also been linked to a feedback mechanism with the upstream transcription factor Mitf, an important master controller of melanocyte survival and proliferation (122). The histology results suggest that the Bw genotype reduces antigen levels on a molecular level with reduced gene expression as well as a macro level with attrition of the melanocyte population. A difference in peripheral antigen levels between WT and Bw mice is significant for central tolerance, particularly in the context of class II antigen presentation. Investigators have shown that dendritic cells from the periphery are capable of acquiring exogenous antigen, migrating to the medullary region of the thymus, and mediating deletion of corresponding antigen specific T cells (70). A marked reduction in class II epitope levels in the periphery would correspond to a decrease in trafficking of the epitope to the thymus for presentation and tolerization of cognate TCR-bearing thymocytes.

Besides dendritic cells carrying peripheral epitopes to the thymus, many studies (described in the introduction) have implicated the medullary thymic epithelium as the central mediator in promiscuous gene expression of self antigens and elimination of corresponding thymocytes with self-reactive TCRs. For example,

several models of mTEC mediated tolerance have centered around the APECED syndrome (autoimmune polyendocrinopathy) associated with infiltrates of autoimmune T cells in endocrine tissues. The causality for this disease has been linked to a defect in the prominent mTEC transcription factor, Aire. Normally, Aire promotes promiscuous gene expression of endocrine family genes such as insulin, mucin-6 and thyroglobulin. Loss of Aire and its associated downstream targets is attributed to the autoimmune syndrome. We investigated whether an analogous Aire:TRP-1 transcription axis exists in thymic epithelium. To do so, we isolated mTECs from WT mice to analyze RNA samples to probe for TRP-1 gene expression. Interestingly, we found that TRP-1 mRNA was detectable in WT mTECs. Furthermore, we utilized immunofluorescent staining with an anti-TRP-1 Ab and were able to image detectable levels of TRP-1 protein in a subset of mTEC cells. This finding is consistent with reported patterns of promiscuous gene expression in the literature which suggests that, often, only a fraction of mTECs ectopically express a given host antigen (123). Importantly, our discovery that TRP-1 is expressed in mTECs implies a role for central tolerance as modulator of the TRP-1-specific T cell repertoire.

Having found that TRP-1 is expressed by mTECs, our next focus was to test whether activation of the tyrp1 gene could be attributed to a particular transcription factor. Aire is conventionally denoted as the master promoter of promiscuous gene activation in mTECs, as it has been shown to promote expression of various tissue associated antigens. At the same time, Mathis and

colleagues have conducted genome-wide array analyses of RNA samples from Aire$^{-/-}$ mTECs which showed that some genes are promiscuously expressed independently of Aire and others appear to be elevated in the absence of Aire (124). To expand the experiment beyond Aire, we also considered the Mitf protein which is the central controller of TRP-1 expression in peripheral melanocytes. With Aire and Mitf as targets of consideration, we isolated mTEC RNA from Aire$^{-/-}$ and Mitf$^{-/-}$ mice to compare with WT and Bw mTEC RNA in qRT-PCR gene expression assays.

First, we tested for expression of TRP-1 and found that RNA copy number in the Mitf$^{-/-}$ sample was indistinguishable from the WT. This result was not unexpected since there is little evidence in the literature of mTECs utilizing conserved transcriptional programs from peripheral tissues for promiscuous expression antigens. The second notable result was that TRP-1 levels in the Aire$^{-/-}$ sample were actually elevated compared to the WT by approximately 7-fold. This could imply that TRP-1 expression is suppressed by the Aire protein, whether through direct regulation or an indirect pathway.

The next question posed by these findings is what transcription factors or gene modification processes are responsible for upregulating expression of TRP-1 in mTECs. With no apparent role of the conventional transcriptional program seen in melanocytes or of the transcription factor Aire, one possible explanation is chromatin modifications. Investigators have recently turned their attention to the

role of histone acetylation/deacetylation of mTEC DNA in permitting promiscuous expression to occur (125). By loosening gene promoter sites, which permits RNA polymerase cofactors to bind and initiate a transcription complex, histone modifications in mTECs could efficiently enable the promiscuous expression of tissue antigens including TRP-1. This is an area of mTEC biology that is only beginning to be revealed.

Besides probing the Tyrp1 gene, we performed additional qRT-PCR assays with the mTEC samples to assess expression of the transcription factors Aire and Mitf. The first notable finding was that the Aire$^{-/-}$ sample exhibited a 2 log increase in Mitf expression compared to the WT. It is not clear whether there is a unified mechanism responsible for the concomitant increase in TRP-1 and Mitf in this population (such as de-repression of both genes in the absence of Aire). An alternative possibility is that Mitf is independently de-repressed in the Aire$^{-/-}$, and its increased expression leads to upregulation of the Tyrp1 gene. Results from the Mitf$^{-/-}$ mTEC sample were also unexpected. There was a log-fold decrease in Aire expression compared to the WT sample, and an assay for expression of Insulin showed a log-fold decrease in transcript levels relative to the WT. These results are interesting when considered in the context of the flow cytometry data for the respective mTEC population sorts. The flow sort of the WT population returned 10-20% of the input cells matching the mTEC staining profile, whereas the Mitf$^{-/-}$ sort returned only 2% of input cells. Though we hypothesized that Mitf would promote TRP-1 expression in mTECs, these results may imply a role for

Mitf in regulating Aire expression and possibly mTEC maturation at the cellular level.

After investigating the transcriptional profile of TRP-1 and its potential regulators in mTECs, we next sought to analyze the developmental fate of TRP-1 specific T cells in the thymus. We utilized donor mice expressing a transgenic I-$A^b$ restricted TRP-1-specific TCR. Whole bone marrow was transplanted into lethally irradiated WT, Bw and DR4 Tg (I-$A^{b-/-}$) recipients which were subsequently analyzed after reconstitution. It was hypothesized that WT mice, with TRP-1 expressing mTECs, would preferentially delete developing TRP-1-specific thymocytes resulting in fewer cells with the transgenic TCR surviving to the $CD4^+$ SP stage. We performed flow cytometry on thymocytes from the transplanted mice and found, contrary to our hypothesis, that the percentage of $V\beta14^+$ $CD4^+$ SP cells (TCR transgene positive) was statistically indistinguishable between all three groups with a range of 42-44%. Furthermore, Annexin V staining was performed to characterize the degree of apoptosis in $V\beta14^+$ $CD4^+$ SP cells, and the differences between the populations were statistically insignificant (in the range of 6-7%). Splenocytes were also harvested and analyzed to determine the prevalence of $V\beta14^+$ $CD4^+$ T cells in the periphery. The WT population showed a slightly lower prevalence of transgenic T cells compared to Bw and DR4Tg (44% compared to 50% and 51%, respectively), but the differences were not statistically significant.

