THE EFFECT OF SELF-ANTIGEN SPECIFIC CD4+ T CELL PRECURSOR FREQUENCY ON THE ANTI-TUMOR IMMUNE RESPONSE

A Dissertation

Presented to the Faculty of the Weill Cornell Graduate School

of Medical Sciences

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

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February 2016

THE EFFECT OF SELF-ANTIGEN SPECIFIC CD4+ T CELL PRECURSOR FREQUENCY ON THE ANTI-TUMOR IMMUNE RESPONSE

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Cornell University 2016

Many of the current approaches to cancer immunotherapy aim to exploit and enhance the natural T cell response against cancer. However, these treatment modalities may fall short of their potential due to the elimination of self-tumor antigen specific T cells from the immune repertoire to guard against autoimmunity. To investigate how the precursory frequency of self-antigen specific T cells shapes therapeutic outcome, our lab developed a model system in which the precursor number of T cells specific for a self/melanoma antigen could be manipulated in mice bearing melanoma tumors through the adoptive transfer of tumor specific T cells. Using this model we had previously determined that bringing the frequency of CD8+ T cells within or slightly above a normal physiologic range favored proliferation and generation of polyfunctional effector T cells and anti-tumor immunity, while dramatically exceeding this threshold resulted in intraclonal competition and an impaired immune response. We adapted this model to investigate CD4+ T cells specific for the tumor-self antigen TRP-1 and demonstrated that clonal abundance dictates the development of CD4+ T cell mediated anti-tumor immunity as well. Tumor specific CD4+ T cells operate within the constraints imposed by intraclonal competition, despite ubiquitous expression of cognate antigen. Unlike CD8+ T cells, the defects in activation and proliferation are uncoupled from the development of effector function. Low physiologic precursor frequencies of self-antigen specific T cells support rapid expansion of the population at the expense of the generation of effector function due to onset of irreversible T cell exhaustion. Despite decreased

expansion at high precursor frequencies, tumor specific effector CD4+ T cells accumulate in the periphery and tumor in greater numbers, with a lower proportion of TRP-1 specific Foxp3+ regulatory T cells within the population. Through a mechanism of population-induced positive feedback involving paracrine IFNγ sharing and traditional T cell help, we observe intraclonal cooperation resulting in strong Th1 differentiation, increased T cell cytotoxicity, and potent anti-tumor responses. These findings assert that the differential effects of T cell clonal abundance on phenotypic outcome should be considered during the design of adoptive T cell therapies, including the use of engineered T cells.

BIOGRAPHICAL SKETCH

Nicole Malandro earned her Bachelors of Science degree in Cellular and Molecular Biology from the Johns Hopkins University in 2008. In September of 2008, Nicole matriculated in the Immunology and Microbial Pathogenesis (IMP) Ph.D. program at Weill Graduate School of Medical Sciences.

While enrolled as an undergraduate at the Johns Hopkins University, Nicole joined the laboratory of Dr. Richard B. Roden as an undergraduate research assistant at the Johns Hopkins School of Medicine, where she helped design a concatenated peptide vaccine against oncogenic forms of the human papillomavirus for future use in animal studies and investigated the neutralizing antibody titers necessary to confer protection against bovine papillomavirus in cows. After completion of her degree requirements, Nicole interned at the biotechnology company Qiagen, where she worked full-time in the Research and Development department on the development of a second-generation diagnostic assay for HPV. While at Weill Cornell Graduate School of Medical Sciences, Nicole has pursued her doctoral research under the mentorship of Dr. Jedd Wolchok.

In 2011 Nicole was awarded a Cancer Research Institute Pre-Doctoral Fellowship. From 2011 to 2013 Nicole was a Teaching Assistant for the yearlong graduate course Fundamental Immunology. She has given talks on her work at the International Graduate Student Immunology Conference in Boston, MA, in 2012, the American Association of Immunologists Annual Conference in Honolulu, Hawaii in 2013, and at the 35th Annual Vincent du Vigneaud Research Symposium at Weill Cornell Medical College in 2015. Nicole has also presented posters on her research at the DNA Vaccines Conference in New Orleans, LA in 2010 and CRI Annual International Cancer Immunotherapy Symposium in New York, NY in 2012 and 2013.

ACKNOWLEDGMENTS

I would first like to thank my mentors. I could not imagine finding a mentor that cares more deeply for his cause than Jedd Wolchok. Thank you for your support, guidance, and consistent enthusiasm over the years. Immense gratitude is owed to my mentor Taha Merghoub. Thank you for believing in my abilities and supporting me through the setbacks. This would not have been possible were it not for your wisdom and encouragement.

Thank you to my thesis committee members Grégoire Altan-Bonnet, Marcel van den Brink, and Michel Sadelain. Your insight and suggestions have been invaluable to the completion of this work. My committee member, collaborator, and un-official mentor Grégoire Altan-Bonnet deserves special recognition. Thank you for making me feel welcome in your lab, for pushing me, and for indelibly shaping my interpretation of things.

I am grateful for the support of the current and former members of the Wolchok laboratory: Cailian Liu, Ceyhan Elipenahli, Czrina Cortez, Roberta Zappasodi, Lauren Boucher, Billel Gasmi, Yanyun Li, Dmitriy Zamarin, Alexander Lesohkin, Maggie Callahan, Rikke Holmgaard, Daniel Hirschhorn-Cymerman, Beatrice Yin, Shakuntala Tiwari, Yuka Maeda, Jacob Ricca, Tamar Plitt, Hong Zhong, Xia Yang, Kyle Draleau, Feng Zhao, and Maria Alexander. I would also like to thank past lab members: Andre Burey, Karim Lakehal, Arvin Yang, and Francesca Avogadri-Connnors. Sadna Budhu and David Schaer have been especially wonderful mentors over the years. Thank you to Gabrielle Rizzuto for laying the foundation for this project. I also appreciate the contributions of rotating student Nicholas Kuhn to this work. Many of you have not only been my co-workers, but also my friends, travel companions, co-conspirators, and confidants.

Thank you to the Cancer Research Institute for the pre-doctoral fellowship. I feel

lucky to have been a part of the IMP community at Weill Cornell and MSKCC. The passion of the faculty and the students has made this a wonderful place to develop as a scientist. Thank you also to the members of RARC and the Flow Cytometry Core who have positively shaped my experience here with their compassion and expertise, especially Lynne Rudwick and Melissa Nashat.

I am especially grateful to have been able to share this adventure with my best friend and former lab mate Judy Murphy. Thank you for everything – you have been there through it all and I cannot imagine having done it without you. I also have my friends and colleagues to thank: Courtney, Beth, Andre, Czrina, Lauren, Kathryn, Jamie, Nury, Welby, and Nick for the laughs and technical expertise, often at the same time. A big thank you to Altan-Bonnet lab collaborators and friends Karen Tkach, Jen Oyler-Yaniv, and Alon Oyler-Yaniv. You have each taught me so much and I have immensely enjoyed my time in your qBio world.

Lastly, I would like to thank my family. Thank you to my parents and my brother Michael, for encouraging me to pursue higher education and teaching me the value of hard work and persistence. Thank you to my husband Chris for keeping me whole throughout this process. Thank you all for enduring the many years of my education and for your love and support the entire way.

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LIST OF ABBREVIATIONS

ACT: adoptive cell therapy

BTLA: B and T lymphocyte attenuator

CAR: chimeric antigen receptor

CD: cluster of differentiation

CTLA-4: cytotoxic T lymphocyte antigen-4

CTV: Cell Trace Violet

D: diversity

DTR: diphtheria toxin receptor

EOMES: eomesodermin

FDA: Food and Drug Administration

FliCA: fluorescent caspase and apoptosis detection assay

Foxp3: forkhead box P3

G-CSF: granulocyte-colony stimulating factor

GFP: green fluorescent protein

HLX: H2.0 like-homeobox protein

ICOS: inducible T-cell costimulator

ICS: intracellular staining

IFN-γ: Interferon-gamma

IL: interleukin

iNOS: inducible nitric oxide synthase

i.p.: intraperitoneal

IPEX: immune dysregulation polyendocrinopathy enteropathy X-linked

ITIM: immunoreceptor tyrosine-based inhibition motif

ITSM: immunoreceptor tyrosine-based switch motif

i.v: intravenous

KO: knockout

LAG-3: lymphocyte activation gene -3

LCMV: lymphocytic choriomeningitis virus

MCA: methylcholanthrene

MCP-1: monocyte chemoattractant protein -

MHC: major histocompatibility complex

OVA: ovalbumin

PD-1: programmed death 1

PD-L1: programmed death ligand 1

PLSR: partial least squares regression

PMA: phorbol 12-myristate 13-acetate

Pmel-1: pre-melanosome protein -1

pMHC: peptide-major histocompatibility complex

qRT-PCR: quantitative reverse transcriptase polymerase chain reaction

RAG: recombination activating gene

SCID: severe combined immunodeficiency

SHP: Src-homology domain-containing phosphatase

SOCS: suppressors of cytokine signaling

STAT: signal transducers and activators of transcription

ThPOK: Th inducing POZ-Kruppel Factor

TCR: T cell receptor

TGF-β: transforming growth factor-beta

Th: T helper

TIL: tumor infiltrating lymphocytes

TNF: tumor necrosis factor

Treg: regulatory T cell

TRP-1: tyrosinase related protein 1

CHAPTER 1: INTRODUCTION

1.1. Cancer

In general, cancer is a disease typified by aberrant cell growth, which becomes increasingly dangerous when primary lesions spread to new locations via metastasis. While each of the individual malignancies that fall under the umbrella classification of cancer has its own unique characteristics, common hallmarks of cancer have become evident (Hanahan and Weinberg 2011). One fundamental difference between normal and cancerous tissue is the manifestation of sustained abnormal cell division, often driven by unabated proliferative signaling. This signaling can occur in a number of ways: through the aberrant production of growth factors, conferred hypersensitivity, or through the complete circumvention of the ligand-receptor interaction via constitutive activation of downstream mediators. One example of this is a mutation that is found in many melanomas, which causes constitutive activation of the B-Raf molecule and downstream signaling through the MAP kinase pathway (Davies and Samuels 2010). Another hallmark of cancer is insensitivity to antigrowth signals. These signals are normally important for blocking cells from proliferating and enable their transition into a quiescent or post-mitotic differentiated state. A primary example of deregulation of this pathway comes from disruption of retinoblastoma protein signaling, which is normally responsible for integrating extracellular cues into a growth decision (Burkhart and Sage 2008). Cancer also is able to promote angiogenesis, or blood vessel formation, in order to enable its own survival. The growth factor VEGF-A, which is normally involved in blood vessel formation during embryonic development, can be activated during hypoxic conditions, such as those found in the tumor (Gabhann and Popel 2008). Cancer possesses the unique ability to metastasize, which is regulated by genes involved in cell-to-cell or cell-to-extracellular matrix adhesion. Intravasation of cells from a primary lesion into blood vessels or lymphatics enables their systemic dissemination and seeding of other tissues.

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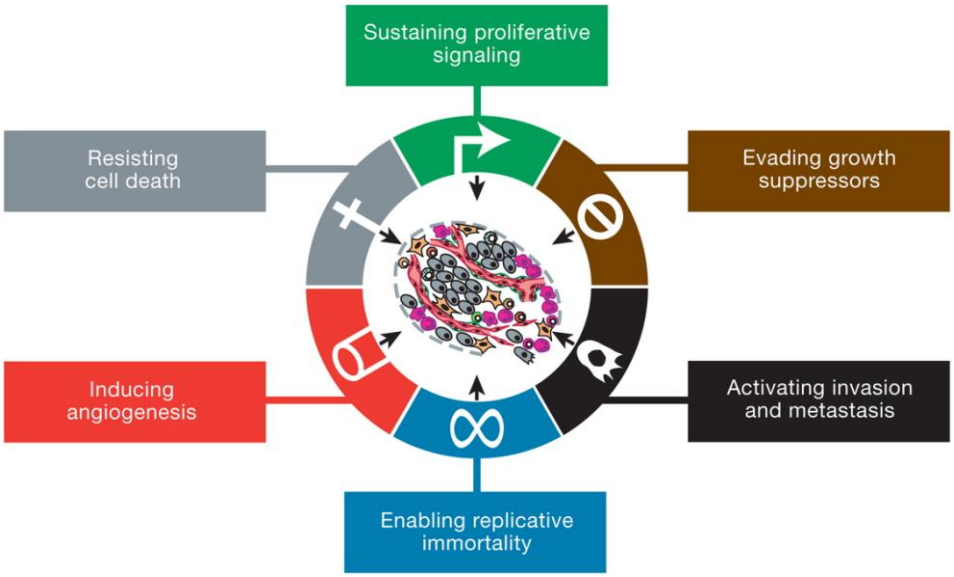

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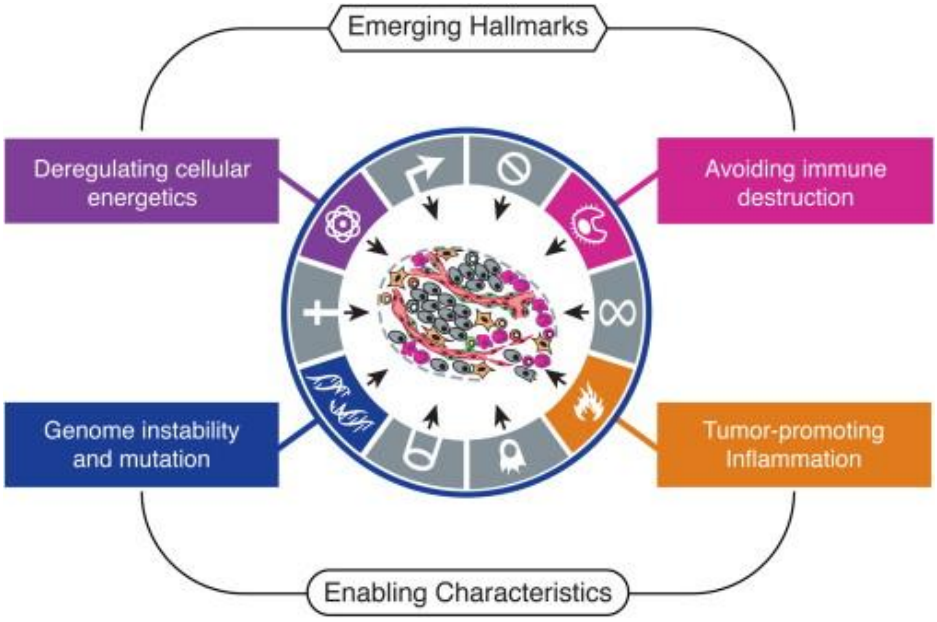


Figure 1.1. Evolution of the hallmarks of cancer.
(A) Although cancer takes many different forms and represents multiple disease states, a number of unifying hallmarks have been established. (B) Over the past decade, the contribution of the immune system to cancer development and control has become universally recognized as a recurrent cancer hallmark. Adapted from (Hanahan and Weinberg, 2011).

Tumor associated macrophages are now known contribute to the process of metastatic invasion (Gocheva et al. 2010). While these are some of the more traditional hallmarks of cancer, the more recent hallmarks recognize the ability of the immune system to both promote and eradicate tumors (Hanahan and Weinberg 2011). Inflammation is capable of enabling small neoplasms to develop into full-blown tumors; additionally inflammatory mediators such as reactive oxygen species, can directly drive mutagenesis (Shalapour and Karin 2015). Tumors can avoid and attenuate immune destruction through multiple pathways, which include the downregulation of antigen presentation and the recruitment and induction of suppressive cell subsets. This thesis will focus specifically on one particularly immunogenic model of cancer, melanoma, and the regulation of the CD4+ T cell immune response this cancer elicits.

1.2 Melanoma and Murine Melanoma Models

Melanoma is the deadliest form of skin cancer and causes an estimated 9,000 deaths each year in the United States (Guy et al. 2015). It is a cancer of the pigment cells, or melanocytes, that are found throughout the skin, but are also present in other tissues such as the inner ear and eye. Melanoma became a model for tumor immunology partly due the tumor cells being easily adaptable to tissue culture (Houghton, Gold, and Blachere 2001). However, melanoma also showed surprising susceptibility to immunologic intervention, such as IL-2 therapy, despite being somewhat resistant to traditional cancer treatments such as chemotherapy and radiation. These early clinical observations concerning the responsiveness of melanoma to immune manipulation paved the way for the current success of immunotherapies including the recent FDA approvals of checkpoint blockade targeting anti-CTLA-4 and anti-PD-1.

Many of the first characterizations of tumor-associated antigens stemmed from interrogation of the immune responses of patients with melanoma. One class of antigen that was identified through the study of melanoma consists of “tumor specific antigens”,

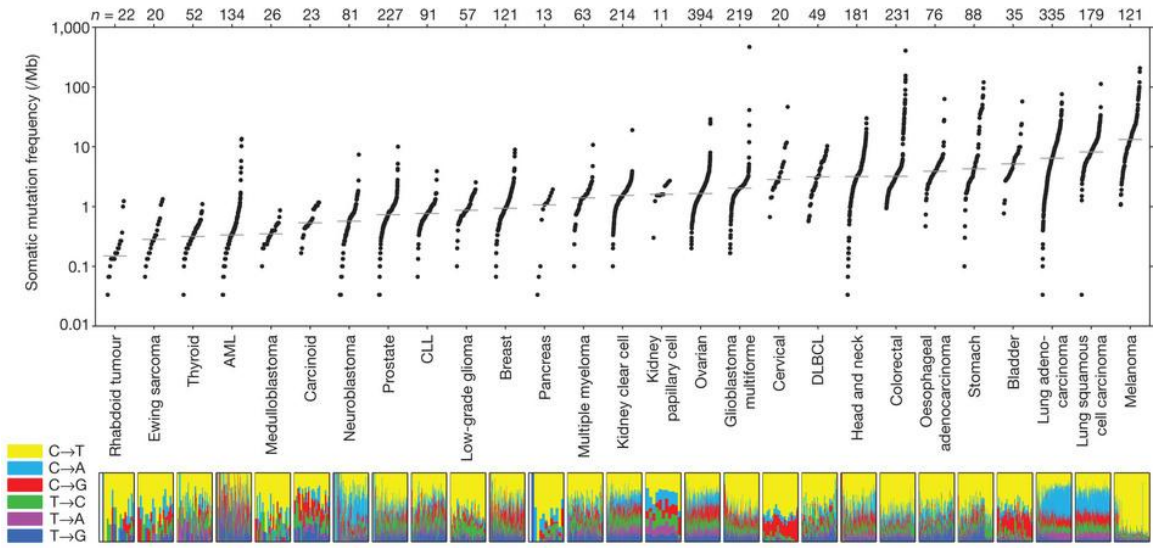


Figure 1.2. Melanoma has a high frequency of somatic mutation compared to other cancers.

Each dot represents a tumor compared to normal tissue sample in which the frequency of somatic mutations was quantified by whole exome sequencing and is plotted according to height. Tumors induced by carcinogens cluster together with the highest level of somatic mutations. Adapted from (Lawrence et al., 2013).

which are now referred to as a "neo-antigens", that stem from the immune recognition of novel T cell epitopes formed by somatic mutation (Lurquin et al. 1989). Although there have been many subsequent studies of neo-antigens in the time since they were first reported, the importance of neo-antigens has not been fully appreciated until recently. Following the introduction of checkpoint blockade, there have been numerous reports of anti-tumor responses directed against antigens of this nature (Gubin et al. 2014; van Rooij et al. 2013; Snyder et al. 2014). In addition to this, it was recently demonstrated that the frequency of somatic mutations in melanoma is extremely high (Figure 1.2) (Lawrence et al. 2013). This has lead to the hypothesis that the primary reason melanoma is so amenable to immunotherapy is because of the high abundance of neo-antigens generated through mutation that can be targeted by the immune system. In support of this, anti-PD-1 therapy has shown clinical success in the treatment of lung cancer, another type of cancer with a high mutagenic load. Even more striking is that the responses initiated by anti-PD-1 therapy of lung cancer have been shown to target neo-antigens as well (Rizvi et

al. 2015). In addition to immunotherapy, the highly mutagenized landscape of melanoma has rendered it susceptible to small molecule inhibitors. Around 40% of human melanomas contain activating mutations in the B-Raf protein, resulting in constitutive signaling of the MAP-kinase pathway (Davies and Samuels 2010). Vemurafenib, which targets the V600E mutation of B-Raf, was also recently approved by the FDA for the treatment of melanoma.

Two other types of antigens that immune responses against melanoma can be directed towards are cancer testes antigens and melanoma differentiation antigens. Cancer testes antigens are normally only expressed by spermatozoa in the immune privileged site of the testes. While normally silenced in germ-line tissue, expression of these proteins can be induced during cancer. MAGE-1 was the first cancer testes antigen that was shown to be recognized by CD8+ T cells (van der Bruggen et al. 1991). Other members of this class of antigen include MAGE-3, GAGE, and NY-ESO-1; many of these antigens are not only the targets of CD8+ and CD4+ T cell responses, but also elicit antibody responses as well. Melanoma differentiation antigens are not only expressed by melanomas, but are also found in healthy melanocytes. Tyrosinase related protein-1 was the first identified of these antigens and is the target of the tumor specific TCR transgenic CD4+ T cells utilized in our model system (Vijayasaradhi, Bouchard, and Houghton 1990). Tyrosinase related protein -1 or TRP-1 is encoded by the *brown* locus and is a membrane glycoprotein with oxidative activity responsible for the conversion of 5,6-dihydroxyindole-2-carboxylic acid (DHICA) into a carboxylated indole-quinone, which is necessary for the black coat color of mice (Jiménez-Cervantes et al. 1994). Other antigens of this class are also involved in melanin synthesis and include gp100, tyrosinase, tyrosinase related protein -2, and MART-1.

Although melanoma differentiation antigens have been the focus of many therapies, both pre-clinical and clinical, caution must be taken when targeting an antigen expressed by both tumor and healthy tissue. In mouse models utilizing implantation of

syngeneic B16F10 melanoma cells, development of immunity against melanoma is frequently accompanied by the development of vitiligo (Hara, Takechi, and Houghton 1995). Vitiligo is also observed following use of immunotherapies in the clinic, however, more severe autoimmunity directed against melanocytes, such as that observed during uveitis, has also been described. For these reasons differentiation antigens may not represent an ideal clinical target, but have been invaluable for the investigation of how the immune system recognizes and responds to cancer.

1.3 Cancer Immunotherapy

Although some of the first evidence of the immune involvement in cancer came over 100 years ago, it is only in recent history that it has become generally accepted that the immune system possesses the ability to recognize and eradicate cancer. In the 1890s, William B. Coley, a surgeon specializing in bone malignancies at the New York Cancer Hospital, which would later become Memorial-Sloan Kettering, began to notice that patients with infections occasionally experienced spontaneous tumor regression (McCarthy 2006). In an attempt to elicit feverous infections and the accompanying anti-tumor responses, Coley began injecting his patients with infectious agents. In the beginning, Coley favored the use of live streptococcal bacteria and noted significant tumor shrinkage in his first treated patient; however, the next two patients Coley treated succumbed to infection, rather than cancer, and Coley began to refine his approach (Coley 1891). Using a combination of heat killed streptococcus and *Serratia marcescens* Coley went on to treat almost 1000 cases over the course of his career and described therapeutic successes primarily in bone and soft-tissue sarcomas. Eventually, Coley's Toxins were manufactured for use by all physicians and were a popular form of treatment for over 30 years.

However, even in his own time Coley's methods were questioned and criticized because of inconsistencies. The documentation of his studies was often incomplete due

to poor patient follow-up and other physicians had difficulty replicating his clinical successes. This was possibly due to the vast number of different formulations of Coley's Toxins that were available, as well as the numerous routes of administration possible. Due to both scientific and inter-personal biases, this modality of treatment began to fall out of favor and was replaced by the growing fervor for radiation therapy.

The possibility that the immune system may recognize cancer began to re-emerge in the 1950s. Using the reagent methylcholanthrene (MCA) to induce sarcoma tumors, it was demonstrated that upon transplantation of these tumors into syngeneic animals, these animals would become immunized against the tumor and subsequent transplantation attempts (Foley 1953; Baldwin 1955; Klein et al. 1960). These results supported the idea that there existed tumor associated antigens that could be recognized by the immune system (Parish 2003). However, these findings appeared to be in direct contrast with contemporary theories of cellular immunity. Frank Macfarlane Burnet's theory of immunological tolerance suggested that early exposure to an antigen would induce specific tolerance to that antigen and work by Peter Medawar expanded on this theory to show that tolerance could be acquired through repeated antigen exposure (Burnet 1949; Billingham et al. 1952). Thus, it seemed more likely that the immune system would be tolerant to cancer, as opposed to responsive to it. Although an unlikely champion for the immune recognition of cancer, Burnet came to believe that lymphocytes were constantly patrolling tissues and eliminating malignant cells. Integrating the concept first conceived by Paul Ehrlich in 1909, Burnet together with Lewis Thomas, formulated the theory of cancer immunosurveillance (Burnet 1957b; Thomas 1959). This theory would not be elaborated upon until the 1990s, as the field began to lose popularity again due to a number of scientific setbacks.

Mounting evidence arose contradicting the theory of cancer immunosurveillance. Athymic nude mice were shown to develop a comparable number of primary tumors relative to immunocompetent mice, although it would later be demonstrated that these

mice still possessed a sizable population of functional T cells (Stutman 1979; Maleckar and Sherman 1987). Contrary to carcinogen induced cancers, it was demonstrated that spontaneously arising tumors were not recognized upon transplantation (Hewitt, Blake, and Walder 1976). And as the mechanisms of T cell thymic development began to be elucidated, it seemed unlikely that tumor specific T cells could escape negative selection. However, over time the balance began to shift back in support of cancer immunosurveillance. Self-reactive T cells were shown to exist in the periphery in a state of perpetual tolerance (Arnold, Schönrich, and Hämmerling 1993). Individual tumor associated antigens were characterized, including melanoma differentiation antigens and cancer testes antigens (Houghton 1994). Work in a variety of immunocompromised mouse models, including RAG, IFN-γ, IFN-γ receptor, perforin, and STAT1 deficient mice, demonstrated that these animals were more prone to MCA induced, and sometimes spontaneous tumors (Shankaran et al. 2001; van den Broek et al. 1996; Smyth et al. 2000; Kaplan et al. 1998; Dighe et al. 1994). This work validated the theory of cancer immunosurveillance, which was further adapted into the modern concept of cancer immunoediting.

Cancer immunoediting is comprised of three phases: elimination, equilibrium, and escape (Figure 1.3) (Dunn, Old, and Schreiber 2004). Elimination encompasses the classic theory of cancer immunosurveillance; that the immune system is capable of recognizing and eliminating malignant self. The equilibrium phase is a period of tumor latency occurring between the elimination phase and the escape phase. During this time the immune system dynamically constrains the growth of a heterogeneous population of tumor cells. Immunogenic tumor is readily eliminated through immune mechanisms, but evolutionary pressure allows the emergence of resistant tumor variants. Evidence of the immune system's ability to sculpt the immunogenicity of tumors was demonstrated in series of seminal experiments utilizing immunodeficient mice. When MCA induced tumors were derived in immunodeficient RAG2 knockout mice or wildtype mice, and

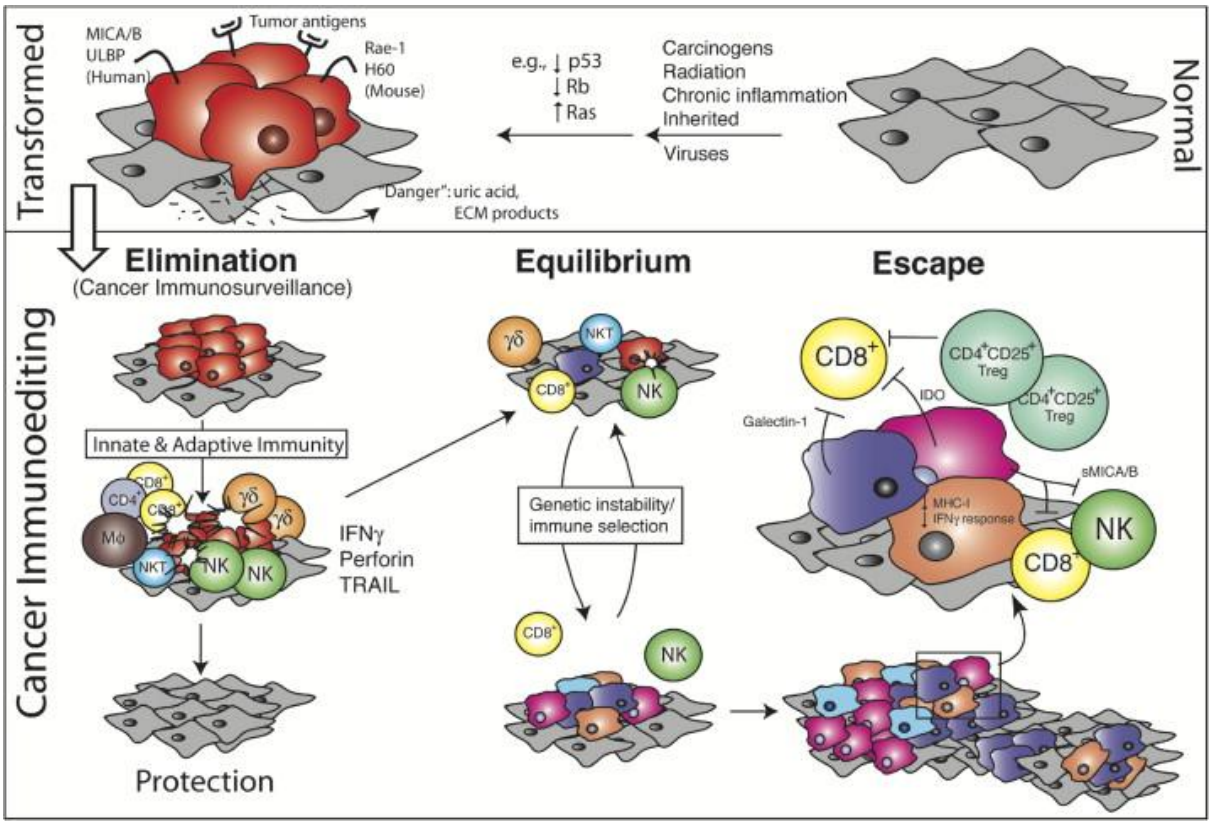


Figure 1.3. Cancer immunoediting expands upon the theory of immunosurveillance. The theory of immunosurveillance proposes that the immune system is capable of recognizing and eliminating cancerous malignancies. The theory of cancer immunoediting expands upon this idea, by describing two additional phases of the host defense against cancer. Elimination encompasses classic immunosurveillance. The equilibrium phase describes a latent period between elimination and escape where tumor growth is actively constrained. Evolutionary pressure exerted during this phase allows the escape of less immunogenic tumor variants. The escape phase describes the outgrowth of edited tumor and immunological mechanisms that curtail anti-tumor immunity. Adapted from (Dunn et al., 2004).

subsequently transplanted into RAG2 knockout hosts, all tumors were capable of progressive growth. However, when these same tumors were transplanted into wildtype hosts the tumors induced in wildtype animals were able to grow, but a subset of the tumors derived from the RAG2 knockout hosts were rejected (Shankaran et al. 2001). Similar observations were made in experiments using tumors derived from nude and SCID mice (Svane et al. 1996; Engel et al. 1996). These results suggested that the tumors arising from immunodeficient animals were more immunogenic, or less "edited",

and implied that tumors of this quality would be eliminated under normal immune surveillance. The final phase of cancer immunoediting is the escape phase, in which edited tumor is able to grow without immune restraint. Support for this came from humans, in which tumor variants have been characterized that lack important components contributing to their immune sensitivity; these include loss of HLA class I proteins or TAP, which reduces the presentation of tumor antigen, as well as loss of factors involved in the IFN-γ signaling pathway (Algarra, Cabrera, and Garrido 2000; Seliger, Maeurer, and Ferrone 2000; Kaplan et al. 1998). The adoption of the cancer immunoediting hypothesis embodied a broader paradigm shift within the field. No longer was there a question of whether or not the immune system was capable of responding to cancer. A new impetus was placed on understanding how this interaction occurred and ultimately how this process could be controlled to eradicate human disease.

1.4 Adoptive Cell Therapy

The general definition of adoptive cell therapy (ACT) describes the transfer of any lymphocyte population with the intention of mediating a specific effector function. However, the modality that has been most thoroughly investigated is the transfer of conventional αβ T cells for the treatment of cancer. Three primary approaches to adoptive cell therapies have been the focus of the majority of clinical trials. The first involves endogenously occurring tumor specific T cells isolated directly from a patient's tumor, also called tumor infiltrating lymphocytes or TILs. The other two approaches employ T cells engineered to recognize tumor through the expression of chimeric antigen receptors (CARs) or tumor specific T cell receptors. The following sections will detail each of these treatment modalities.

1.4.1. Tumor Infiltrating Lymphocytes

In this form of adoptive cell transfer a tumor is resected from a patient, dissociated, and then grown in IL-2. Within a few weeks the T cells that had infiltrated the tumor have undergone extensive expansion and can be tested for tumor reactivity. Tumor reactive isolates are then further expanded in culture until around 10^{11} lymphocytes are obtained for direct infusion back into the original patient. This treatment approach was first extended into the clinic in 1988 and showed that T cells in combination with IL-2 therapy were capable of mediating tumor regression in patients with metastatic melanoma (Rosenberg et al. 1988). However, for many years this approach had limited success – tumor regressions were not sustained primarily due to loss of the T cells shortly after infusion.

However in 2002, the efficacy of this therapy was dramatically improved when T cell transfer was combined with a pre-conditioning regimen that included lymphodepletion using nonmyeloablative chemotherapy (Dudley et al. 2002). The optimization of this pre-conditioning regimen continues to be investigated with clinical success also achieved following the addition of total body irradiation (Rosenberg et al. 2011). It has not been determined exactly how lymphopenia improves clinical efficacy, but it is likely through the same mechanisms that contribute to the immune activation observed in mice after lymphodepletion. These mechanisms include the depletion of suppressor cells, an increased abundance of homeostatic cytokines, and the release of commensal bacteria.

To better understand the ideal antigens to target in order to initiate tumor regression much effort has focused on characterizing the antigens being recognized by the isolated TILs. Of the first antigens identified were MART-1 and gp100, both melanoma differentiation antigens. However, current attention has focused primarily on identification of tumor neo-antigens due to the increased reactivity against these targets observed after checkpoint blockade (Gubin et al. 2014; van Rooij et al. 2013). It was

recently determined, that as in other immunotherapies, neo-antigens are a driver of melanoma recognition during adoptive cell therapy as well, with 45 distinct epitopes recognized by tumor isolated lymphocytes already identified (Lu et al. 2014; Robbins et al. 2013). Melanoma is not the only cancer that is able to be targeted by adoptive cell therapy against tumor neo-antigens, as a successful response has been demonstrated in the treatment of an epithelial cancer with neo-antigen specific T cells as well (Tran et al. 2014).

The majority of adoptive cell therapy trials have focused on the use of autologous tumor infiltrating CD8+ T cells. However, the idea of transferring CD4+ T cells, in combination with CD8+ T cells or as a single agent, has been gaining traction as CD4+ T cells are increasingly being recognized as not only necessary to maintain CD8+ immunity but also as effector cells in their own right. In 2008, Cassian Yee's group described the use of CD4+ T cells specific for the antigen NY-ESO1 in adoptive cell therapy for a patient with metastatic melanoma, which resulted in durable remission (Hunder et al. 2008). These T cells were shown to persist for three months and induced responses against other tumor antigens. However the eight other patients in the same clinical trial failed to respond. In an effort to simplify the T cell expansion protocol prior to adoptive cell therapy others have investigated whether removal of CD4+ T cells is warranted and have shown no significant difference in efficacy for mixed transfers (Dudley et al. 2013). However, even in un-enriched transfer recipients, CD4+ T cells only comprised a median of 8% of the total transferred population. One consideration that must be taken into account with CD4+ T cells that is not of concern with CD8+ T cells is which helper subset mediates the best response against tumors.

Although lymphocytes have been isolated from other types of cancer, few have been as tumor reactive as the ones derived from melanoma. Moreover, this type of therapy requires tumor resection, which is not always feasible. These and other limitations have lead to the development of engineered T cells.

1.4.2. Chimeric Antigen Receptor T cells

The first generation of chimeric antigen receptors was described in 1989, when the variable region of an antibody containing both heavy and light chains was conjugated to the CD3-zeta signaling motif utilized by T cell receptors (Gross, Waks, and Eshhar 1989). Through this approach, antigen recognition is mediated by an antibody domain and is independent of antigen processing and presentation on MHC molecules, increasing the range of antigens that could be targeted. Second and third generation chimeric antigen receptors have improved upon the original design by adding additional signaling domains from co-stimulatory molecules such as CD28, alone or in combination with other signaling domains, derived from other co-stimulatory molecules such as 4-1BB and OX40 (Maher et al. 2002; Sadelain, Brentjens, and Riviere 2013). This has lead to the greater persistence and potency of CAR T cells. However, in order for T cells to be engineered to stably express CARs they must be expanded in culture and transduced. This raises questions concerning the optimal method of transduction and state of T cell differentiation that should be utilized.

The most well characterized CAR T cell to date is that targeting the CD19 antigen on B cell leukemias and lymphomas. It was the first to demonstrate efficacy in pre-clinical mouse models and has since been the focus of numerous clinical trials (Brentjens et al. 2003). These clinical trials have proven the efficacy of this therapeutic modality, although a range of responses have been observed and can likely be attributed to differences in pre-conditioning regimens, the CARs utilized, tumor burden, as well as T cell dose. Although adoptive cell therapy using patient isolated TILs has usually been safe, on-target and off-target reactivity of chimeric antigen receptor T cells has posed some safety concerns. While CAR targets have been carefully validated and identified there are few antigens with expression solely restricted to tumors. In the case of CD19 specific CARs this off-tumor targeting has lead to the development of B cell aplasias

(Brentjens et al. 2011). However for other CAR T cells, such as those targeting PMSA, off-tumor effects have not been observed. Additionally, safety concerns have stemmed from the development of cytokine storm following CAR T cell infusion. Patients develop fever and potentially lethal low blood pressure, however treatment with steroids, vasopressors, and anti-IL-6 has been shown to relieve these symptoms. Nevertheless, much of the current research on CAR T cells has focused on improving both their safety and their efficacy. This is being accomplished through the development of novel CAR approaches, such as CARs containing "kill switches", inhibitory CARs, dual antigen recognition CARs, as well as CAR T cells that secrete cytokines.

1.4.3. Tumor Specific T cell Receptors

The first clinical trial using T cells engineered to express low affinity, tumor specific T cell receptors targeted the melanoma antigen MART-1 and resulted in some partial regressions in treated patients (Morgan et al. 2006). However, when trials were extended to the use of higher affinity T cell receptors, targeting either MART-1 or gp100, improved objective responses were accompanied by severe off tumor effects in the skin, eye, and ear (Johnson et al. 2009). Similar off tumor effects were observed in trials using TCRs specific for carbonic anhydrase 9 and MAGE-A3, due to the expression of antigen or cross-reactive epitopes in healthy tissues. In addition to this, efforts to improve T cell receptor affinity through mutagenesis have resulted in conferring additional cross reactivity resulting in lethality. These studies underscore the need for better target identification, understanding of TCR cross-reactivity, and of how T cell receptor affinities shape the potency of adoptive cell therapies.

1. 5 T cell Homeostasis and Memory

The survival and composition of the peripheral T cell pool is under the control of complex homeostatic mechanisms. Different combinations of factors are necessary

depending on the T cell differentiation state; although, largely these mechanisms are mediated by the interaction of T cells with self-peptide MHC and signaling through common gamma chain cytokines. It is generally accepted that naïve T cells are dependent on tonic signaling through the T cell receptor to enable survival. This was established through a series of experiments utilizing T cells lacking T cell receptors or receptor proximal kinases and further supported by the observation of intraclonal competition amongst T cells of the same specificity and the absence of competition for high affinity polyclonal T cell populations (Labrecque et al. 2001; Seddon and Zamoyska 2002; Hataye et al. 2006; Moses et al. 2003). Efforts to address the survival of naïve CD4+ T cells in MHC-II deficient mice have yielded mixed results. Some groups have demonstrated a swift decline of T cells in this environment, while other groups have shown persistence; interpretation has been confounded by an increase in cytokines resulting from lymphopenia in MHC-II deficient mice (Rooke et al. 1997; Dorfman et al. 2000). As a whole it is generally accepted that naïve CD4+ T cells are dependent on TCR signaling, though to a lesser extent than CD8+ T cells.

The signaling that results from this tonic TCR engagement is believed to occur below the threshold required for T cell activation and is thought to be the result of "tuning" of the T cell receptors following positive selection in the thymus (Grossman and Singer 1996). This is especially important for high affinity and auto-reactive T cell receptors, which are sensitive to homeostatic proliferation and have the highest potential to become activated during lymphopenia (Kieper, Burghardt, and Surh 2004). Loss of T cell tuning has been shown to occur upon transfer of CD4+ T cells into MHC-II deficient hosts, implying that MHC engagement is vital for modulating responsiveness to antigen (Fischer et al. 2007).

Both naïve CD4+ and CD8+ T cells are also dependent on IL-7 signaling and die within 2 weeks following transfer into IL-7 deficient hosts (Surh and Sprent 2008). IL-7 signaling promotes expression of anti-apoptotic factors and regulates T cell metabolism

(Khaled and Durum 2002). This cytokine is primarily produced by non-lymphoid stromal cells and is found at high levels in the thymus, as well as in lymph nodes and bone marrow (Shalapour et al. 2010). Responsiveness to IL-7 is downregulated through a negative feedback loop where signaling reduces receptor expression, which is regulated by the transcription factor Foxo1, which also regulates the expression of lymph node homing molecules such as CD62L and CCR7 (Ouyang et al. 2009).

Immune recovery though homeostatic proliferation following severe lymphopenia has offered important insights into the homeostatic mechanisms governing the immune system (Figure 1.5). It has been observed that following transfer of naïve T cells into lymphopenic hosts most T cells will undergo a slow proliferation, while a small subset will experience more rapid division and adopt a memory phenotype. This slow proliferation has been termed lymphopenia-induced proliferation, can be induced by irradiation, and is driven primarily by increased access to pMHC and IL-7 (Surh and Sprent 2008). Evidence for the importance of self-peptide MHC during lymphopenia stems from models demonstrating the proliferation of TCR transgenic T cells following transplant into lymphopenic hosts lacking cognate antigen (Ernst et al. 1999). It has been shown that T cell receptor avidity is the primary determinant for the rate of lymphopenia induced proliferation (Kieper, Burghardt, and Surh 2004). Inhibitory receptors that limit signal strength have also been shown influence lymphopenia driven proliferation, including LAG-3 and BTLA, with mice lacking these receptors displaying increased T cell accumulation and altered memory (Workman and Vignali 2005; Krieg et al. 2007). Homeostatic proliferation can also be driven by cytokines other than IL-7, such as IL-2 and IL-15. However, homeostatic proliferation induced by IL-2 and IL-15 is rare outside of hosts deficient in receptors for these cytokines, with the exception of IL-2, as it is an important cytokine for the homeostasis of regulatory T cells. Another type of homeostatic proliferation is experienced in chronically lymphopenic hosts, such as RAG deficient mice. Upon T cell transfer into these hosts most cells will divide in response to

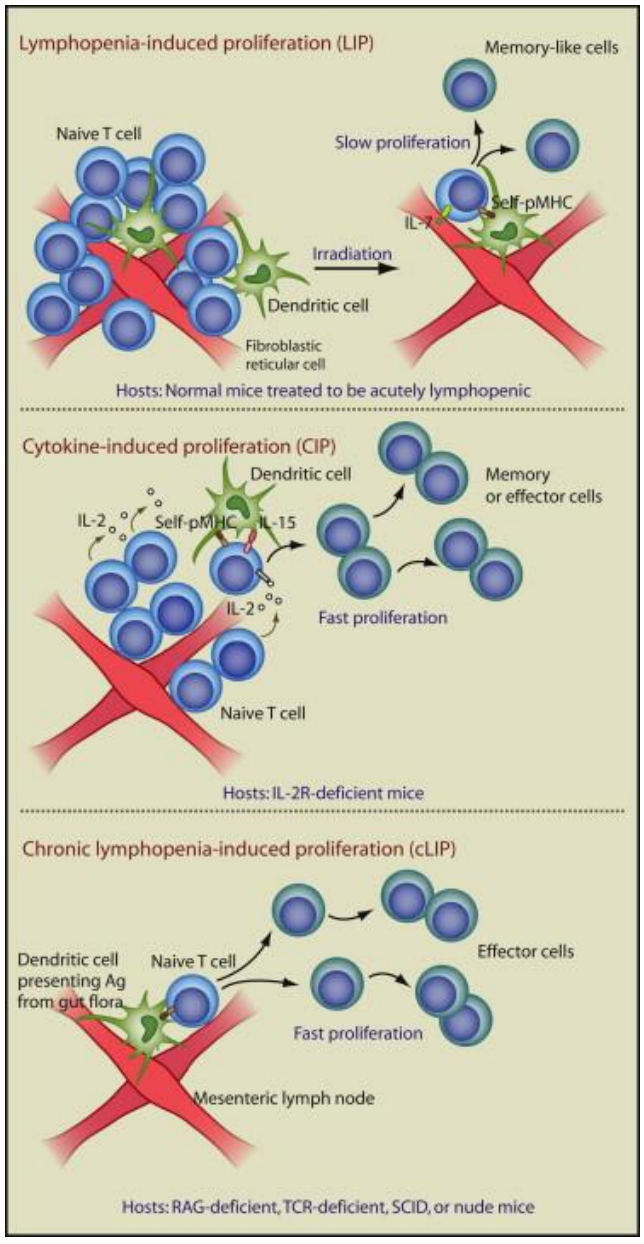


Figure 1.4. There are distinct types of homeostatic proliferation.
Lymphopenia induce proliferation, such as that observed following irradiation, progresses at a slow rate and is primarily driven by IL-7 signaling and T cell receptor engagement. IL-2 and IL-15 are capable of driving homeostatic proliferation, which is also dependent on T cell receptor engagement. Chronic lymphopenia induced proliferation is independent of IL-7 signaling and driven by reactivity against commensal antigens. This is the fast paced proliferation observed in RAG deficient mice. Adapted from Sprent and Surh, 2008.

IL-7 and pMHC as in lymphopenia induced proliferation. However, a small subset of cells will undergo fast paced proliferation in response to commensal antigens, termed chronic lymphopenia induced proliferation or "spontaneous" proliferation. This type of proliferation is diminished when mice are raised in a germ free environment (Kieper et al. 2005). Chronic lymphopenia induced proliferation is independent of IL-7, but dependent on CD28 signaling (Min et al. 2005).

Over the course of the normal immune response, T cells transition their dependence on different homeostatic cytokines and regulate the surface expression of adhesion molecules based on their activation state. Naïve CD4+ T cells express the IL-7 receptor, characterized by expression of the CD127 subunit, and their survival is dependent on IL-7 signaling in combination with TCR engagement via self-peptide MHC (Surh and Sprent 2008). Upon antigen encounter T cells transiently produce the cytokine IL-2, which is shortly followed by the upregulation of CD25 and downregulation of CD127; during this time T cells also down regulate expression of CD62L to facilitate egress from the lymph node to the site of infection and are characterized as T effector cells (von Andrian and Mackay 2000). Following elimination of the pathogen and contraction of the T effector response, memory T cells persist and are primarily characterized by their tissue localization. Memory CD4+ subsets express high levels of CD127, but low levels of CD122; although they rely on both IL-7 and IL-15 signaling, TCR signaling is no longer necessary for survival. Although IL-15 deficient mice possess normal numbers of memory cells, basal levels of contact with IL-15 are required for long-term maintenance of memory T cells (Purton et al. 2007).

T central memory cells express CD62L and CCR7 and are poised in the lymph node to expand upon secondary antigen encounter. In contrast, effector memory T cells are located at the sites of inflammation and do not express CD62L, but maintain the ability to provide swift effector responses. It has been suggested that in chronic infection, where repeated antigen exposure is anticipated, memory formation favors the effector

memory subset. (Appay et al. 2002) While some effector memory cells remain migratory, another more newly defined subset of resident memory T cells remains at the site of infection and adapts a tissue specific phenotype, including the upregulation of CD103, which is dependent on TGF-β signaling.(El-Asady et al. 2005) Although tissue resident effector memory has been most well characterized as contributing to mucosal immunity in the gut, is has also been implicated in other areas of barrier immunity, such as the skin (Masopust and Picker 2012).

1.6 T cell Precursor Frequency

Beginning when Burnet first postulated his theory of clonal selection in 1957, it has become a central tenet of immunology that the immune system has evolved in such a way as to promote repertoire diversity while limiting self reactivity (Burnet 1957a, 1959). Balance is achieved by maintaining a varied repertoire of adaptive immune cells of unique specificity, which expand upon encounter with their cognate antigen in a process called clonal expansion; while self-reactivity is prevented by eliminating clones that recognize self from the immune repertoire early in development through negative selection and subsequently peripheral tolerance. In the time since Burnet, it has been estimated that the number of theoretical T cell antigen receptor combinations approaches 10^{15} (Davis and Bjorkman 1988). Repertoire diversity in both B cells and T cells is maintained through the process of V(D)J recombination. Each variable, diversity, and joining segment is present in the germ line in multiple largely unique iterations. During T cell receptor formation, one of each of these segments is combined through a series of somatic recombination events, resulting in the formation of the antigen receptor β chain. Diversity arises from the myriad of potential combinations of V(D)J segments and is further enhanced by random nucleotide additions and exclusions by DNA polymerases and exonucleases. Ultimately the β chain is paired with a similarly generated α chain, comprised of only V and J segments. The region where V(D) and J segments intersect in

the TCR β chain is the most highly variable region of the T cell receptor and is known as complementarity determining region 3 (CDR3). It is this domain that directly interacts with peptide in the context of a major histocompatibility molecule.

Following rearrangement of the T cell receptor β chain and pairing with a successfully rearranged α chain the fully formed T cell receptor is tested for the ability to recognize self-peptide in the context of a major histocompatibility molecule (MHC). This process is termed positive selection and is mediated by thymic epithelial cells in the cortex. Cortical thymic epithelial cells are equipped with a unique proteasome and other antigen processing machinery that enables them to present "private" peptides; these peptides are believed to enable the selection of lower affinity T cells; however this may confer a disadvantage if these private peptides are not representative of the self-peptides T cells encounter in the periphery during homeostasis (Klein et al. 2014). More than 90% of T cell precursors possess a non-functional TCR and will experience death by neglect. Following recognition of a pMHC T cells will transition from expression of both CD4 and CD8 co-receptors into a single positive state. This process is believed to be instructed by TCR signal strength, with higher affinity reactions favoring the development of CD4+ T cells. While there is evidence that some negative selection can occur in the cortex, negative selection primarily takes place in the thymic medulla. During this process, medullary thymic epithelial cells present tissue specific self-antigens that are ectopically expressed by AIRE (Anderson et al. 2002). T cells that are too self-reactive are clonally deleted, while T cells of more moderate affinities survive. There is new evidence that a somewhat large pool of self-reactive T cells remain following negative selection to prevent exploitable holes in the repertoire (Yu et al. 2015).

Following positive and negative selection, the theoretical TCR repertoire diversity is reduced to 10^{13} possibilities (Nikolich-Zugich, Slifka, and Messaoudi 2004). However, this might actually be a gross underestimate, as it is becoming increasingly apparent that T cell receptors can have cross reactivity for both self and foreign epitopes (Birnbaum et

al. 2014). Each repertoire is further constrained by the number of T cells that can be physiologically supported, 10^8 T cells in mice and 10^{12} T cells in humans, as well as by some redundancy in antigen specificity. Many groups have shown that T cells specific for the epitopes of common antigens can be maintained in the repertoire at precursor frequencies that range from only a few clones to pools numbering in the thousands (Blattman et al. 2002; Whitmire, Benning, and Whitton 2006; Rizzuto et al. 2009; Jenkins and Moon 2012). For example, the precursor pool of CD8+ T cells specific for the model antigen ovalbumin has been estimated at 600 cells, while vaccinia virus specific CD8+ T cells can number around 1500 (Jenkins and Moon 2012). Generally the precursor pools of CD4+ T cells are slightly lower compared to that of CD8+ T cells. Variance in the endogenous precursor frequency of foreign antigen specific T cells impacts the magnitude of the response to pathogen (Butz and Bevan 1998; Blattman et al. 2002; Moon et al. 2007; Jenkins and Moon 2012). Patterns of immunodominance are affected by precursor frequencies, with a conferred advantage to larger precursor pools and higher affinity T cells (Kotturi et al. 2008). Adoptive transfer experiments utilizing TCR transgenic T cells at supra-physiologic clonal abundance failed to recapitulate the responses of endogenous T cells of the same specificity, showing both altered function and kinetics as a result of precursor frequency; when T cells were at greater precursor frequencies the immune response displayed an earlier peak response (Badovinac, Haring, and Harty 2007). When endogenous naive CD4+ T cells precursor pools were explored using tetramer enrichment techniques similar results were obtained, with larger endogenous precursor populations favoring early peak responses, yet higher amplitude, and more immediate contraction (Moon et al. 2007). Although heterogeneity in the size of precursor populations exists, frequency is maintained within a relatively narrow physiological range. When T cells far exceed this range, their survival and ability to expand in response to antigen are impaired through a process called intraclonal competition (Hataye et al. 2006).

1.7 Intraclonal Competition

To ensure the development of the most high affinity antibody responses, B cells compete against each other for access to antigen during the process of affinity maturation. In the germinal center, the B cell receptor is subjected to a series of random mutations, which confer a stronger or weaker affinity for antigen. Only the B cells that bind to antigen with strong affinity receive survival signals, allowing for differentiation into plasma cells (De Silva and Klein 2015). Similar to B cells, T cells depend on competition for antigen to generate optimal responses. Evidence of T cell interclonal competition for antigen comes from experiments establishing that T cells specific for the immunodominant epitope of an antigen can prevent responses directed against sub-dominant or unrelated epitopes (Kedl et al. 2000). These patterns of immunodominance were eliminated when mice received antigen presenting cells that had been pulsed with each antigen separately. These findings implied that interclonal competition was due to competition for antigen at the level of the APC. This may be a factor, but is not the only reason for development of immunodominance; it is now known that immunodominance is also influenced by factors such as precursor frequency and T cell receptor avidity (Kotturi et al. 2008).

Although the exact mechanism of intraclonal competition has yet to be completely elucidated, it is widely believed that competition for antigen during engagement with antigen presenting cells is at least partly responsible (Kedl et al. 2000; Smith, Wikstrom, and Fazekas de St. Groth 2000; Willis, Kappler, and Marrack 2006). Using two-photon intra-vital microscopy it was determined that interactions between CD4+ T cells and dendritic cells were infrequent when monoclonal T cells were highly abundant, but prolonged 2 day contacts were formed when the T cells were present at lower frequencies (Garcia et al. 2007). It was concluded that T cells were competing for a limited number of sites that allowed for prolonged dendritic cell contacts. For T cells

present at high precursor frequencies, this competition results in a decreased initial proliferative burst and impaired overall expansion, as well as deficiencies in the induction of effector function and generation of memory (Marzo et al. 2005; Blair and Lefrançois 2007; Badovinac, Haring, and Harty 2007). In a model of *Listeria monocytogenes* expressing the model antigen ovalbumin it was demonstrated that OT-II CD4+ T cells experience reduced proliferation when at a high clonal abundance. Production of IFNγ and clearance of bacteria from the liver and spleen were also shown to be impaired when OT-II cells were highly abundant (Foulds and Shen 2006). The Paul and Grossman groups comprehensively investigated the underlying factors contributing to the reduced clonal expansion occurring at high precursor frequencies. They determined that on a logarithmic scale, for every log increase in the precursor number of a clonal population the factor of expansion of that population was reduced by half a log. It was also established that changes in antigen, number of dendritic cells, homeostatic cytokines, regulatory T cells and expression of Fas and CTLA-4 had little to no effect on factor of expansion; rather, variables such as increased antigen could alter the overall amplitude of the responses, but in a manner relative to the initial precursor frequencies of the population. The authors instead propose a model whereby the highly differentiated cells within an individual cluster restrict the expansion of the less differentiated nearby cells (Quiel et al. 2011; Bocharov et al. 2011). Restrained activation of bystander cells has been described before, however, this was accomplished by anergic CD4+ T cells rather than by effector T cells (Vendetti et al. 2000). An alternate interpretation proposed by a separate group implicates the acquisition of peptide-MHC by the CD4+ T cells themselves as a potential limiting mechanism (De Boer and Perelson 2013). However, in models where antigen may not be a limited resource, such as when the cognate antigen is a ubiquitously expressed self-molecule as in cancer, it is less well understood to what extent competition influences immunity.

1.8 Characterizing Antigen Specific T cell Responses In Vivo

Two approaches are commonly undertaken to investigate the antigen specific T cell response to infection or cancer. The first approach relies on probing the endogenous T cell response; antigen specific T cells can be identified through the use of tetramers or by their reactivity to the antigen of interest. The second approach utilizes exogenous TCR transgenic T cells of a known specificity to approximate the endogenous response. Both of these approaches have been adapted to quantify the precursor pool of antigen specific T cell populations, in addition to use in the characterization of T cells during an ongoing immune response. However, each of these methods has limitations and are more often successfully employed during the study of CD8+ rather than CD4+ T cells.

1.8.1. Tetramers

The use of tetramers for the identification of antigen specific CD4+ T cells is complicated by a number of factors (Nepom 2012; Vollers and Stern 2008). Tetramers were originally developed to investigate CD8+ T cell recognition of peptide in the context of MHC-I (Altman et al. 1996). A peptide of interest is bound to the groove of a MHC molecule to form a peptide MHC complex. Unlike MHC-I molecules, many MHC-II molecules are unstable in the absence of peptide binding. In order to form stable peptide-MHC-II complexes these MHC-II molecules must be folded in the presence of the peptide of interest or a removable filler peptide such as CLIP. This can be aided by conjugation of the MHC-II molecule to the peptide through covalent linkers and can be combined with the addition of leucine zipper or Ig-domain tails to promote αβ assembly (Kozono et al. 1994; Scott et al. 1996). The covalent linker approach not only requires the characterization of the class II binding epitopes of the antigen of interest, but also requires the synthesis of new expression constructs for each epitope. Even with covalently linked peptide approaches, peptide loading is not always successful.

Following the loading of peptide, the pMHCs are coupled to a scaffolding protein, most commonly streptavidin. Streptavidin forms a tetravalent linkage with biotin labeled pMHC and is frequently conjugated to a fluorophore for use in flow-cytometry studies. The multivalency of the tetramer is required for the successful binding to TCR and has lead to the development of higher order multimers, such as octamers, decamers, and pMHC conjugated beads. The avidity of the tetramer for the TCR must be high enough to prevent the dissociation of the complex, which favors the detection of high avidity interactions (Sabatino et al. 2011). This can be especially problematic for the study of auto-antigens, in which the spectrum of TCR avidities can range from low to high due to the negative selection of strongly self-reactive clones. Additionally, self-antigen specific clones are present at low enough frequencies that detection via tetramer labeling alone presents technical challenges and often requires *ex vivo* expansion or tetramer enrichment via magnetic beads (Moon et al. 2009). Tetramer enrichment allows for the detection of rare clones without the need for immunologic boosting of the response through infection, vaccination, or *ex vivo* expansion with antigen. Thus, it is the superior method for the quantification and phenotypic analysis of naïve antigen specific T cell populations.

1.8.2. Reactivity to Antigen

Other techniques rely on the T cell response to antigen to identify antigen specificity– the most notable of these being limiting dilution assays, ELISPOT, and intracellular cytokine staining (Strijbosch et al. 1987; Czerkinsky et al. 1983). While limiting dilution assays and ELIPSOT can be used to accurately quantify rare antigen specific T cells, they provide no basis for phenotypic analysis besides the enumeration of cells secreting the cytokine or product of interest. Intracellular cytokine staining, as well as cytokine capture, can be used to detect the cytokine production of lymphocytes and combined with other phenotypic characterization during flow cytometry analysis. When cytokine is detected directly *ex vivo* or after non-specific re-stimulation, such as through

use of CD3 and CD28 specific antibodies or PMA and ionomycin, specificity cannot be determined and T cell reactivity can only be tenuously attributed to the pathogen, vaccine, or disease state being studied. However, re-stimulation can be used to demonstrate antigen specificity if a peptide of interest, such as the known immune-dominant epitope, is used. These approaches are less sensitive and most useful for the detection of previously activated and expanded populations, making it difficult to phenotype T cells lacking robust cytokine production such as naïve, anergic, or quiescent T cell populations. Additionally, re-stimulation can alter the T cell phenotype and not accurately reflect *in vivo* cytokine production depending on the method used. Lastly, because CD4+ T cells can differentiate into a number of different helper phenotypes, each producing a unique cytokine profile, identification of antigen specificity on the basis of cytokine production would have to be done systematically. The evaluation of cytokine production can also be combined with tetramer labeling or use of TCR transgenic T cells for characterization of effector function in a population of known antigen specificity.

1.8.3. TCR Transgenic T cells

T cell receptor transgenic T cells provide an alternative method to study antigen specific T cells, especially in situations where the endogenous T cell response is difficult to probe (Lafaille 2004). While tetramer positive T cells share antigen specificity, their individual TCRs frequently reflect a polyclonal population. TCR transgenic T cells provide a method to restrict analysis to a monoclonal antigen specific population if the transgene is maintained on a RAG knockout background to prevent recombination and alternate pairing. Detection of TCR transgenic T cells can be simplified through the use of reporters and congenic markers. However, because TCR transgenic T cells can be administered exogenously their responses may incorrectly mimic those of endogenous T cells if unphysiologic precursor numbers are used (Hataye et al. 2006). Using adoptive transfer, TCR transgenic T cells have been used to estimate the endogenous precursor

frequencies of T cells sharing the same antigen specificity. Assuming the endogenous T cells share the same affinity as the TCR transgenic T cell clone, when the TCR transgenic T cells are adoptively transferred at an identical frequency to that of the endogenous precursor pool they will contribute equally to the immune response against their cognate antigen (Blattman et al. 2002). Additionally, because TCR transgenic animals are historically laborious to create, the range of antigens that can be investigated is restricted to the specificity of the available mice. Due to this limitation, model antigens, such as chicken ovalbumin, have been engineered into pathogens, tumors, or healthy tissue to create model systems to investigate T cells responses in infections, cancer, and autoimmunity (Kurts et al. 1996; Foulds et al. 2002; Schüler and Blankenstein 2003). However, the use of model antigens in place of self-antigens for the investigation of autoimmune disease and cancer limits their relevance. Self-antigens are ubiquitously expressed, induce both central and peripheral tolerance, and have been shown to have TCRs of different affinities than those specific for foreign antigen.

CHAPTER 2:

MATERIALS AND METHODS

Mice and tumors

All mouse procedures were performed in accordance with institutional protocol guidelines at Memorial Sloan-Kettering Cancer Center (MSKCC) under an approved protocol. C57BL/6J, RAG$^{-/-}$, IFNγ$^{-/-}$ (Stock 002287), IFNγR$^{-/-}$ (Stock 003288) MHC-II$^{-/-}$ 6–8-wk-old males were obtained from The Jackson Laboratory. TRP-1 CD4^{+} TCR and pmel-1 TCR transgenic mice were obtained from the N. Restifo laboratory (National Institutes of Health, Bethesda, MD). TRP-1 CD4^{+} TCR-Luciferase and TRP-1 CD4+TCR-Foxp3-GFP reporter mice were obtained from the J. Allison laboratory (MD Anderson Cancer Center, Houston, TX). All TRP-1 CD4^{+} TCR transgenic were maintained on a RAG1$^{-/-}$ TRP-1$^{-/-}$ CD45.1 background. OT-II RAG+/+ and OT-II RAG-/- TCR transgenic mice were originally obtained from the Jackson Laboratory and bred in house. The B16-F10 mouse melanoma line was originally obtained from I. Fidler (M.D. Anderson Cancer Center, Houston, TX). Tumor implantation was via intradermal injection of a 50 µl bolus containing 2.5 x 10^5 B16-F10 and PBS in the flank of shaved recipients. Tumor growth was tracked every 3-5 days via caliper measurement of diameter. Mice received adoptive transfer of tumor specific T cells on day 14 after implantation. Mice were euthanized when tumors reached 2 cm in diameter.

Irradiation and adoptive transfer

Recipient mice received 600 cGy total body irradiation from a ^{137}Cs source several hours prior to adoptive transfer. Donor cells were isolated from lymph node and spleen of male TRP-1, OT-I, OT-II, Pmel-1 TCR transgenic mice (6-12 week old) or TRP-1 TCR-Luciferase reporter mice when indicated. Purification was by positive selection magnetic cell sorting using CD4 beads (L3T4) (Miltenyi Biotech) according to the manufacturer's instructions unless indicated otherwise. Dose groups were washed twice with PBS, re-

suspended at 30 million cells per 200 µl, and injected intravenously via tail vein. Open repertoire splenocytes were derived from the ACK lysis buffer (Lonzo) incubated spleens of naïve donors. When indicated, CD4+ T cells were labeled with 5 µM CFSE or CellTraceViolet (Life technologies) prior to transfer according to manufacturers instructions.

In Vivo and *Ex Vivo* killing assays

For *in vivo* killing assays splenocytes from C57BL/6J mice were labeled with 5 or 0.5 µM CFSE. $CFSE^{high}$ splenocytes were loaded with 20 µM of TRP-1 peptide (Genemed Synthesis, Inc) for 2 hours at 37°C. On day 6 after adoptive transfer 5×10^5 cells of a 50:50 mixture of TRP-1 peptide pulsed and un-pulsed splenocytes were transferred via tail vein injection in a 200 µl bolus. The following day, mice were sacrificed and spleens were removed, and the percentage of loaded and unloaded splenocytes was analyzed by flow cytometry. Cytotoxicity was calculated using the following equation:

In vivo killing percentage =

$100\%\ (1-((\text{unloaded/loaded})_{\text{control}}/(\text{unloaded/loaded})_{\text{experimental}}))$.

For clonogenic *ex vivo* killing assays wells in a 48-well tissue culture plate were filled sequentially with 5 µl PBS containing 0.1 U thrombin, 100 µl PBS containing 1 mg/ml of human fibrinogen, 1 mg/ml of rat tail collagen I, 10% FBS, and B16 cells, with or without TRP-1 cells at a 1:10 ratio. The plates were incubated for 20 min at 37°C in a 95% air/5% CO_2 humidified atmosphere to allow the fibrin to gel. Gels were overlaid with 0.5 ml RPMI 1640 supplemented with 10% FCS, 1× nonessential amino acids, 1 mM sodium pyruvate, 2 mM l-glutamine, and 50 µM β-mercaptoethanol and incubated at 37°C in a 95% air/5% CO_2 humidified atmosphere. These gels are 0.1 ml in volume and ~1,500 µm in height. 24 hours later, the gels were lysed by sequential collagenase (2.5 mg/ml) and trypsin (2.5 mg/ml; Sigma-Aldrich) digestion. The lysed gels were then diluted and the recovered melanoma cells were plated in 6-well plates for colony

formation. After 7 days in culture, the plates were fixed with formaldehyde, stained with 2% methylene blue, and the colonies were counted manually. For the preparation of targets, B16 cells were incubated overnight with 10 ng/ml of recombinant IFNγ (PeproTech) and single cell suspension were prepared using Cellstriper (Cellgro) before the assay. Target cells killing was calculated using the equation: 1 − [melanoma + T cells]/[melanoma alone]. The killing coefficient *k* was calculated applying the equation: $b = b_0 e^{-(kp-g)t}$ in which b is the tumor concentration at any time, b_0 is the initial tumor concentration, p is the T cell concentration, *k* is the second-order rate constant for T cell killing of tumor, and *g* is the first-order rate constant for tumor growth. (Li et al. 2004)

***In vitro* regulatory T cell suppression assay**

Plates were coated overnight at 4°C with anti-CD3 antibody (145-2C11) at a concentration of 2.3 µg/well. Foxp3-GFP+ and Foxp3-GFP- TRP-1 CD4 T cells were isolated from TRP-1 TCR transgenic mice by FACs sort. GFP- target cells were labeled with CellTraceViolet according to manufacturers instructions. Labeled effectors were plated at a density of 50,000 cells per well with 25,000 irradiated splenocytes feeder cells. The indicated ratios of GFP+ regulatory T cells were co-plated with effectors in the presence of 10µg/ml anti-CD28 antibody (37N1). Proliferation of target cells was assessed 5 days later by flow cytometry.

***In vitro* TRP-1 CD4+ and CD4+CD8+ T cell differentiation assays**

TRP-1 CD4+ T cells were isolated as previously described by CD4 positive separation on MACS columns. 50,000 TRP-1 CD4+ T cells were co-plated with 25,000 irradiated splenocytes that had been pulsed with TRP-1 peptide for 2 hours at 37°C and washed twice, in RPMI 1640 supplemented with 10% FCS, 1× nonessential amino acids, 1 mM sodium pyruvate, 2 mM l-glutamine, and 50 µM β-mercaptoethanol and incubated at 37°C in a 95% air/5% CO_2 humidified atmosphere. In experiments where contribution of

common gamma chain cytokines were not being investigated 20 U/ml of rhIL-2 was added to culture media. Neutralization by antibody and/or addition of cytokines was conducted as described, with assays being evaluated 5 days after initiation of culture. FLiCA kit (LifeTechnologies) was used according to manufacturers instructions.

In vivo neutralization via monoclonal antibody

Anti-PD-1(RMP1-14), anti–CTLA-4 (9D9,) anti–IFNγ (XMG1.2), anti-IL-12p40 (C17.8), anti-CD25 (PC61), ant-IL-2 (JES6-1A12), anti-IL-7R (A7R34) anti-MHC-II (M5/114) and respective isotype controls (2A3, HRPN, MPC-11, LTF-2) were purchased from BioXCell. For cytokine neutralization experiments mice received intra-peritoneal injection of 200µg/dose of isotype control, anti-IFNγ, or anti-IL-12p40 antibody with the exception of the wildtype control group. Treatment was initiated the day before adoptive transfer and was administered every other day. For checkpoint blockade experiments mice received intra-peritoneal injection of 250 µg/dose of anti-PD-1, 100 µg/dose of anti-CTLA-4, or their respective isotype controls, with the exception of the 10^6 adoptive transfer control group. Treatment was initiated the day of adoptive transfer and was administered every 3 days.

DNA Immunization

DNA immunization by helium-driven gold particle bombardment via the gene gun has been previously reported(Ross et al. 1997). Plasmid DNA was coated on 1.0-µm-diameter gold particles (Bio-Rad, Hercules, CA) and precipitated on bullets of Teflon tubing (McMaster-Carr, East Rutherford, NJ). Mice were anesthetized and abdominal hair was removed as necessary with Nair depilatory cream (Church & Dwight Co.). Gold particles containing 1 µg of plasmid DNA were delivered to each abdominal quadrant of a mouse using a helium-driven gene gun (Accell; PowderJect Vaccines, Madison, WI) for a total of four injections.

Peptide Immunization

Wildtype splenocytes were incubated for 2 hours at 37°C in complete RPMI media containing 0, 0.5 μg, or 5 μg of TRP-$1_{106-130}$ peptide (SGHNCGTCRPGWRGAACNQKILTVR). The splenocytes were washed 3x with PBS and then co-transferred intravenously with 10^5 or 10^6 TRP-1 CD4+ T cells into irradiated hosts.

***In vivo* T cell imaging**

Fully shaved mice were anaesthetized and injected retro-orbitally 2 minutes prior to imaging with 1.5 mg D-Luciferin K+ reconstituted according to manufacturers instructions (PerkinsElmer). Mice were imaged every 2-3 days on an IVIS 200 (Xenogen). Data was acquired and analyzed using LivingImage software (Xenogen).

***Ex vivo* restimulation, quantification, and flow cytometry**

Cells from tumor draining lymph nodes, spleens, and tumors were prepared by mechanical dissociation in 40 μM filters and red blood cells were removed by incubation in ACK Lysing Buffer (Lonza). When tumor mass exceeded .1 grams live lymphocytes were isolated using Percoll (GE Healthcare) gradient centrifugation. Pre- and post Percoll isolation samples were taken to determine total enrichment. Sample cellularity was quantified prior to enrichment steps using a Guava EasyCyte (EMD Millipore) and CytoSoft software. (EMD Millipore). Absolute number was determined by gating on the population of interest, back calculating, and multiplying by absolute number. For samples receiving re-stimulation, cells were stimulated in RPMI 1640 supplemented with 10% FCS, 1× nonessential amino acids, 1 mM sodium pyruvate, 2 mM l-glutamine, and 50 μM β-mercaptoethanol with 500 ng/ml PMA and 1 μg/ml ionomycin and incubated at 37°C. After 2 hours, 10 μg/ml monensin and 1x GolgiPlug (BD Biosciences) were

added to the culture and incubated for an additional 3 hours at 37°C. Surface staining was performed with an initial 1 hour incubation FcBlock biotin (clone 2.4G2; BD Biosciences) and CD45.1 biotin (clone 2.4G2; BD). A secondary surface stain with fluorophore conjugated streptavidin and antibodies followed for 45 minutes. All intracellular staining was conducted using the Foxp3 fixation/permeabilization buffer (eBioscience) according to the manufacturer's instructions. Flow cytometry was performed on an LSRII (BD Biosciences). FlowJo software (version 9.4.10; Tree Star) was used for all flow cytometry analysis. FACS sorting was conducted on a FACSAria II cell sorter (BD Biosciences).

Epitope spreading assays

On day 28 post-adoptive transfer, mice were euthanized and cells from tumor draining lymph nodes and spleens were prepared by mechanical dissociation in 40 μM filters and red blood cells were removed by incubation in ACK Lysing Buffer (Lonza). Cells were stimulated in RPMI 1640 supplemented with 10% FCS, 1× nonessential amino acids, 1 mM sodium pyruvate, 2 mM l-glutamine, and 50 μM β-mercaptoethanol with 20μM of peptide representing the CD8+ immunodominant epitopes of melanoma differentiation antigens and incubated at 37°C. After 2 hours, 10 μg/ml monensin and 1x GolgiPlug (BD Biosciences) were added to the culture and incubated for an additional 3 hours at 37°C. Intracellular staining of IFNγ was assessed by flow cytometry as described above.

Flow cytometry antibodies

Biolegend – Biotin CD45.1, Pe-Cy7 PDL1, APC-Cy7 MHC-II (I-A/I-E)
BD Biosciences - FITC Ki67, FITC Streptavidin, FITC Cd11b, FITC CD44, Alexa488 TNF, PE Streptavidin, PE - CD80, PerCP CD3e, PerCP CD4, PerCP CD8, Pe-Cy7 CD4, Pe-Cy7 Cd11c, Pacific Blue CD8a, V450 CD80, Alexa700 IFNγ, APC-Cy7 CD45

eBioscience - FITC ICOS, PE-BTLA, PE Eomesodermin, PE CD86, PeCy7 PD-1, eFluor 450 LAG-3, eFluor 450 MHC-II (I-A/I-E), eFluor 450 Eomesodermin, APC IL-21, APC CTLA-4, APC eFluor 780 CD27, APC eFluor780 CD8a, APC eFluor780 CD62L
LifeTechnologies - PE-TexasRed Granzyme B

Lymph node and tumor cytokine quantification

Inguinal tumor draining lymph node was isolated and homogenized using a Retsch Mixer Mill in 150 µl PBS containing Complete EDTA-free protease inhibitor (Roche) resuspended according to the manufacturer instructions. 500 µl of tumor volume was massed and homogenized in 500 µl PBS + protease inhibitor via Retsch Mixer Mill. Homogenate supernatant was clarified twice via centrifugation at 2000 rpm. Cytokine was quantified on a Luminex Magpix using a Milliplex MAP Kit (Millipore). For tumor samples cytokine was normalized to mass of isolated tissue.

***In vitro* supernatant cytokine quantification**

The 1 ml of media overlaying the collagen-fibrin gels of *in vitro* clonogenic killing assays was collected at 24 or 48 hours prior to digesting gels. The supernatant was frozen down and thawed to assess cytokine production. Cytokine was quantified using a CBA Th1/Th2/Th17 Kit (BD Biosciences) on the LSRII (BD Biosciences).

RT-PCR

CD4+ T cells were sorted on a FACS AriaII (BD Biosciences) directly into Trizol LS (Life technologies) and total RNA was obtained via Trizol purification according to the manufacturers instructions. cDNA was synthesized using High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to manufacturer's instructions. All primers and probes were from TaqMan Gene Expression Assays (Applied Biosystems). Real-time PCR reactions were prepared with 3 µl cDNA according to the manufacturer's

instructions. All amplifications were done using the ABI 7500 Real Time PCR system (Applied Biosystems). Each gene was amplified in triplicate and cDNA concentration differences were normalized to GAPDH. Relative gene expression of the target genes were shown by $2 - \Delta Ct$ (ΔCt = Ct(target gene) − Ct(GAPDH)) using mean Ct (threshold cycle) of triplicates.

Statistical Analysis

All values shown in graphs represent the mean ± SEM, with the exception of *ex vivo* clonogenic killing assays, which represent the mean ± SD. In graphs comparing titration of 4 different precursor frequencies, significance is only shown for extreme groups for ease of interpretation. Statistical differences were determined using ANOVA and *post hoc* Bonferroni adjustment for multiple comparisons. Statistics for comparing 2 groups were determined by a 2-tailed Student's *t* test. * $P < 0.05$ was considered statistically significant. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Significance of survival curves was evaluated by log rank (Mantel Cox) test. All graphs and statistical calculations were done using Prism software (GraphPad Software). PLSR and heat maps were generated using MatLab (MathWorks).

CHAPTER 3:

ESTABLISHING A MODEL SYSTEM TO INVESTIGATE THE ROLE OF TUMOR SPECIFIC CD4+ T CELL PRECURSOR FREQUENCY ON THE ANTI-TUMOR IMMUNE RESPONSE

Introduction

Although the size of naïve precursor pools had been quantified for a number of T cell clones specific for foreign antigens, little was known about the relative abundance of T cells specific for self-derived melanoma differentiation antigens and how the initial precursor size of this population impacted therapeutic outcome. Our laboratory had hypothesized, that as a result of mechanisms of negative selection these T cells would be present at lower frequencies in the immune repertoire and that their lower frequency relative to T cell clones specific for foreign antigens partly contributed to challenges in provoking efficacious anti-tumor responses (Cao, Myers-Powell, and Braciale 1996). To investigate this hypothesis, our group developed a model system in which the precursor number of CD8+ T cells specific for the self/melanoma antigen gp100/pmel-1 could be manipulated in mice bearing melanoma tumors through the adoptive transfer of tumor specific T cells (Rizzuto et al. 2009).

Using a previously described method pioneered by Blattman et al., the endogenous precursor frequency of T cells specific for the antigen gp100 was estimated to be around only 5 CD8+ T cells per mouse (Blattman et al. 2002). A number of mice failed to generate an endogenous response against the gp100 antigen, suggesting that the precursor frequency of T cells specific for gp100 is heterogeneous and reaches a functional zero in some repertoires. When the T cell receptor beta chain usage of the endogenous gp100 specific T cells was analyzed the population was found to be oligoclonal. To better understand how this low endogenous clonal frequency affected the induction of effective anti-tumor immunity the response of TCR transgenic pmel-1 CD8+ T cells at varied initial clonal abundances was evaluated.

In this model, mice received an intradermal injection of B16 melanoma, which was allowed to grow for 4 days. On the fourth day following tumor implantation, mice were treated with 600 cGy of sub-lethal irradiation. Irradiation was used to ablate the pre-existing immune repertoire and replicate the pre-conditioning regimen administered to patients receiving adoptive cell therapy. Several hours following irradiation mice were reconstituted with a polyclonal repertoire of 30 million total cells containing splenocytes isolated from naïve donor mice and varied numbers of the TCR transgenic T cell of interest. This approach ensured that the only manipulated variable was the frequency of the T cell of interest in the context of a diverse polyclonal T cell population. Beginning on the day after adoptive transfer, mice were vaccinated weekly, three times in total, to activate the T cells *in vivo*. The expression of a congenic marker on the pmel-1 CD8+ T cells allowed them to be easily characterized over the course of the anti-tumor response (Figure 3.1).

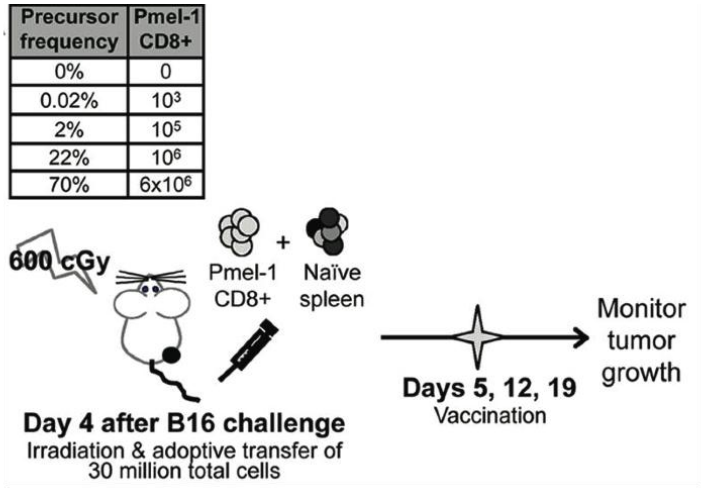


Figure 3.1. Schematic of adoptive transfer model developed to study self-antigen specific CD8+ T cell response against melanoma.
C57BL/6J mice were implanted with 1x 10^5 B16 cells. Four days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of the indicated number of Pmel-1 CD8+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. On the day following adoptive transfer mice were vaccinated via gene gun with plasmid DNA encoding hgp100, weekly for three vaccinations in total. Adapted from (Rizzuto et al., 2009).

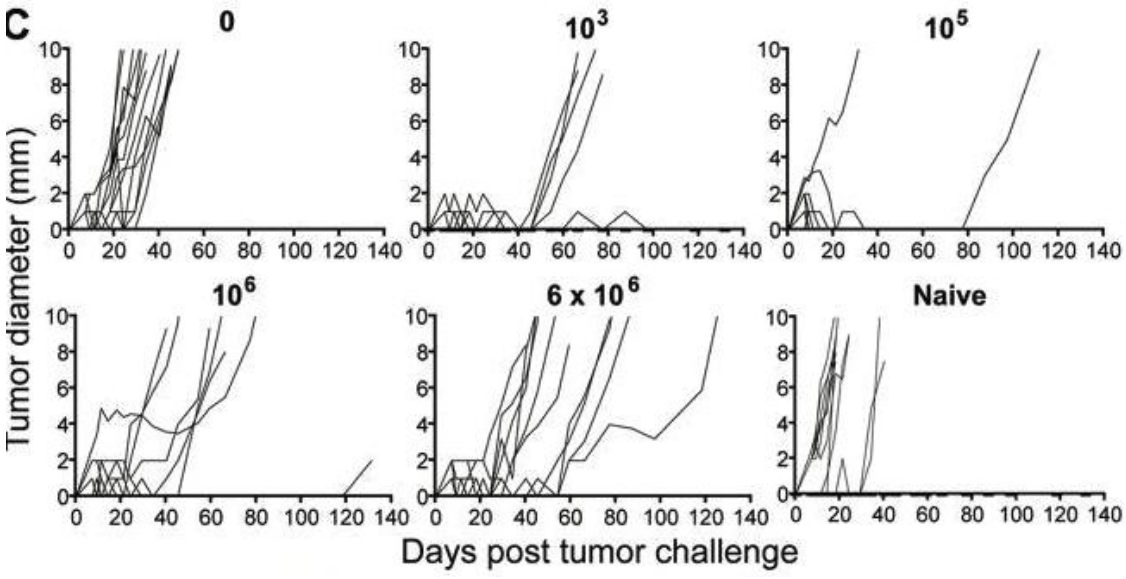


Figure 3.2. Anti-tumor immunity declines when Pmel-1 CD8+ T cells far exceed physiological precursor frequencies.
C57BL/6J mice were implanted with 1x 10^5 B16 cells. Four days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of the indicated number of Pmel-1 CD8+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. On the day following adoptive transfer mice were vaccinated via gene gun with plasmid DNA encoding hgp100, weekly for three vaccinations in total. Every 2-3 days tumors were measured by caliper and represented on the graph by individual lines. Adapted from (Rizzuto et al., 2009).