Overall, the transplant findings did not reveal the expected difference in central tolerance to TRP-1 between the WT and Bw populations that we hypothesized based on the vaccination studies. One explanation for this finding could be a transplant effect – hematopoietic depletion by irradiation leads to a systemic equilibrium shift in the host that induces IL-7 release to facilitate substantial repopulation of blood cells (126). This shift may be more permissive for cell development in the thymus and, to some extent, override thymic selection. A second possibility is that the avidity of the transgenic I-$A^b$ TCR used in the transplant experiment was below the threshold for negative selection. As shown in the WT and Bw vaccination studies, TRP-1-specific T cells do expand in a vaccinated WT population, but they are of markedly diminished potency which is likely indicative of lower avidity cells surviving negative selection in the thymus. Repeating these studies with competitor non-transgenic bone marrow would possibly allow for contrasting survival of the TRP-1-specific thymocytes with a general thymocyte population. Finally, communicated data from the Restifo lab strongly suggests that the Vβ14 chain of the transgenic can mispair with de novo alpha chains produced by the T cell, thus creating a different composite TCR than the TRP-1 moiety. The T cells bearing such TCRs, if not specific for TRP-1 or another self antigen, would escape negative selection. Our flow cytometry analysis staining for anti-V β14 would not have distinguished between the transgenic and mispaired TCR bearing T cells.

Our final analysis was a pilot study to assess promiscuous gene expression in DCs. Bevan and colleagues have already shown that DCs may be the primary mediators of negative selection of thymocytes with class II restricted TCRs (67). This includes a subpopulation that differentiates from resident lymphoid progenitors in the thymic medulla, cells which are thought to acquire and present exogenous antigens provided locally by promiscuously expressing mTECs (69). Additionally, DCs inhabiting various peripheral tissues migrate to the thymic medulla carrying antigen from the periphery; they present peripheral epitopes to developing thymocytes thereby mediating deletion of self-reactive cells. Given the growing interest in the contribution of DCs to central tolerance, we peformed a qRT-PCR assay to assess whether they can express the Tyrp1 gene themselves. In a pilot experiment with RNA from WT mTECs and DCs ($CD11c^+$ MHC class $II^+$) sorted from thymic, splenic and lymph node tissue, we compared relative transcript levels of TRP-1 among the samples. Interestingly, our preliminary results show that TRP-1 transcript is expressed in all of the DC populations. Notably, the differences between each of the samples are within a 4-fold range. Based on these intriguing findings, we will expand our investigation of central tolerance to consider the impact of DCs on the TRP-1 T cell repertoire. Additionally, we will continue to investigate the hypothesis of a DC contribution to central tolerance that includes not only antigen acquisition and presentation, but also promiscuous antigen expression (Figure 33).

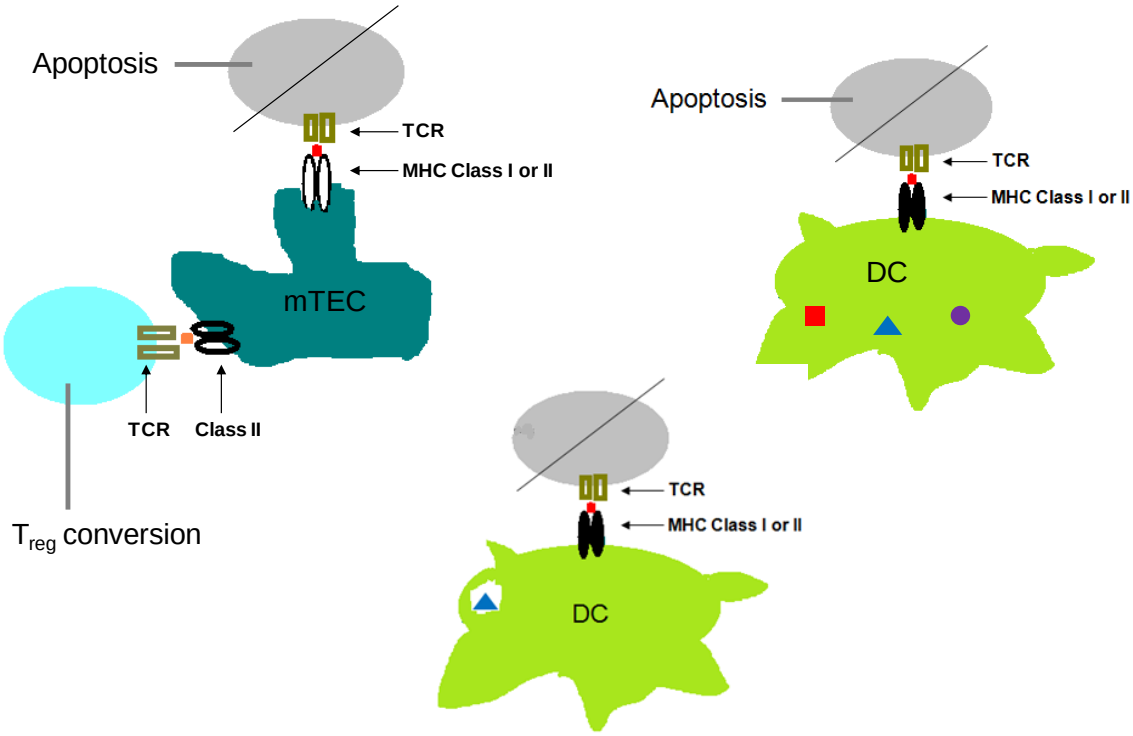


**Figure 33. Theoretical model for promiscuous antigen expression in central tolerance.** mTECs have already been shown to express an array of self antigens, and DCs are known to contribute to deletion of $CD4^+$ SP cells. A potential new role for DC expression of self-antigens is suggested by the data and will be analyzed in future studies.

## FUTURE OBJECTIVES

Chapter 1 experiments showed the potency of TRP-1 specific T cells derived from Bw mice. They displayed a characteristic $T_h1$ phenotype with secretion of IFN- γ cytokine, even at diminishing peptide antigen concentrations. Adoptive transfer experiments with tumor challenged mice illustrated the melanoma treatment efficacy of these $CD4^+$ T cells. The experiments controlled for antigen specificity in the tumor response and showed that $CD8^+$ cells in the recipient hosts were necessary for the tumor treatment effect. These experiments did not prove IFN- γ was the operative cytokine directly stimulating the anti-melanoma immune response. To better answer this question, we will employ IFN- $\gamma^{-/-}$// Bw mice for vaccination and derivation of an IFN- γ deficient population of TRP-1 specific T cells. Utilizing these cells for repeat adoptive transfer experiments in mice challenged with B16 melanoma will reveal whether, and to what extent, this cytokine is responsible for the tumor treatment efficacy observed in the original experiment.