Intriguingly, the most successful anti-tumor responses were observed when pmel-1 CD8+ T cells were present at precursor frequencies within or more modestly above the normal physiologic range observed for foreign antigen specific T cells (Rizzuto et al. 2009). When the initial precursor frequency of T cells greatly exceeded this range, a reduction in tumor immunity was observed (Figure 3.2). Using a competition assay in which pmel-1 specific CD8+ T cells were co-transferred with monoclonal OT-I CD8+ T cells, it was determined that the expansion of pmel-1 CD8+ T cells was impaired by intraclonal competition at supra-physiologic precursor frequencies. In addition to reduced levels of expansion, intraclonal competition at high precursor frequencies of pmel-1 CD8+ T cells resulted in diminished development of effector function and a lower degree of polyfunctionality (Figure 3.3). The formation of T cell memory was also

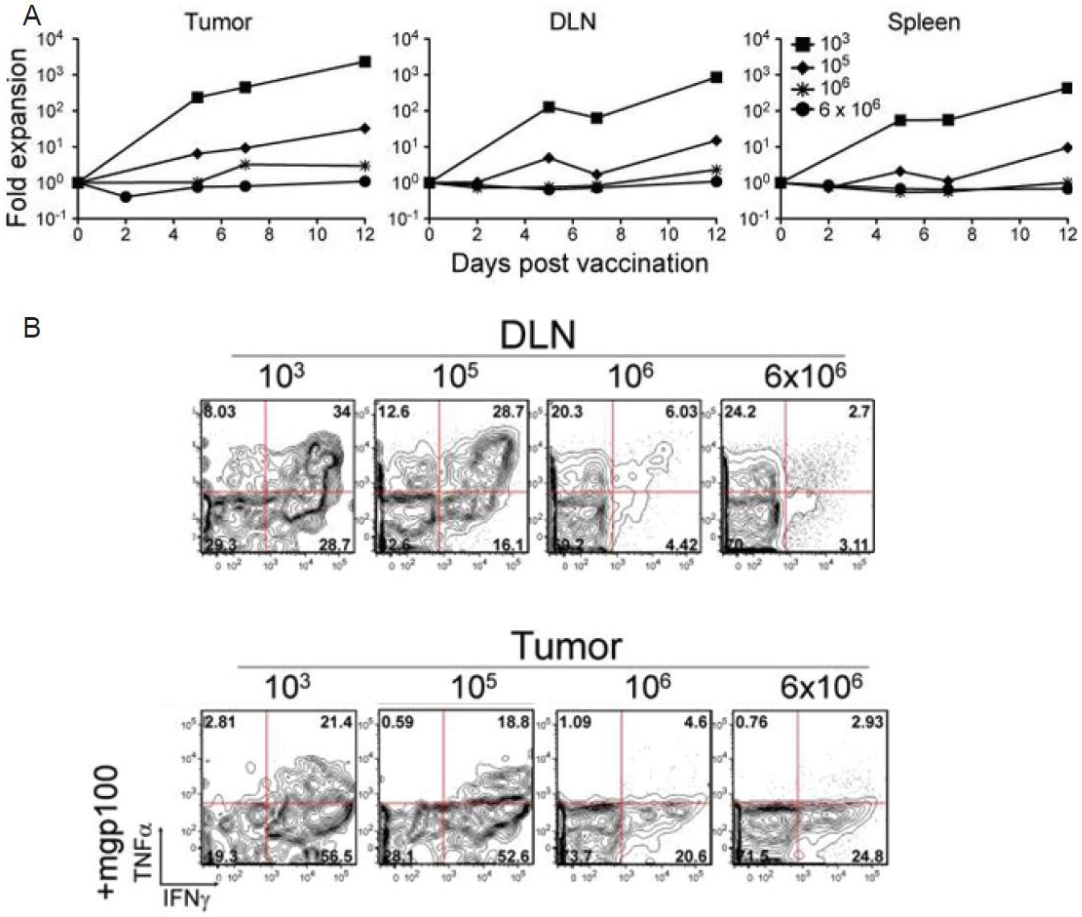


Figure 3.3. Population expansion and development of T cell effector function are impaired at high precursor frequencies of Pmel-1 CD8+ T cells.

(A) Fold expansion of Pmel-1 CD8+ T cells was determined at time points indicated in each organ relative to initial precursor number. (B) Effector function of Pmel-1 CD8+ T cells evaluated 5 days after vaccination following restimulation with peptide. Adapted from (Rizzuto et al., 2009).

negatively impacted in conditions where initial clonal abundance far exceeded physiologic levels. This significant study confirmed that the precursor frequency of self-antigen specific CD8+ T cells instructs the magnitude and quality of an immune response and revealed a capacity for intraclonal competition even in the presence of ubiquitous cognate antigen. The decreased anti-tumor immunity observed in conditions where T cells were present at high clonal abundance countered conventional wisdom that more is always better.

We were interested in determining if the clonal responses of CD4+ T cells were similarly instructed by their initial precursor frequency within the context of tumor immunity. To study this, we aimed to adapt the adoptive transfer system used to investigate the role of CD8+ T cell precursor frequency to allow us to address how clonal abundance affected tumor specific CD4+ T cells. We pursued the development of two different adoptive transfer models using TCR transgenic CD4+ T cells in which precursor frequency could be manipulated in tumor bearing mice. The first model used OT-II T cells specific for the I-A^b binding immunodominant epitope of the model antigen ovalbumin. These T cells were used in combination with a B16 melanoma cell line stably transfected to express ovalbumin, termed M04. The other adoptive transfer model used T cells specific for the self/tumor-antigen TRP-1 in combination with wildtype B16 (Muranski et al. 2008). The development of two separate models would allow for the results obtained using the surrogate and self-antigen models to be compared and contrasted. In addition to this, we sought to synthesize a MHC-II tetramer containing the same peptide epitope recognized by the TRP-1 transgenic CD4+ T cells in order to provide a means to corroborate findings made using TCR transgenic T cells with the endogenous CD4+ T cell response, as well as to estimate the endogenous precursor

frequency of this clone. However, this tetramer was unable to be synthesized (personal communication).

In this chapter we detail the development of both the OT-II and TRP-1 CD4+ T cell adoptive transfer models. Altering the clonal abundance of OT-II CD4+ T cells did not result in any discernible differences in anti-tumor immunity due to the failure of these T cells to mediate appreciable responses, even after vaccination; this was likely due to the inability of these T cells to expand upon transfer. For this reason, we decided to ultimately proceed with only the TRP-1 specific CD4+ T cell model. In contrast to OT-II CD4+ T cells, TRP-1 CD4+ T cells undergo vigorous proliferation upon adoptive transfer into lymphopenic hosts. As in the pmel-1 CD8+ T cell model, a reduced initial proliferative burst and impaired clonal expansion are observed at high precursor frequencies. Surprisingly, intraclonal competition does not preclude anti-tumor immunity and tumor regression is enhanced at high clonal abundance. The anti-tumor responses generated in this model confer long lasting protective immunity and are TRP-1 CD4+ T cell dependent, though CD8+ T cell responses are also enhanced.

Results and Discussion

3.1 Establishing an adoptive transfer model utilizing ovalbumin as a surrogate tumor antigen

OT-II T cells express a TCR specific for the I-A^b-restricted epitope of ovalbumin $OVA_{323–339}$ (Barnden et al. 1998). Many contributions to our understanding of CD4+ T cell mediated immunity have been made utilizing OT-II CD4+ T cells in combination with tumors and foreign pathogens engineered to express ovalbumin as a surrogate antigen. This model has been used to address a wide range of subjects within tumor immunology, including investigation of which T helper fate delivers the greatest therapeutic efficacy, the mechanisms underlying anergy induction by tumors, and the

dendritic cell subsets responsible for antigen processing and engagement with CD4+ T cells during anti-tumor responses (Purwar et al. 2012; Abe et al. 2012; Kalergis and Ravetch 2002; Dudziak et al. 2007). Although the OT-II CD4+ T cell model is a tool of immeasurable importance to immunology, it was unsuitable for our investigation due to low levels of quantifiable tumor immunity and the impaired proliferation of these cells.

3.1.1. Assessing the OT-II CD4+ T cell mediated anti-tumor response

In order to adapt the pmel-1 CD8+ T cell adoptive transfer model to investigate tumor specific CD4+ T cells, we replicated the established adoptive transfer protocol, with additional modifications, using the OT-II CD4+ TCR transgenic T cells. OT-II CD4+ T cells recognize ovalbumin (OVA), which is a foreign antigen not normally expressed by wildtype B16 melanoma. To facilitate tumor recognition, the M04 tumor cell line, which is B16F10 stably transfected to express ovalbumin, was transplanted into adoptive hosts in lieu of wildtype B16. Additionally, the vaccination step was removed from the initial investigations because it had not been empirically determined if *in vivo*

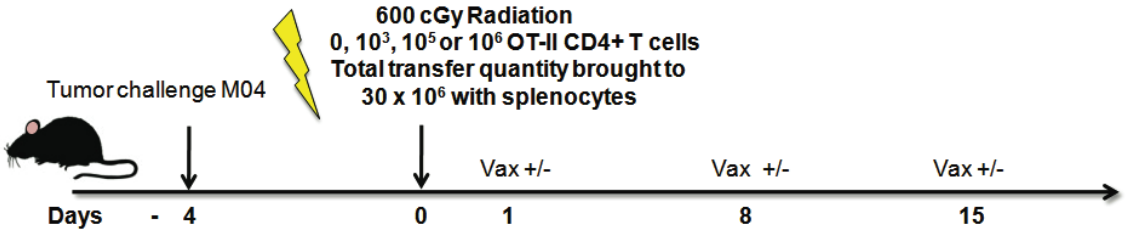


Figure 3.4. Schematic of OT-II CD4+ T cell adoptive transfer model investigating immunity against a surrogate tumor antigen.
C57BL/6J mice were implanted with 2.5 x 10^5 M04 cells. Four days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of the indicated number of OT-II CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. In experiments that included vaccination, on the day following adoptive transfer and every 7 days for 3 doses in total, mice were vaccinated via gene gun with plasmid DNA encoding ovalbumin. Tumor diameters were measured every 3-5 days by caliper.

activation would be necessary to facilitate an anti-tumor response in this model (Figure 3.4).

To determine the clonal abundance of OT-II CD4+ T cells necessary to observe regression of established M04 tumors, different quantities (0, 10^3, 10^5, 10^6, and 10^7) of OT-II CD4+ T cells were adoptively transferred, together with naïve open repertoire splenocytes, into hosts bearing day 4 M04 tumors. At no clonal abundance examined were the OT-II CD4+ T cells capable of mediating consistent tumor regression (Figure 3.5). To investigate whether the absence of a productive anti-tumor response was due to the lack of *in vivo* activation, we repeated the experiment with vaccination against ovalbumin via gene gun beginning on the day following adoptive transfer, continuing once every week for three weeks. Even with the addition of vaccination, no consistent reduction in tumor growth or increase in the incidence of tumor regression was observed at any clonal abundance (Figure 3.6). When the composite tumor growth over time of vaccinated and unvaccinated recipients of OT-II CD4+ T cells was compared side by side no clear trend in anti-tumor efficacy was observed (Figure 3.7). It is possible that tumor growth was slowed in groups receiving greater than 10^5 OT-II CD4+ T cells, but the differences between groups were too subtle to pursue and obscured by the addition of vaccination.

To better understand the underlying factors that contributed to the absence of anti-tumor activity mediated by the OT-II CD4+ T cells, we examined the proliferative capacity of the T cells following adoptive transfer and their ability to produce effector cytokines. Unlike other T cells, which undergo homeostatic proliferation upon adoptive transfer into a lymphopenic environment, no proliferation was observed in the transferred population of 10^5 CFSE labeled OT-II CD4+ T cells (Figure 3.8). This finding was in accordance with previously published observations and has been attributed to the weak affinity of the OT-II TCR for self-peptide MHC (Ernst et al. 1999). Self-antigen specific T cells are generally recognized to possess a high affinity for self-peptide MHC, which

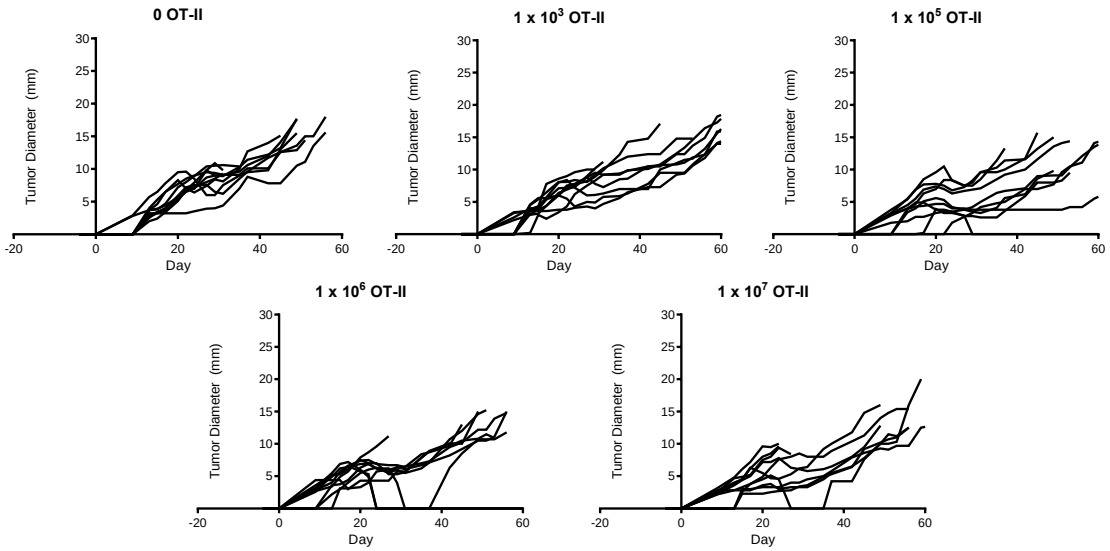


Figure 3.5. Varying the clonal abundance of OT-II CD4+ T cells does not significantly alter tumor immunity.

C57BL/6J mice were implanted with 2.5 x 10^5 M04 cells. Four days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of the indicated number of OT-II CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. Tumor diameters were measured every 3-5 days by caliper and represented on the graph by individual lines. Mice were euthanized when tumor diameter reached 1.5 cm (n= 9-10 mice per group).

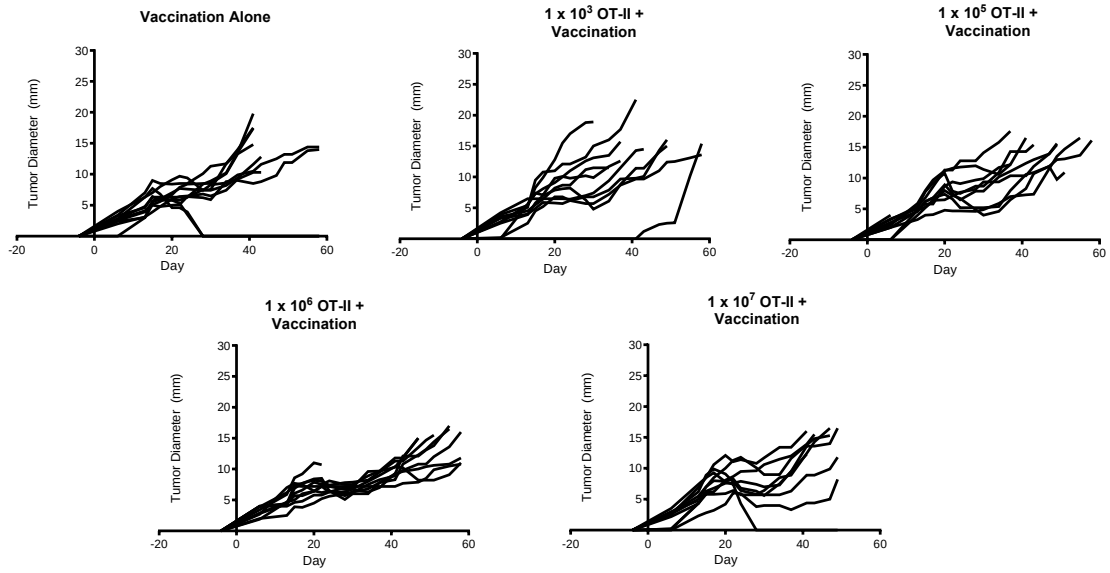


Figure 3.6. Combining adoptive transfer of OT-II CD4+ T cells with vaccination against ovalbumin does not improve anti-tumor efficacy.
C57BL/6J mice were implanted with 2.5 x 10^5 M04 cells. Four days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of the indicated number of OT-II CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. On the day following adoptive transfer and every 7 days for 3 doses in total, mice were vaccinated via gene gun with plasmid DNA encoding ovalbumin. Tumor diameters were measured every 3-5 days by caliper and represented on the graph by individual lines. Mice were euthanized when tumor diameter reached 1.5 cm (n= 9-10 mice per group).

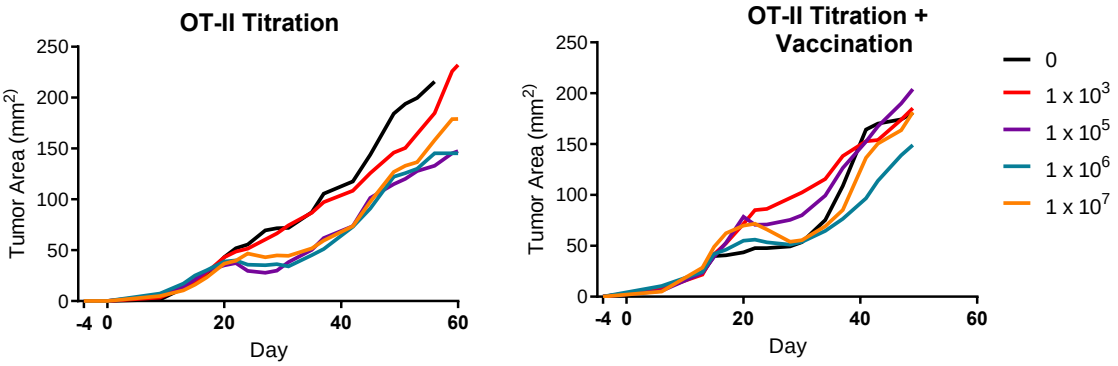


Figure 3.7. Composite tumor growth comparison between unvaccinated and vaccinated mice receiving adoptive transfer of OT-II CD4+ T cells.
C57BL/6J mice were implanted with 2.5 x 10^5 M04 cells. Four days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of the indicated number of OT-II CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. On the day following adoptive transfer and every 7 days for 3 doses in total, one cohort of mice was vaccinated via gene gun with plasmid DNA encoding ovalbumin. Tumor diameters were measured every 3-5 days by caliper. Measurements were squared and the mean tumor area of each group is represented on the graph by individual lines. Composite data was derived from Figures 3.4 and 3.5 (n= 9-10 mice per group).

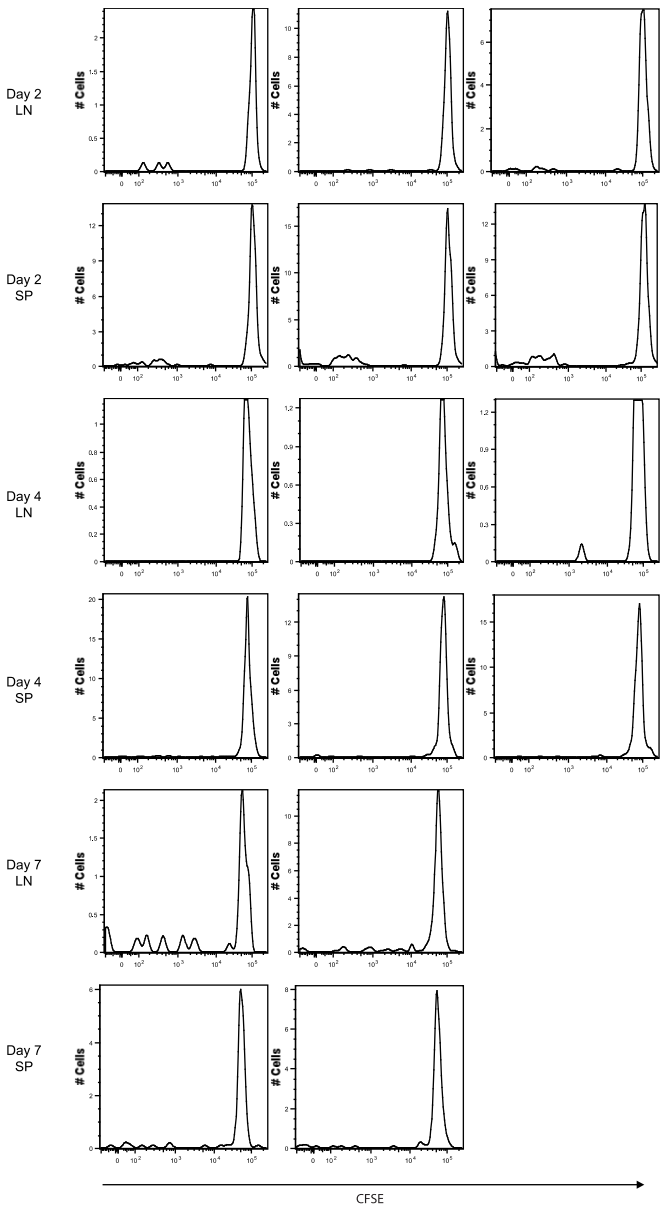


Figure 3.8. OT-II CD4+ T cells adoptively transferred into lymphopenic hosts do not undergo homeostatic proliferation.
C57BL/6J mice were implanted with 2.5 x 10^5 M04 cells. Four days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 1x10^5 CFSE labeled OT-II CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. On the day following adoptive transfer, mice were vaccinated via gene gun with plasmid DNA encoding ovalbumin. On indicated days mice were euthanized and CFSE dilution of OT-II CD4+ T cells was assessed in draining lymph node and spleen (n = 2-3 mice/group).

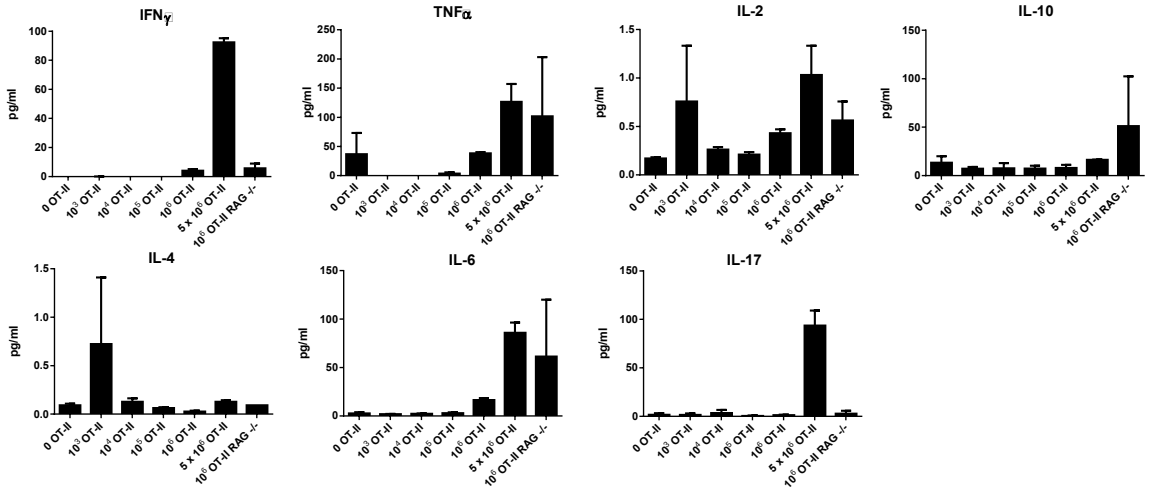


Figure 3.9. OT-II CD4+ T cells are capable of producing a variety of cytokines *in vitro*.

OT-II CD4+ T cells were isolated by MACs separation from RAG sufficient or deficient mice. The indicated numbers of OT-II CD4+ T cells were co-embedded with a fixed number of 50,000 M04 tumor cells in *in vitro* clonogenic killing assays in triplicate. At 48 hours the assay supernatant was collected and cytokine production was assessed with a CBA kit. Data are represented as mean ± SEM.

facilitates their negative selection. Thus, it is questionable whether a low-affinity T cell such as the OT-II is an appropriate surrogate antigen for the study of self-reactive T cells. We next examined effector cytokine production in the supernatants of OT-II CD4+ T cells co-cultured at varying frequencies with M04 tumor cells. We found that accumulation of effector cytokines, such as IFNγ, TNFα, IL-17, and IL-6, occurred primarily in the culture condition containing the highest quantity ($5x10^6$) of OT-II CD4+ T cells (Figure 3.9). Interestingly, if the OT-II TCR was expressed on a RAG deficient background it conferred a different cytokine profile, favoring production of high levels of IL-10. These results suggest that the lack of anti-tumor efficacy may be attributable to the inability of the OT-II CD4+ T cells to undergo the expansion necessary to reach the critical concentration of T cells required to mediate direct tumor killing or facilitate the accumulation of cytokines allowing T cell differentiation or tumor recognition.

One approach that is commonly taken to improve the resolution of differences in anti-tumor immunity is to change the size of the tumor being treated to create a wider range of observed responses. In this model, responses may have been improved if the size of the tumor being treated were reduced. However, one caveat is that a tumor requires around 7 to 10 days of growth after implantation to become palpable. Tumor implantation could not be moved closer than the 4 days prior to adoptive transfer used in this system, because it would have effectively created a prophylactic model of anti-tumor immunity rather than a therapeutic one. These initial experiments clearly illustrated that OT-II CD4+ T cells were incapable of mediating sufficient anti-tumor immunity to resolve differences in the effector functions mediated by varying initial precursor frequencies.

3.1.2. Characterization of OT-II CD4+ T cell helper function

Although the OT-II CD4+ T cells showed little anti-tumor activity as effectors, we wanted to explore if this adoptive transfer model was suitable for investigating OT-II CD4+ T cell helper function. We first wanted to assess if the adoptive transfer of OT-II CD4+ T cells resulted in CD8+ T cell epitope spreading. Epitope spreading is the phenomenon in which disease epitopes, distinct from the inducing epitope, become targets of an ongoing immune response (Vanderlugt and Miller 2002). Control mice implanted with B16 tumors and experimental mice implanted with M04 tumors received adoptive transfer of 10^5 OT-II CD4+ T cells and naïve splenocytes. One cohort of M04 tumor bearing mice was vaccinated three times against ovalbumin via gene gun, beginning the day following adoptive transfer. On day 28, reactivity against the CD8 immunodominant epitopes of a variety of melanoma differentiation antigens was tested. Naïve animals and animals implanted with B16 melanoma showed no evidence of epitope spreading or reactivity against ovalbumin (Figure 3.10). Mice vaccinated against ovalbumin bearing M04 tumors showed strong CD8+ T cell reactivity against the

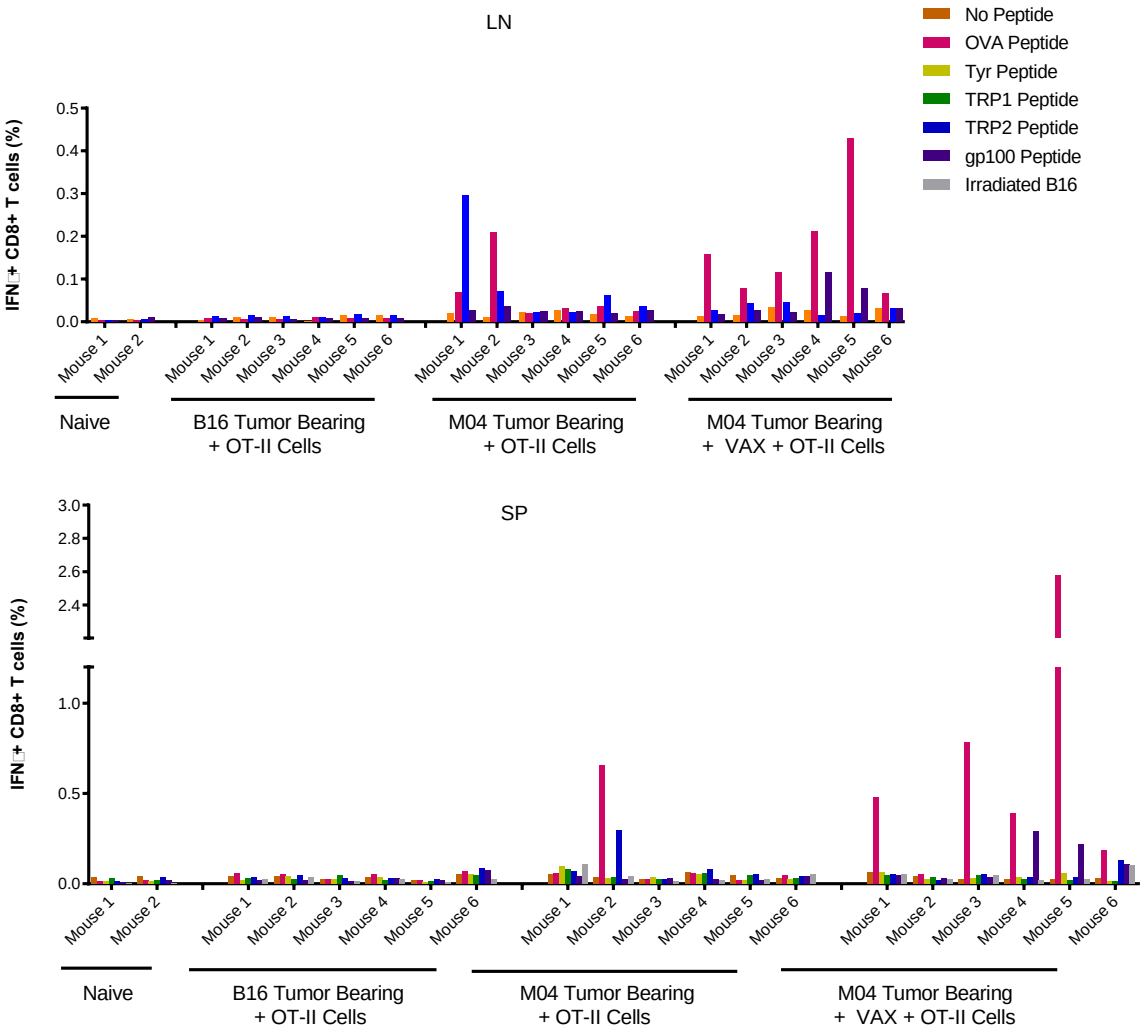


Figure 3.10. CD8+ T cell epitope spreading occurs sporadically in mice bearing M04 tumors.

C57BL/6J mice were implanted with 2.5 x 10^5 B16 or M04 cells. Four days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 1x10^5 OT-II CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells, with the exception of the naïve group. On the day following adoptive transfer and every 7 days for 3 doses in total, one cohort of mice was vaccinated via gene gun with plasmid DNA encoding ovalbumin. On day 28 post adoptive transfer, mice were euthanized and draining lymph node and spleen were isolated. Single cell suspensions were restimulated with peptides representative of the CD8+ immunodominant epitopes of melanoma differentiation antigens. Intracellular cytokine staining for IFNγ after 5 hours was used to assess antigen reactivity (n = 2-6 mice per group).

SIINFEKL epitope of OVA, as would be expected following vaccination against an inducing epitope. Sporadic epitope spreading was observed in both vaccinated and unvaccinated mice. Within the group of unvaccinated mice, one occurrence of strong reactivity against the antigen TRP-2 was observed in both the lymph node and spleen, and additional weak TRP-2 responses were observed in the lymph node. In the mice receiving vaccination, at least two CD8+ T cell responses were observed against gp100 in both the lymph node and spleen. It is interesting that vaccination appears to change the dominant CD8+ T cell response against melanoma differentiation antigens. It is possible that the OVA specific CD8+ T cell response in vaccinated mice forces competition for CD8+ T cell immunodominance and favors the expansion of different subsets of tumor specific CD8+ T cells. There has been recent concern over whether vaccination against the wrong targets can misdirect the total immune response, as was debatably observed in the gp100 + ipilimumab arm of the clinical trials for ipilimumab (Hodi et al. 2010). Future experiments could be conducted in this model to explore this possibility and its potential impact on anti-tumor efficacy.

The epitope spreading experiment demonstrated strong CD8+ T cell reactivity against ovalbumin in mice receiving adoptive transfer combined with vaccination. In order to determine if the number of helper OT-II CD4+ T cells impacted the CD8+ T cell response we performed a co-transfer experiment. Mice bearing M04 tumors received adoptive transfer of 10^5 OVA specific OT-I CD8+ T cells co-transferred with varied numbers (0, 10^3, 10^5, 10^6, 10^7) of OT-II CD4+ T cells. Mice were vaccinated against ovalbumin on the day following adoptive transfer; tumor growth and overall survival of the groups were monitored. The greatest anti-tumor efficacy was observed when both populations of OT-I and OT-II cells were adoptively transferred at equivalent frequencies of 10^5 (Figure 3.11). When OT-II CD4+ T cells were transferred at a clonal abundance greater than 10^5 the improvement in tumor growth and overall survival was abolished. This result was noteworthy because a similar reduction in efficacy was observed at high

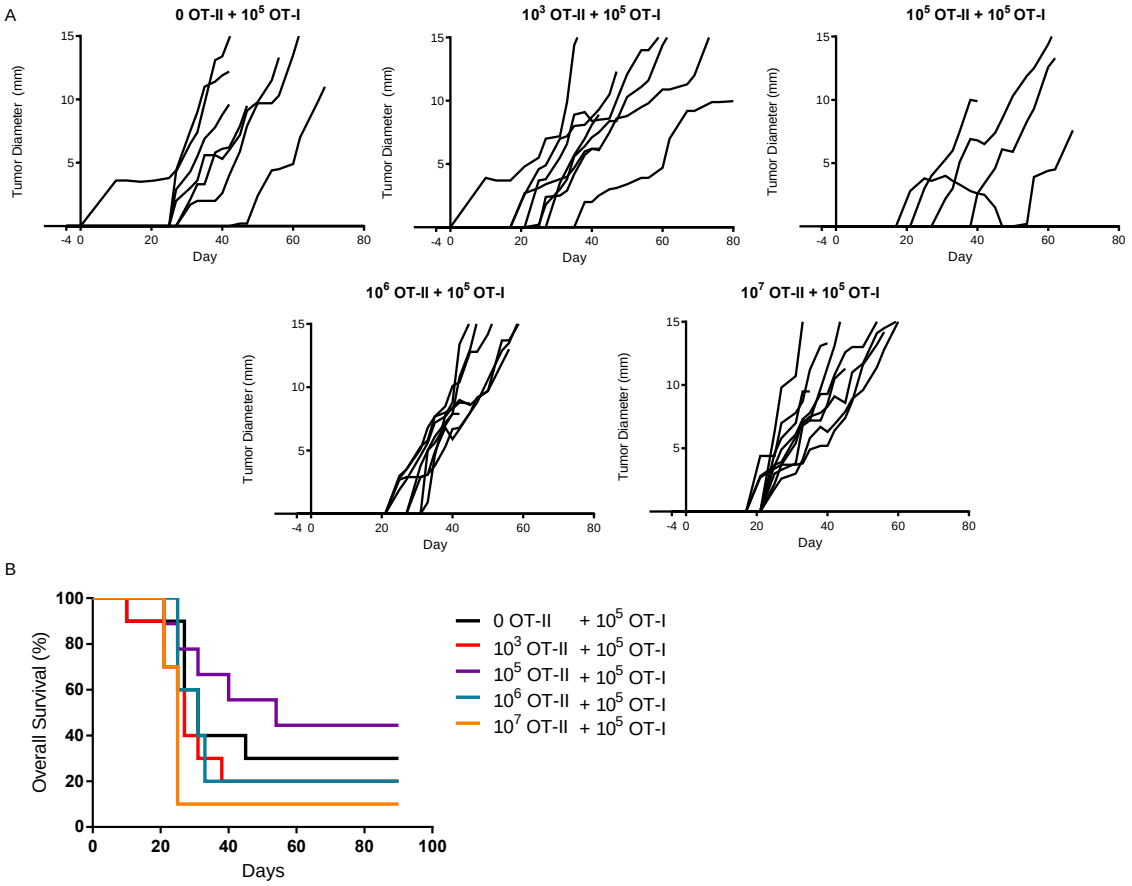


Figure 3.11. OT-II CD4+ T cells co-transferred with equivalent numbers of OT-I CD8+ T cells show the greatest anti-tumor efficacy.

C57BL/6J mice were implanted with 2.5 x 10^5 M04 cells. Four days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of the indicated number of OT-II CD4+ T cells co-transferred with 10^5 OT-I CD8+ T cells and naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. On the day following adoptive transfer mice were vaccinated via gene gun with plasmid DNA encoding ovalbumin. (A) Tumor diameter was measured every 3-5 days by caliper and is represented on the graph by individual lines. (B) The overall survival of each group was plotted. Mice were euthanized when tumor diameter reached 1.5 cm (n= 9-10 mice per group).

precursor frequencies during the study of self-antigen specific pmel-1 CD8+ T cells (Rizzuto et al. 2009). However, the mechanism behind the improved anti-tumor immunity in this condition was not investigated further. Additionally, it is unclear how the frequency of OT-I CD8+ T cells contributes to this observation. A reciprocal experiment varying the frequency of OT-I CD8+ T cells would have to be performed to address this matter. While it appeared that the M04 co-transfer model was a favorable one to evaluate CD4+ T cell helper function, we were more interested in evaluating the contribution of precursor frequency to the development of effector CD4+ T cells. This objective drove us to solely pursue another model, the TRP-1 CD4+ T cell adoptive transfer model.

3.2 Establishing an adoptive transfer model specific for the self-tumor antigen TRP-1

Use of surrogate tumor antigens and investigation of the endogenous T cell response has aided the study of how CD4+ T cells contribute to the generation of a tumor specific immune response. However, in order to examine how tumor-self antigen specific CD4+ T cells and their clonal abundance affects the immune response in hosts bearing both ubiquitous cognate antigen and implantable melanoma, T cells specific for a native self antigen are required. A CD4+ T cell TCR transgenic mouse line specific for the melanoma differentiation antigen tyrosinase related protein 1 (TRP-1) had been recently developed and provided the ideal model (Muranski et al. 2008). One unique feature of this model is that anti-TRP-1 TCR transgenic T cells are negatively selected in mice expressing TRP-1, such that these TCR transgenic mice must also be maintained on a TRP-1 deficient background. These conditions imply that this T cell clone is strongly selected against in the thymus and likely present at very low quantities, if at all, in the normal immune repertoire. In support of this assumption, Aire deficient mice display lower levels of TRP-1 expression in the thymus during negative selection. As a result,

TRP-1 specific T cells escape negative selection in Aire deficient mice and display elevated responses against melanoma (Zhu, Nagavalli, and Su 2013). Taking into account the low abundance of TRP-1 specific T cells after normal thymic development, we examined how the relative paucity of this clone affects its ability to mount a successful response to B16 melanoma.

3.2.1.At high precursor frequencies, tumor-specific CD4+ T cells experience impaired expansion and activation

We first determined how precursor frequency impacted the initial proliferative burst of the tumor specific CD4+ T cells. TRP-1 CD4+ T cells were labeled with CFSE and co-transferred at titrated quantities of 10^3, 10^5 or 10^6 cells, with open repertoire, meaning un-enriched and unbiased, naïve splenocytes (Figure 3.12). At days 5 and 7, proliferation was assessed in the tumor draining lymph nodes (LN) and spleens of the treated animals (Figure 3.13A). At all frequencies, proliferation was observed, as expected after adoptive transfer into a lymphopenic host. However, only at the lowest

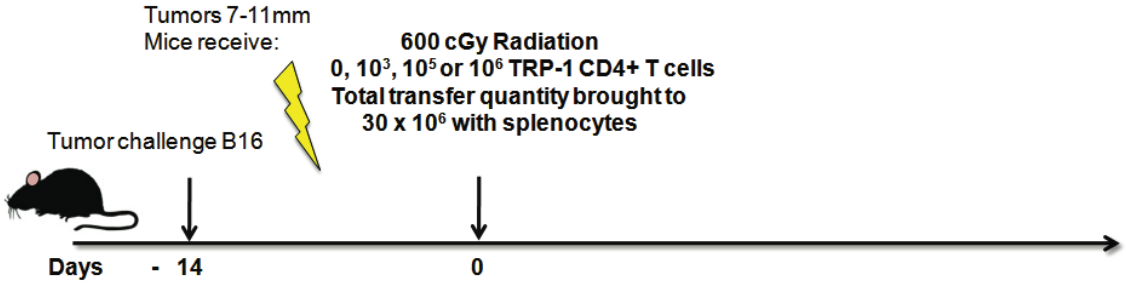


Figure 3.12. TRP-1 CD4+ T cell adoptive transfer model developed to study self-antigen specific CD4+ T cell response against melanoma.
C57BL/6J mice were implanted with 1 x 10^5 B16 tumor cells. Fourteen to eighteen days post tumor challenge, when tumors were around 7-11 mm in diameter, mice received 600 cGy of irradiation, followed by tail vein injection of TRP-1 CD4+ T cells at varied precursor frequencies co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells.