Our second future aim is to clarify the impact of $T_{regs}$ on the tumor treatment efficacy of TRP-1 specific T cells. Adoptive transfer of cells derived from the WT cohort was consistent with the literature in that depletion of $T_{regs}$ in the tumor challenged hosts with PC61 Ab resulted in a significantly enhanced tumor treatment efficacy. However, the cohorts receiving Bw cells had the same treatment outcomes, irrespective of depletion with PC61 Ab or administration of control IgG. Data in the literature suggests a role for host $T_{regs}$ in suppressing the

tumor response. For example, they have been shown to migrate to tumors in large numbers by following a CCL22 gradient (127). Within the tumor or tumor draining lymph node, they become activated and expand, and more than one clinical study has shown that elevated $T_{regs}$ in tumor tissue correlates with a poorer prognosis (128,129). Our immunotherapy studies showed that even the most optimally treated mice still had an average of 5 pulmonary tumor nodules at the end of the experiment, leaving room for improving the efficacy. A suppressor effect from residual $T_{regs}$ or even phenotypic conversion of a subset of the adoptively transferred cells to $Foxp3^{+}$ are two possible avenues for $T_{reg}$ suppression following adoptive transfer of high-avdity $T_{effectors}$. We plan to test this question with an absolute reduction of $T_{reg}$ numbers by utilizing $Foxp3^{-/-}$ tumor challenged hosts. The tumor treatment studies with Bw donor cells will be repeated with two cohorts: WT and Foxp3 knockout mice versus WT mice. This experiment will assess if the high potency T cells achieve superior efficacy in the complete absence of $T_{reg}$ cells.

Future experiments will also identify which mechanisms of tolerance suppress the immune response to TRP-1. Vaccination studies described in Chapter 1 illustrated the significance of the host's tissue expression profile in determining the immune potency of responding T cells. Additionally, analyses of thymic tissue in Chapter 2 revealed that the TRP-1 antigen is expressed in mTECs of WT mice. These findings implicate a contribution of negative selection to the immune tolerance manifested towards this antigen. To unify these concepts, we

will perform vaccination studies on WT and Bw mice that have been thymectomized and transplanted with a Bw or WT thymus, respectively. With four groups (listed as host-donor thymus): WT-WT, Bw-Bw, WT-Bw, Bw-WT, we will be able to isolate the impact of negative selection by thymic deletion from all other tolerance mechanisms mediated by the host. By comparing the cytokine release profiles from the four groups, we will be able to quantify the direct effect of central tolerance on the TRP-1 specific T cell repertoire.

Besides isolating the impact of central tolerance, the aforementioned experiment will reveal if, and to what extent, other tolerance mechanisms of the host are affecting the TRP-1 T cell response. For example, a result where mice in the WT-Bw cohort produce lower potency T cells than the TRP-1 Bw-Bw cohort would illustrate that other processes besides thymic deletion, such as peripheral tolerance in the lymph nodes, should be examined. More recent studies have examined a newly defined population of epithelial cells that appear to play a role in mediating deletion of T cells expressing TCRs specific for host antigens (130,131). We plan to analyze these eTACS (extrathymic Aire-expressing cells) for gene expression, in a similar manner as our mTEC studies to determine if they are comparable to mTECs in their levels of TRP-1, Aire and Mitf. These studies will further expand our scope of understanding about where and how the host tolerizes its T cell repertoire with the goal of better understanding the mechanisms limiting tumor immunotherapy.

# REFERENCES


1. Davis, M. M., and P. J. Bjorkman. 1988. T-cell antigen receptor genes and T-cell recognition. *Nature* 334: 395-402.

2. Lin, J., M. J. Miller, and A. S. Shaw. 2005. The c-SMAC: sorting it all out (or in). *J. Cell Biol.* 170: 177-182.

3. Donermeyer, D. L., K. S. Weber, D. M. Kranz, and P. M. Allen. 2006. The study of high-affinity TCRs reveals duality in T cell recognition of antigen: specificity and degeneracy. *J. Immunol.* 177: 6911-6919.

4. Weiss, A., and D. R. Littman. 1994. Signal transduction by lymphocyte antigen receptors. *Cell* 76: 263-274.

5. Finco, T. S., T. Kadlecek, W. Zhang, L. E. Samelson, and A. Weiss. 1998. LAT is required for TCR-mediated activation of PLCgamma1 and the Ras pathway. *Immunity.* 9: 617-626.

6. Barda-Saad, M., A. Braiman, R. Titerence, S. C. Bunnell, V. A. Barr, and L. E. Samelson. 2005. Dynamic molecular interactions linking the T cell antigen receptor to the actin cytoskeleton. *Nat. Immunol.* 6: 80-89.

7. Janeway, C. A., Jr., and K. Bottomly. 1994. Signals and signs for lymphocyte responses. *Cell* 76: 275-285.

8. He, X., K. Park, H. Wang, X. He, Y. Zhang, X. Hua, Y. Li, and D. J. Kappes. 2008. CD4-CD8 lineage commitment is regulated by a silencer element at the ThPOK transcription-factor locus. *Immunity.* 28: 346-358.

9. Bouvier, M. 2003. Accessory proteins and the assembly of human class I MHC molecules: a molecular and structural perspective. *Mol. Immunol.* 39: 697-706.

10. Pamer, E., and P. Cresswell. 1998. Mechanisms of MHC class I--restricted antigen processing. *Annu. Rev. Immunol.* 16: 323-358.

11. Haring, J. S., V. P. Badovinac, and J. T. Harty. 2006. Inflaming the CD8+ T cell response. *Immunity.* 25: 19-29.

12. Heusel, J. W., R. L. Wesselschmidt, S. Shresta, J. H. Russell, and T. J. Ley. 1994. Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells. *Cell* 76: 977-987.

13. Berke, G. 1995. The CTL's kiss of death. *Cell* 81: 9-12.

14. Ashkenazi, A., and V. M. Dixit. 1998. Death receptors: signaling and modulation. *Science* 281: 1305-1308.

15. Zhou, L., M. M. Chong, and D. R. Littman. 2009. Plasticity of CD4+ T cell lineage differentiation. *Immunity.* 30: 646-655.

16. Murphy, K. M., and B. Stockinger. 2010. Effector T cell plasticity: flexibility in the face of changing circumstances. *Nat. Immunol.* 11: 674-680.

17. Clark, W. H., Jr., D. E. Elder, D. Guerry, L. E. Braitman, B. J. Trock, D. Schultz, M. Synnestvedt, and A. C. Halpern. 1989. Model predicting survival in stage I melanoma based on tumor progression. *J. Natl. Cancer Inst.* 81: 1893-1904.

18. Clemente, C. G., M. C. Mihm, Jr., R. Bufalino, S. Zurrida, P. Collini, and N. Cascinelli. 1996. Prognostic value of tumor infiltrating lymphocytes in the vertical growth phase of primary cutaneous melanoma. *Cancer* 77: 1303-1310.

19. Ogg, G. S., D. P. Rod, P. Romero, J. L. Chen, and V. Cerundolo. 1998. High frequency of skin-homing melanocyte-specific cytotoxic T lymphocytes in autoimmune vitiligo. *J. Exp. Med.* 188: 1203-1208.

20. Bobisse, S., M. Rondina, A. Merlo, V. Tisato, S. Mandruzzato, M. Amendola, L. Naldini, R. A. Willemsen, R. Debets, P. Zanovello, and A. Rosato. 2009. Reprogramming T lymphocytes for melanoma adoptive immunotherapy by T-cell receptor gene transfer with lentiviral vectors. *Cancer Res.* 69: 9385-9394.

21. Overwijk, W. W., A. Tsung, K. R. Irvine, M. R. Parkhurst, T. J. Goletz, K. Tsung, M. W. Carroll, C. Liu, B. Moss, S. A. Rosenberg, and N. P. Restifo. 1998. gp100/pmel 17 is a murine tumor rejection antigen: induction of "self"-reactive, tumoricidal T cells using high-affinity, altered peptide ligand. *J. Exp. Med.* 188: 277-286.

22. Williams, M. A., B. J. Holmes, J. C. Sun, and M. J. Bevan. 2006. Developing and maintaining protective CD8+ memory T cells. *Immunol. Rev.* 211: 146-153.

23. Ossendorp, F., E. Mengede, M. Camps, R. Filius, and C. J. Melief. 1998. Specific T helper cell requirement for optimal induction of cytotoxic T lymphocytes against major histocompatibility complex class II negative tumors. *J. Exp. Med.* 187: 693-702.

24. Sun, J. C., M. A. Williams, and M. J. Bevan. 2004. CD4+ T cells are required for the maintenance, not programming, of memory CD8+ T cells after acute infection. *Nat. Immunol.* 5: 927-933.

25. Williams, M. A., B. J. Holmes, J. C. Sun, and M. J. Bevan. 2006. Developing and maintaining protective CD8+ memory T cells. *Immunol. Rev.* 211: 146-153.

26. Hung, K., R. Hayashi, A. Lafond-Walker, C. Lowenstein, D. Pardoll, and H. Levitsky. 1998. The central role of CD4(+) T cells in the antitumor immune response. *J. Exp. Med.* 188: 2357-2368.

27. Greenberg, P. D. 1991. Adoptive T cell therapy of tumors: mechanisms operative in the recognition and elimination of tumor cells. *Adv. Immunol.* 49: 281-355.

28. Dighe, A. S., E. Richards, L. J. Old, and R. D. Schreiber. 1994. Enhanced in vivo growth and resistance to rejection of tumor cells expressing dominant negative IFN gamma receptors. *Immunity.* 1: 447-456.

29. Williamson, B. D., E. A. Carswell, B. Y. Rubin, J. S. Prendergast, and L. J. Old. 1983. Human tumor necrosis factor produced by human B-cell lines: synergistic cytotoxic interaction with human interferon. *Proc. Natl. Acad. Sci. U. S. A* 80: 5397-5401.

30. Coughlin, C. M., K. E. Salhany, M. S. Gee, D. C. LaTemple, S. Kotenko, X. Ma, G. Gri, M. Wysocka, J. E. Kim, L. Liu, F. Liao, J. M. Farber, S. Pestka, G. Trinchieri, and W. M. Lee. 1998. Tumor cell responses to IFNgamma affect tumorigenicity and response to IL-12 therapy and antiangiogenesis. *Immunity.* 9: 25-34.

31. Ndejembi, M. P., J. R. Teijaro, D. S. Patke, A. W. Bingaman, M. R. Chandok, A. Azimzadeh, S. G. Nadler, and D. L. Farber. 2006. Control of memory CD4 T cell recall by the CD28/B7 costimulatory pathway. *J. Immunol.* 177: 7698-7706.

32. Soroosh, P., S. Ine, K. Sugamura, and N. Ishii. 2006. OX40-OX40 ligand interaction through T cell-T cell contact contributes to CD4 T cell longevity. *J. Immunol.* 176: 5975-5987.

33. Behrens, G., M. Li, C. M. Smith, G. T. Belz, J. Mintern, F. R. Carbone, and W. R. Heath. 2004. Helper T cells, dendritic cells and CTL Immunity. *Immunol. Cell Biol.* 82: 84-90.

34. Chang, A. E., B. G. Redman, J. R. Whitfield, B. J. Nickoloff, T. M. Braun, P. P. Lee, J. D. Geiger, and J. J. Mule. 2002. A phase I trial of tumor lysate-pulsed dendritic cells in the treatment of advanced cancer. *Clin. Cancer Res.* 8: 1021-1032.

35. Dudley, M. E., J. Wunderlich, M. I. Nishimura, D. Yu, J. C. Yang, S. L. Topalian, D. J. Schwartzentruber, P. Hwu, F. M. Marincola, R. Sherry, S. F. Leitman, and S. A. Rosenberg. 2001. Adoptive transfer of cloned melanoma-reactive T lymphocytes for the treatment of patients with metastatic melanoma. *J. Immunother.* 24: 363-373.

36. Morgan, R. A., M. E. Dudley, J. R. Wunderlich, M. S. Hughes, J. C. Yang, R. M. Sherry, R. E. Royal, S. L. Topalian, U. S. Kammula, N. P. Restifo, Z. Zheng, A. Nahvi, C. R. de Vries, L. J. Rogers-Freezer, S. A. Mavroukakis, and S. A. Rosenberg. 2006. Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science* 314: 126-129.

37. Gajewski, T. F. 2007. Failure at the effector phase: immune barriers at the level of the melanoma tumor microenvironment. *Clin. Cancer Res.* 13: 5256-5261.

38. Lin, J. Y., and D. E. Fisher. 2007. Melanocyte biology and skin pigmentation. *Nature* 445: 843-850.

39. Michaeli, Y., G. Denkberg, K. Sinik, L. Lantzy, C. Chih-Sheng, C. Beauverd, T. Ziv, P. Romero, and Y. Reiter. 2009. Expression hierarchy of T cell epitopes from melanoma differentiation antigens: unexpected high level presentation of tyrosinase-HLA-A2 Complexes revealed by peptide-specific, MHC-restricted, TCR-like antibodies. *J. Immunol.* 182: 6328-6341.

40. de Vries, T. J., A. Fourkour, T. Wobbes, G. Verkroost, D. J. Ruiter, and G. N. van Muijen. 1997. Heterogeneous expression of immunotherapy candidate proteins gp100, MART-1, and tyrosinase in human melanoma cell lines and in human melanocytic lesions. *Cancer Res.* 57: 3223-3229.

41. Vijayasaradhi, S., B. Bouchard, and A. N. Houghton. 1990. The melanoma antigen gp75 is the human homologue of the mouse b (brown) locus gene product. *J. Exp. Med.* 171: 1375-1380.

42. Overwijk, W. W., D. S. Lee, D. R. Surman, K. R. Irvine, C. E. Touloukian, C. C. Chan, M. W. Carroll, B. Moss, S. A. Rosenberg, and N. P. Restifo. 1999. Vaccination with a recombinant vaccinia virus encoding a "self" antigen induces autoimmune vitiligo and tumor cell destruction in mice: requirement for CD4(+) T lymphocytes. *Proc. Natl. Acad. Sci. U. S. A* 96: 2982-2987.

43. Hallsson, J. H., J. Favor, C. Hodgkinson, T. Glaser, M. L. Lamoreux, R. Magnusdottir, G. J. Gunnarsson, H. O. Sweet, N. G. Copeland, N. A. Jenkins, and E. Steingrimsson. 2000. Genomic, transcriptional and mutational analysis of the mouse microphthalmia locus. *Genetics* 155: 291-300.