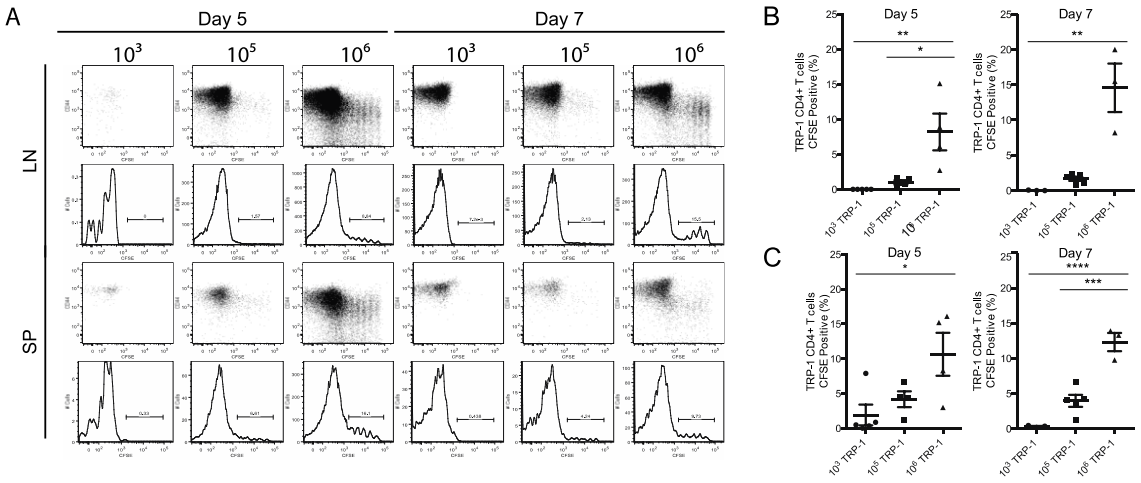


Figure 3.13. The initial proliferative burst of tumor specific CD4+ T cells is reduced at high precursor frequencies.

C57BL/6J mice were implanted with 1 x 10^5 B16 tumor cells. Fourteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 10^3, 10^5, or 10^6 CFSE labeled TRP-1 CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. On days 5 and 7, CFSE dilution of the TRP-1 CD4+ T cells was assessed in the lymph node and spleens of host mice. (A) Representative flow plots of CFSE dilution. (B) Percentage of undivided $CFSE^{hi}$ TRP-1 CD4+ T cell population in LN and C. spleen (n=3-5 mice/group). Data are represented as mean ± SEM.

frequency (10^3), did almost all TRP-1 CD4+ T cells undergo robust proliferation (Figure 3.13B,C). Notably, in the 10^6 group, between 10-15% of the population showed only partial proliferation. This population increased in the LN between day 5 and 7, possibly due to egress of divided cells from the LN to tumor. To determine if this early defect in proliferation at high clonal abundance resulted from incomplete activation of the population, we examined T cell activation markers CD44 and CD62L in tumor draining LNs (Figure 3.14A,B). In the lower frequency groups, a greater proportion of T cells displayed an activated $CD44^{hi}CD62L^{lo}$ phenotype compared to groups with higher precursor frequencies. However, all T cells that infiltrate the tumor had already undergone activation and no difference was observed (Figure 3.14C). This asserts that during the priming phase, self-antigen specific T cells adoptively-transferred at high numbers experience intraclonal competition, which limits their early activation and expansion. Yet the tumor environment is enriched for the properly activated T cells, despite any deficiencies of activation in the lymph node.

We quantified the absolute number of TRP-1 CD4+ T cells after transfer and measured proliferation over the course of the immune response. The accumulation of total TRP-1 T cells was greatest when cells were initially transferred at larger numbers, with the number of cells peaking at day 9 in the highest frequency group and at day 11 at the lower frequencies (Figure 3.15A). However, at the highest clonal abundance (10^6), the transferred population had expanded only 2.5 fold at the height of the immune response, while the population of 10^5 cells had expanded 15 fold, and 10^4 TRP-1 CD4+ T cells had expanded over 75 fold (Figure 3.15B). The increased overall expansion at lower precursor frequencies correlated with elevated and sustained Ki67 staining of the TRP-1 specific CD4+ T cells (3.16). These data support previous findings demonstrating that precursor frequency is inversely related to the ability of the population to expand (Quiel et al. 2011).

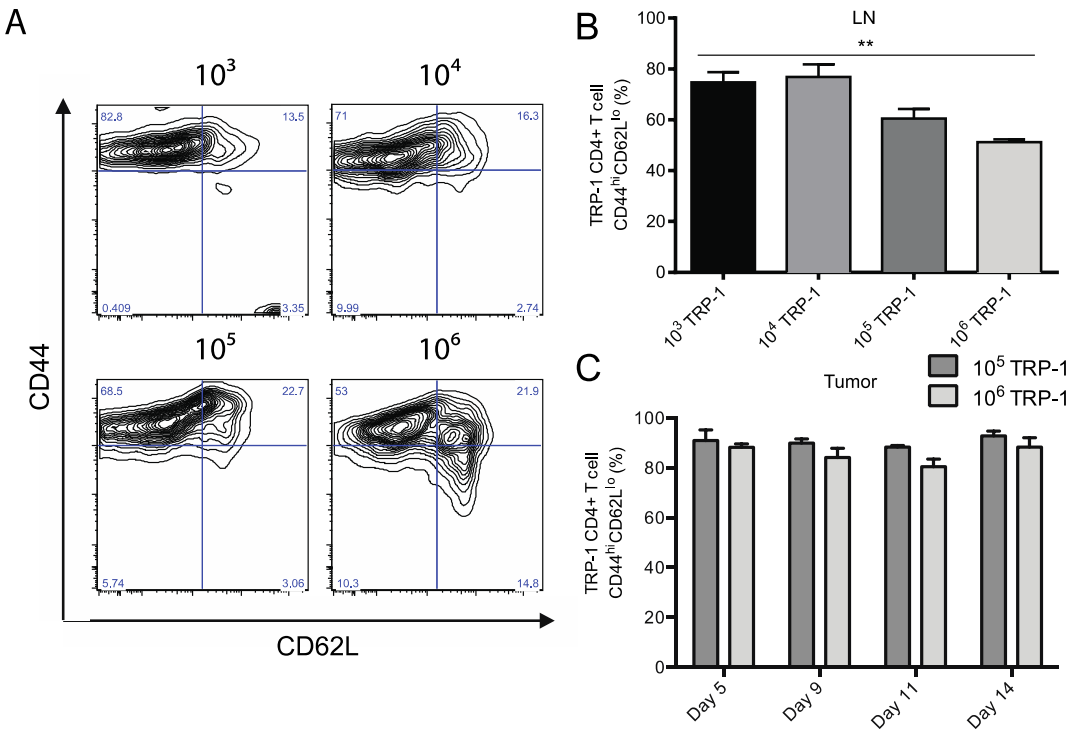


Figure 3.14. At high precursor frequencies the early activation of tumor specific CD4+ T cells is impaired.

C57BL/6J mice were implanted with 1 x 10^5 B16 tumor cells. Fourteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 10^4, 10^5, or 10^6 TRP-1 CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. (A) Representative flow plots of day 7 activation of TRP-1 CD4+ T cells in LN. (B) Summary of TRP-1 CD4+ T cell activation in LN and C. tumor (n=3-5 mice/group). Data are represented as mean ± SEM.

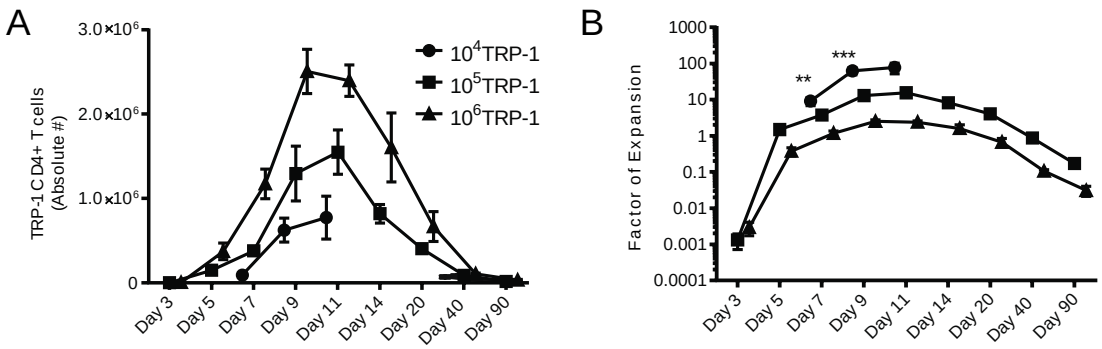


Figure 3.15. Lower precursor frequencies of TRP-1 specific CD4+ T cells undergo greater clonal expansion.

C57BL/6J mice were implanted with 1 x 10^5 B16 tumor cells. Fourteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 10^4, 10^5, or 10^6 TRP-1 CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. (A) On indicated days the absolute number of TRP-1 CD4+ T cells per mouse was determined by quantifying the population in LN, SP, and tumor by flow cytometry. (B) Factor of expansion as determined by dividing absolute number by original precursor frequency (n=5 mice/group). Data are represented as mean ± SEM.

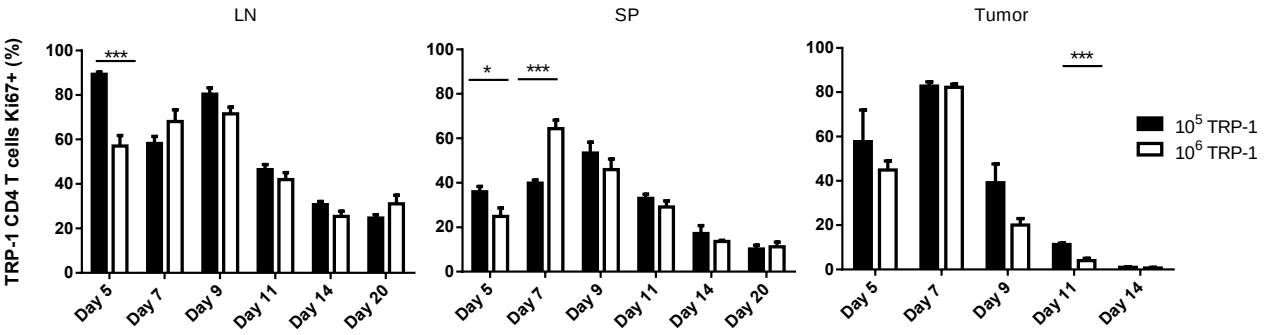


Figure 3.16. Higher levels of proliferation as measured by Ki67 are sustained at lower precursor frequencies.

C57BL/6J mice were implanted with 1 x 10^5 B16 tumor cells. Fourteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 10^5 or 10^6 TRP-1 CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. On indicated days the expression of Ki67 by the TRP-1 CD4+ T cell population was evaluated in the lymph node, spleen, and tumor (n=5 mice/group). Data are represented as mean ± SEM.

3.2.2. Intraclonal competition of tumor specific CD4+ T cells does not preclude a successful anti-tumor immune response

We then quantified the anti-tumor response to B16 melanoma, by measuring tumor growth over time in mice reconstituted with variable numbers of TRP-1 CD4+ T cells. In other reports utilizing irradiated or immunodeficient mice, it had been demonstrated that TRP-1 CD4+ T cells were able to mediate potent anti-tumor immunity (Quezada et al. 2010; Xie et al. 2010). For this reason, it was important to begin with a large established tumor that had been allowed to grow for 14-18 days, until the initial size at adoptive transfer was approximately 1 cm. These animals then received sub-lethal irradiation to eliminate their existing immune repertoire and were reconstituted via adoptive transfer of naïve polyclonal splenocytes supplemented with varied frequencies (0, 10^3, 10^5, 10^6, 10^7) of the TRP-1 specific CD4+ T cell clone of interest. The total cell transfer quantity was fixed to 30 million cells, ensuring that the only manipulated

variable is the number of TRP-1 CD4+ T cells. Tumor growth was tracked every 3 to 5 days with caliper measurements.

In groups where no TRP-1 specific CD4+ T cells were transferred and at the lowest precursor frequency of 10^3 TRP-1 CD4+ T cells, little anti-tumor immunity and no complete tumor regressions were observed (Figure 3.17A). However, when the frequency was increased to 10^5 T cells, a bi-modal response occurred and 30-60% of tumors were eradicated. When frequency was further increased to levels where intraclonal competition was most apparent, at a clonal abundance of 10^6 and 10^7 TRP-1 CD4+ T cells, the greatest anti-tumor response was observed with 100% tumor rejection and no incidents of recurrence (Figure 3.17B). These results demonstrated that the TRP-1 specific CD4+ T cell model could resolve dramatic differences in anti-tumor immunity mediated by varying the clonal abundance of tumor specific T cells. More importantly, these findings were in direct contrast to results obtained in other models, in which high clonal abundance impaired the immune response against foreign pathogen and tumors (Foulds and Shen 2006; Rizzuto et al. 2009). The disparate findings suggested that there was a unique mechanism contributing to the disparities between the models, one that warranted intense investigation and is the focus of Chapter Four.

3.2.3. Tumor regression mediated by high precursor frequencies of TRP-1 CD4+ T cells confers long lasting protective immunity

In the previous experiment it was determined that TRP-1 CD4+ T cells were capable of regressing large established tumors when present at a high clonal abundance. This resulted in the long-term survival of 100% of the mice in the group possessing TRP-1 specific CD4+ T cells at a clonal abundance of 10^6; alternatively, in the group possessing a clonal abundance of 10^5 TRP-1 CD+ T cells, only 40% of the mice survived (Figure 3.17B). To examine whether the surviving animals had developed long-lasting anti-tumor immunity, mice were re-challenged with B16 melanoma to assess if they

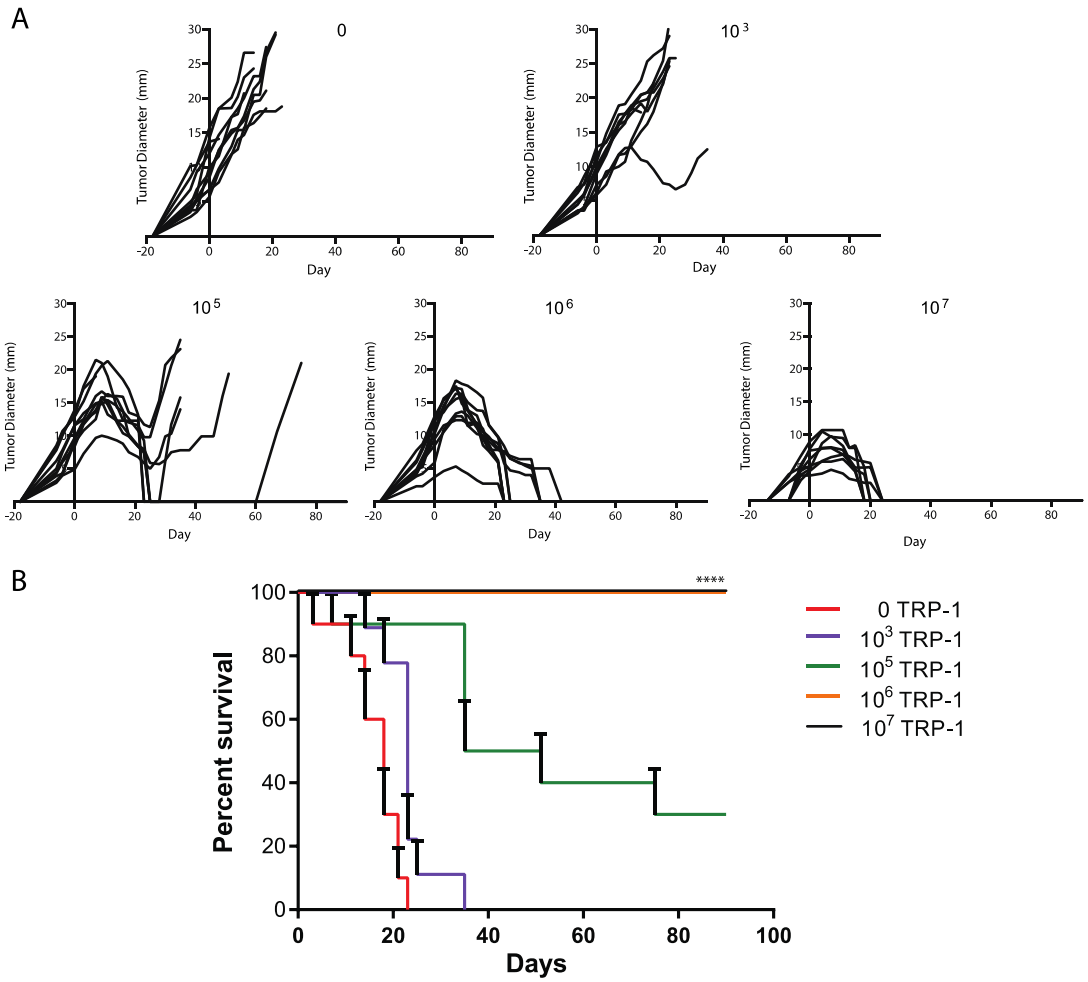


Figure 3.17. Precursor frequency of self-antigen specific CD4+ T cells dictates anti-tumor immunity.

C57BL/6J mice were implanted with 1 x 10^5 B16 tumor cells. Eighteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 0, 10^3, 10^5, 10^6, or 10^7 TRP-1 CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. (A) Tumor diameter was measured every 3-5 days by caliper and is represented on the graph by individual lines. (B) The overall survival of each group was plotted. Mice were euthanized when tumor diameter reached 3 cm. Representative data of at least 3 separate experiments (n= 9-10 mice per group).

could reject subsequent tumor development. The kinetics of initial tumor regression were heterogeneous among the animals, so it was not possible to standardize the time between tumor regression and tumor re-challenge. However, all mice were re-challenged 5 months after the initial adoptive transfer of TRP-1 CD4+ T cells and as a control a naïve cohort of mice was implanted with tumor. The naïve mice and mice possessing an initial clonal abundance of 10^5 TRP-1 CD4+ T cells showed no protection against tumor re-challenge (Figure 3.18A,B). However, over 50% of mice that had initially received the higher clonal abundance of 10^6 TRP-1 CD4+ T cells were protected from tumor implantation. This long-lived immunity was likely not mediated directly by the TRP-1 CD4+ T cells, which were undetectable in the blood prior to and after re-challenge (Figure 3.18C). One explanation could be that antigen released during tumor eradication lead to the development of tumor specific antibodies, which conferred protection upon re-challenge. Another possibility, which is investigated in later sections, is the development of a CD8+ T cell anti-tumor response facilitated through the process of epitope spreading.

3.2.4. Vaccination does not improve the TRP-1 CD4+ T cell anti-tumor response at low clonal abundance

Although TRP-1 specific CD4+ T cells present at a high clonal abundance were capable of mediating a successful anti-tumor response without other immunologic intervention, we wanted to evaluate whether the addition of vaccination could improve the responses of TRP-1 CD4+ T cells present at low clonal abundance. Mice bearing established B16 tumors were irradiated and received adoptive transfer of 0, 10^3 or 10^5 TRP-1 CD4+ T cells and naïve splenocytes. One cohort from each group that had received TRP-1 CD4+ T cells, in addition to a vaccine only control group, were vaccinated against full length TRP-1 via gene gun beginning on the day following

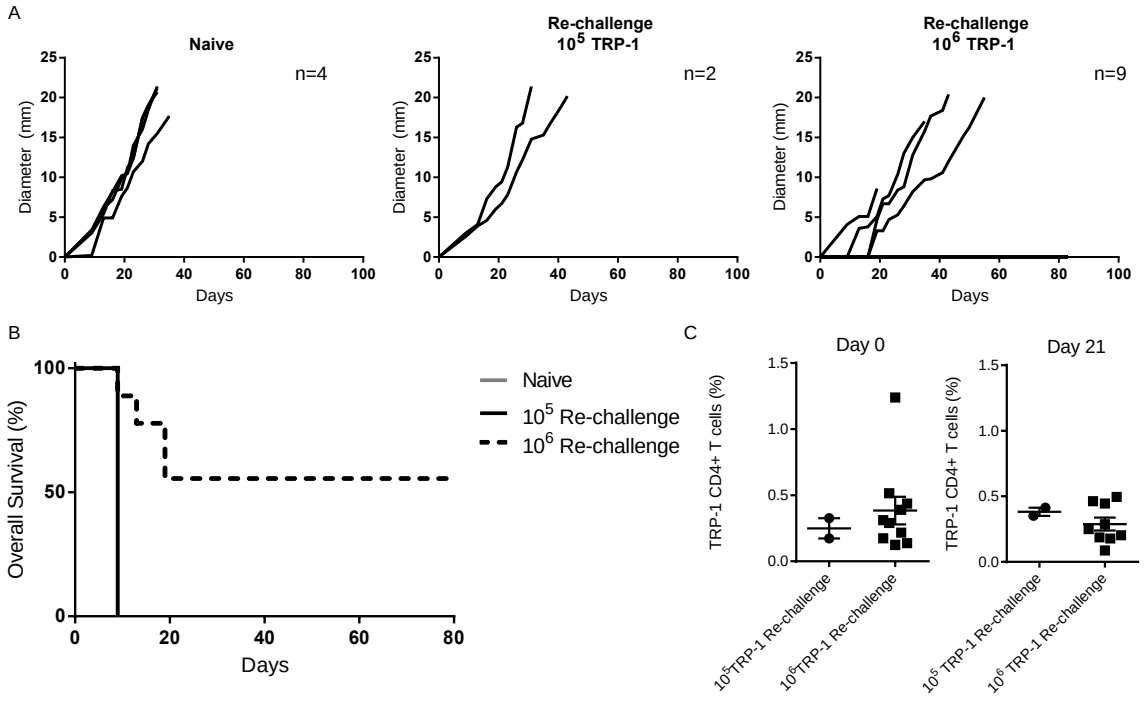


Figure 3.18. Tumor regression mediated by high precursor frequencies of TRP-1 CD4+ T cells confers long lasting protective immunity.

Surviving C57BL/6J mice from the previous experiment (Figure 3.17) that had undergone tumor regression and control naïve mice were re-challenged with 1 x 10^5 B16 tumor cells 5 months after the initial adoptive transfer. (A) Tumor diameter was measured every 3-5 days by caliper and is represented on the graph by individual lines. (B) The overall survival of each group was plotted. Mice were euthanized when tumor diameter reached 2.5 cm. (C) On days indicated TRP-1 CD4+ T cells were detected by flow cytometry as a percentage of total CD4+ T cells within peripheral blood (n= 2-9 mice per group as indicated). Data are represented as mean ± SEM.

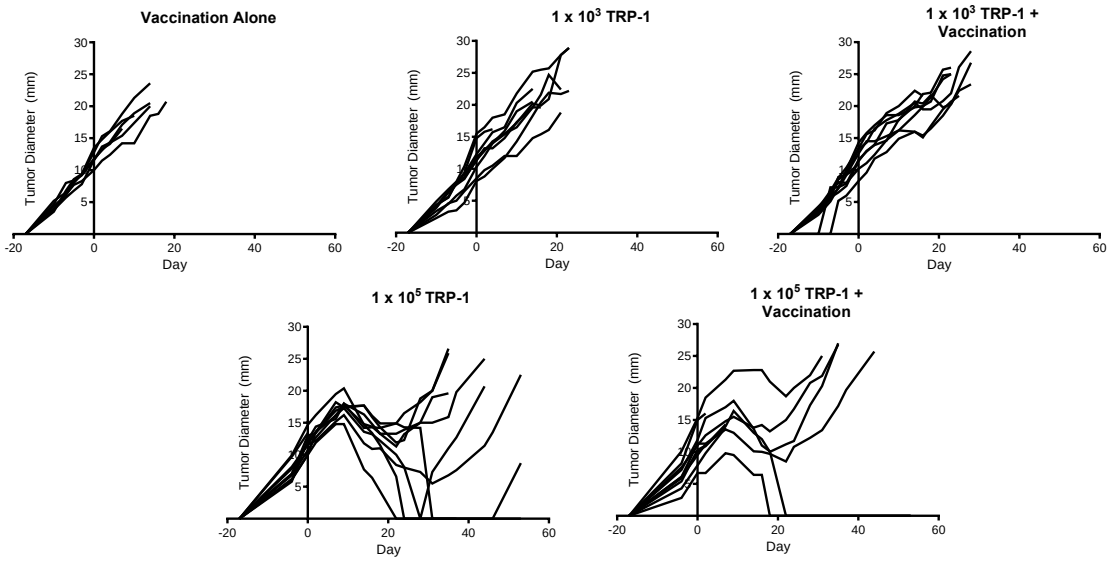


Figure 3.19. Vaccination does not improve the TRP-1 CD4+ T cell anti-tumor response at low clonal abundance.

C57BL/6J mice were implanted with 1 x 10^5 B16 tumor cells. Eighteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 0, 10^3, or 10^5 TRP-1 CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. On the day following adoptive transfer and every 7 days for 3 doses in total, the indicated cohorts of mice were vaccinated via gene gun with plasmid DNA encoding TRP-1. Tumor diameter was measured every 3-5 days by caliper and is represented on the graph by individual lines. Mice were euthanized when tumor diameter reached 2.5 cm (n= 9-10 mice per group).

adoptive transfer and continuing every seven days for three weeks. No improvement in anti-tumor immunity was observed in any of the groups receiving the additional vaccination step (Figure 3.19). These findings indicated that the reduced anti-tumor immunity observed when tumor specific T cells are at low clonal abundance was not due to a deficiency in *in vivo* activation or insufficient antigen exposure, but was occurring through another mechanism, which we determined to be T cell exhaustion and is detailed more completely in Chapter Five.

3.2.5. TRP-1 CD4+ T cells are sufficient to mediate anti-tumor responses in the absence of adaptive immunity

Other reports utilizing TRP-1 CD4+ T cells have maintained that these T cells act as the primary effectors of the anti-tumor response, as opposed to engaging other arms of adaptive immunity (Quezada et al. 2010; Xie et al. 2010). Taking into consideration that the TRP-1 CD4+ T cells were likely not directly responsible for the long-lived immunity observed after re-challenge of the tumor regressors (Figure 3.18), we questioned the ability of the co-transferred open repertoire splenocytes to contribute to the anti-tumor immune response. To explore this, TRP-1 CD4+ T cells were transferred at varied frequencies (0, 10^3, 10^5, 10^6) into irradiated tumor bearing hosts, with and without the co-transfer of open repertoire splenocytes. Due to the smaller tumor size upon adoptive transfer, the observed tumor regression was skewed, but re-capitulated the previously observed trend. We found that in mice receiving adoptive transfer of TRP-1 CD4+ T cells co-transferred with open repertoire splenocytes, tumor growth was slowed (Figure 3.20). Conversely, when splenocytes were transferred without TRP-1 CD4+ T cells, some tumors showed faster growth. This was possibly due to the restoration of suppressive cell subsets, such as regulatory T cells and myeloid suppressor cells that had been removed during irradiation. Although the impact of removing splenocytes from the

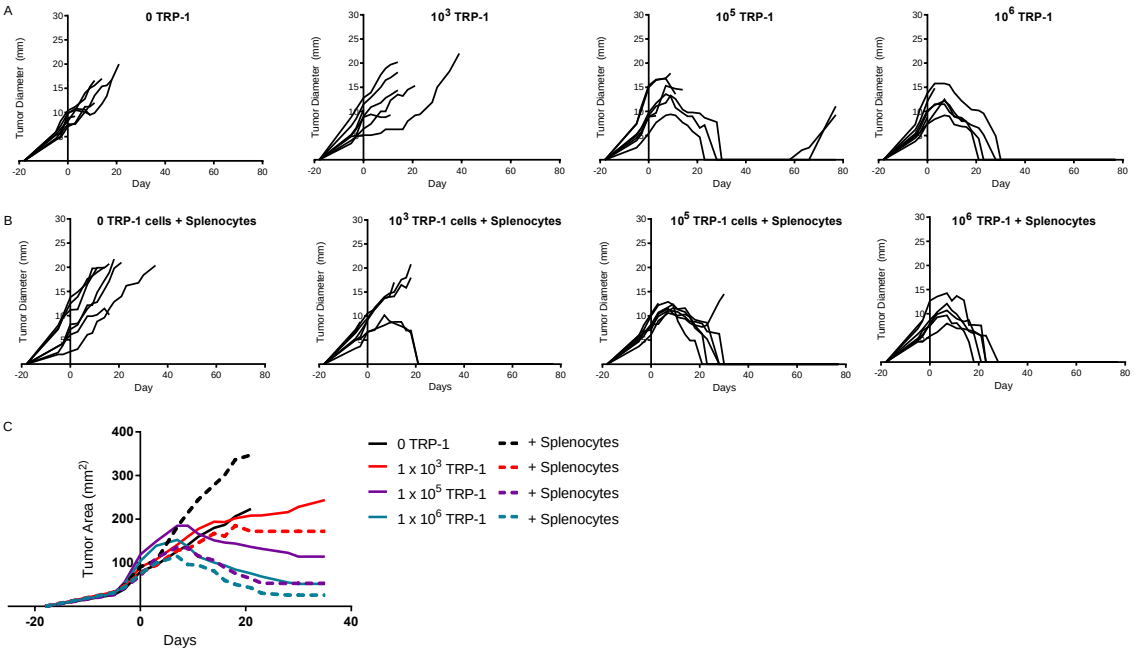


Figure 3.20. Although unnecessary, open repertoire splenocytes contribute to TRP-1 CD4+ T cell mediated anti-tumor immunity.

C57BL/6J mice were implanted with 1 x 10^5 B16 tumor cells. Eighteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 0, 10^3, 10^5, or 10^6 TRP-1 CD4+ T cells. Indicated cohorts received either (A) No co-transferred splenocytes or (B) Co-transfer with open repertoire splenocytes to bring the total transfer quantity to 30 x 10^6 cells. Tumor diameter was measured every 3-5 days by caliper and is represented on the graph by individual lines. (C) Measurements were squared and the composite mean tumor area of each group is represented on the graph by individual lines. Mice were euthanized when tumor diameter reached 2 cm (n= 5-7 mice per group).

co-transfer was subtle, the results suggested that other immune cells may act during the anti-tumor response and encouraged further investigation.

To more exhaustively determine the contribution of the endogenous adaptive immune system we transferred varied quantities (0, 10^3, 10^5, 10^6, 10^7) of TRP-1 specific CD4+ T cells without the co-transfer of splenocytes into RAG deficient hosts. Mice that lack RAG recombinase fail to undergo V(D)J recombination and do not develop mature B or T cells (Shinkai et al. 1992). In the groups where TRP-1 CD4+ T cells were at a suboptimal clonal abundance (10^3 and 10^5) to achieve complete tumor regression in wildtype animals, more robust initial anti-tumor responses were observed in RAG deficient animals (Figure 3.21). This may be due to the lack of endogenous regulatory T

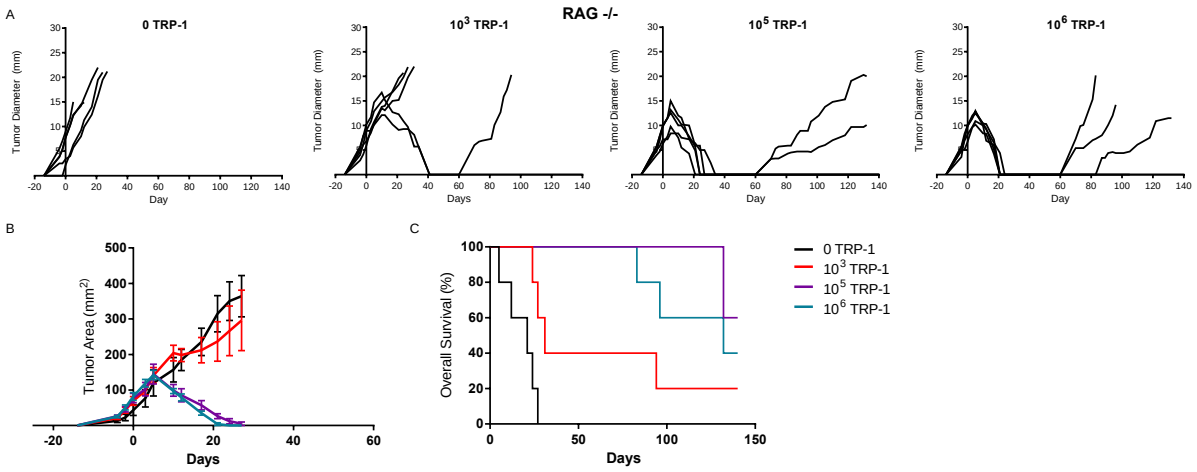


Figure 3.21. TRP-1 CD4+ T cells act as singular mediators of initial tumor regression, but fail to prevent long-term tumor recurrence.
RAG deficient C57BL/6J mice were implanted with 1 x 10^5 B16 tumor cells. Eighteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 0, 10^3, 10^5, or 10^6 TRP-1 CD4+ T cells with no co-transferred splenocytes. (A) Tumor diameter was measured every 3-5 days by caliper and is represented on the graph by individual lines. (B) Measurements were squared and the composite mean tumor area of each group is represented on the graph by individual lines. (C) The overall survival of each group was plotted. Mice were euthanized when tumor diameter reached 2 cm. Representative data of at least 3 independent experiments (n= 5 mice per group). Data are represented as mean ± SEM.

cells in the RAG knockout mice. Alternatively, the profound lymphopenia may allow for improved proliferation of the TRP-1 CD4+ T cells, due to the lack of homeostatic cytokine sinks, which would result in greater accumulation of the T cells present at lower precursor frequencies. These hypotheses are explored further in Section 4.7, which examines the effector phase of the TRP-1 CD4+ T cell response, and Section 6.5, which investigates the contribution of regulatory T cells. As in wildtype animals, TRP-1 CD4+ T cells mediated complete tumor regression when at high clonal abundance (10^6). These results validated TRP-1 CD4+ T cells as the primary mediators of the initial anti-tumor response.

However, unlike what was observed in wildtype animals, some tumors that had experienced complete regression re-emerged after a period of latency lasting around 40 days. It was possible that the tumor regrowth observed in the RAG knockout animals was due to the emergence of antigen loss variants. The theory of cancer immunoediting describes the equilibrium period as a latent period in which the immune system dynamically constrains tumor growth; escape occurs when less immunogenic tumor variants arise due to immunologic selection. Antigen loss has been previously described following immunotherapy and in this model selective pressure resulting in the loss of TRP-1 expression would allow tumor to evade immune detection by the TRP-1 CD4+ T cells (Yee et al. 2002). To examine if loss of TRP-1 expression could account for the escape tumors, these tumors were isolated from the RAG deficient mice and adapted into a cell line. Both PCR and intracellular staining of the tumor cells were used to evaluate TRP-1 expression. However, all escape variants continued to express TRP-1, indicating that antigen loss was not responsible for the tumor recurrence (Figure 3.22A,B).

During the normal immune response to acute infection after a pathogen is eliminated and antigen no longer persists, the adaptive immune response undergoes a phase of contraction. A majority of the responding antigen specific effector T cells die and self-renewing memory T cells persist to produce a rapid recall response to a

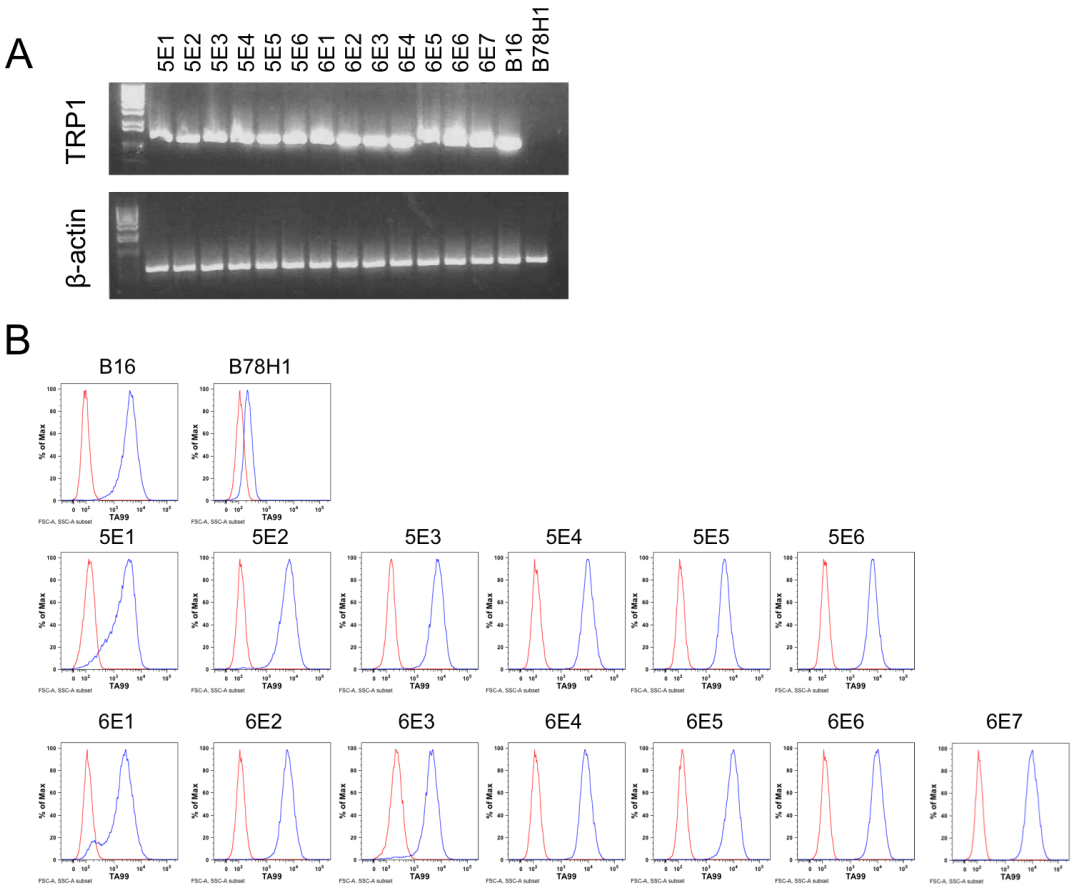


Figure 3.22. Antigen loss variants are not responsible for the tumor escape observed in RAG deficient mice.

Cell lines were established from tumors that had re-emerged after a period of initial regression in RAG deficient mice following transfer of 10^5 (5E) or 10^6 (6E) TRP-1 CD4+ T cells. (A) RNA isolated from the tumor cell lines was made into cDNA and PCR was used to detect TRP-1 expression. (B) TRP-1 expression was evaluated in tumor cell lines by flow cytometry via intracellular staining for TRP-1 using the antibody TA99. In both experiments B16 was used as a positive control and B78H1, which does not express TRP-1, provided a negative control.

secondary challenge. It is possible that the TRP-1 CD4+ T cells had undergone defective memory T cell development, and experienced an impaired recall response to tumor regrowth, due to either alterations in T cell activation or differentiation caused by the profound lymphopenia of the RAG deficient host or the lack of bystander cells. Other groups that have directly investigated tumor recurrence in RAG deficient hosts attribute tumor growth to the accumulation of regulatory and exhausted TRP-1 CD4+ cells (Goding et al. 2013). Their conclusions align with the idea of dysfunctional T cell memory generation.

The tumor recurrence observed in RAG deficient mice calls into question observations made during the investigation of long-term immunity in wildtype hosts. Considering that no evidence of a TRP-1 CD4+ T cell recall response was observed upon tumor re-challenge of wildtype mice that had previously regressed tumors (Figure 3.18), TRP-1 CD4+ T cells may undergo intrinsically restrained memory differentiation, or development of exhaustion, regardless of environment. To examine the possibility of deficient memory, the development of memory was characterized in wildtype tumor bearing hosts that had received adoptive transfer of 10^5 or 10^6 TRP-1 specific CD4+ T cells. In a previous experiment assessing absolute numbers of TRP-1 CD4+ T cells over time, we had found that after the resolution of the immune response the T cells had undergone a profound contraction independent of initial precursor frequency (Figure 3.15). Of the remaining TRP-1 specific T cells at later time points, most exhibited an effector memory phenotype, with low expression of CD62L, suggesting that they were a migratory population that has low proliferative potential but a high immediate effector response to a secondary challenge (Figure 3.23)(Masopust and Picker 2012). TRP-1 CD4+ T cells from the more clonally abundant population were skewed more towards this phenotype, which implies that there is more diversity in the memory population of T cells from lower precursory frequencies, a trait that is beneficial for the development of a complex immune response. Considering the low abundance of central memory T cells, it

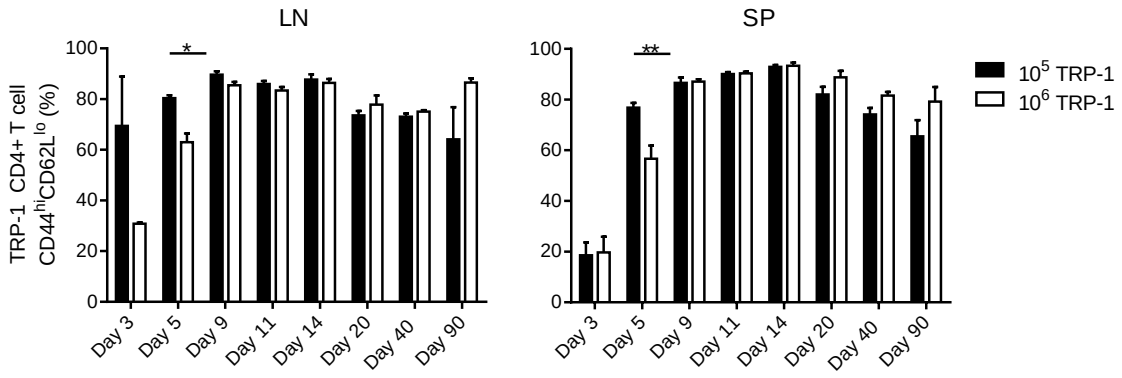


Figure 3.23. Within the limited subset of TRP-1 CD4+ T cells persisting in the lymphoid organs after retraction most display an effector memory phenotype. C57BL/6J mice were implanted with 1 x 10^5 B16 tumor cells. Fourteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 10^5 or 10^6 TRP-1 CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. On indicated days the expression of CD44 and CD62L by the TRP-1 CD4+ T cell population was evaluated in the lymph node and spleen (n=5 mice/group). Data are represented as mean ± SEM.

becomes more understandable why little expansion of the TRP-1 subset was observed upon tumor re-challenge (Figure 3.18). Unless the site of the tumor challenge itself was directly probed it would be difficult to attribute protection to the effector memory subset of TRP-1 CD4+ T cells. However, due to the profound contraction and effector memory phenotype of the TRP-1 CD4+ T cells, we cannot exclude the possibility that protection against a secondary encounter may be dependent upon the development of sufficient humoral and CD8+ T cell immunity. The latter hypothesis will be discussed further in Sections 3.2.7 and 3.2.8, which examine the helper potential of the TRP-1 CD4+ T cell population.