44. Goding, C. R. 2000. Mitf from neural crest to melanoma: signal transduction and transcription in the melanocyte lineage. *Genes Dev.* 14: 1712-1728.

45. Lowings, P., U. Yavuzer, and C. R. Goding. 1992. Positive and negative elements regulate a melanocyte-specific promoter. *Mol. Cell Biol.* 12: 3653-3662.

46. Tachibana, M., K. Takeda, Y. Nobukuni, K. Urabe, J. E. Long, K. A. Meyers, S. A. Aaronson, and T. Miki. 1996. Ectopic expression of MITF, a gene for Waardenburg syndrome type 2, converts fibroblasts to cells with melanocyte characteristics. *Nat. Genet.* 14: 50-54.

47. Amiel, J., P. M. Watkin, M. Tassabehji, A. P. Read, and R. M. Winter. 1998. Mutation of the MITF gene in albinism-deafness syndrome (Tietz syndrome). *Clin. Dysmorphol.* 7: 17-20.

48. Moore, K. J. 1995. Insight into the microphthalmia gene. *Trends Genet.* 11: 442-448.

49. Le Douarin, N. M., E. Houssaint, F. V. Jotereau, and M. Belo. 1975. Origin of hemopoietic stem cells in embryonic bursa of Fabricius and bone marrow studied through interspecific chimeras. *Proc. Natl. Acad. Sci. U. S. A* 72: 2701-2705.

50. Le Douarin, N. M., and F. V. Jotereau. 1975. Tracing of cells of the avian thymus through embryonic life in interspecific chimeras. *J. Exp. Med.* 142: 17-40.

51. Liu, C., F. Saito, Z. Liu, Y. Lei, S. Uehara, P. Love, M. Lipp, S. Kondo, N. Manley, and Y. Takahama. 2006. Coordination between. *Blood* 108: 2531-2539.

52. Li, J., N. Iwanami, V. Q. Hoa, M. Furutani-Seiki, and Y. Takahama. 2007. Noninvasive intravital imaging of thymocyte dynamics in medaka. *J. Immunol.* 179: 1605-1615.

53. Jung, D., and F. W. Alt. 2004. Unraveling V(D)J recombination; insights into gene regulation. *Cell* 116: 299-311.

54. Irving, B. A., F. W. Alt, and N. Killeen. 1998. Thymocyte development in the absence of pre-T cell receptor extracellular immunoglobulin domains. *Science* 280: 905-908.

55. Miyazaki, M., K. Miyazaki, M. Itoi, Y. Katoh, Y. Guo, R. Kanno, Y. Katoh-Fukui, H. Honda, T. Amagai, L. M. van, H. Kawamoto, and M. Kanno. 2008. Thymocyte proliferation induced by pre-T cell receptor signaling is maintained through polycomb gene product Bmi-1-mediated Cdkn2a repression. *Immunity.* 28: 231-245.

56. Koltsova, E. K., M. Ciofani, R. Benezra, T. Miyazaki, N. Clipstone, J. C. Zuniga-Pflucker, and D. L. Wiest. 2007. Early growth response 1 and NF-ATc1 act in concert to promote thymocyte development beyond the beta-selection checkpoint. *J. Immunol.* 179: 4694-4703.

57. Michie, A. M., and J. C. Zuniga-Pflucker. 2002. Regulation of thymocyte differentiation: pre-TCR signals and beta-selection. *Semin. Immunol.* 14: 311-323.

58. Gray, D. H., A. L. Fletcher, M. Hammett, N. Seach, T. Ueno, L. F. Young, J. Barbuto, R. L. Boyd, and A. P. Chidgey. 2008. Unbiased analysis, enrichment and purification of thymic stromal cells. *J. Immunol. Methods* 329: 56-66.

59. Witt, C. M., S. Raychaudhuri, B. Schaefer, A. K. Chakraborty, and E. A. Robey. 2005. Directed migration of positively selected thymocytes visualized in real time. *PLoS. Biol.* 3: e160.

60. Huseby, E. S., J. White, F. Crawford, T. Vass, D. Becker, C. Pinilla, P. Marrack, and J. W. Kappler. 2005. How the T cell repertoire becomes peptide and MHC specific. *Cell* 122: 247-260.

61. Zamoyska, R., A. Basson, A. Filby, G. Legname, M. Lovatt, and B. Seddon. 2003. The influence of the src-family kinases, Lck and Fyn, on T cell differentiation, survival and activation. *Immunol. Rev.* 191: 107-118.

62. Hernandez-Hoyos, G., S. J. Sohn, E. V. Rothenberg, and J. berola-Ila. 2000. Lck activity controls CD4/CD8 T cell lineage commitment. *Immunity.* 12: 313-322.

63. Ueno, T., F. Saito, D. H. Gray, S. Kuse, K. Hieshima, H. Nakano, T. Kakiuchi, M. Lipp, R. L. Boyd, and Y. Takahama. 2004. CCR7 signals are essential for cortex-medulla migration of developing thymocytes. *J. Exp. Med.* 200: 493-505.

64. Kappler, J. W., N. Roehm, and P. Marrack. 1987. T cell tolerance by clonal elimination in the thymus. *Cell* 49: 273-280.

65. Ashton-Rickardt, P. G., A. Bandeira, J. R. Delaney, K. L. Van, H. P. Pircher, R. M. Zinkernagel, and S. Tonegawa. 1994. Evidence for a differential avidity model of T cell selection in the thymus. *Cell* 76: 651-663.

66. Gotter, J., B. Brors, M. Hergenhahn, and B. Kyewski. 2004. Medullary epithelial cells of the human thymus express a highly diverse selection of tissue-specific genes colocalized in chromosomal clusters. *J. Exp. Med.* 199: 155-166.

67. Gallegos, A. M., and M. J. Bevan. 2004. Central tolerance to tissue-specific antigens mediated by direct and indirect antigen presentation. *J. Exp. Med.* 200: 1039-1049.

68. Gray, D., J. Abramson, C. Benoist, and D. Mathis. 2007. Proliferative arrest and rapid turnover of thymic epithelial cells expressing Aire. *J. Exp. Med.* 204: 2521-2528.

69. Koble, C., and B. Kyewski. 2009. The thymic medulla: a unique microenvironment for intercellular self-antigen transfer. *J. Exp. Med.* 206: 1505-1513.

70. Bonasio, R., M. L. Scimone, P. Schaerli, N. Grabie, A. H. Lichtman, and U. H. von Andrian. 2006. Clonal deletion of thymocytes by circulating dendritic cells homing to the thymus. *Nat. Immunol.* 7: 1092-1100.

71. Derbinski, J., A. Schulte, B. Kyewski, and L. Klein. 2001. Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. *Nat. Immunol.* 2: 1032-1039.