3.2.6. Tumor rejection is dependent upon TRP-1 CD4+ T cell interaction with MHC-II

CD4+ T cells are restricted to recognizing antigen presented in the context of major histocompatibility complex-II (MHC-II). We utilized mice deficient in MHC-II to probe how TRP-1 CD4+ T cells are mediating tumorcidal activity and to explore whether

direct target recognition is necessary in our model. Tumor bearing MHC-II deficient hosts received adoptive transfer of TRP-1 CD4+ T cells at varied clonal abundance (0, 10^5, and 10^6) accompanied by co-transfer of MHC-II deficient splenocytes. Higher clonal abundances were used to increase the likelihood of tumor regression. In the MHC-$II^{-/-}$ model, antigen presentation to the TRP-1 CD4+ T cells is abolished in the periphery, which would result in defective activation and homeostatic proliferation. B16 tumor is derived from a mouse replete in MHC-II expression, so recognition of antigen presented directly by the tumor is unaffected. However, to disrupt direct recognition of tumor cells, cohorts of mice received blocking antibody against the MHC-II molecule utilized by the TRP-1 CD4+ T cells, IA^b, or an IgG control. In IgG control recipients, where target recognition was intact, TRP-1 CD4+ T cells were capable of mediating tumor regression (Figure 3.24). In recipients receiving TRP-1 CD4+ T cells and blockade of IA^b on the tumor, tumor regression was diminished. One tumor had regressed in each group receiving blocking antibody, however in these instances regression began after day 21, when treatment with blocking antibody had ceased. Interestingly, some spontaneous tumor regression was also observed in groups that had not received TRP-1 CD4+ T cells; this was attributed to the adoptive transfer of CD8+ T cells into hosts that lack regulatory T cells due to the MHC-II deficiency. These findings demonstrated that direct recognition of the tumor cells through antigen presentation by MHC-II, was required for the TRP-1 CD4+ cell anti-tumor response.

3.2.7. TRP-1 CD4+ T cells initiate CD8+ T cell epitope spreading

TRP-1 CD4+ T cells were capable of initiating effective anti-tumor immunity in the absence of other components of the adaptive immune system. Although evident that CD8+ T cells are unnecessary for the primary tumor regressions observed in this model, we wanted to evaluate the potential of the TRP-1 CD4+ T cells to augment the anti-tumor CD8+ T cell response. Similar to the OT-II model, we first wanted to investigate the

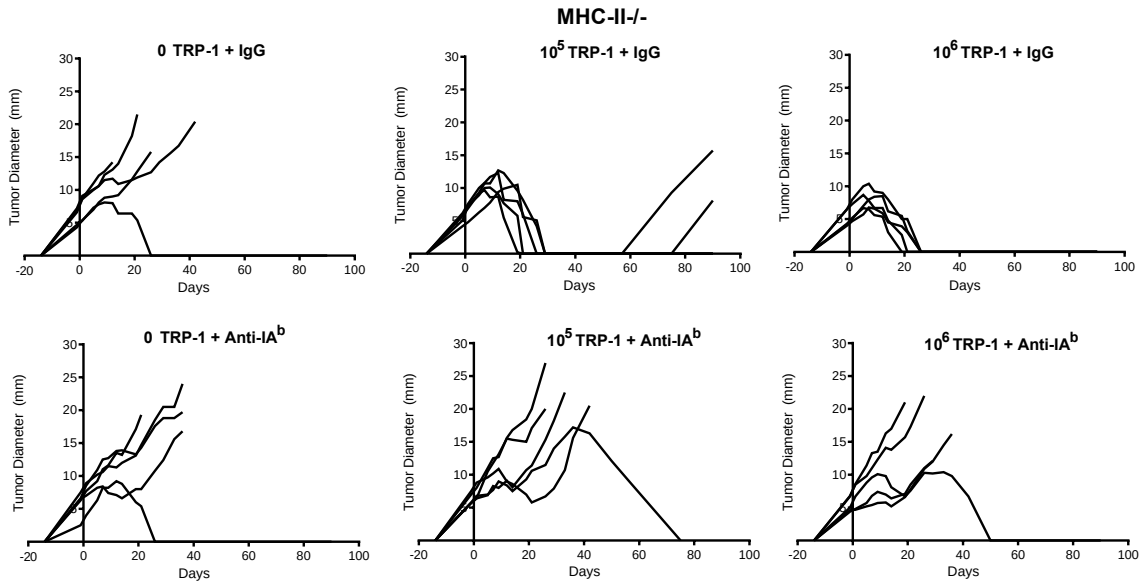


Figure 3.24. Tumor rejection is dependent on TRP-1 CD4+ T cell interaction with MHC-II.

MHC-II deficient C57BL/6J mice were implanted with 2.5 x 10^5 B16 tumor cells. Eighteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 0, 10^5, or 10^6 TRP-1 CD4+ T cells with co-transferred splenocytes derived from MHC-II deficient mice. Mice received intra-peritoneal injection of 200 μg of an MHC-II (IA^b) blocking antibody or an IgG control beginning on the day prior to adoptive transfer and every other day until 21 days post-transfer. Tumor diameter was measured every 3-5 days by caliper and is represented on the graph by individual lines. Mice were euthanized when tumor diameter reached 2.5 cm. Representative data of at least 3 independent experiments (n= 5 mice per group)

occurrence of epitope spreading after adoptive transfer of TRP-1 CD4+ T cells. Mice bearing an established B16 melanoma received adoptive transfer of 10^5 TRP-1 CD4+ T cells and naïve splenocytes. On day 28 after adoptive transfer, mice were sacrificed to characterize the melanoma specific CD8+ T cell response. At this time point it appears that the tumor had not yet fully regressed in the treated animals; however this can be attributed the use of Matrigel as a tumor scaffold in these injections (Figure 3.25). Due to the misleading residual tumor plug that remains and the technical difficulties associated with injection, this was the only experiment in which Matrigel was used. When the reactivity of CD8+ T cells to different melanoma antigens was evaluated, as previously described, we found a high occurrence of epitope spreading. The most dominant responses were against the melanoma differentiation antigen TRP-2, which were accompanied by some weak responses against gp100. This observation is similar to the one made in the OT-II model, in which unvaccinated mice responded primarily to TRP-2 and supports TRP-2 as the dominant melanoma antigen recognized by CD8+ T cells during a CD4+ T cell mediated anti-tumor response (van Elsas et al. 2001). It is also interesting that almost no reactivity against the CD8+ epitope of TRP-1 was observed. This demonstrates that a CD4+ T cell response against a target antigen will not necessarily cause a CD8+ T cell response against the same target.

To determine if clonal abundance impacted the extent of epitope spreading we repeated the previous experiment, but varied the quantities of TRP-1 CD4+ T cells the B16 tumor bearing hosts received. We chose to look for epitope spreading on day 21 instead of day 28, because mice that do not regress their tumors in the groups receiving low precursor frequencies of TRP-1 CD4+ T cells are likely to die before this time point. Unfortunately, this made the results difficult to interpret, as the extent of epitope spreading in the group that received 10^5 TRP-1 CD4+ T cells was less dramatic than that observed at day 28 (Figure 3.26). Similar to the previous experiments the most dominant response was against the TRP-2 CD8+ T cell epitope; however, unlike previous epitope

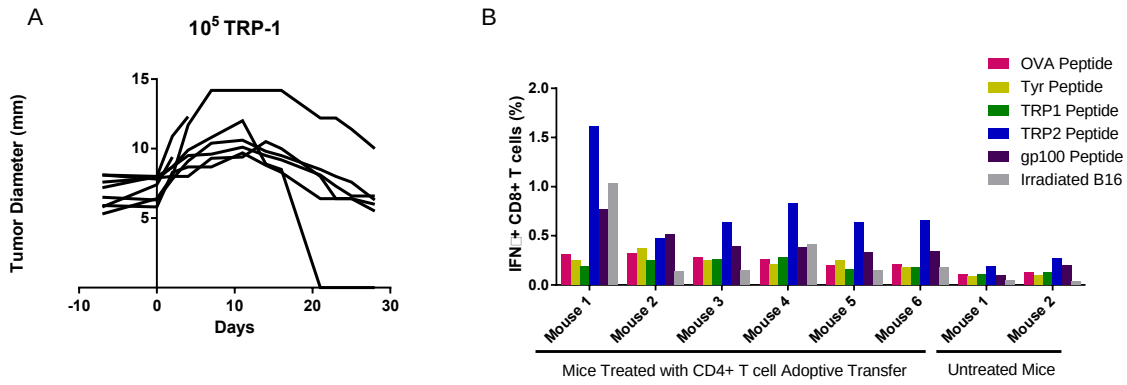


Figure 3.25. A high occurrence of epitope spreading is observed in mice receiving TRP-1 CD4+ T cells.

C57BL/6J mice were implanted with 1 x 10^5 B16 cells resuspended in Matrigel tissue scaffold. Fourteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 1x10^5 TRP-1 CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. (A) Tumor diameter was measured every 3-5 days by caliper and is represented on the graph by individual lines. (B) On day 28 following adoptive transfer, mice were euthanized and spleen was isolated. Single cell suspensions were restimulated with peptides representative of the CD8+ immunodominant epitopes of melanoma differentiation antigens. Intracellular cytokine staining for IFNγ after 5 hours was used to assess antigen reactivity (n = 2-6 mice/group).

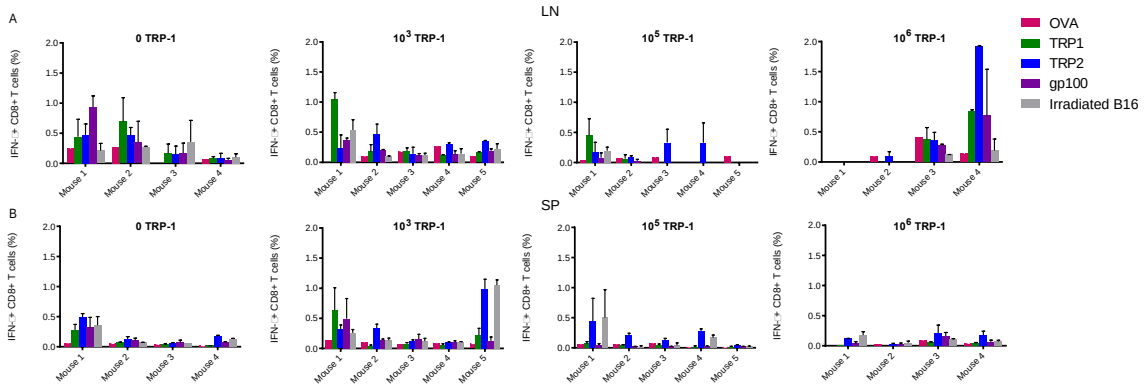


Figure 3.26. Clonal abundance of TRP-1 CD4+ T cells is not correlated with the degree of epitope spreading.

C57BL/6J mice were implanted with 1 x 10^5 B16 tumor cells. Fourteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 0, 10^3, 10^5 or 10^6 TRP-1 CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. On day 21 following adoptive transfer, mice were euthanized and tumor draining lymph node and spleen were isolated. Single cell suspensions were restimulated with peptides representative of the CD8+ immunodominant epitopes of melanoma differentiation antigens in duplicate. Intracellular cytokine staining for IFNγ after 5 hours was used to assess antigen reactivity. (A) Epitope spreading as assessed in the lymph node. (B) Epitope spreading as assessed in spleen (n = 4-5 mice/group). Data are represented as mean ± SEM.

spreading experiments some responses against the TRP-1 CD8+ T cell epitope were also observed. There did not appear to be any clear correlation between clonal abundance and the degree of epitope spreading, although this is arguable and may be attributed to the insufficient time allowed for epitope spreading due to the tumor mediated fatality.

While we did not further investigate the mechanism responsible for the epitope spreading observed we believe that there are two likely contributing factors. One is that CD4+ T cell mediated tumor destruction releases antigen and increases the presentation of CD8+ epitopes. The other is T cell help provided to the CD8+ T cells in the form of antigen presenting cell maturation and cytokine production. Thorough investigation of this hypothesis would be complicated by the need to separate T cell help from tumor antigen release, but could be accomplished within this model system through the use of genetic knockouts targeting T cell help and bi-lateral tumors experiments addressing the need for tumor death. Regardless of the mechanism, TRP-1 CD4+ T cells induced CD8+ T cell responses in tumor bearing mice that may augment the initial tumor regression and contribute to the formation of a memory response.

3.2.8. TRP-1 CD4+ T cell help does not moderate Pmel-1 CD8+ T cell intraclonal competition

We were interested in further examining the ability of the TRP-1 CD4+ T cells to reinforce a CD8+ T cell response by performing a co-transfer experiment. We chose to use the pmel-1 TCR transgenic mouse, specific for gp100, which has been well characterized in our laboratory and by others. Additionally, by using the pmel-1 CD8+ T cell model we were able to address an unanswered question from the previous study investigating CD8+ T cell intraclonal competition (Rizzuto et al. 2009). Can CD4+ T cell help alter the threshold at which CD8+ T cell intraclonal competition is observed? While the exact mechanism of intraclonal competition has yet to be completely elucidated, it is widely believed that competition for antigen during engagement with

antigen presenting cells is at least partly responsible (Kedl et al. 2000; Quiel et al. 2011; Smith, Wikstrom, and Fazekas de St. Groth 2000; Willis, Kappler, and Marrack 2006). If CD4+ T cell mediated maturation of antigen presenting cells increases the presentation of CD8+ T cell epitopes it may result in an increase in the threshold at which intraclonal competition manifests.

To address these questions, 10^5 TRP-1 CD4+ T cells were co-transferred with varied quantities (0, 10^3, 10^5, 10^6) of pmel-1 CD8+ T cells into mice bearing established B16 tumors. This quantity of TRP-1 CD4+ T cells was used because it produced sub-optimal tumor regression (~50%) and had demonstrated CD8+ T cell epitope spreading (Figure 3.25). In contrast to the previous study investigating the effects of clonal abundance on pmel-1 CD8+ T cells, we excluded the vaccination step to evaluate the ability of the TRP-1 CD4+ T cells to induce activation of the CD8+ T cells *in vivo*. We found that vaccination was unnecessary for the synergistic increase in anti-tumor efficacy observed when TRP-1 CD4+ T cells were co-transferred with pmel-1 CD8+ T cells (Figure 3.27). It was striking that the threshold at which intraclonal competition was observed, 10^6 pmel-1 CD8+ T cells, and the frequency with the greatest anti-tumor efficacy, 10^5 pmel-1 T cells, coincided with what was observed both in the pmel-1 experiments and the previously described OT-I/OT-II co-transfer experiment (Figure 3.11) (Rizzuto et al. 2009). Additionally when pmel-1 CD8+ T cell memory formation was examined, it also mirrored previous observations, with the highest percentage of the population becoming $CD44^{hi}CD62L^{lo}$ memory T cells in the group at the lowest clonal abundance (Rizzuto et al. 2009). We can conclude that CD4+ T cell help does not impact CD8+ T cell intraclonal competition, however tumor antigen release through tumor killing is important. These findings support observations made in Quiel et al., in which the maturation of dendritic cells by Flt-3 ligand and increased quantities of antigen showed no impact on the occurrence of competition as measured by factor of expansion, but instead modified the amplitude of the effect (Quiel et al. 2011). Further research will

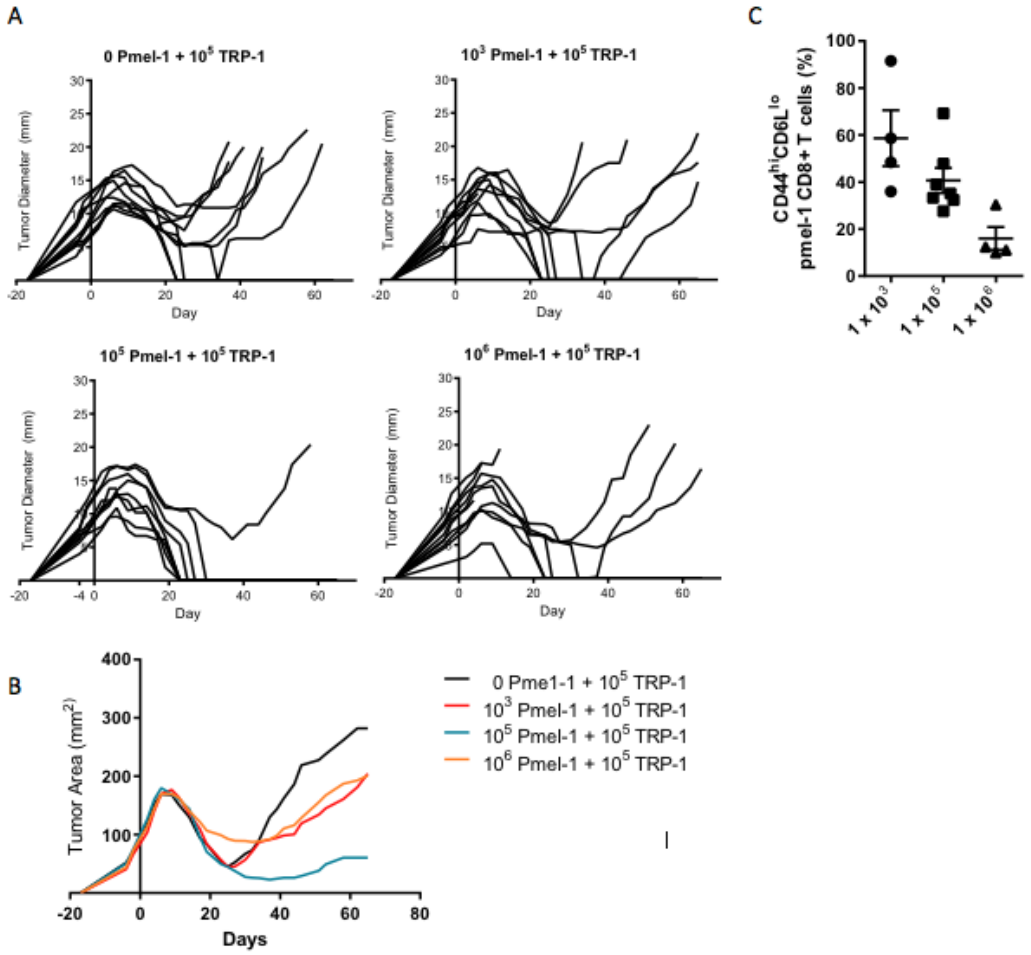


Figure 3.27. Co-transfer of TRP-1 CD4+ T cells with pmel-1 CD8+ T cells does not mitigate CD8+ T cell intraclonal competition.
C57BL/6J mice were implanted with 1 x 10^5 B16 tumor cells. Fourteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of the indicated number of pmel-1 CD8+ T cells co-transferred with 10^5 TRP-1 CD4+ T cells and naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. (A) Tumor diameter was measured every 3-5 days by caliper and is represented on the graph by individual lines. (B) Measurements were squared and the composite mean tumor area of each group is represented on the graph by individual lines. Mice were euthanized when tumor diameter reached 2 cm (n= 9-10 mice per group). (C) On day 100 after adoptive transfer spleen was isolated from the remaining mice (n= 4-7) and flow cytometry was used to assess pmel-1 CD8+ T cell memory formation. Data are represented as mean ± SEM.

be required to identify the physiological reason behind the impaired response observed when CD8+ T cells surpass the recurrent threshold of 10^5, including the possibility that it is reflects the entirety of MHC-I availability, the point at which steric hindrance prevents CD8+ T cells from accessing MHC-I molecules, or the optimal abundance to prevent identical clones from joining the response.

CHAPTER 4: HIGH CLONAL ABUNDANCE OF TUMOR SPECIFIC CD4+ T CELLS FAVORS DEVELOPMENT OF EFFECTOR FUNCTION

Introduction

The constrained activation and proliferation observed within the high precursor frequency population of self antigen specific CD4+ T cells is consistent with observations made in models investigating foreign antigen specific CD4+ T cells and our previous findings regarding self-antigen specific CD8+ T cells. However, in the TRP-1 CD4+ T cell model the impaired proliferative capacity was uncoupled from the generation of an efficacious anti-tumor immune response. One of the primary stipulations of Burnet's theory of clonal selection is that rare clones expand upon encounter with their cognate antigen; it is following this activation event that these clones undergo robust division, but also transition from a naïve to an effector state.

In contrast to CD8+ T cells, CD4+ T cells are a very heterogeneous population. CD4+ T cells are involved in many aspects of the immune response, including providing help to B cells during antibody production, supporting the responses of CD8+ T cells, activating myeloid cells, and behaving in a regulatory capacity. To accomplish this diverse array of functions, CD4+ T cells differentiate into a number of specialized T helper phenotypes following activation. Development into each specific T helper lineage is dependent on the cytokine milieu encountered during activation, which reinforces the expression of lineage specific master regulatory transcription factors. A number of T helper lineages have been defined and evaluated for anti-tumor efficacy. TRP-1 CD4+ T cells have been demonstrated to mediate highly effective anti-tumor responses following *in vitro* differentiation into Th17 cells (Muranski et al. 2008). However, these T cells showed considerable plasticity following transfer and acquired more of a Th1 phenotype *in vivo*. Thus, for our purposed we primarily examined cytotoxic responses and effector function consistent with the Th1 lineage.

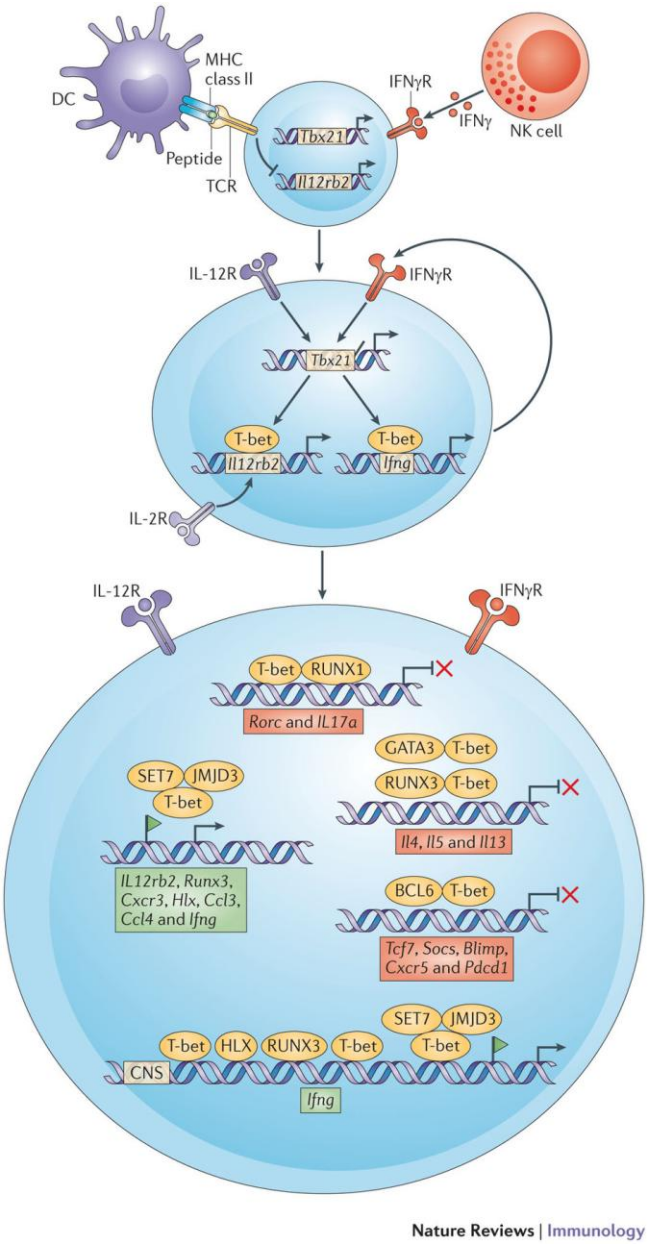


Figure 4.1. Th1 differentiation is regulated through expression of T-bet and signaling via IFNγ and IL-12.

T cell receptor signaling in combination with transient signaling through the IFN-γ receptor induces the expression of the Th1 lineage master transcription factor T-bet. Following cessation of TCR signaling, T-bet initiates IL-12RB2 expression and Th1 differentiation transitions from IFN-γ dependence to being sustained by IL-12. Adapted from Lazarevic, Glimcher, and Lord 2013.

Th1 differentiation begins upon the initial encounter with antigen (Figure 4.1). T cell receptor signaling in combination with transient signaling through the IFN-γ receptor induces the expression of the Th1 lineage master transcription factor T-bet. Following cessation of TCR signaling, T-bet initiates IL-12RB2 expression and Th1 differentiation transitions from IFN-γ dependence to being sustained by IL-12 (Lazarevic, Glimcher, and Lord 2013). Signaling through STAT4 maintains T-bet expression, which acts in concert with Runx3 and HLX to maintain optimal IFNγ expression. T-bet not only regulates genes required for the Th1 fate, but actively suppresses differentiation into Th17 cells and Th2 cells through chromatin modification and gene silencing and by sequestering transcription factors, such as GATA3. The ability of effector T cells to secrete cytokines has long been linked to their progression through the cell cycle (Bird et al. 1998). However, in these experiments T cells were used at physiologic frequencies and all had been allowed to progress through the cell cycle. Interestingly, it is more common for this strict linkage between division and differentiation in the CD4+ subset to break down when precursor frequency exceeds physiological levels; some T cells remained un-activated yet were capable of producing IFNγ (Laouar and Crispe 2000). It has also been described that large antigen doses can overcome barriers to initial activation at high precursor frequencies and it is possible that this is occurring in our model due to the ubiquitous expression of the cognate antigen (Catron et al. 2006).

One of the most important ways in which CD4+ and CD8+ T cells differ is in how they recognize antigen. CD8+ T cells primarily recognize intracellular antigens that are presented on MHC-I molecules, which are ubiquitously expressed but also frequently downregulated by tumors during immune evasion. Although CD8+ T cells can recognize exogenous antigen on antigen presenting cells through a process called cross-presentation, this capacity is restricted to specialized cells. CD4+ T cells predominantly recognize exogenous antigens that are phagocytosed, processed, and presented principally by professional antigen presenting cells. Priming via an antigen presenting

cells is advantageous because antigen recognition is accompanied by co-stimulation and tertiary cytokine signals instructing T helper differentiation. While tumors can be directly recognized by CD4+ T cells through the upregulation of MHC-II in response to inflammation eradication of tumor that does not express MHC can be orchestrated by CD4+ T cell recruitment and activation of innate lymphocytes (Mumberg et al. 1999).

In this chapter, we further examine how the generation of anti-tumor immunity was uncoupled from the deficiencies arising from a supra-physiologic precursor frequency by thoroughly examining the development of T cell effector function. We demonstrate that high clonal abundance of tumor-specific CD4+ T cells facilitate increased tumor infiltration and generation of effector function. We show that the improvement in T cell effector function that occurs when TRP-1 specific T cells are present at a high clonal abundance occurs not only on a population level, but also through an increase in the tumor killing capacity on a per cell basis as measured by the killing co-efficient *k*. This cell intrinsic increase in cytotoxicity is associated with increased expression of Th1 differentiation markers, including the transcription factor T-bet (*TBX21*). We demonstrate that the increased Th1 polarization observed is dependent on TRP-1 CD4+ T cell derived IFN-γ and also correlates with improved dendritic cell maturation and increased MHC-II upregulation within the tumor. Upon neutralization of IFN-γ, effector cytokine production, T-bet (*TBX21*) expression, and cytotoxicity are reduced in the lymph node. However, a reciprocal and paradoxical increase in effector function is observed in the tumor. The enhanced TRP-1 effector function as a result of IFN-γ neutralization does not result in any improvement in anti-tumor efficacy and is not associated with decreased apoptosis. It may instead reflect a transient reversal of IFN-γ mediated negative feedback loops through secondary mediators, expression of inhibitory ligands, or regulation of IFN-γ receptor expression.

Results and Discussion

4.1. Clonal abundance regulates the infiltration and regulatory T cell composition of the TRP-1 CD4+ T cell population

In order to determine how the differential accumulation of self-antigen specific T cells might impact the anti-tumor response, tumor infiltration of the TRP-1 CD4+ T cells was evaluated. At days 7, 9, and 11 the absolute number of TRP-1 CD4+ T cells infiltrating the tumor was quantified and determined to be greater in the cohort receiving 10^6 compared to 10^5 precursors (Figure 4.2A). The increased tumor infiltration was validated in a separate experiment, which utilized TRP-1 specific CD4+ T cells expressing a luciferase reporter. Following adoptive transfer of the luciferase reporter T cells into tumor bearing hosts, mice were imaged over the entirety of the immune response and the radiance of the tumor infiltrating TRP-1 specific CD4+ T cells was quantified. Higher radiance was observed in the tumor when the TRP-1 CD4+ T cells were at greater clonal abundance (Figure 4.3). Additional characterization of the tumor infiltrating TRP-1 CD4+ T cells revealed that lower precursor frequencies gave rise to a population containing a higher proportion of TRP-1 specific Foxp3+ regulatory T cells (Figure 4.2B). The increased abundance of regulatory T cells in these conditions resulted in an unfavorable effector to regulatory T cell ratio compared to the higher precursor frequency group (Figure 4.2C). The intra-tumor balance of effector to regulatory T cells, as measured by their ratio, can be correlated with development of anti-tumor immunity, with a higher ratio favoring a more efficacious response (Quezada et al. 2006).

4.2. Small changes in the kinetics of TRP-1 CD4+ T cell tumor infiltration differentiate tumors undergoing regression versus progression

Although the differences in tumor regression observed in mice containing the highest clonal abundance of TRP-1 CD4+ T cells could be partly attributed to increased tumor infiltration, it remained unclear why a bi-modal response was occurring when

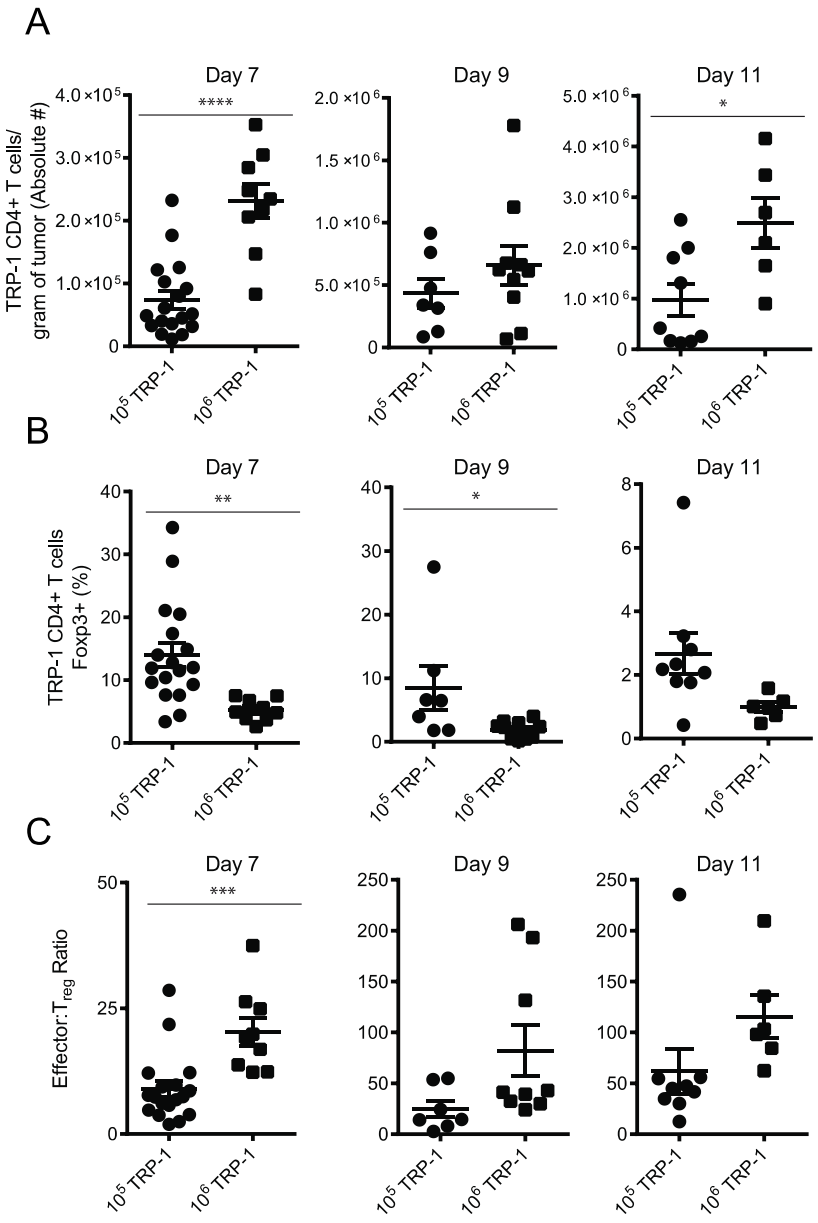


Figure 4.2. Clonal abundance regulates the infiltration and regulatory T cell composition of the TRP-1 CD4+ T cell population.

C57BL/6J mice were implanted with 1 x 10^5 B16 tumor cells. Fourteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 10^4, 10^5, or 10^6 TRP-1 CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. (A) Absolute number of TRP-1 CD4+ T cells infiltrating the tumor on indicated days as determined by flow cytometry quantification and and normalizing to tumor mass. (B) Percentage of Foxp3+ T cells within tumor infiltrating TRP-1 CD4+ T cell population. (C). Ratio of absolute number of TRP-1 CD4+ T cell effectors to absolute number of TRP-1 CD4+ regulatory T cells. Composite data of 3 independent experiments. Data are represented as mean ± SEM.

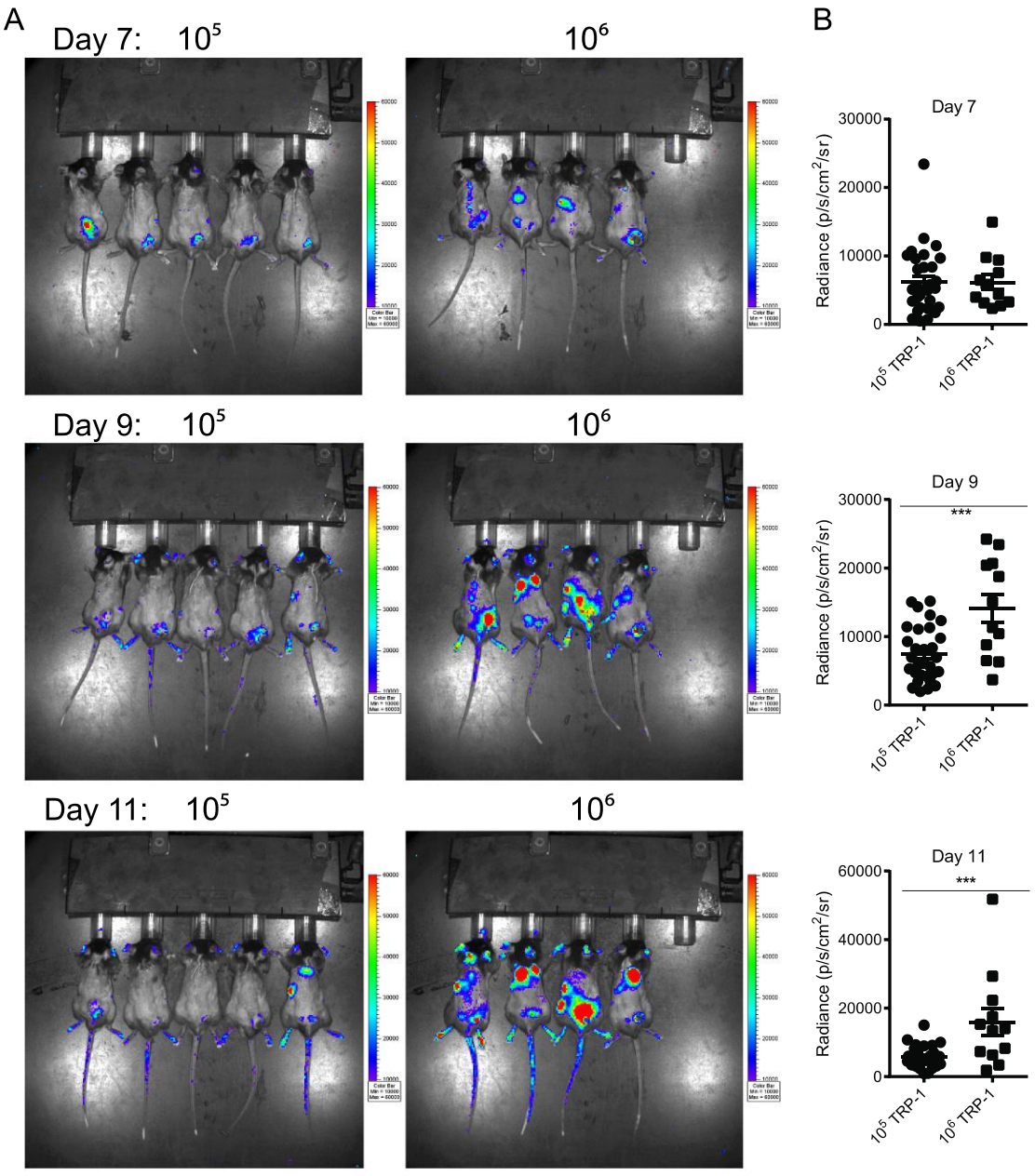


Figure 4.3. During the peak of the anti-tumor immune response T cell infiltration into the tumor is greatest when TRP-1 CD4+ T cells are present at high clonal abundance.

10^5 or 10^6 TRP-1 CD4+ T cells expressing a luciferase reporter were adoptively transferred I.V. into C57BL6 mice bearing 14 day old B16 melanoma tumors. Total cell quantity transferred was normalized to 30 x 10^6 per mouse using naïve splenocytes. On days 7, 9, and 11 mice were injected with D-luciferin substrate and imaged on an IVIS 200. (A) Representative images. (B) Summary of radiance quantified within the tumor region of interest. (n = 10-15 mice/group). Data are represented as mean ± SEM.

clonal abundance was reduced to 10^5. Within the two populations that arise from the 10^5 and 10^6 precursor frequencies, depending on the attributes examined, there is considerable phenotypic overlap. These intersects may be used to explain the variation observed in tumor response arising from a clonal abundance of 10^5, which would result in better identification of the vital factors underlying tumor regression. To identify a truly predictive element, tumor-bearing mice must be followed until the response outcome is determined and immune correlates retrospectively determined and prospectively validated. However, the methods of characterizing the immune response in living mice are limited. To address whether differences in the infiltration of TRP-1 CD4+ T cells into the tumor could account for the observed bi-modal response we used TRP-1 CD4+ T cells expressing a luciferase reporter to image tumor infiltration *in vivo*. Tumor bearing hosts received TRP-1 CD4+ T cells at a clonal abundance of 10^6 or 10^5 co-transferred with open repertoire splenocytes. These two frequencies were examined in order to provide a comparison of the tumor infiltration observed in mice that had undergone tumor regression at both higher and lower clonal abundances. Within the cohort of 30 mice receiving TRP-1 CD4+ T cells at a clonal abundance of 10^5, a true bi-modal distribution occurred with fourteen tumors experiencing regression and sixteen tumors continuing to progress. Tumor infiltration was semi-quantitatively determined by measuring the radiance of the T cells within the tumor region of interest. When infiltration was compared between the two groups only subtle differences were observed. The kinetics of infiltration within all tumors undergoing regression, regardless of the initial precursor frequency of TRP-1 CD4+ T cells, were similar with the response reaching the peak mean radiance at day 13 and contracting following that time point (Figure 4.4A). Conversely, tumors that had progressed showed an early peak in infiltration, with most mice experiencing peak infiltration on days 7 or 9, compared to the more even distribution of peak infiltration at later time points in the tumors undergoing regression (Figure 4.4C). Although the kinetics showed a small variance, the peak

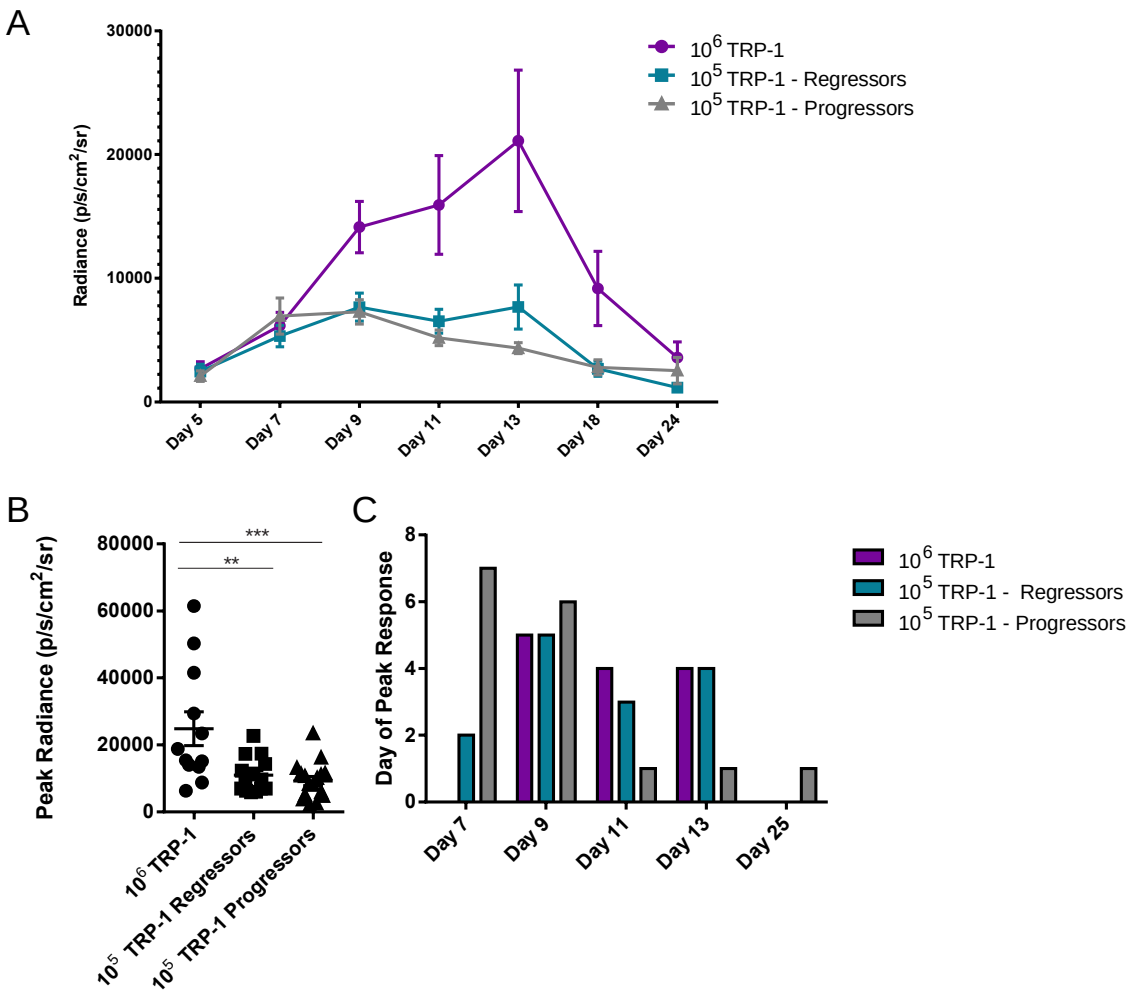


Figure 4.4. Small differences in the kinetics of TRP-1 CD4+ T cell tumor infiltration were observed in tumors undergoing regression versus progression.
C57BL/6J mice were implanted with 1 x 10^5 B16 tumor cells. Eighteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 10^5 or 10^6 luciferase expressing TRP-1 CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. (A) On the indicated days, mice were imaged on the IVIS and tumor infiltration as measured by radiance was determined in the area of the tumor. The cohort receiving 10^5 TRP-1 CD4 T cells was split into two groups on the basis of tumor regression and mean radiance of each group was plotted (10^6 n =13, 10^5 regressors n=14, 10^5 progressors n=14). (B) The peak radiance of each individual mouse was plotted for each group to quantify changes in response amplitude. (C) The number of mice achieving their peak response as measured by radiance at each time point plotted. Data are represented as mean ± SEM.

radiance of each response showed no significant difference between the progressor and regressor groups (Figure 4.4B).

It remains unclear how the early infiltration of TRP-1 CD4+ T cells may contribute to an impaired anti-tumor immune response. Previous experiments have established that regulatory T cells are at the highest percentage within the intra-tumor TRP-1 CD4+ T cell population at early time points, specifically day 7, and that this percentage decreases as the anti-tumor response progresses (Figure 4.2). We speculate that early infiltration of high numbers of regulatory T cells may establish an immunosuppressive environment within the tumor, which would contribute to the early decline of TRP-1 CD4+ T cell accumulation in the tumor. Other studies of regulatory T cell kinetics within the B16F10 tumor model implicate the early emergence of regulatory T cells in the creation of a tolerogenic environment and attenuated immunity (Darrasse et al. 2009). We had previously established that the phenotype of TRP-1 CD4+ T cells within peripheral blood did not accurately recapitulate what was observed in either lymph node or tumor (data not shown), so it has not been possible to characterize the regulatory T cell population within the tumor and correlate it with response outcome in imaging experiments. The development of reagents that would allow the manipulation and characterization of Foxp3+ TRP-1 CD4+ T cells, such as a regulatory T cell specific reporter mouse line, would be necessary for the examination of this hypothesis.

4.3. At high clonal abundance tumor specific CD4+ T cells differentiate into polyfunctional effector cells

In other models of CD8+ and CD4+ T cell competition, the failure to mount an effective immune response was correlated with the impaired development of polyfunctional effector T cells (Foulds and Shen 2006; Rizzuto et al. 2009). To investigate whether intraclonal competition had affected the differentiation of the tumor specific CD4+ T cells, we focused on day 7 during the initiation of the anti-tumor

response. We chose to examine the earliest phase of the response because variability increases at later stages due to the heterogeneous kinetics of regression and levels of tissue necrosis. T cells were harvested from tumors and draining LNs, re-stimulated *ex vivo* and assessed for the production of effector cytokines IFNγ and TNFα, as well as the expression of granzyme B, given the cytotoxic nature of TRP-1 CD4+ T cells (Muranski et al. 2008; Quezada et al. 2010; Xie et al. 2010). In lymph nodes, tumor specific CD4+ T cells differentiated into the most potent effector T cells when their clonal abundance was highest (Figure 4.5A), and up to 25% of the population produced both IFNγ and TNFα. The tumor specific T cells at lower clonal abundance produced little IFNγ and TNFα and did not upregulate expression of granzyme B (Figure 4.5B). A reciprocal effector phenotype was observed in the tumor infiltrating TRP-1 CD4+ T cell population (Figure 4.5C,D). These results demonstrate that high clonal abundance favors generation of polyfunctional effector CD4+ T cells and that the extent of T cell differentiation established during priming in the lymph node is preserved during the effector phase.

Although extensive division may be unnecessary for the generation of effector function, we observe that the vast majority of tumor specific CD4+ T cells that have acquired effector function have also undergone proliferation (data not shown). Even though a smaller percentage of the total T cell population is activated and recruited into the response at any single time point at high precursor frequencies, when the greater cumulative size of this population is taken into account a larger number of cells absolutely are engaged.

4.4. Clonal abundance is directly correlated with killing efficiency and Th1 differentiation

While production of effector cytokines and expression of granzyme B are useful surrogates for effector function, we wanted to establish if clonal abundance influenced the development of CD4+ T cell cytotoxic function. We first examined the direct

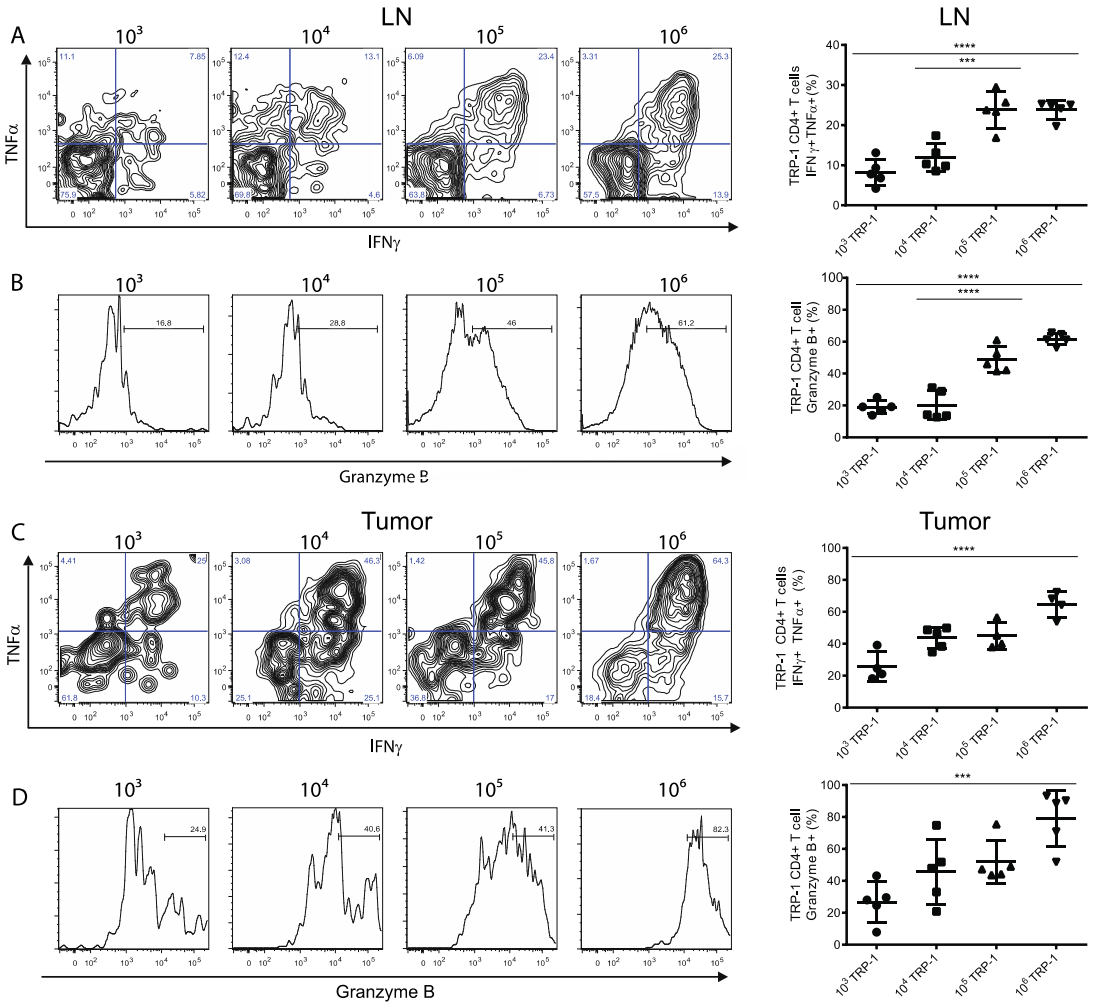


Figure 4.5. At high precursor frequencies tumor specific CD4+ T cells differentiate into polyfunctional effector cells.

C57BL/6J mice were implanted with 1 x 10^5 B16 tumor cells. Fourteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 10^3, 10^4, 10^5, or 10^6 TRP-1 CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. (A) On day 7 post-transfer lymphocytes isolated from lymph node and tumor were restimulated with PMA/ionomycin, representative flow plots of IFNγ and TNFα expression in TRP-1 CD4+ T cell population (left), summary of percentage of IFNγ+ TNFα+ of TRP-1 CD4+ T (right) in lymph node (B) Representative histogram plots of granzyme-B expression in TRP-1 CD4+ T cell population (left), summary of percentage of granzyme B producing population (right) in lymph node. (C) Representative flow plots of IFNγ and TNFα expression in TRP-1 CD4+ T cell population (left), summary of percentage of IFNγ+ TNFα+ of TRP-1 CD4+ T (right) in tumor (D) Representative histogram plots of granzyme-B expression in TRP-1 CD4+ T cell population (left), summary of percentage of granzyme B producing population (right) in tumor (n=4-5 mice/group). Data are represented as mean ± SEM.

contribution of T cell density to the killing ability of the TRP-1 CD4+ T cells through the use of a clonogenic killing assay (Budhu et al. 2010). This assay allowed us to isolate the variable of quantity alone to model how different concentrations of tumor infiltrating T cells could alter the potential for tumor regression. TRP-1 CD4+ T cells were isolated directly from TRP-1 TCR transgenic mice and were then co-embedded at varied concentrations with identical quantities of B16 melanoma cells in collagen-fibrin gels. At 24, 48, and 72 hours the gels were lysed and plated to form colony-forming units, which were counted 7 days later. We observed the greatest level of killing in the condition where the T cell concentration was the highest (Figure 4.6A). In the groups with high concentrations of TRP-1 CD4+ T cells, 10^6 and 5 x 10^6, tumor killing was sustained over time, with the best responses seen at 72 hours. However, at the lower concentrations, tumor growth began to outpace killing, and lower responses were seen at 72 hours compared to the peak killing at 48 hours (Figure 4.6B). This observation is reminiscent of what occurs *in vivo*, with some tumors slowing and showing the beginnings of regression, but ultimately returning to progressive growth. These findings implicate T cell concentration within the tumor as one of the factors that shifts that balance.

The collagen-fibrin gel based killing assay is more sensitive than a traditional chromium release assay in its measurement of tumor killing. It is also more physiologically relevant than two-dimensional killing assays, because it allows T cells to migrate naturally on a collagen matrix, thus providing a better surrogate for the actual tumor microenvironment. However, one limitation is that it is not a true cytolytic assay. The clonogenic assay measures overall cytotoxicity, which can be mediated by direct cell-to -cell killing via granzymes and perforin, but alterations in killing can also result from factors arising from differences in T cell migration, serial killing, and the accumulation of cytotoxic cytokines. In order to assess the role of cytokine accumulation, the media overlaying the gel-based killing assay was collected at 48 hours

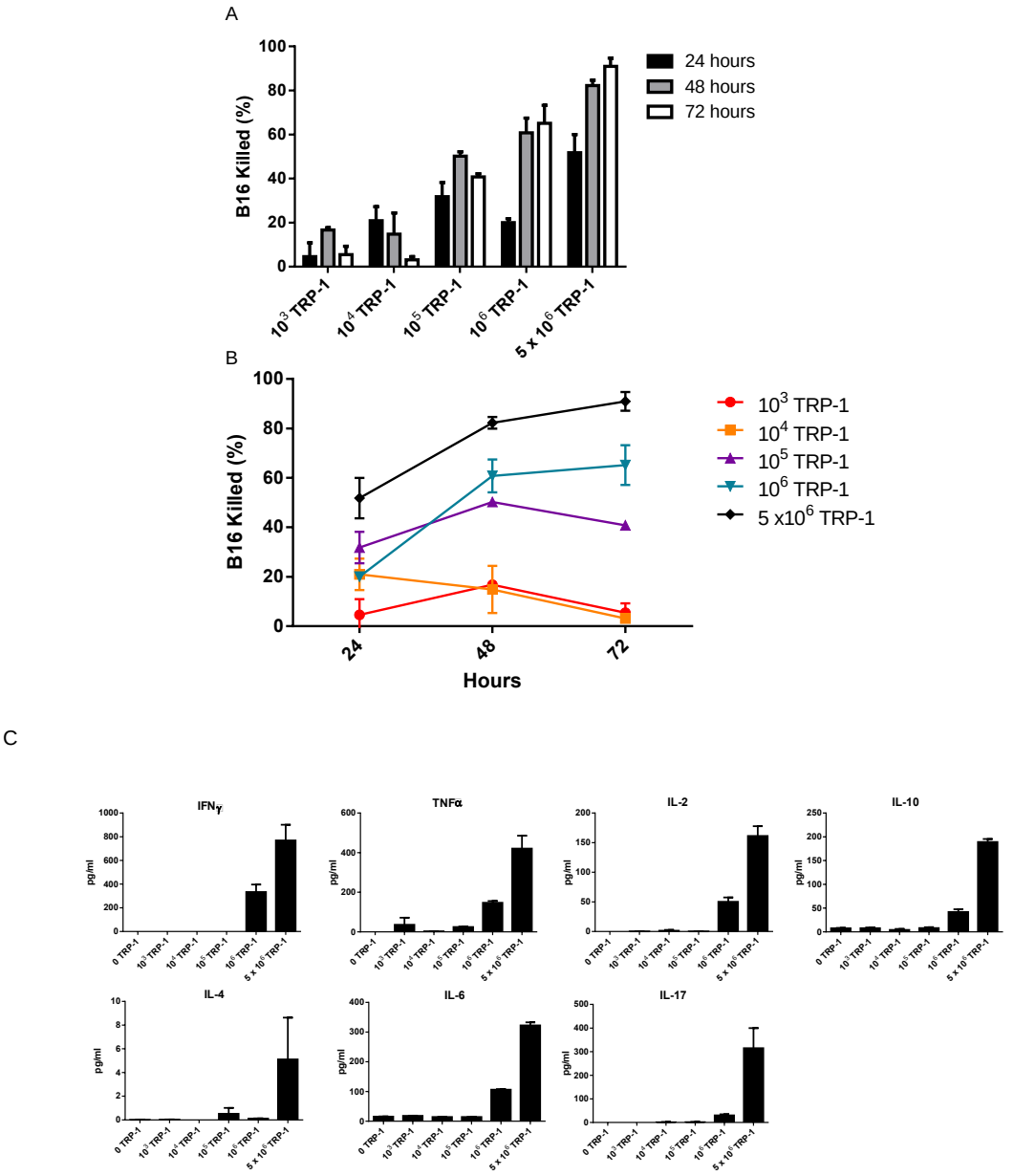


Figure 4.6. Large quantities of TRP-1 CD4+ T cells sustain high levels of B16 killing over time.

TRP-1 CD4+ T cells were isolated via MACS purification from TRP-1 TCR transgenic mice and directly co-embedded at indicated quantities with equivalent numbers of B16 targets (50,000) in a collagen fibrin gel based *ex vivo* killing assay. At 24, 48, and 72 hours gels were lysed, plated for B16 colony forming units, and killing was evaluated by staining for CFU 7 days later. (A) Percentage of B16 killed as a function of concentration (B) Percentage of B16 killed as a function of time. (C) Supernatants overlaying the gel based killing assay were collected upon gel lysis at 48 hours. Using a CBA kit, cytokine concentrations in the supernatants were quantified. Data are represented as mean ± SEM.

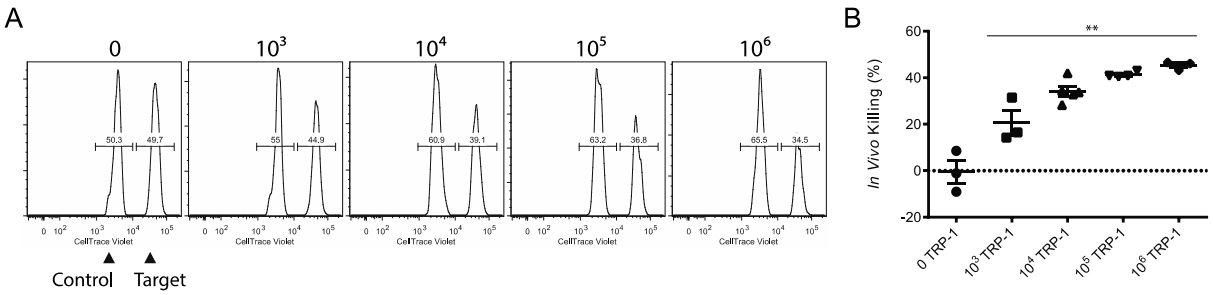


Figure 4.7. *In vivo* killing is enhanced as the clonal abundance of TRP-1 CD4+ T cells increases.
C57BL/6J mice were implanted with 1 x 10^5 B16 tumor cells. Fourteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 0, 10^3, 10^5, or 10^6 TRP-1 CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. On day 6, mice were transferred with 500,000 CellTraceViolet (CTV) labeled *in vivo* killing assay targets (250,000 unpulsed:250,000 antigen pulsed). (A) Representative flow plots of day 7 *in vivo* killing of TRP-1 peptide loaded CTV^{hi} populations compared to unloaded CTV^{lo} control population in spleen. (B) Summary of *in vivo* killing as percentage of targets killed. Data are represented as mean ± SEM.

and analyzed. All of the cytokines examined, which included IFN-γ, TNF-α, IL-2, IL-4, IL-6, IL-10 and IL-17, had the most considerable concentrations when the TRP-1 CD4+ T cells were also the most plentiful (Figure 4.6C). The concentration of IFN-γ was found to be within an active range in the highest condition, according to the EC50, making a direct effect on tumor cells possible. However, this likely manifested itself through upregulation of MHC-II on the tumor cells, rather than direct killing. We can conclude that the cytokines are not directly killing the tumor by examining the 10^5 TRP-1 CD4+ T cell concentration specifically. In the killing assay almost 50% of the co-embedded B16 tumor cells are killed however, no accumulation of cytokine is observed. Thus, these T cells are likely mediating tumor killing through direct cytolysis.

To assess how differences in the clonal abundance of TRP-1 CD4+ T cells alters the killing of targets *in vivo*, we performed an *in vivo* killing assay, in which splenocytes were divided into two populations and labeled CellTrace Violet $(CTV)^{hi}$ or CTV^{lo}. The CTV^{hi} population was loaded with the MHC-II restricted peptide recognized by the TRP-1 CD4+ T cells and the CTV^{lo} cell population was used as control. Both populations

were transferred intravenously and killing was evaluated by comparing the ratio of peptide-loaded targets to unloaded controls. Killing of targets *in vivo* increased in direct proportion to the clonal abundance of the antigen specific T cells (Figure 4.7A,B). While the *in vivo* killing assay clearly shows that a higher clonal abundance of tumor-antigen specific CD4+ T cells results in better killing of target cells, it cannot be used to differentiate the individual contributions of T cell quantity versus quality.

To examine the quality of the response by determining the impact of clonal abundance on the development of cytotoxic function on a per cell basis, we once again employed the clonogenic killing assay. On this occasion, it was used to evaluate *ex vivo* the cytotoxicity of T cells during the height of an ongoing immune response. Titrated quantities of 10^4, 10^5 or 10^6 TRP-1 CD4+ T cells with open repertoire naïve splenocytes were co-transferred into irradiated recipients bearing established B16 melanoma. On day 7, the TRP-1 CD4+ T cells were sorted directly from tumor draining LNs and tumor, and then co-embedded at identical T cell frequencies with identical numbers of B16 melanoma cells in collagen-fibrin gels. Killing percent and killing coefficient *k* were determined by comparing the killing rate of each group to the empirically determined tumor growth rate (Figure 4.8A,B). We found that the *per cell* killing capability of the effector cells increased with increasing clonal abundance of the tumor specific CD4+ T cells.

To understand the underlying factors driving this increase in effector function, we investigated candidate transcriptional regulators of cytotoxicity by RT-PCR. The expression of the master regulator of the Th1 lineage, T-bet or *TBX21*, directly correlated with the increase in killing function (Figure 4.9). Reciprocally, expression of the T-box transcription factor Eomesodermin (*Eomes*) was increased at low clonal abundances –a hallmark of terminal differentiation and exhaustion (Paley et al. 2012). Additionally, we found that *IL-12RB2* expression correlated with T-bet expression and the initial precursor frequency of the tumor specific CD4+ T cells (Figure 4.9). These findings suggest that as

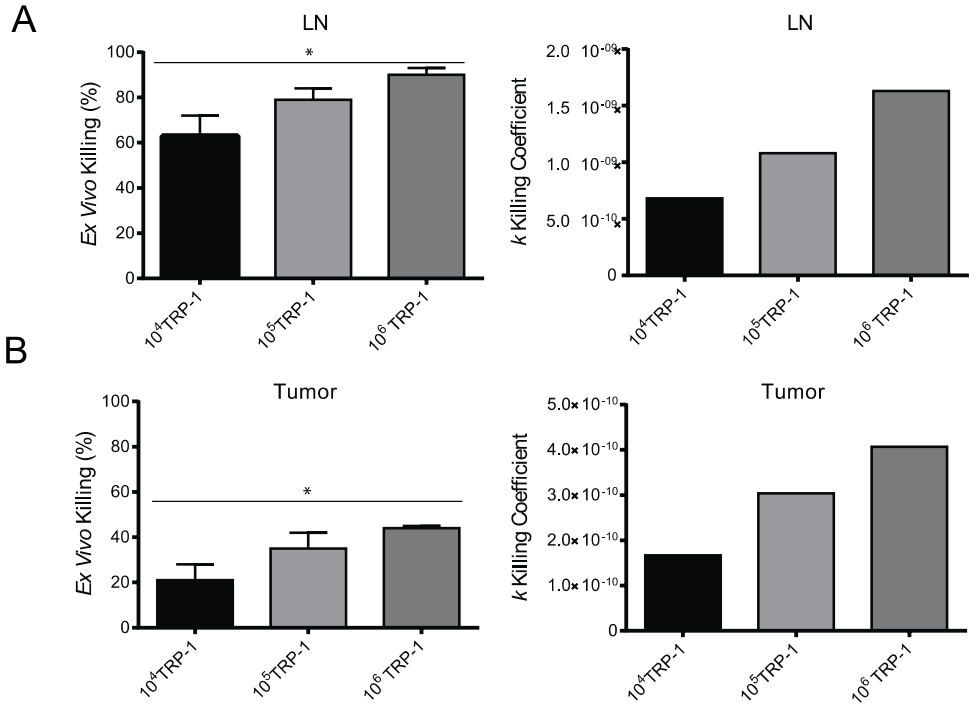


Figure 4.8. Cytotoxic potential increases on a per cell basis when TRP-1 CD4+ T cells are at a high clonal abundance.

C57BL/6J mice were implanted with 1 x 10^5 B16 tumor cells. Fourteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 10^4, 10^5, or 10^6 TRP-1 CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. On day 7, TRP-1 CD4+ T cells were sorted from LN and tumor and co-embedded with B16 tumor cells in an *ex vivo* collagen-fibrin gel based killing assay. (A) Percentage of B16 killed (left), *k* coefficient of killing was calculated using empirically determined tumor killing and growth rates (right) for TRP-1 CD4+ T cells isolated from the lymph node. (B) Percentage of B16 killed (left), *k* coefficient of killing was calculated using empirically determined tumor killing and growth rates (right) for TRP-1 CD4+ T cells isolated from the lymph node. Each condition was performed in triplicate. Data are represented as mean ± SEM.

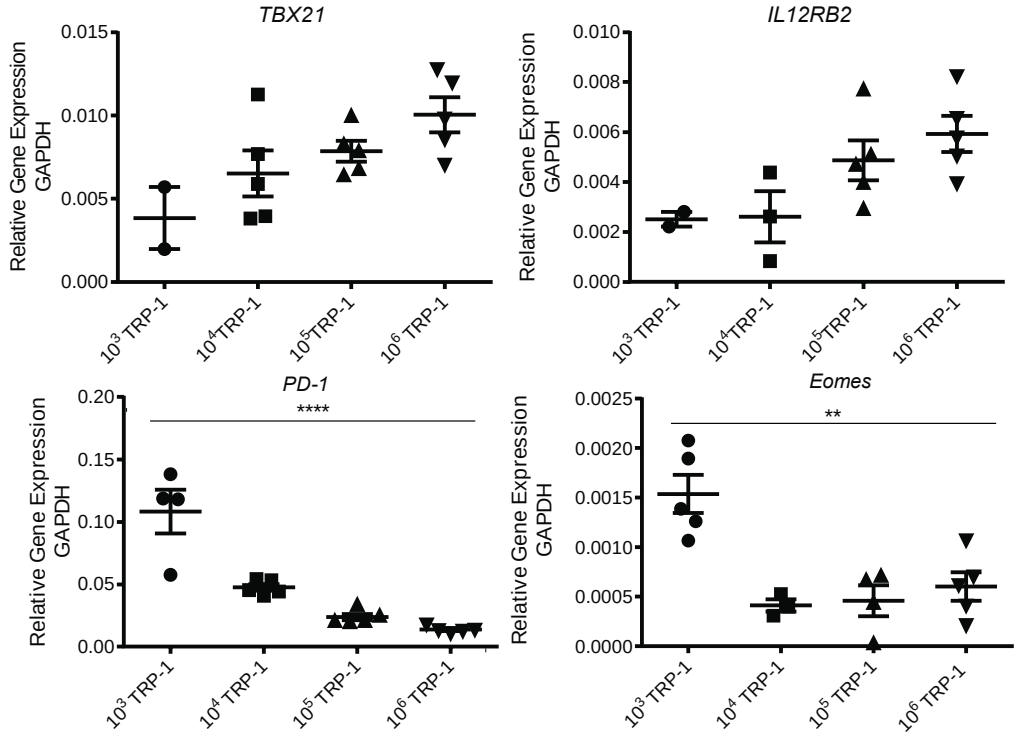


Figure 4.9. The expression of Th1 differentiation markers parallels increases in T cell clonal abundance.

C57BL/6J mice were implanted with 1 x 10^5 B16 tumor cells. Fourteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 10^3, 10^4, 10^5, or 10^6 TRP-1 CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. On day 7 post-transfer TRP-1 CD4+ T cells were sorted from lymph nodes directly into Trizol for RNA extraction. qRT-PCR analysis of targets *TBX21*, *IL-12RB2*, *PD-1*, and *Eomes* is reported as transcript relative to GAPDH (n=5 independent analytes, resulting from independent or pooled animals). Data are represented as mean ± SEM.

clonal abundance of antigen specific CD4+ T cells increases, the population more efficiently drives its own lineage commitment. We also found that PD-1 expression was inversely correlated with T-bet expression in the TRP-1 specific CD4+ T cells, which implies T-bet mediated suppression of PD-1 expression (Kao et al. 2011). The high Eomes and PD-1 expression and reduced T-bet expression occurring in T cells derived from low clonal abundances strongly suggested that the impaired development of effector function could be the consequence of T cell exhaustion, a hypothesis that is explored in further detail in Chapter Five.

4.5. Generation of polyfunctional effector phenotype is associated with T cell help and population sharing of IFN-γ

Traditionally, CD4+ T cells are known to orchestrate an immune response through the maturation of antigen presenting cells and the secretion of cytokines (Bevan 2004). We first looked for evidence of dendritic cell maturation in the lymph nodes of animals with TRP-1 specific CD4+ T cells of different precursor frequencies. In CD11b+CD11c+ dendritic cells, we observed increased expression of both CD80 and MHC-II as the frequency of tumor specific CD4+ T cells increased (Figure 4.10A) while CD86 expression was unchanged (data not shown). The increase in MHC-II expression correlated with an increased concentration of IFNγ found in lymph node extracts above the threshold of functional significance (Figure 4.10C). The cytokine IFNγ has been described to induce MHC-II expression on cells that are devoid of MHC-II at homeostasis, including B16 melanoma. This tumor specific expression has been shown to be vital to the cytotoxic function of TRP-1 CD4+ T cells (Quezada et al. 2010). We investigated if this could be a mechanism of positive feedback within the tumor. We found that MHC-II expression on both CD45- tumor cells, as well as the CD45+ lymphocytic infiltrate is highest when the tumor specific T cells are at the greatest clonal abundance (Figure 4.10B). This increase in MHC-II expression correlated with

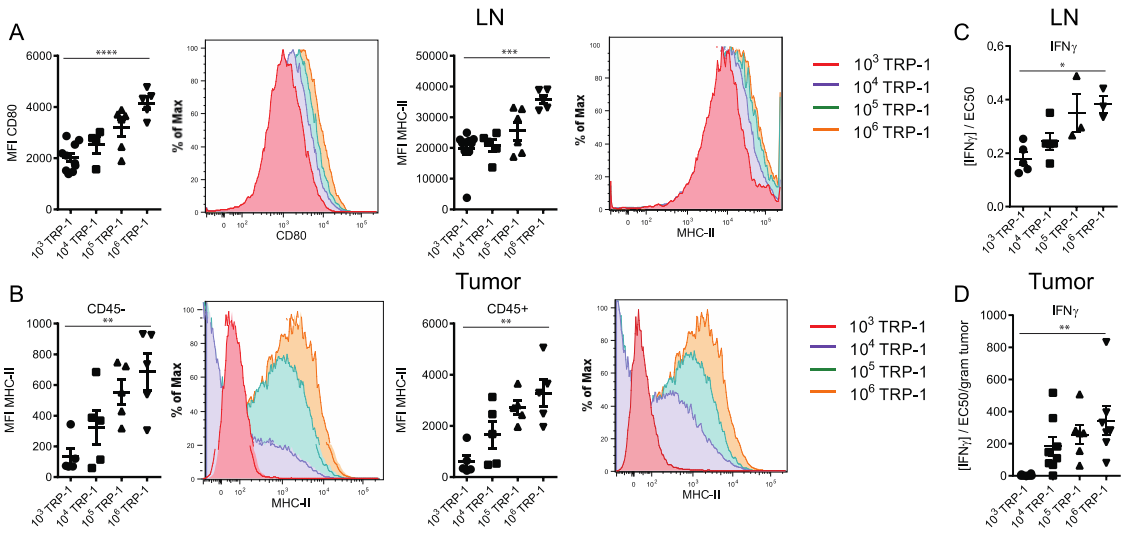


Figure 4.10. Dendritic cell maturation and MHC-II upregulation correlated with IFN-γ accumulation and clonal abundance of TRP-1 CD4+ T cells.
C57BL/6J mice were implanted with 1 x 10^5 B16 tumor cells. Fourteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 10^3, 10^4, 10^5, or 10^6 TRP-1 CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. On day 7 post-transfer (A) MFI of CD80 and MHC-II expression of lymph node CD11b+CD11c+ dendritic cells was evaluated, summary and representative histogram plots. (B) MFI of MHC-II expression on CD45- and CD45+ tumor populations, summary and representative histogram plots. (C) Quantification of IFNγ concentration in LN and (D) tumor isolates, calculated to demonstrate activity as a function of half-maximal response (n=3-5 mice/group). Data are representative of three independent experiments. Data are represented as mean ± SEM.

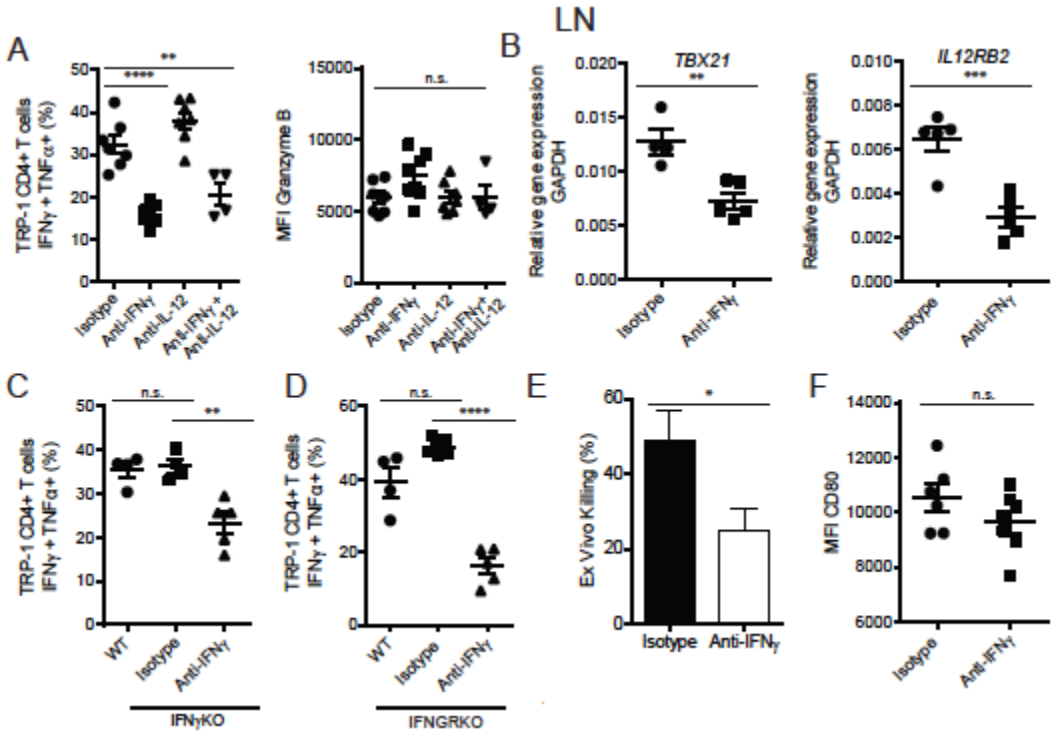


Figure 4.11. Paracrine derived IFN-γ promotes Th1 differentiation and cytotoxicity within the TRP-1 CD4+ T cell population.

C57BL/6J, IFN-γKO, or IFNGRKO mice were implanted with 2.5 x 10^5 B16 tumor cells. Fourteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 10^6 TRP-1 CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. Mice received intra-peritoneal injection of 200 μg of an antibody targeting IFN-γ, IL-12, or a mixed IgG control beginning on the day prior to adoptive transfer and every other day until lymphocyte isolation on day 7. Isolated lymphocytes from LN of treated mice were re-stimulated with PMA/ionomycin. (A) Summary of percentage of IFNγ+ TNFα+ TRP-1 CD4+ T cells, MFI granzyme-B respectively from WT mice. (B) TRP-1 CD4+ T cells were sorted from LN of treated WT mice directly into Trizol for RNA extraction and qRT-PCR analysis of targets *TBX21*, *IL-12RB2*. (C) IFNγKO mice and (D) IFNGRKO tumor bearing mice, summary of percentage of IFNγ+ TNFα+ TRP-1 CD4+ T cells. (E) TRP-1 CD4+ T cells were sorted from pooled LN of treated WT mice on day 7 and co-embedded with B16 in an *ex vivo* killing assay, percentage of B16 killed. (F) CD80 expression on LN CD11b+CD11c+ dendritic cells of WT treated mice (n=4-5 mice/group. Data are representative of three independent experiments. Data are represented as mean ± SEM.

accumulation of higher levels of IFN-γ as measured in tumor extract (Figure 4.10D). It is likely the total accumulation of activation signals that antigen presenting cells are integrating from the tumor specific T cell population, through co-stimulatory and cytokine signaling, that contributes to their increased maturation and antigen presentation. Consequently, this would reduce the threshold of activation for the tumor specific CD4+ T cell population as a whole.

IFNγ is also the canonical effector cytokine of the Th1 lineage; lineage commitment begins during T cell priming when IFN-γ signaling initiates early expression of T-bet. T-bet induction promotes expression of IL-12 receptor, which then shifts Th1 differentiation into a primarily IL-12 driven process (Lazarevic, Glimcher, and Lord 2013). To address the role of Th1 polarization in the development of effector function we sought to neutralize the cytokines IFN-γ and IL-12 *in vivo*, alone and in combination. In the two groups receiving IFN-γ neutralizing antibodies, the generation of effectors capable of producing both IFN-γ and TNF-α was impaired, but granzyme B expression was not significantly altered (Figure 4.11A). IL-12 did not contribute to the effector differentiation of these tumor specific T cells, so was not investigated further. We then embedded the T cells that had differentiated *in vivo* in the presence and absence of IFN-γ neutralization to assess *ex vivo* killing capacities. These T cells were less cytotoxic when differentiation had occurred in the absence of IFN-γ (Figure 4.11E). The observed decrease in effector function was associated with decreased Th1 polarization, as measured by expression of *TBX21* and *IL-12RB2* (Figure 4.11B).

To address if paracrine IFN-γ produced by the TRP-1 CD4+ T cell population was driving their own differentiation, we performed the same adoptive transfer of 10^6 TRP-1 specific CD4+ T cells, using splenocytes and hosts deficient in IFN-γ production. In this model, IFN-γ could only be derived from the transferred TRP-1 transgenic CD4+ T cells or the B16 tumor itself, which has not been described to produce IFN-γ. Beginning the day before adoptive transfer, mice received an isotype control or an IFN-γ

neutralizing antibody, with the exception of the control group. Formation of a polyfunctional population of tumor specific CD4+ T cells was the same in wild-type and IFNγ deficient hosts (Figure 4.11C), demonstrating that IFNγ produced by the TRP-1 specific CD4+ T cell population alone is sufficient to drive their own differentiation. When IFNγ is blocked in this experiment, the development of effector function is decreased, but not completely extinguished. While IFNγ is an important clonally derived mediator of the population's differentiation, it is not the only necessary factor. To elucidate how IFNγ might be regulating cell extrinsic factors important to the differentiation of the CD4+ T cells, we performed a similar experiment utilizing IFNγ receptor (IFNGR) knockout tumor bearing hosts. In this experiment, the only cells expressing IFNGR, and thus directly responsive to IFNγ, were the TRP-1 CD4+ T cells and the implanted tumor. When IFNγ was neutralized in these mice, differentiation is still not completely abolished and is maintained at concentrations similar to previous experiments asserting that the other factors contributing to the differentiation of the T cells are not directly regulated by IFNγ (Figure 4.11D). In support of this, we observe that IFNγ neutralization has no effect on the expression of CD80 (Figure 4.11F) in the lymph node, implicating other mechanisms of T cell help, likely through co-stimulation. These findings were further validated in *in vitro* experiments, where IFNγ was neutralized during the expansion of TRP-1 CD4+ T cells in culture. In these experiments the neutralization of IFNγ resulted in even greater decreases in effector function compared to what was observed *in vivo,* with the exception of TNF-α production (Figure 4.12A-D). This suggests that our culture conditions exclude the additional variables that moderate the development of effector function *in vivo.* The decrease in effector function observed *in vitro* was accompanied by a reciprocal downregulation of T-bet and Eomesodermin, transcription factors associated with cytotoxic function (Figure 4.12E,F).

We believe the improved effector function observed at a high clonal abundance is dependent upon a combination of CD4+ T cell help and paracrine IFN-γ signaling.