72. Anderson, M. S., E. S. Venanzi, L. Klein, Z. Chen, S. P. Berzins, S. J. Turley, B. H. von, R. Bronson, A. Dierich, C. Benoist, and D. Mathis. 2002. Projection of an immunological self shadow within the thymus by the aire protein. *Science* 298: 1395-1401.

73. Mathis, D., and C. Benoist. 2004. Back to central tolerance. *Immunity.* 20: 509-516.

74. Anderson, M. S., E. S. Venanzi, Z. Chen, S. P. Berzins, C. Benoist, and D. Mathis. 2005. The cellular mechanism of Aire control of T cell tolerance. *Immunity.* 23: 227-239.

75. Gavanescu, I., B. Kessler, H. Ploegh, C. Benoist, and D. Mathis. 2007. Loss of Aire-dependent thymic expression of a peripheral tissue antigen renders it a target of autoimmunity. *Proc. Natl. Acad. Sci. U. S. A* 104: 4583-4587.

76. Koh, A. S., A. J. Kuo, S. Y. Park, P. Cheung, J. Abramson, D. Bua, D. Carney, S. E. Shoelson, O. Gozani, R. E. Kingston, C. Benoist, and D. Mathis. 2008. Aire employs a histone-binding module to mediate immunological tolerance, linking chromatin regulation with organ-specific autoimmunity. *Proc. Natl. Acad. Sci. U. S. A* 105: 15878-15883.

77. Guerau-de-Arellano, M., D. Mathis, and C. Benoist. 2008. Transcriptional impact of Aire varies with cell type. *Proc. Natl. Acad. Sci. U. S. A* 105: 14011-14016.

78. Cloosen, S., J. Arnold, M. Thio, G. M. Bos, B. Kyewski, and W. T. Germeraad. 2007. Expression of tumor-associated differentiation antigens, MUC1 glycoforms and CEA, in human thymic epithelial cells: implications for self-tolerance and tumor therapy. *Cancer Res.* 67: 3919-3926.

79. Touloukian, C. E., W. W. Leitner, P. F. Robbins, Y. F. Li, X. Kang, R. Lapointe, P. Hwu, S. A. Rosenberg, and N. P. Restifo. 2002. Expression of a "self-"antigen by human tumor cells enhances tumor antigen-specific CD4(+) T-cell function. *Cancer Res.* 62: 5144-5147.

80. Smyth, I. M., L. Wilming, A. W. Lee, M. S. Taylor, P. Gautier, K. Barlow, J. Wallis, S. Martin, R. Glithero, B. Phillimore, S. Pelan, R. Andrew, K. Holt, R. Taylor, S. McLaren, J. Burton, J. Bailey, S. Sims, J. Squares, B. Plumb, A. Joy, R. Gibson, J. Gilbert, E. Hart, G. Laird, J. Loveland, J. Mudge, C. Steward, D. Swarbreck, J. Harrow, P. North, N. Leaves, J. Greystrong, M. Coppola, S. Manjunath, M. Campbell, M. Smith, G. Strachan, C. Tofts, E. Boal, V. Cobley, G. Hunter, C. Kimberley, D. Thomas, L. Cave-Berry, P. Weston, M. R. Botcherby, S. White, R. Edgar, S. H. Cross, M. Irvani, H. Hummerich, E. H. Simpson, D. Johnson, P. R. Hunsicker, P. F. Little, T. Hubbard, R. D. Campbell, J. Rogers, and I. J. Jackson. 2006. Genomic anatomy of the Tyrp1 (brown) deletion complex. *Proc. Natl. Acad. Sci. U. S. A* 103: 3704-3709.

81. Wakeland, E., L. Morel, K. Achey, M. Yui, and J. Longmate. 1997. Speed congenics: a classic technique in the fast lane (relatively speaking). *Immunol. Today* 18: 472-477.

82. Ito, K., H. J. Bian, M. Molina, J. Han, J. Magram, E. Saar, C. Belunis, D. R. Bolin, R. Arceo, R. Campbell, F. Falcioni, D. Vidovic, J. Hammer, and Z. A. Nagy. 1996. HLA-DR4-IE chimeric class II transgenic, murine class II-deficient mice are susceptible to experimental allergic encephalomyelitis. *J. Exp. Med.* 183: 2635-2644.

83. Barnden, M. J., J. Allison, W. R. Heath, and F. R. Carbone. 1998. Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. *Immunol. Cell Biol.* 76: 34-40.

84. Kurts, C., W. R. Heath, F. R. Carbone, J. Allison, J. F. Miller, and H. Kosaka. 1996. Constitutive class I-restricted exogenous presentation of self antigens in vivo. *J. Exp. Med.* 184: 923-930.

85. Touloukian, C. E., W. W. Leitner, S. L. Topalian, Y. F. Li, P. F. Robbins, S. A. Rosenberg, and N. P. Restifo. 2000. Identification of a MHC class II-restricted human gp100 epitope using DR4-IE transgenic mice. *J. Immunol.* 164: 3535-3542.

86. Touloukian, C. E., W. W. Leitner, P. F. Robbins, S. A. Rosenberg, and N. P. Restifo. 2001. Mining the melanosome for tumor vaccine targets: P.polypeptide is a novel tumor-associated antigen. *Cancer Res.* 61: 8100-8104.

87. Surman, D. R., M. E. Dudley, W. W. Overwijk, and N. P. Restifo. 2000. Cutting edge: CD4+ T cell control of CD8+ T cell reactivity to a model tumor antigen. *J. Immunol.* 164: 562-565.

88. Muranski, P., A. Boni, P. A. Antony, L. Cassard, K. R. Irvine, A. Kaiser, C. M. Paulos, D. C. Palmer, C. E. Touloukian, K. Ptak, L. Gattinoni, C. Wrzesinski, C. S. Hinrichs, K. W. Kerstann, L. Feigenbaum, C. C. Chan, and N. P. Restifo. 2008. Tumor-specific Th17-polarized cells eradicate large established melanoma. *Blood* 112: 362-373.

89. Overwijk, W. W., M. R. Theoret, S. E. Finkelstein, D. R. Surman, L. A. de Jong, F. A. Vyth-Dreese, T. A. Dellemijn, P. A. Antony, P. J. Spiess, D. C. Palmer, D. M. Heimann, C. A. Klebanoff, Z. Yu, L. N. Hwang, L. Feigenbaum, A. M. Kruisbeek, S. A. Rosenberg, and N. P. Restifo. 2003. Tumor regression and autoimmunity after reversal of a functionally tolerant state of self-reactive CD8+ T cells. *J. Exp. Med.* 198: 569-580.

90. Wang, H. Y., D. A. Lee, G. Peng, Z. Guo, Y. Li, Y. Kiniwa, E. M. Shevach, and R. F. Wang. 2004. Tumor-specific human CD4+ regulatory T cells and their ligands: implications for immunotherapy. *Immunity.* 20: 107-118.

91. Baecher-Allan, C., and D. E. Anderson. 2006. Immune regulation in tumor bearing hosts. *Curr. Opin. Immunol.* 18: 214-219.