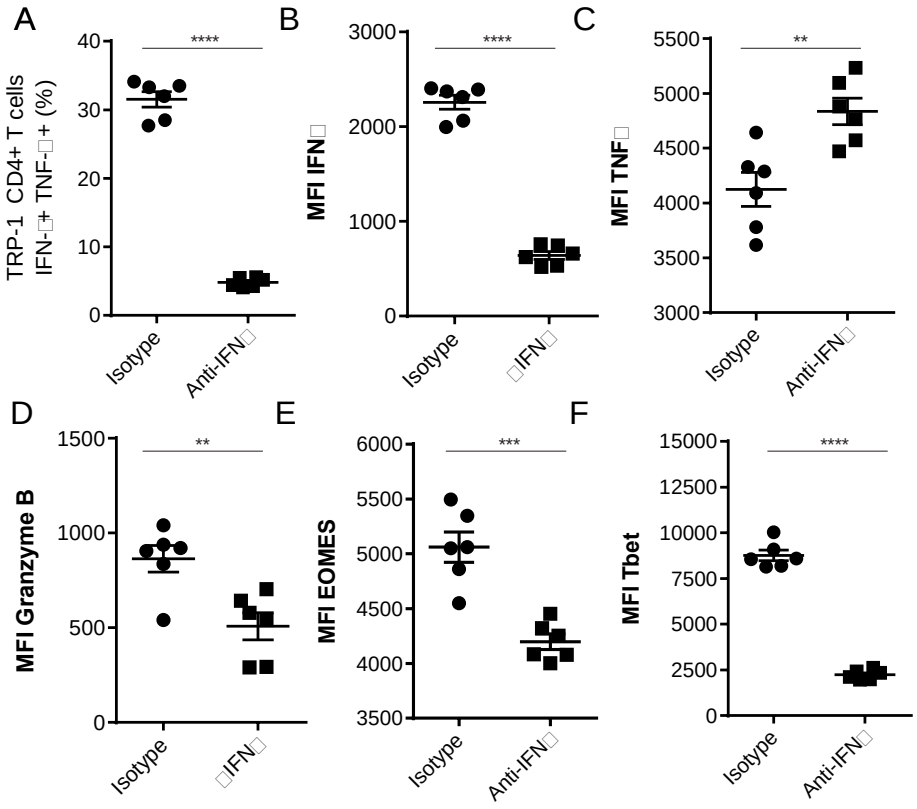


Figure 4.12. Neutralization of IFN-γ *in vitro* decreases Th1 differentiation and effector function.

Irradiated feeder APCs were pulsed for 2 hours with TRP-1 peptide and plated with MACS separated naïve TRP-1 CD4+ T cells in media containing IFN-γ neutralizing antibody or an isotype control in a 96 well plate. 5 days after initiation of culture lymphocytes were stimulated with PMA/ionomycin and evaluated for effector function and transcription factor expression. (A) Summary of percentage of IFNγ+ TNFα+ TRP-1 CD4+ T cells (B) MFI IFNγ (C) MFI TNFα (D) MFI Granzyme B (E) MFI EOMES (F) MFI Tbet. Each culture condition was performed with six replicates. Data are representative of two independent experiments. Data are represented as mean ± SEM.

Historically, CD4+ T cell help has been described in the context of the CD8+ T cell response. T cell help is indispensible to the success of the cytotoxic T cell response to infection and has also been implicated in the prevention of CD8+ T cell exhaustion during chronic infection (Bevan 2004; Zajac et al. 1998). While not as much is known in regards to help provided to CD4+ T cells themselves, there is evidence for cooperation between CD4+ T cell clones of different specificities (Creusot et al. 2003). The upregulation of CD80 expression on lymph node dendritic cells observed in our model is likely due to increased engagement with CD40 or MHC-II by the tumor specific T cells at high clonal abundance (Nabavi et al. 1992; Ranheim 1993). This increased CD80 expression may be contributing to the residual effector differentiation occurring during IFNγ neutralization. During normal conditions the increased antigen presentation and co-stimulation would be acting in synergy with the accumulation of paracrine cytokines, such as IFNγ and possibly IL-2. This population-mediated cooperation is likely deterring the development of exhaustion, through the expression of protectively high levels T-bet.

4.6. IFN-γ neutralization within the tumor results in a paradoxical stimulation of effector function

Although neutralization of IFN-γ resulted in a decrease in T cell differentiation in the lymph nodes, the effector phase of the immune response in the tumor was also altered. The neutralization of IFN-γ resulted in a paradoxical increase in the population of T cells producing effector cytokines and the total production of IFN-γ upon restimulation of cells isolated from the tumors of wildtype, as well as IFN-γ knockout and IFNGR knockout hosts (Figure 4.13A-C). This observation in the IFN-γ knockout model implies TRP-1 CD4+ T cell produced IFN-γ is restraining T cell activation during the effector phase. The maintenance of this suppressive effect in the IFNGR knockout hosts demonstrates that this effect is not mediated by IFNGR signaling and feedback from bystander cells but is perhaps due to direct signaling on TRP-1 cells or feedback

from tumor. This increase in cytokine production only marginally altered the overall *ex vivo* cytotoxicity of the T cells isolated from wild-type hosts, possibly due to the reciprocal decrease in granzyme B production (Figure 4.13D). All attempts to demonstrate a reversal of IFN-γ induced suppression by neutralizing IFN-γ *in vitro* resulted in a reduction in the Th1 phenotype as in Figure 4.12, even when neutralizing antibody was added at late time points during culture to better mimic the effect on differentiated cells (data not shown).

In addition to its role as the initiator of Th1 differentiation, IFN-γ has also been shown to act in a homeostatic manner through the regulation of contraction during later stages of the immune response. In one study using IFNγ deficient mice and receptor deficient mice it was shown that Th1 cells limit their own activity through IFNγ induced iNOS; however, it was the number of antigen specific effector T cells that was affected by the regulation of IFN-γ and not their intrinsic ability to produce effector cytokines (Feuerer et al. 2006). Other investigations that have revealed a key role for IFN-γ in immune contraction directly implicate IFN-γ in the apoptosis of Th1 cells via the mitochondrial damage pathway and caspase-9; other studies have shown that IFN-γ also has the capacity to mediate the induction of activation induced cell death (Li et al. 2007; Refaeli et al. 2002).

We sought to determine if the increase in effector activity observed upon neutralization of IFNγ in our model was due to the prevention of apoptosis. When we examined FLiCA staining in mice that had received IFNγ neutralization or an isotype control no differences were observed (data not shown). We performed an *in vitro* assay to determine the concentration of exogenously added IFNγ necessary for the induction of apoptosis. Although we investigated a wide range of concentrations, the highest of which exceeded levels observed i*n vivo* almost five-fold, we did not see any alteration in apoptosis of the TRP-1 CD4+ T cells. However, there were a larger proportion of dead cells specifically at the highest culture concentration. While IFNγ neutralization reduced

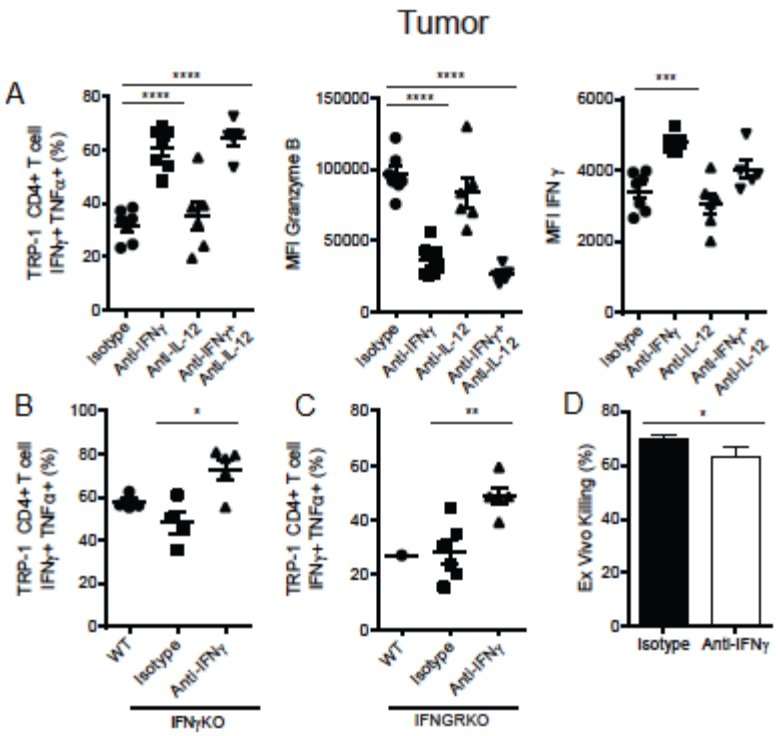


Figure 4.13. Neutralization of IFN-γ paradoxically stimulates effector function within the intra-tumor TRP-1 CD4+ T cell population.
C57BL/6J, IFN-γKO, or IFNGRKO mice were implanted with 2.5 x 10^5 B16 tumor cells. Fourteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 10^6 TRP-1 CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. Mice received intra-peritoneal injection of 200 μg of an antibody targeting IFN-γ, IL-12, or a mixed IgG control beginning on the day prior to adoptive transfer and every other day until lymphocyte isolation on day 7. Isolated lymphocytes from tumor of treated mice were re-stimulated with PMA/ionomycin. (A) Summary of percentage of IFNγ+ TNFα+ TRP-1 CD4+ T cells, and MFI IFN-γ, granzyme-B respectively from wildtype tumor infiltrating lymphocytes. (B) Day 7 tumor infiltrating lymphocytes from IFN-γKO treated mice and (C) IFNGRKO mice; summary of percentage of IFNγ+ TNFα+ TRP-1 CD4+ T cells (n=4-5 mice/group). (D) TRP-1 CD4+ T cells were sorted from pooled tumors of treated WT mice on day 7 and co-embedded with B16 in an *ex vivo* killing assay, percentage of B16 killed. Data are representative of three independent experiments. Data are represented as mean ± SEM.

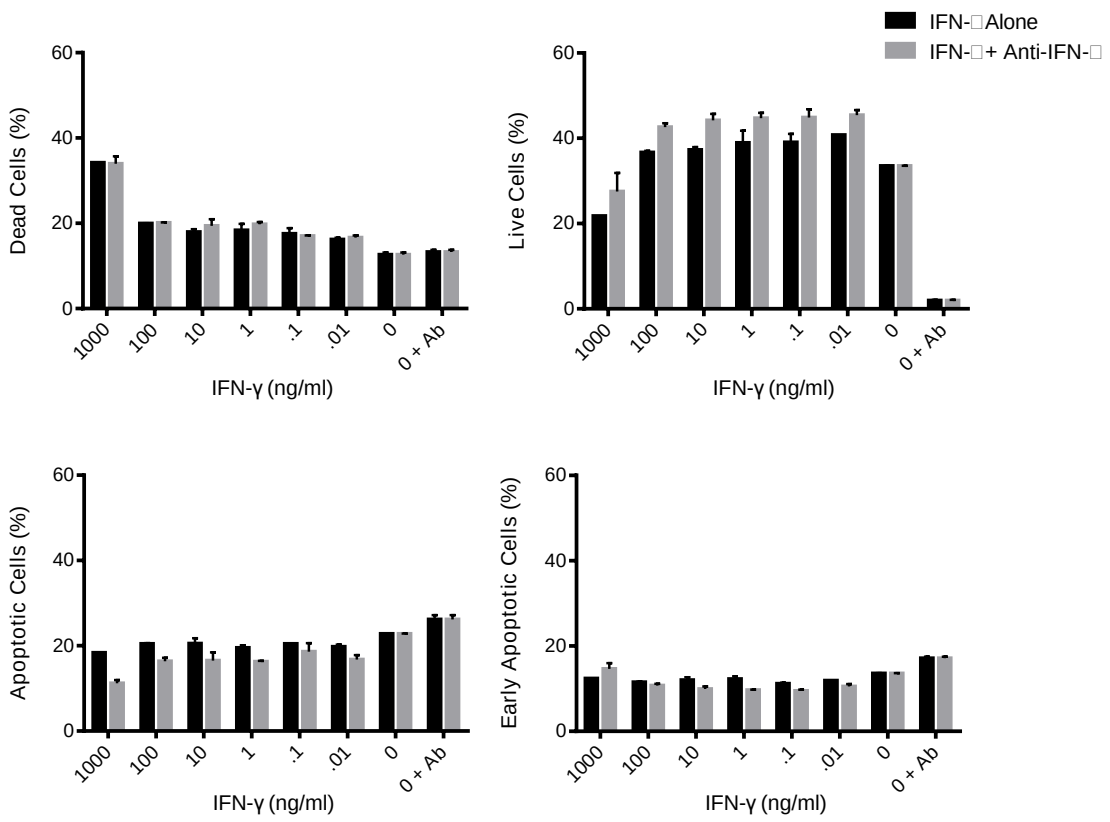


Figure 4.14. A gradient of IFN-γ combined with neutralization does not demonstrate dramatic reversal of apoptosis.

Irradiated feeder APCs were pulsed for 2 hours with TRP-1 peptide and plated with MACS separated naïve TRP-1 CD4+ T cells in media containing IFN-γ cytokine at the indicated concentrations in the presence or absence of IFN-γ neutralizing antibody in a 96 well plate. Apoptosis was characterized by FLiCA 5 days after initiation of culture. Each culture condition was performed in triplicate. Data are representative of two independent experiments. Data are represented as mean ± SEM.

the levels of apoptosis that were observed, it was not to a high enough degree that would explain the observations *in vivo* (Figure 4.14). Based on this, we do not believe the increased effector function observed after IFN-γ neutralization in the tumor was due to alterations in apoptosis.

Neutralization of IFN-γ affects the regulation of sources of both positive and negative feedback within the tumor. Blocking IFN-γ causes MHC-II to be downregulated in both CD45- and CD45+ tumor populations, which would impair the recognition and destruction of the tumor (Figure 4.15A). Alternatively, by removing IFN-γ from the tumor microenvironment, we are also decreasing IFN-γ mediated immunosuppression. This could occur in a T cell intrinsic manner, such as through decreased production of IL-10, but may also involve an indirect reduction of other immunosuppressive mediators, such as IDO, iNOS, and arginine (Taylor and Feng 1991; Xie, Whisnant, and Nathan 1993; Drapier, Wietzerbin, and Hibbs 1988). Additionally, IFNγ could be regulating inhibitory ligands on the tumor cells themselves. When we examined the effect of IFN-γ neutralization on the expression of the inhibitory ligand PD-L1 on the CD45- tumor we found it to be decreased, which would release negative feedback on the T cells and possibly result in restored effector function (Figure 4.15B). Although responsible for T cell activation, MHC-II is also a known ligand of LAG-3 receptor; downregulation of MHC-II by IFN-γ neutralization could also provide an additional mechanism for the release of T cell inhibition (Huard et al. 1994).

IFN-γ itself regulates the expression of its own receptor to reduce cytokine sensitivity and prevent apoptosis in Th1 cells (Pernis et al. 1995). In our model, we have found that as a result of IFN-γ blockade the expression of IFNGR is increased (Figure 4.16). It is possible that within the tumor, this increase in IFNGR expression raises the threshold of sensitivity of the TRP-1 specific CD4+ T cells to autocrine IFN-γ or to IFN-γ that has not been neutralized due to high intra-tumor concentrations, but not to such an extent as to result in apoptosis.

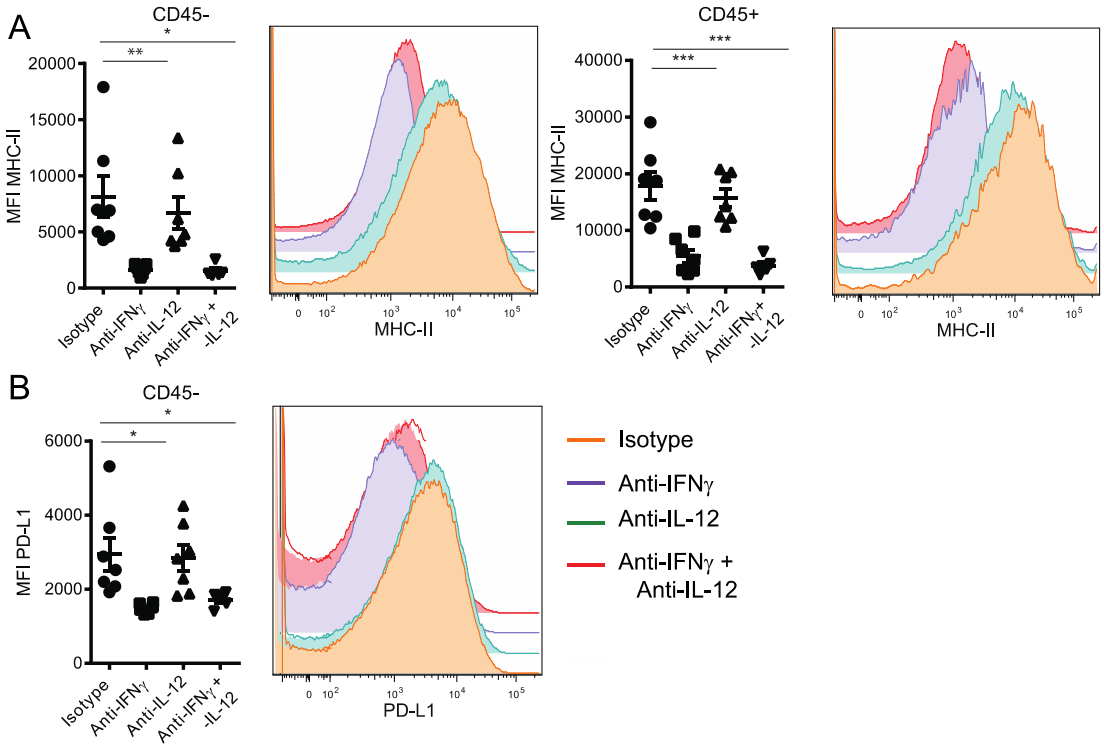


Figure 4.15. Neutralization of IFN-γ within the tumor inhibits the expression of MHC-II and inhibitory ligands such as PD-L1.

C57BL/6J mice were implanted with 2.5 x 10^5 B16 tumor cells. Fourteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 10^6 TRP-1 CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. Mice received intra-peritoneal injection of 200 μg of an antibody targeting IFN-γ, IL-12, or a mixed IgG control beginning on the day prior to adoptive transfer and every other day until lymphocyte isolation on day 7. (A) MFI of MHC-II on CD45- and CD45+ tumor populations. (B) MFI of PD-L1 on CD45- tumor population. Data are representative of three independent experiments. Data are represented as mean ± SEM.

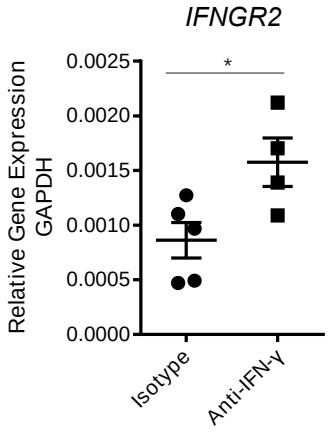


Figure 4.16. IFN-γ receptor is upregulated in response to IFN γ neutralization. C57BL/6J mice were implanted with 2.5 x 10^5 B16 tumor cells. Fourteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 10^6 TRP-1 CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. Mice received intra-peritoneal injection of 200 μg of an antibody targeting IFN-γ or a mixed IgG control beginning on the day prior to adoptive transfer and every other day until lymphocyte isolation on day 7. TRP-1 CD4+ T cells were sorted from LN of treated WT mice directly into Trizol for RNA extraction and qRT-PCR analysis of target *IFNGR2* (n=4-5 mice/group). Data are represented as mean ± SEM.

Despite the complexity of the pleiotropic effects mediated by IFNγ, this cytokine appears to be an integral component of a successful CD4+ T cell mediated anti-tumor response. When IFNγ is neutralized, tumor regression is dramatically impacted. Overall survival of the population drops from 100% to less than 50% and many of the tumors fail to regress (Figure 4.17). Whether this loss of tumor regression is due to IFNγ as an effector cytokine itself, driver of T cell differentiation, regulator of tumor recognition, or more likely a combination of these factors, it is clear that it is a fundamental mediator of the intraclonal cooperation within the tumor-responding CD4+ T cell population.

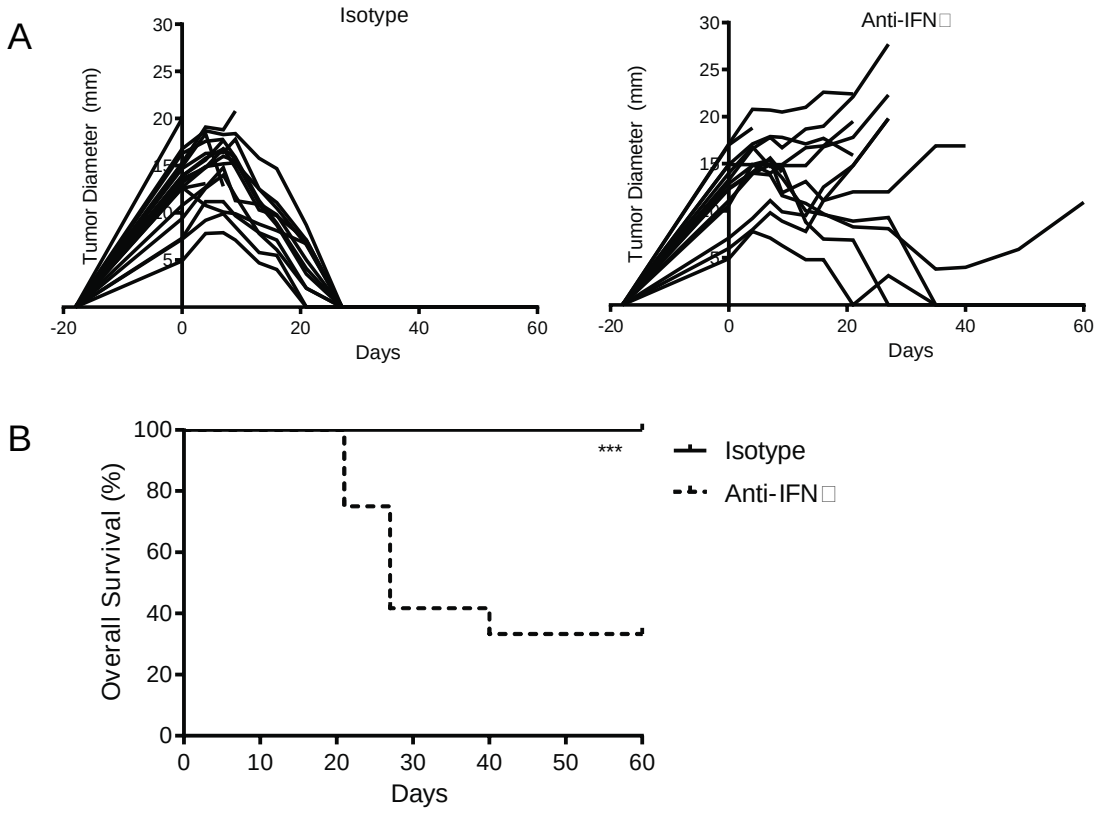


Figure 4.17. Despite enhanced effector function after IFN-γ neutralization the anti-tumor response is greatly diminished.

(A) 10^6 TRP-1 CD4+ T cells were adoptively transferred IV into C57BL6 bearing 14 day old B16 melanoma tumors, following 600 cGy of sub-lethal irradiation. Beginning on the day of adoptive transfer and then every other day mice were injected with isotype control or IFNγ neutralizing antibody. Tumor diameter was measured with calipers every 3-5 days represented on the graph by individual lines. (B) The overall survival of each group was plotted (n = 13-15 mice/group).

4.7. Highly abundant TRP-1 CD4+ T cells experience late-stage intra-tumor T cell dysfunction in RAG deficient mice

We were interested in investigating the increased anti-tumor immunity observed when TRP-1 specific CD4+ T cells were present at a low clonal abundance in immunodeficient RAG knockout animals. Based on our findings investigating the effects of clonal abundance on effector generation in wildtype animals, we had hypothesized that in RAG deficient hosts TRP-1 CD4+ T cells with low initial precursor frequencies would expand and accumulate to a greater abundance due to the more profound lymphopenia and absence of bystander splenocyte transfer; the increased amplitude of the response would allow the T cells to infiltrate the tumor at higher concentrations and differentiate into better effectors, thus shifting the precursor frequency necessary to mediate efficacious anti-tumor responses lower.

When we examined the effector function of TRP-1 CD4+ T cells within RAG knock out hosts we found that as in wildtype hosts effector differentiation in the lymph node was dependent on clonal abundance on both day 5 and 7 (Figure 4.18A). Differentiation of a population producing both IFN-γ and TNF-α appeared to be uniformly higher in the lymph node of RAG knockout mice on day 7 compared to previously conducted experiments in wildtype hosts. The high levels of differentiation in the lymph node were accompanied by elevated levels of proliferation, as measured by Ki67, and greater T cell accumulation (Figure 4.18B,C). However, what was most striking was what was observed in the tumor. The proportion of the population producing effector cytokines in the tumor was greatest in the group with the highest clonal abundance only on day 5. The kinetics of the response within the tumor had greatly shifted by day 7, with dramatic effector function observed at a clonal abundance of 10^4 (Figure 4.18A). This shift in effector function on day 7 was also mirrored by suppression of proliferation within the tumor in groups that had the highest initial precursor frequency, but was not

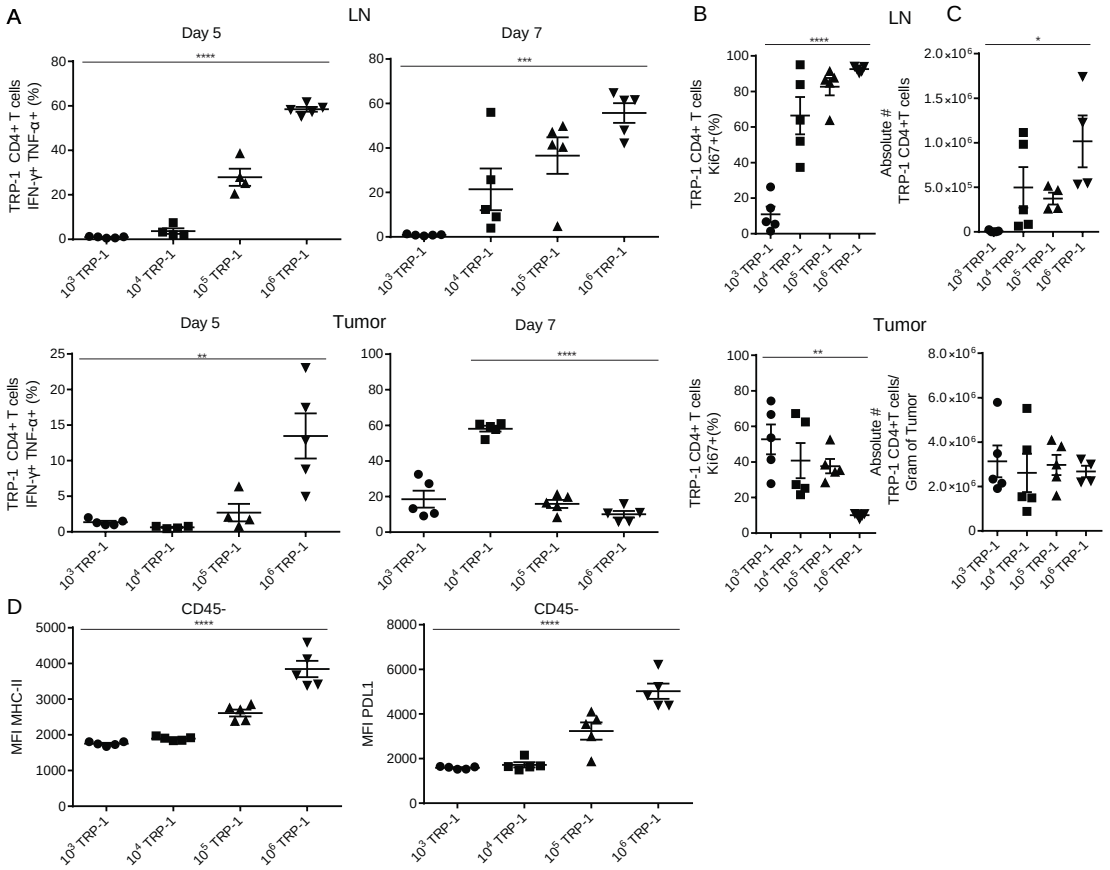


Figure 4.18. Highly abundant TRP-1 CD4+ T cells experience late-stage intra-tumor T cell dysfunction in RAG deficient mice.

RAG deficient mice were implanted with 1 x 10^5 B16 tumor cells. Fourteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 10^3, 10^4, 10^5, or 10^6 TRP-1 CD4+ T cells in the absence of co-transfer. On days 5 and 7, lymphocytes were isolated from the lymph node and tumor for characterization and re-stimulation with PMA/ionomycin. (A) Summary of percentage of IFNγ+ TNFα+ TRP-1 CD4+ T cells from LN and tumor infiltrating lymphocytes on day 5 and 7. (B) Day 7 proliferation of TRP-1 CD4+ T cells as measured by percent Ki67+ staining in lymph node and tumor (C) Absolute number of TRP-1 CD4+ T cells on day 7 in lymph node and tumor (D) MFI of MHC-II and PD-L1 on CD45- tumor population (n=4-5 mice/group). Data are representative of two independent experiments. Data are represented as mean ± SEM.

accompanied by any interpretable increase in tumor infiltration of the T cells (Figure 4.18B,C).

The depressed effector function and proliferation observed on day 7 in the group that had received the highest initial precursor frequency of TRP-1 CD4+ T cells was reminiscent of the IFN-γ mediated attenuation of effector responses that could be reversed though neutralization of IFN-γ. To determine if IFN-γ mediated suppression was involved in the intra-tumor T cell dysfunction we first examined correlates of IFN-γ accumulation. We found that despite the decreased IFN-γ+ TNF-α+ TRP-1 CD4+ T cell population, greater expression of MHC-II and PD-L1 by CD45- tumor cells was observed in the group with the highest precursor frequency, implying higher levels of IFN-γ accumulation (Figure 4.18D). To investigate the actual levels of cytokine accumulation, tumor bearing RAG deficient hosts received adoptive transfer of varied quantities of TRP-1 CD4+ T cells. On day 7 the tumors were isolated and cytokine levels were quantified in tumor extracts. Among the cytokines that were found to be most elevated in the highest precursor frequency group was IFN-γ, which was accompanied by IL-6, MCP-1, and G-CSF (Figure 4.19). The levels of IFN-γ observed in RAG tumors reflect a mean α of 1176, which is not only physiologically active, but also almost threefold higher than what had been observed in wildtype mice. This combination of cytokines implicates that the repression observed may also be attributable to the activity of neutrophils or monocytes. Additionally, we also observed a unique cytokine signature that paralleled the increase in effector function observed at a precursor frequency of 10^4. Cytokines that were found to be elevated at this clonal abundance included the IL-12/IL-23 p40 subunit, IL-13, and common gamma chain cytokines IL-7 and IL-15 (Figure 4.19). Il-7, IL-15, and IL-12 are all known to have supportive or inducing roles in the development of effector function, however IL-13 is most often associated with allergic inflammation and its function in tumor immunity is largely unknown.

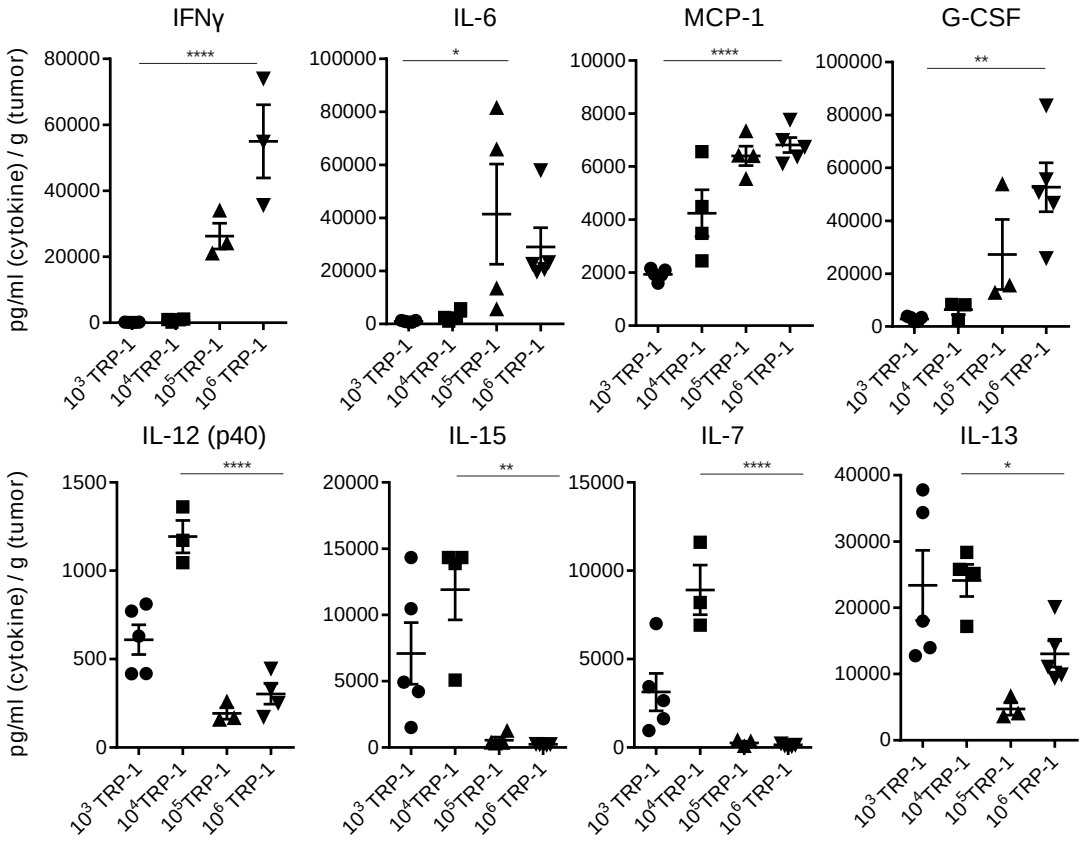


Figure 4.19. Disparate intra-tumor cytokine signatures are associated with the T cell dysfunction observed in RAG deficient mice.

RAG deficient mice were implanted with 1 x 10^5 B16 tumor cells. Fourteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 10^3, 10^4, 10^5, or 10^6 TRP-1 CD4+ T cells in the absence of co-transfer. On day 7, tumor was isolated and homogenized by mixer mill. Quantification of cytokine concentration in tumor isolates. Data are represented as mean ± SEM.

These observations suggest that IFNγ, perhaps acting through neutrophils and monocytes is acting to suppress later stages of inflammation during the anti-tumor response in RAG deficient mice. Considering that tumor regression is not impaired, but may actually be accelerated in RAG deficient hosts, it is possible that this day 7 dysfunction is the beginning of the retraction phase. The anti-tumor response of less clonally abundant T cells could peak later due to the need for more extensive proliferation in order to amass a high level of tumor infiltrate. This could account for the high IL-7 observed within the tumor on day 7 within cohorts containing T cells of a lower clonal abundance, whereas IL-7 production within the tumor is completely absent in the more clonally abundant groups. Considering that IL-7 is derived primarily from stromal cells, its existence suggests that the tumor has not yet been eradicated to the point that stromal cells have been affected at lower precursor frequencies. Whereas the absence of IL-7 implies that the tumor stromal cells may have been destroyed in the more highly abundant groups. Although these ideas have not formally been investigated, the RAG knockout model provides a very unique system to isolate and study the CD4+T cell regulation of innate immune cells and tumor derived factors.

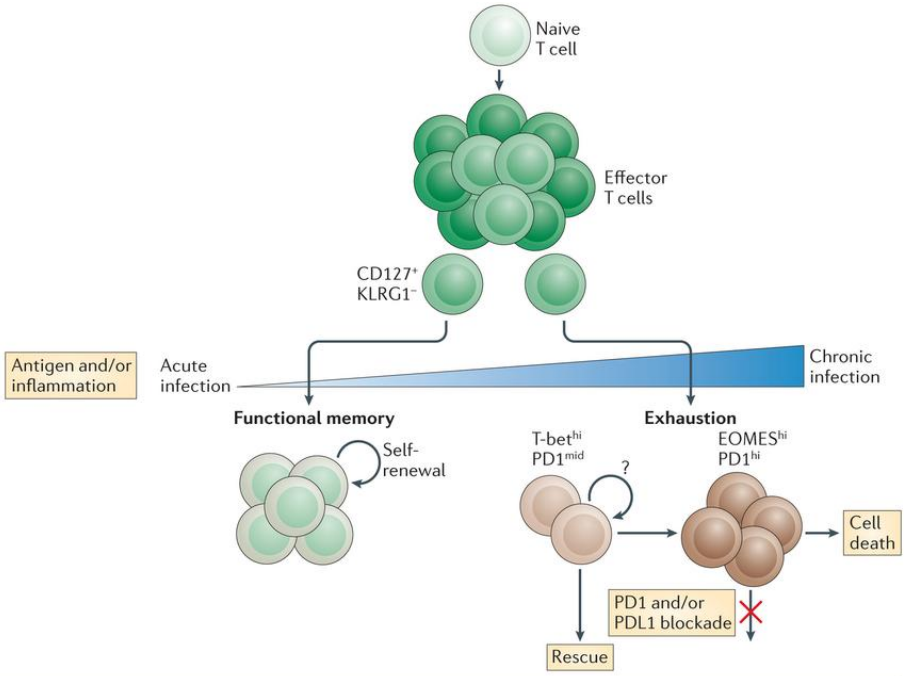
CHAPTER 5: LOW CLONAL ABUNDANCE OF TUMOR SPECIFIC CD4+ T CELLS POTENTIATES T CELL EXHAUSTION

Introduction

During chronic infections and cancer, persistent antigen exposure combined with inflammatory cues has been recognized to lead to state of impaired T cell effector function. This dysfunctional hyporesponsive state has been termed T cell exhaustion (Figure 5.1). In the time since T cell exhaustion was first identified in mouse models of chronic LCMV it has been an area of active investigation and much has been learned about the development, identification, and regulation of exhausted T cells (Zajac et al. 1998). However, the majority of these studies have focused on the exhaustion of cytotoxic CD8+ T cells and only recently has it been appreciated that exhaustion of the CD4+ T cell subset manifests as a distinctly different phenotype.

In models of LCMV infection, as well as in human HIV infection, it has been shown that T cell exhaustion is dependent on the level of antigen stimulation. More abundantly presented epitopes resulted in peripheral deletion, while viral epitopes presented at lower, yet sustained, levels resulted in the onset of T cell exhaustion. Notably, the severity of exhaustion showed a strong correlation with viral load (Wherry et al. 2003). The influence of persistent antigen on the development of T cell exhaustion was perhaps most elegantly demonstrated in studies that removed T cells from the source of chronic stimulation, either through isolation and secondary adoptive transfer or through anti-retroviral therapy (Streeck et al. 2008; Brooks, McGavern, and Oldstone 2006). These studies definitively revealed that exhaustion occurs via a long-term instructive process.

Loss of effector function during the development of T cell exhaustion is believed to occur in a hierarchical manner. First, T cells lose the ability to produce IL-2, which is followed by the impaired production of effector cytokines, such as IFN-γ and TNF-α, and



Feature	Functional memory T cell	Exhausted T cell
Proliferative potential	+++	+/–
Cytokine production	+++	+/–
Memory markers (e.g. CD44, CD62L, CD127 or CXCR3)	+++	+/–
Inhibitory receptors (e.g. PD1, LAG3, CD160 or 2B4)	–	+++
IL-7- and/or IL-15-driven self-renewal	++	–
Antigen dependency	–	+

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Figure 5.1. T cell exhaustion is a distinct state of differentiation that occurs during chronic infection.

T cell exhaustion is unique from the normal development of functional T cell memory. In the context of chronic antigen stimulation, T cells begin to lose effector function in a hierarchical manner, which is accompanied by an increase in the expression of inhibitory receptors. Heterogeneity within the exhausted T cell population can be attributed to the severity of infection and is reflected through the expression of a diverse combination of inhibitory receptors, as well as through the differential regulation of transcription factors contributing to exhaustion. Notably, the pattern of transcription factors expressed can confer sensitivity or resistance to therapeutic intervention via checkpoint blockade. Adapted from Wherry and Kurachi 2015.

eventual loss of cytolytic function (Wherry et al. 2003). T cells also begin to progressively upregulate the expression of non-redundant inhibitory and co-stimulatory molecules associated with exhaustion, with a larger diversity of inhibitory receptors corresponding to increased disease severity (Blackburn et al. 2009). These include PD-1, LAG-3, CTLA-4, and 2B4 on CD8+ T cells. Interestingly, CD4+ T cells have been shown to express a distinct molecular signature from CD8+ T cells. CD4+ T cells and CD8+ T cells share high expression of PD-1 and LAG-3, but elevated expression of CTLA-4 and BTLA was biased towards CD4+ T cells; conversely 2B4 was not strongly associated with CD4+ T cell exhaustion (Crawford et al. 2014). These inhibitory receptors have been theorized to contribute to T cell dysfunction via a variety of different mechanisms (Wherry and Kurachi 2015). Many inhibitory receptors, such as PD-1, contain inhibitory ITIM or ITSM domains, which are known to recruit phosphatases such as SHP1 and SHP2, and are capable of attenuating intracellular signals from activating receptors (Parry et al. 2005). Secondly, some of these receptors, including CTLA-4, have been shown to alter the stabilization of signaling receptors at the immunological synapse (Pentcheva-Hoang et al. 2004). Alternatively, signaling through inhibitory receptors could induce a gene expression program that facilitates T cell exhaustion (Quigley et al. 2010). It is clear that enhanced expression of co-stimulatory molecules also contributes to the exhausted T cell profile. In CD4+ T cells, elevated expression of ICOS, OX40, and CD27 are also observed, whereas CD8+ T cells show biased induction of CD80 (Crawford et al. 2014). The upregulation of co-stimulatory molecules during T cell exhaustion is also not fully understood, but may occur to compensate for the attenuation of downstream signaling by loss of adaptor proteins due to chronic stimulation (Wang et al. 2012).