92. Sutmuller, R. P., L. M. van Duivenvoorde, E. A. van, T. N. Schumacher, M. E. Wildenberg, J. P. Allison, R. E. Toes, R. Offringa, and C. J. Melief. 2001. Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25(+) regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. *J. Exp. Med.* 194: 823-832.

93. Comes, A., O. Rosso, A. M. Orengo, C. E. Di, C. Sorrentino, R. Meazza, T. Piazza, B. Valzasina, P. Nanni, M. P. Colombo, and S. Ferrini. 2006. CD25+ regulatory T cell depletion augments immunotherapy of micrometastases by an IL-21-secreting cellular vaccine. *J. Immunol.* 176: 1750-1758.

94. Sutmuller, R. P., L. M. van Duivenvoorde, E. A. van, T. N. Schumacher, M. E. Wildenberg, J. P. Allison, R. E. Toes, R. Offringa, and C. J. Melief. 2001. Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25(+) regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. *J. Exp. Med.* 194: 823-832.

95. Takahashi, T., T. Tagami, S. Yamazaki, T. Uede, J. Shimizu, N. Sakaguchi, T. W. Mak, and S. Sakaguchi. 2000. Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J. Exp. Med.* 192: 303-310.

96. Matsushita, N., S. A. Pilon-Thomas, L. M. Martin, and A. I. Riker. 2008. Comparative methodologies of regulatory T cell depletion in a murine melanoma model. *J. Immunol. Methods* 333: 167-179.

97. Smyth, I. M., L. Wilming, A. W. Lee, M. S. Taylor, P. Gautier, K. Barlow, J. Wallis, S. Martin, R. Glithero, B. Phillimore, S. Pelan, R. Andrew, K. Holt, R. Taylor, S. McLaren, J. Burton, J. Bailey, S. Sims, J. Squares, B. Plumb, A. Joy, R. Gibson, J. Gilbert, E. Hart, G. Laird, J. Loveland, J. Mudge, C. Steward, D. Swarbreck, J. Harrow, P. North, N. Leaves, J. Greystrong, M. Coppola, S. Manjunath, M. Campbell, M. Smith, G. Strachan, C. Tofts, E. Boal, V. Cobley, G. Hunter, C. Kimberley, D. Thomas, L. Cave-Berry, P. Weston, M. R. Botcherby, S. White, R. Edgar, S. H. Cross, M. Irvani, H. Hummerich, E. H. Simpson, D. Johnson, P. R. Hunsicker, P. F. Little, T. Hubbard, R. D. Campbell, J. Rogers, and I. J. Jackson. 2006. Genomic anatomy of the Tyrp1 (brown) deletion complex. *Proc. Natl. Acad. Sci. U. S. A* 103: 3704-3709.

98. Anderson, M. S., E. S. Venanzi, L. Klein, Z. Chen, S. P. Berzins, S. J. Turley, B. H. von, R. Bronson, A. Dierich, C. Benoist, and D. Mathis. 2002. Projection of an immunological self shadow within the thymus by the aire protein. *Science* 298: 1395-1401.

99. Derbinski, J., J. Gabler, B. Brors, S. Tierling, S. Jonnakuty, M. Hergenhahn, L. Peltonen, J. Walter, and B. Kyewski. 2005. Promiscuous gene expression in thymic epithelial cells is regulated at multiple levels. *J. Exp. Med.* 202: 33-45.

100. Kobayashi, T., K. Urabe, A. Winder, C. Jimenez-Cervantes, G. Imokawa, T. Brewington, F. Solano, J. C. Garcia-Borron, and V. J. Hearing. 1994. Tyrosinase related protein 1 (TRP1) functions as a DHICA oxidase in melanin biosynthesis. *EMBO J.* 13: 5818-5825.

101. Rosenberg, S. A. 2004. Development of effective immunotherapy for the treatment of patients with cancer. *J. Am. Coll. Surg.* 198: 685-696.

102. Gerloni, M., and M. Zanetti. 2005. CD4 T cells in tumor immunity. *Springer Semin. Immunopathol.* 27: 37-48.

103. Pardoll, D. M., and S. L. Topalian. 1998. The role of CD4+ T cell responses in antitumor immunity. *Curr. Opin. Immunol.* 10: 588-594.

104. Knutson, K. L., K. Schiffman, and M. L. Disis. 2001. Immunization with a HER-2/neu helper peptide vaccine generates HER-2/neu CD8 T-cell immunity in cancer patients. *J. Clin. Invest* 107: 477-484.

105. Langlade-Demoyen, P., F. Garcia-Pons, P. Castiglioni, Z. Garcia, S. Cardinaud, S. Xiong, M. Gerloni, and M. Zanetti. 2003. Role of T cell help and endoplasmic reticulum targeting in protective CTL response against influenza virus. *Eur. J. Immunol.* 33: 720-728.

106. Ossendorp, F., E. Mengede, M. Camps, R. Filius, and C. J. Melief. 1998. Specific T helper cell requirement for optimal induction of cytotoxic T lymphocytes against major histocompatibility complex class II negative tumors. *J. Exp. Med.* 187: 693-702.

107. Bowne, W. B., R. Srinivasan, J. D. Wolchok, W. G. Hawkins, N. E. Blachere, R. Dyall, J. J. Lewis, and A. N. Houghton. 1999. Coupling and uncoupling of tumor immunity and autoimmunity. *J. Exp. Med.* 190: 1717-1722.

108. Schoenberger, S. P., R. E. Toes, E. I. van der Voort, R. Offringa, and C. J. Melief. 1998. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* 393: 480-483.

109. Ridge, J. P., R. F. Di, and P. Matzinger. 1998. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. *Nature* 393: 474-478.

110. Imai, H., M. Saio, K. Nonaka, T. Suwa, N. Umemura, G. F. Ouyang, J. Nakagawa, H. Tomita, S. Osada, Y. Sugiyama, Y. Adachi, and T. Takami. 2007. Depletion of CD4+CD25+ regulatory T cells enhances interleukin-2-induced antitumor immunity in a mouse model of colon adenocarcinoma. *Cancer Sci.* 98: 416-423.

111. Antony, P. A., C. A. Piccirillo, A. Akpinarli, S. E. Finkelstein, P. J. Speiss, D. R. Surman, D. C. Palmer, C. C. Chan, C. A. Klebanoff, W. W. Overwijk, S. A. Rosenberg, and N. P. Restifo. 2005. CD8+ T cell immunity against a tumor/self-antigen is augmented by CD4+ T helper cells and hindered by naturally occurring T regulatory cells. *J. Immunol.* 174: 2591-2601.

112. Bai, A., E. Higham, H. N. Eisen, K. D. Wittrup, and J. Chen. 2008. Rapid tolerization of virus-activated tumor-specific CD8+ T cells in prostate tumors of TRAMP mice. *Proc. Natl. Acad. Sci. U. S. A* 105: 13003-13008.

113. Zheng, S. G., J. H. Wang, J. D. Gray, H. Soucier, and D. A. Horwitz. 2004. Natural and induced CD4+CD25+ cells educate CD4+. *J. Immunol.* 172: 5213-5221.

114. Cederbom, L., H. Hall, and F. Ivars. 2000. CD4+CD25+ regulatory T cells down-regulate co-stimulatory molecules on antigen-presenting cells. *Eur. J. Immunol.* 30: 1538-1543.

115. Hori, S., T. Takahashi, and S. Sakaguchi. 2003. Control of autoimmunity by naturally arising regulatory CD4+ T cells. *Adv. Immunol.* 81: 331-371.

116. Barber, D. L., E. J. Wherry, D. Masopust, B. Zhu, J. P. Allison, A. H. Sharpe, G. J. Freeman, and R. Ahmed. 2006. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 439: 682-687.

117. Barger, B. O., R. T. Acton, S. J. Soong, J. Roseman, and C. Balch. 1982. Increase of HLA-DR4 in melanoma patients from Alabama. *Cancer Res.* 42: 4276-4279.

118. Marincola, F. M., P. Shamamian, L. Rivoltini, M. Salgaller, J. Cormier, N. P. Restifo, T. B. Simonis, D. Venzon, D. E. White, and D. R. Parkinson. 1995. HLA associations in the antitumor response against malignant melanoma. *J. Immunother. Emphasis. Tumor Immunol.* 18: 242-252.

119. Southwood, S., J. Sidney, A. Kondo, M. F. del Guercio, E. Appella, S. Hoffman, R. T. Kubo, R. W. Chesnut, H. M. Grey, and A. Sette. 1998. Several common HLA-DR types share largely overlapping peptide binding repertoires. *J. Immunol.* 160: 3363-3373.

120. Touloukian, C. E., W. W. Leitner, P. F. Robbins, Y. F. Li, X. Kang, R. Lapointe, P. Hwu, S. A. Rosenberg, and N. P. Restifo. 2002. Expression of a "self-"antigen by human tumor cells enhances tumor antigen-specific CD4(+) T-cell function. *Cancer Res.* 62: 5144-5147.

121. Jackson, I. J., D. Chambers, E. M. Rinchik, and D. C. Bennett. 1990. Characterization of TRP-1 mRNA levels in dominant and recessive mutations at the mouse brown (b) locus. *Genetics* 126: 451-459.

122. Lin, J. Y., and D. E. Fisher. 2007. Melanocyte biology and skin pigmentation. *Nature* 445: 843-850.

123. Derbinski, J., J. Gabler, B. Brors, S. Tierling, S. Jonnakuty, M. Hergenhahn, L. Peltonen, J. Walter, and B. Kyewski. 2005. Promiscuous gene expression in thymic epithelial cells is regulated at multiple levels. *J. Exp. Med.* 202: 33-45.

124. Johnnidis, J. B., E. S. Venanzi, D. J. Taxman, J. P. Ting, C. O. Benoist, and D. J. Mathis. 2005. Chromosomal clustering of genes controlled by the aire transcription factor. *Proc. Natl. Acad. Sci. U. S. A* 102: 7233-7238.

125. Abramson, J., M. Giraud, C. Benoist, and D. Mathis. 2010. Aire's partners in the molecular control of immunological tolerance. *Cell* 140: 123-135.

126. Mackall, C. L., T. J. Fry, C. Bare, P. Morgan, A. Galbraith, and R. E. Gress. 2001. IL-7 increases both thymic-dependent and thymic-independent T-cell regeneration after bone marrow transplantation. *Blood* 97: 1491-1497.

127. Nishikawa, H., and S. Sakaguchi. 2010. Regulatory T cells in tumor immunity. *Int. J. Cancer* 127: 759-767.

128. Zou, W. 2006. Regulatory T cells, tumour immunity and immunotherapy. *Nat. Rev. Immunol.* 6: 295-307.

129. Yu, P., Y. Lee, W. Liu, T. Krausz, A. Chong, H. Schreiber, and Y. X. Fu. 2005. Intratumor depletion of CD4+ cells unmasks tumor immunogenicity leading to the rejection of late-stage tumors. *J. Exp. Med.* 201: 779-791.

130. Nichols, L. A., Y. Chen, T. A. Colella, C. L. Bennett, B. E. Clausen, and V. H. Engelhard. 2007. Deletional self-tolerance to a melanocyte/melanoma antigen derived from tyrosinase is mediated by a radio-resistant cell in peripheral and mesenteric lymph nodes. *J. Immunol.* 179: 993-1003.

131. Gardner, J. M., J. J. Devoss, R. S. Friedman, D. J. Wong, Y. X. Tan, X. Zhou, K. P. Johannes, M. A. Su, H. Y. Chang, M. F. Krummel, and M. S. Anderson. 2008. Deletional tolerance mediated by extrathymic Aire-expressing cells. *Science* 321: 843-847.

CURRICULUM VITAE

Andrew G. Brandmaier

## Education

2004 B.S. Chemistry Durham, NC
Duke University
2011 Ph.D. Department of Microbiology & Immunology Indianapolis, IN
Indiana University

## Honors, Awards, and Fellowships

2001 Howard Hughes Medical Institute Summer Undergraduate Research Fellowship, Duke University
2003 Howard Hughes Medical Institute Continuing Research Fellowship, Duke University
2003 Summer Undergraduate Research Fellowship, University of Texas Southwestern
2004 Medical Scientist Training Program Fellowship, Indiana University
2007 Best First Year Student Seminar – Student Vote, Department of Microbiology & Immunology, Indiana University
2008 NIH T32 Predoctoral Training Grant – Immunology and Infectious Diseases

## Abstracts Presented and Conferences Attended

2007 American Association of Cancer Research, San Francisco, CA
*Molecular Targets and Therapeutics*: "Tyrp1$^{-/-}$ mice vaccinated with Tyrp1 generate high avidity T cells that are protective against melanoma."

2008 Keystone Symposia, Keystone, CO
*Tolerance in Transplantation and Autoimmunity*: "Tyrp1$^{-/-}$ mice vaccinated with Tyrp1 generate high avidity anti-tumor CD4$^{+}$ T cells."

## Publications

2009 **Brandmaier AG**, Leitner WW, Ha SP, Sidney J, Restifo NP, Touloukian CE. "High-avidity autoreactive CD4$^{+}$ T cells induce host CTL, overcome $T_{regs}$ and mediate tumor destruction." *Journal of Immunotherapy*. 2009 Sep;32(7):677-88.

2007 Sherbet DP, Papari-Zareei M, Khan N, Sharma KK, **Brandmaier A**, Rambally S, Chattopadhyay A, Andersson S, Agarwal AK, Auchus RJ. "Cofactors, redox state, and directional preferences of hydroxysteroid dehydrogenases." *Molecular and Cellular Endocrinology*. 2007 Feb;265-266:83-8.

2006 Papari-Zareei M, **Brandmaier A**, Auchus RJ. "Arginine 276 controls the directional preference of AKR1C9 (rat liver 3 alpha-hydroxysteroid dehydrogenase) in human embryonic kidney cells." *Endocrinology*. 2006 Apr;147(4):1591-7.