While a lineage specific transcriptional regulator of exhausted T cells has not been defined, the contribution of a number of transcription factors to T cell exhaustion has begun to be characterized. Although each transcription factor identified also has

distinct roles in other T cell functions, such as memory development and generation of effector function, the expression pattern of these transcription factors and the genes induced downstream are unique during T cell exhaustion. The balance of expression of the transcription factors T-bet and Eomesodermin defines the terminal differentiation of exhausted T cells. Exhausted CD8+ T cells that are T-bet^{hi} still maintain some proliferative potential, which is partly attributed to repression of PD-1 by T-bet (Paley et al. 2012). Conversely, another subset of exhausted T cells, which comprise a larger proportion of the exhausted T cell population, express high levels of EOMES and display a larger proliferative defect and impairment in cytokine production. During checkpoint blockade therapy targeting PD-1, only the T-bet^{hi} subset has the potential to regain proliferative and effector function; the EOMES^{hi} subset is believed to be terminally differentiated and refractory to therapeutic intervention (Blackburn et al. 2008). Eomesodermin and the transcription factors Blimp-1 and Helios have been identified as important transcriptional regulators of T cell exhaustion specifically within the CD4+ T cell lineage (Crawford et al. 2014). However, expression patterns of these transcription factors have shown considerable heterogeneity; it is still unknown how this heterogeneity is regulated and the functional implications of these findings.

Here we expand upon previous work characterizing exhausted CD4+ T cells during chronic viral infection and demonstrate a similar exhaustion profile during tumor immunity. We establish a novel role for precursor frequency in the generation of T cell exhaustion. We observed impaired generation of effector function within the TRP-1 CD4+ T cell population present at a low clonal abundance. This impaired effector function co-manifested with reduced expression of the transcription factor T-bet and a reciprocal increase in the expression of Eomesodermin and PD-1, a phenotype consistent with T cell exhaustion. In this chapter, we further characterize the TRP-1 CD4+ T cells that arise from a low initial precursor frequency. We determine that these T cells are phenotypically exhausted based on their increased production of IL-21 and the

upregulation of inhibitory and co-stimulatory ligands characteristic of exhausted cells. This phenotype is maintained to a large extent even in the absence of tumor, implicating possible involvement of ubiquitous cognate antigen expression or excessive activation during lymphopenia. By performing a serial co-transfer experiment we demonstrate that previously unexhausted effector T cells can revert to a partially exhausted phenotype upon transfer at a low clonal abundance, further evidence that low clonal abundance can potentiate the development cell exhaustion. The T cell exhaustion developed in this model is resistant to blockade of PD-1 alone or in combination with anti-CTLA-4, likely due to a more terminal phenotype exemplified by high EOMES expression. These findings underscore that caution must be taken when designing trials investigating the combination of adoptive cell therapy with immune modulation, especially when using lymphopenia or self-antigen specific T calls.

Results and Discussion

5.1. Tumor specific CD4+ T cells at low clonal abundance express high levels of T cell exhaustion markers independent of tumor burden

Characterization of T cell exhaustion in chronic viral infection and tumor models has primarily focused on the CD8+ T cell compartment. However, recent studies have identified markers of T cell exhaustion shared with or unique to CD4+ T cells. During our previous investigation of the impact of TRP-1 CD4+ T cell clonal abundance on the production of effector cytokines we had also evaluated IL-21 production. IL-21 production has been described to increase during CD4+ T cell exhaustion, possibly to sustain ongoing CD8+ T cell and B cell responses (Crawford et al. 2014; Elsaesser, Sauer, and Brooks 2009). We observed the greatest levels of IL-21 production in the low precursor frequency TRP-1 specific CD4+ T cells, which implicated that these T cells may be in a state of exhaustion (Figure 5.2). Furthermore, the previously observed high levels of expression of *PD-1* and the transcription factor *EOMES* supported further

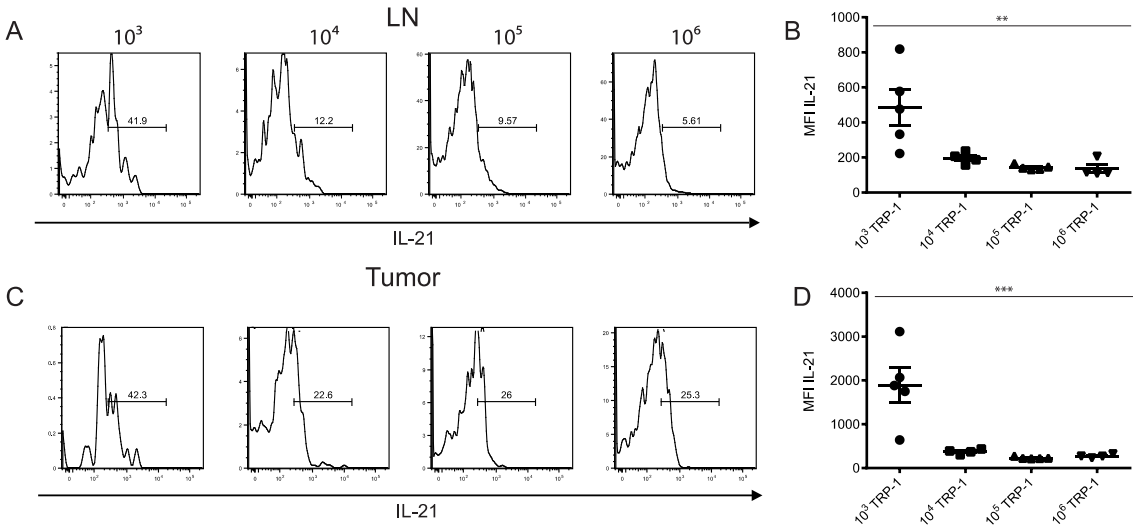

Figure 5.2. At low precursor frequencies tumor antigen specific CD4+ T cells produce IL-21.

C57BL/6J mice were implanted with 1 x 10^5 B16 tumor cells. Fourteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 10^3, 10^4, 10^5, or 10^6 TRP-1 CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. On day 7 post-transfer lymphocytes were isolated from lymph node and tumor and restimulated with PMA/ionomycin. (A) Representative histograms of IL-21 expression in TRP-1 CD4+ T cell LN population. (B) MFI of IL-21 in TRP-1 CD4+ T cell LN population. (C) Representative histograms of IL-21 expression in tumor infiltrating TRP-1 CD4+ T cell population. (B) MFI of IL-21 in tumor infiltrating TRP-1 CD4+ T cell population (n=4-5 mice/group). Data are representative of three independent experiments. Data are represented as mean ± SEM.

investigation of T cell exhaustion (Figure 4.9). To address if the impaired effector phenotype of tumor specific CD4+ T cells at low clonal abundance was linked to T cell exhaustion we investigated surface expression of the inhibitory and co-stimulatory molecules PD-1, CTLA-4, LAG-3, ICOS, BTLA, and CD27, which are components of the CD4+ T cell exhaustion profile (Crawford et al. 2014). We found that in the LN, exhaustion markers were upregulated on tumor specific T cells derived from low clonal abundance, compared to T cells from high clonal abundance (Figure 5.3A). The expression pattern of T cell exhaustion markers was similar, although attenuated, in the tumor, with the exception of LAG-3 (Figure 5.3A). This is not the first time exhaustion has been described using an adoptive transfer model of CD4+ T cells specific for the TRP-1 antigen. However, previous studies addressed the occurrence of exhaustion during tumor recurrence in RAG deficient mice, whereas we investigated effector dysfunction during the primary response (Goding et al. 2013). Of the overlapping inhibitory ligands investigated similar phenotypes were observed, with the exception of LAG-3, although it is unclear whether LAG-3 expression was examined in the lymph node or the tumor in their experiments, hindering direct comparison. It is not clear why the regulation of LAG-3 is distinct from other markers in the tumor. However, expression of this marker has been previously associated with the potential to produce high levels of IFNγ as opposed to immune dysfunction (Annunziato et al. 1996). LAG-3 may be acting to tune sensitivity to chronic cytokine exposure, which is most severe in the tumor of the groups with highly abundant tumor specific T cells.

It may seem counter-intuitive that T cells would be susceptible to exhaustion by day 7 of the anti-tumor immune response; however, early T cell dysfunction appears to be a characteristic of CD4+ T cell exhaustion and has been previously observed during chronic LCMV infection (Brooks et al. 2005; Crawford et al. 2014). One could argue that the T cells at low precursor frequencies are displaying an activated phenotype, as all exhaustion markers are also markers initially upregulated upon T cell activation.

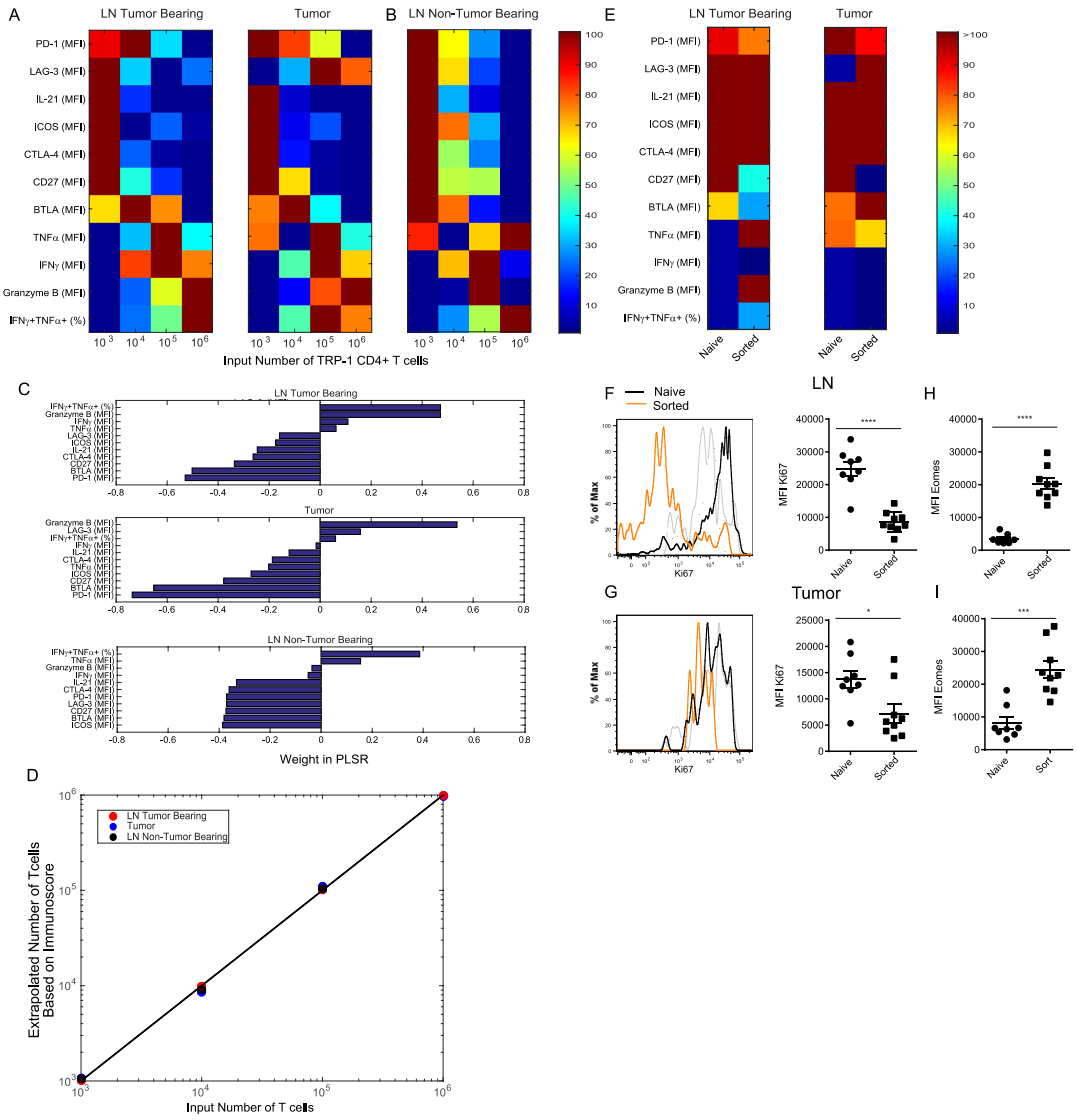

Figure 5.3. At low precursor frequencies tumor specific CD4+ T cells express high levels of T cell exhaustion markers independent of tumor burden.

(A) Heat maps for measures of activation and exhaustion on TRP-1 CD4+ T cells 7 days post transfer in lymph nodes and tumor of tumor bearing (B) and naïve hosts. (C) PLSR weights for activation/exhaustion measures are consistent across tissues. (D) PLSR of the precursor frequencies of TRP-1 T cells as a function of activation/exhaustion measures leads a PLSR score that captures from 99, 95 and 92% of the variability in precursor frequency and 70, 75 and 76% of the variability of activation/exhaustion measures. (E) Heat maps for measures of activation and exhaustion on TRP-1 CD4+ T cells on day 7 in LN and tumor in tumor bearing hosts that had received T cells originally sorted from TRP-1 TCR transgenic donors or from day 7 recipients of 10^6 TRP-1 CD4+ T cells. (F) Representative histogram and summary of Ki67 expression on TRP-1 CD4+ T cell in lymph node and (G) tumor. (H) Summary of Eomes expression of TRP-1 CD4+ T cell in lymph node and (I) tumor. Data are represented as mean ± SEM.

However, the T cells derived from low clonal abundance also show the impaired production of IFN-γ and TNF-α and increased IL-21 production that are characteristic of exhausted CD4+ T cells. It is tempting to speculate that induction of exhaustion in self-antigen specific CD4+ T cells at low precursor frequencies could act as a failsafe mechanism to enforce peripheral tolerance for highly self-reactive clones that have escaped negative selection. Further studies will be required to determine if pools of exhausted self-antigen specific clones exist in the periphery and if their IL-21 production contributes to the development of the aberrant autoantibody production occurring in early stage autoimmune disease.

To understand if the T cell exhaustion was dependent on tumor burden or potentiated by the higher proliferative potential of T cells at low initial abundance, we examined development of exhaustion in tumor free mice. Once again, expression of T cell exhaustion markers was higher when tumor specific CD4+ T cells were present at the low physiologic frequency (Figure 5.3B). The ability of the T cells to produce effector cytokines had a similar trend to that in tumor bearing mice (Figure 5.3B). CD4+ T cells in low abundance favor IL-21 production but reduce TNF-α production. However, the impairment in effector phenotype is not as marked in naïve animals, likely due to the lack of tumor-derived antigen in the lymph nodes driving the extreme polarization. These results suggest that presence of the tumor was unnecessary for the generation of the exhausted T cell phenotype in this model. It is possible that the TRP-1 CD4+ T cells are experiencing chronic antigen exposure in the absence of tumor, due to the fact that TRP-1 is a self-antigen ubiquitously expressed in mouse melanocytes. However, another possibility is that placing these T cells into the lymphopenic environment induced by sub-lethal irradiation is exacerbating the TCR stimulation received from self peptide/MHC-II during homeostatic proliferation. Interestingly, in many of the model systems commonly used for examining T cell exhaustion such as HIV and LCMV infection, some degree of lymphopenia accompanies the chronic infection (Corbeau and Reynes 2011; Walsh et al.

2010). Regardless of the source of the chronic stimulation, it seems that while the intraclonal competition of T cells may impair proliferation it can also protect against exhaustion by limiting repeated antigen exposure.

To quantitatively estimate the multifactorial aspects of T cell exhaustion, we created an exhaustion score compiling the overall quality of T cell responses, and demonstrated its dependence on precursor frequency. To do so, we normalized the variable abundance of markers (M_i) on the surface of T cells harvested from the LN of tumor bearing and tumor free mice, as well as tumor, derived from different clonal abundances. We then applied a partial-least square regression (PLSR) against the logarithm of the number of input T cells in order to generate an exhaustion immunoscore, $Y_{T\ cells}$: $Y_{T\ cells} = \beta_0 + \Sigma_{i,j}\ \beta_{i,j}.X_{i,j,k}$ where $X_{i,j,k} = \alpha_0 + \Sigma_{i,j}\ \alpha_{i,j}.log_{10}(M_{i,j,k})$. as a weighted sum of the abundance of markers.

PLSR is a supervised statistical method that best identifies the latent variables necessary to predict the multivariate immune response in our system, while avoiding the pitfall of overfitting (Wold, Sjöström, and Eriksson 2001). The PLSR weights correlated positively for effector functions and negatively for exhaustion markers in all conditions; Granzyme B and PD-1 expression were the greatest phenotypic contributors to exhaustion scoring in tumor bearing mice (Figure 5.3C). Our exhaustion immunoscore captured 95, 92, and 99% of the variance in the number of cells and 75, 76, and 70% of the variance in activation and exhaustion marker expression respective to the tissue examined (Figure 5.4). The linear correlation between exhaustion score $Y_{T\ cells}$ and initial precursor frequency was excellent ($R > 0.999$) (Figure 5.3D).

To further characterize the impact of initial precursor frequency on development of exhaustion, we examined whether exhaustion could be induced in the high frequency CD4+ T cells. To accomplish this, we adoptively transferred 10^6 TRP-1 CD4+ T cells into tumor bearing recipients and on day 7 sorted the TRP-1 CD4+ T cells directly from tumor draining LNs. These cells were co-transferred at a frequency of 10^3 cells into new

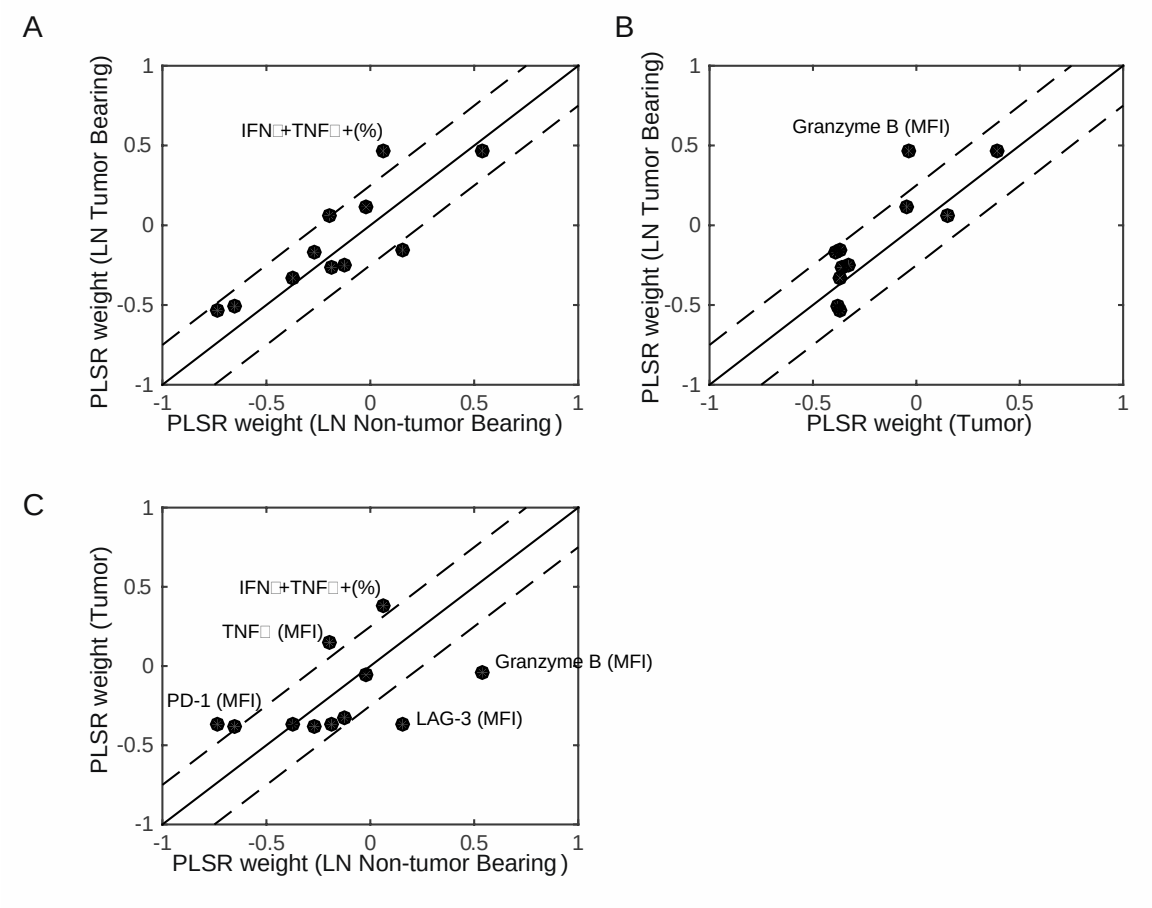

Figure 5.4. PLSR weights contributing to the exhaustion immunoscore are comparable among examined tissues.
(A) Comparison of PLSR weights of T cells isolated from the LNs of tumor bearing and non-tumor bearing mice. (B) Comparison of PLSR weights of T cells isolated from the LN of tumor bearing mice and tumor tissue. (C) Comparison of PLSR weights of T cells isolated from the LNs of non-tumor bearing and tumor tissue.

host animals bearing established B16 melanoma. Upon adoptive transfer, the T cells from the previously adoptively transferred mice show a strong proliferative defect characteristic of terminal differentiation (Figure 5.3F). Compared to naïve TRP-1 CD4+ cells transferred at a clonal abundance of 10^3, the sorted cells from the high frequency condition display comparable or increased expression of exhaustion markers, but retain some effector function characteristic of high clonal abundance; this is accompanied by increased EOMES expression (Figure 5.E,H). This is notable, because it is known that exhausted T cells lose effector function in a hierarchical manner beginning with IL-2 and

IFNγ production. Thus, it is not surprising that the remaining effector functions of these cells are those functions lost last and only during the most severe exhaustion. This supports that these T cells are becoming exhausted, yet might not become as severely exhausted or exhausted as quickly as naïve cells due to their previous state of effector differentiation. These results demonstrate that exhaustion is influenced both by clonal abundance and previous states of differentiation.

5.2. The T cell exhaustion phenotype is irreversible by checkpoint blockade

Considering the recent successes of checkpoint blockade for the treatment of advanced malignancies, we tested whether this approach could reverse the exhaustion observed in our model (Hirano et al. 2005; Barber et al. 2006). We chose to target PD-1 due to its high PLSR weight and the established clinical efficacy of PD-1 blockade (Topalian, Drake, and Pardoll 2015). Tumor bearing mice with a clonal abundance of 10^3 TRP-1 CD4+ T cells received PD-1 blockade every 3 days beginning on the day of adoptive transfer and were compared to mice with a clonal abundance of 10^6 due to their ability to mount a successful anti-tumor response. In the LNs and tumors of mice treated with PD-1 blockade, the tumor antigen specific CD4+ T cells failed to show any increase in effector function (Figure 5.5A-D). Additionally, the TRP-1 CD4+ T cells receiving PD-1 blockade showed upregulation of Eomes expression, which increased in proportion to T-bet (Figure 5.5G,H). When the TRP-1 CD4+ T cells were sorted from the lymph nodes and tumor and then embedded at identical numbers in a clonogenic *ex vivo* killing assay, there was a reduction in cytotoxicity on a per cell basis in the group that received PD-1 blockade (Figure 5.5E,F). To assess if PD-1 blockade could be improved through combination with another form of checkpoint blockade, we combined PD-1 blockade with anti-CTLA-4 therapy. This target was chosen after a pilot experiment comparing LAG-3 blockade to CTLA-4 blockade had demonstrated similar efficacy (data not

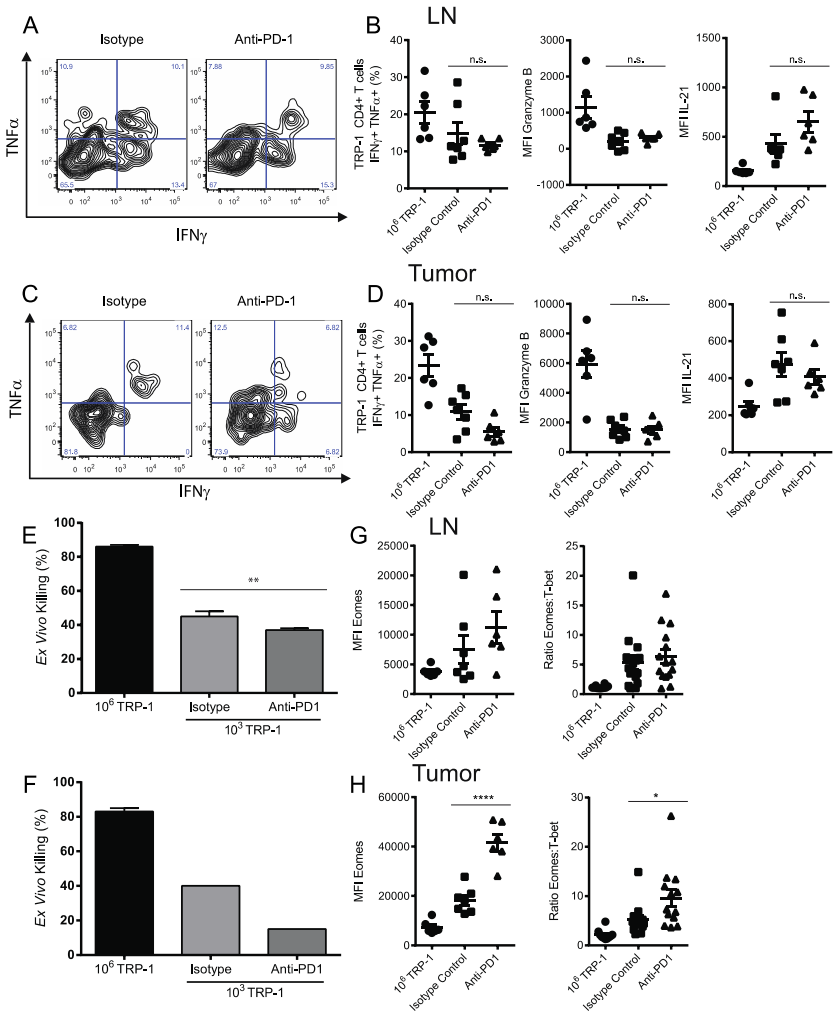

Figure 5.5. The T cell exhaustion phenotype is refractory to PD-1 blockade.
C57BL/6J mice were implanted with 1 x 10^5 B16 tumor cells. Fourteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 10^3 or 10^6 TRP-1 CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. Mice receiving transfer of 10^3 TRP-1 CD4+ T cells were treated with anti-PD-1 antibody or an isotype control every 3 days beginning on the day of adoptive transfer. On day 7 lymphocytes were isolated from LN and tumor of treated tumor bearing mice and restimulated with PMA/ionomycin. (A) Representative plots of IFNγ+ TNFα+ TRP-1 CD4+ T cells in lymph node and (C) tumor. (B) Summary of percentage of IFNγ+ TNFα+ TRP-1 CD4+ T cells, MFI of granzyme-B and IL-21 respectively in LN and (D) tumor (n=5-7 mice/group). (E) TRP-1 CD4+ T cells were sorted from LN or tumor (F) of treated mice on day 7 and co-embedded with B16 in an *ex vivo* killing assay, percentage of B16 killed. (G) MFI of Eomes of TRP-1 CD4+ T cells in day 7 LN and tumor (H) and as a ratio in comparison of MFI of T-bet within population from pooled duplicate experiments. Data are representative of three independent experiments. Data are represented as mean ± SEM.

shown). Yet, no improvement in effector function was observed in any treatment condition (Figure 5.6).

Heterogeneity within the overarching phenotype of T cell exhaustion exists not only between CD4+ and CD8+ T cell lineages, but also within each lineage. One way in which exhaustion is characterized is by examining the ratio of the T-box transcription factors T-bet and Eomes (Paley et al. 2012; Buggert et al. 2014). It is largely believed that within the exhausted CD8+ T cell subset $Eomes^{hi}Tbet^{lo}PD\text{-}1^{hi}$ T cells have less proliferative potential and are more refractory to checkpoint blockade than exhausted populations expressing higher levels of T-bet. Within this context, it is more understandable why checkpoint blockade has failed in our model. The tumor antigen specific CD4+ T cells at low precursor frequencies express low levels of T-bet with Eomes levels comparable to or greater than T cells at high precursor frequencies. This combination suggests they would be resistant to checkpoint blockade. Indeed, we observe that blocking PD-1 results in an increase in the expression of Eomes, supporting that checkpoint blockade is pushing the population further towards terminal differentiation. It would be interesting to determine if checkpoint blockade can rescue the effector function of TRP-1 CD4+ T cells that were previously effector cells transferred at a low clonal abundance as in Figure 5.3. One would hypothesize that considering their less exhausted phenotype they might be more sensitive to this approach. The occurrence of exhaustion and the failure of checkpoint blockade to reverse it have profound implications for the use of CD4+ T cells in adoptive cell therapy for cancer patients. While it would be tempting to use small numbers of tumor specific T cells for adoptive cell therapy, due to the speed at which they can be derived, and compensate by combining ACT with immune modulation, our findings would caution against this approach.

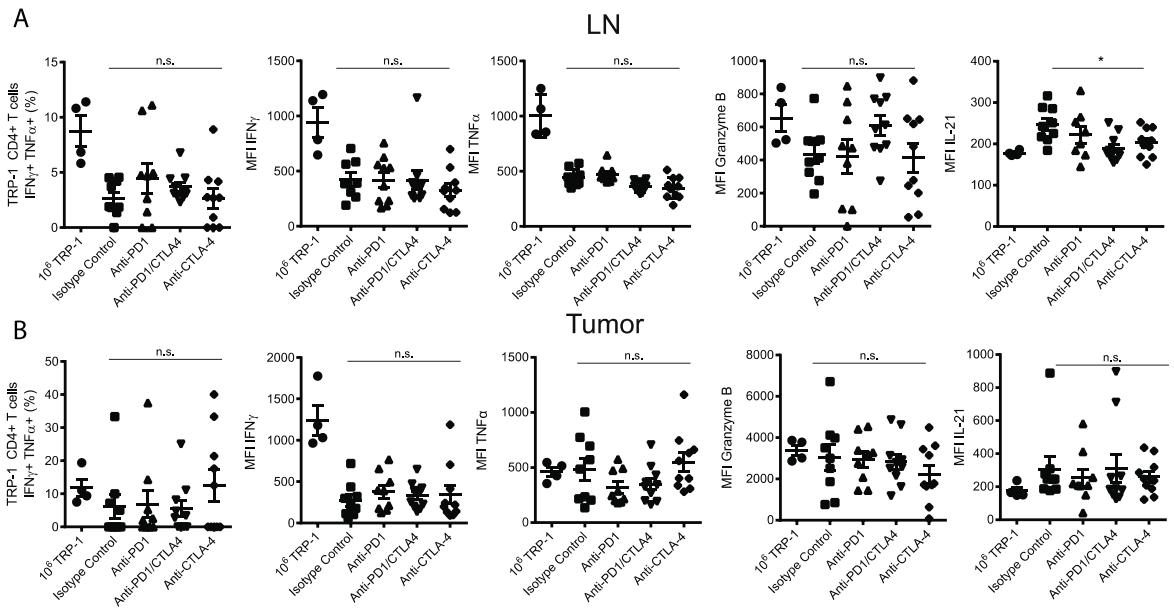

Figure 5.6. Combining PD-1 blockade with anti-CTLA-4 does not improve effector function in the exhausted tumor specific CD4+ T cells.

Mice with established tumors received transfer of 10^3 or 10^6 TRP-1 CD4+ T cells. Beginning on the day of transfer, mice in the 10^3 group received anti-PD-1 alone or in combination with anti-CTLA-4 or an isotype control every 3 days. On day 7 post-transfer lymphocytes were from the lymph node and tumor were restimulated with PMA/ionomycin. (A) Summary of percentage of IFNγ+ TNFα+ TRP-1 CD4+ T cells and MFI of IFNγ, TNFα, granzyme-B and IL-21 expression in TRP-1 CD4+ T cell population respectively from LN and (B) tumor. Data are representative of two independent experiments. Data are represented as mean ± SEM.

CHAPTER 6: THE BALANCE AND FUNCTION OF REGULATORY TRP-1 CD4+ T CELLS

Introduction

Regulatory T cells or Tregs are crucial for the prevention of autoimmunity and maintenance of tolerance to self and commensal derived antigen. Scurfy mice and humans with IPEX syndrome were found to possess a loss of function mutation in the transcription factor *Foxp3*, which results in a fatal T cell mediated lymphoproliferative disorder characterized by symptoms of autoimmune disease and cytokine storm (Brunkow et al. 2001; Bennett et al. 2001). These initial observations lead to the identification of *Foxp3* as the master transcriptional regulator of the regulatory T cell lineage (Hori, Nomura, and Sakaguchi 2003; Fontenot, Gavin, and Rudensky 2003). Expression of Foxp3 protein has been shown to be essential for the suppressive capability of regulatory T cells (Wan and Flavell 2007). Additionally, regulatory T cells have demonstrated an important role in the maintenance of immune homeostasis. In experiments utilizing a Foxp3-DTR transgenic mouse strain, in which regulatory T cells are depleted following administration of diphtheria toxin, loss of regulatory T cells in healthy adult mice was shown to result in fatal lymphoproliferative disease dependent on CD4+ T cell activation (Kim, Rasmussen, and Rudensky 2007). This study affirmed that regulatory T cells are responsible for the restraint of self-reactive CD4+ T cells throughout the lifetime of an animal.

There are at least two distinct subsets of regulatory T cells characterized primarily by developmental origin: thymic derived or "natural" Tregs and peripherally induced regulatory T cells. Thymic derived regulatory T cells are believed to be instructed by strong TCR signaling during lineage determination, as loss of downstream attenuators of TCR signaling results in greater regulatory T cell formation (Carter et al. 2005). It has also been proposed that regulatory T cells are selected based on their recognition of self-antigen. In support of this hypothesis, the TCRα-chain repertoires of effector T cells and

thymic derived regulatory CD4+ T cells display little overlap, while significant similarity exists between the repertoires of thymic and peripherally induced regulatory T cells (Hsieh et al. 2006). In addition, it has been demonstrated that TCR transgenic regulatory T cells fail to develop in the absence of cognate antigen expression in the thymus (Apostolou et al. 2002). The antigen specificity of regulatory T cells has also been shown to influence their survival. Using transgenic mouse lines expressing TCRs derived from regulatory T cell clones, it was revealed that very low numbers of regulatory T cells developed in the thymus of these mice. Intraclonal competition within the thymus was determined to regulate a "saturable niche" and prevent the formation of regulatory T cells of identical specificity to promote repertoire diversity (Bautista et al. 2009).

In contrast to thymically derived regulatory T cells, peripherally derived regulatory T cells or induced Tregs, are derived from naive conventional CD4+ T cells. During conditions of low-dose chronic antigen stimulation, in the presence of cytokines such as IL-2 and TGF-β, as well as environmental factors such as retinoic acid, T cells can be induced to express Foxp3 (Kretschmer et al. 2005). These induced regulatory T cells are often localized to barrier membranes and are believed to have a role in the development of tolerance to environmental antigens such as commensal bacteria and food.

The thymic development and peripheral homeostasis of regulatory T cells are partly dependent on signaling via IL-2 receptor. The first evidence supporting a role for IL-2 in regulatory T cell homeostasis came from IL-2 deficient mice, which exhibit severe autoimmunity and a phenotype similar, albeit attenuated, to that observed in scurfy mice (Sadlack et al. 1993). Foxp3 directly controls the expression of CD25, resulting in high levels of IL-2 receptor expression on regulatory T cells (Chen et al. 2006); however, Foxp3 in complex with NFAT, suppresses IL-2 expression rendering regulatory T cells incapable of producing IL-2 themselves and dependent on paracrine IL-2 derived from

activated T cells (Wu et al. 2006). The importance of signaling via the co-stimulatory receptor CD28 for the homeostasis of regulatory T cells has also been established and experiments utilizing double deficient IL-2 knockout/CD28 knockout mice have revealed additive non-redundant roles for these pathways (Hoyer et al. 2007). Access to IL-2 has been shown to control the balance between effectors and regulatory T cells during immune homeostasis and responses against foreign pathogens. During lymphopenia-induced proliferation, T cells expand until available homeostatic niches are re-populated. When the regulatory T cell niche is re-established in lymphopenic mice it occurs to the same extent independent of the number of T cells initially transferred. This occurs through a mechanism where regulatory T cells are "indexed" to the population of activated IL-2 producing CD4+ T cells (Almeida, Zaragoza, and Freitas 2006). However, in models of *Toxoplasma gondii*, *Listeria monocytogene*s, and vaccinia virus infection the frequency and total number of regulatory T cells have been shown to be reduced due to excessive consumption and limited production of IL-2 by responding effector T cells. This shift was necessary for the potent induction of Th1 mediated immunity, which was accompanied by expression of T-bet and IFN-γ production in regulatory T cells during *T. gondii* infection (Benson et al. 2012; Oldenhove et al. 2009). It is also well recognized that the regulatory T cell subset is quite homogenous in both function and regulation. Some sub-populations of regulatory T cells, specifically $CD44^{lo}CCR7^{hi}CD62L^{hi}$ regulatory T cells, which are located preferentially in secondary lymphoid tissues, are dependent on IL-2 for homeostasis and survival. Other tissue-localized sub-populations appear to be relatively IL-2 independent, display a $CCR7^{lo}CD62L^{lo}$ phenotype and are dependent ICOS signaling for survival (Smigiel et al. 2014).

Regulatory T cells have been described to suppress immunity through various distinct mechanisms. It is likely that these mechanisms are both context dependent and overlapping, making it difficult to parse out exactly how regulatory T cells might suppress a complex immune response such as that observed against cancer. A contact

dependent granzyme B mediated method of suppression has been described to induce apoptosis of effector T cells (Gondek et al. 2005). Immunomodulatory cytokines derived from regulatory T cells, such as IL-10 and IL-35, are important for attenuating autoimmunity in models of colitis and directly impact the expansion of effector T cells (Collison et al. 2007; Asseman et al. 1999). TGF-β is not only important for the induction of suppressor cells, but TGF-β expressed by regulatory T cells has also been shown to mediate direct suppressive function (Tang et al. 2004). Regulatory T cells are also known to limit immune responses through their consumption of IL-2, a mechanism that can control both T cell and NK cell responses (Pandiyan et al. 2007; Sitrin et al. 2013). Aside from scavenging IL-2, regulatory T cells can induce the expression of molecules such as indoleamine 2,3-dioxygenase (IDO), which scavenge essential amino acids from the environment, thus limiting T cell expansion (Cobbold et al. 2009). Additionally, regulatory T cells express high levels of a number of inhibitory receptors, such as LAG-3, TIGIT, and the most thoroughly characterized, CTLA-4. LAG-3 and TIGIT have been shown to modulate the cytokine production and reduce the activation state of dendritic cells (Yu et al. 2009). However, the function of CTLA-4 within the regulatory T cell lineage had long been a source of controversy. CTLA-4 deficient mice develop a lethal lymphoproliferative syndrome, which made use of the model difficult to interpret (Waterhouse et al. 1995). However, selective loss of CTLA-4 in regulatory T cells was found to result in defective suppression by regulatory T cells, autoimmunity, and decreased regulation of co-stimulatory molecules on dendritic cells depending on the genetic background (Wing et al. 2008).

There have been a number of studies investigating the balance between regulatory T cells and effector T cells with identical TCRs specificities. Using DO11.10 T cells transferred into mice immunized with ovalbumin or expressing ovalbumin under the rat insulin promoter, it was shown that regulatory T cells proliferate in response to vaccination and endogenous antigen, whereas effector CD4+ T cells failed to accumulate

(Walker et al. 2003). The same group, again using DO11.10 cells, in combination with a mouse expressing soluble OVA, demonstrated the development of autoimmunity resembling graft versus host disease following transfer into lymphopenic hosts. IL-2 was shown to mediate the balance between the generation of effector T cells and the eventual recovery of regulatory T cells, which resulted in an amelioration of symptoms (Knoechel et al. 2005). In the BDC2.5 TCR transgenic mouse the accumulation of regulatory T cells within the transgenic T cell subset as the mice aged was correlated with lower islet reactivity and decreased development of diabetes (Thomas et al. 2007). However, the balance of a monoclonal T cell population within the context of tumor immunity has not yet been addressed.

Understanding how regulatory T cells facilitate immune evasion by tumors is of profound clinical relevance. Regulatory T cells have not only been implicated in the attenuation of anti-tumor immunity, but can contribute to tumor angiogenesis and metastasis (Facciabene et al. 2011; Tan et al. 2011). Intra-tumor accumulation of regulatory T cells occurs both through the peripheral conversion of CD4+ T cells and by chemokine mediated recruitment (Facciabene et al. 2011; Liu et al. 2007). Depletion of regulatory T cells through antibody targeting of CD25 has facilitated tumor regression in a number of tumor models (Onizuka et al. 1999). However in established B16 melanoma, regulatory T cell depletion, combined with anti-CTLA-4 blockade and GVAX, showed little efficacy in the treatment of established tumors due to impaired infiltration of effectors into the tumor (Quezada et al. 2008). In a model of B16 tumor recurrence utilizing TRP-1 specific CD4+ T cells it was demonstrated that regulatory T cells had accumulated in mice with tumor recurrence. Depletion of this subset was unsuccessful at restoring anti-tumor immunity until combined with PD-L1 blockade (Goding et al. 2013). Although the authors implicate an importance for regulatory TRP-1 CD4+ T cells in this model, the role of these T cells in the initial anti-tumor response is not addressed. More importantly, how clonal abundance in the periphery affects the

regulation of monoclonal regulatory T cell population has not been investigated, in tumor immunity or any other context.

In our model system, around 8-10% of TRP-1 TCR transgenic CD4+ T cells prior to adoptive transfer express the master regulatory factor Foxp3+. These regulatory T cells were found to comprise a substantial proportion of the tumor infiltrating population of TRP-1 specific T cells. In this chapter we describe the emergence and function of these self-antigen specific regulatory T cells. We establish that the Foxp3+ TRP-1 CD4+ T cells are present throughout the immune response and comprise the largest percentage of the population early in the initiation phase. The abundance of these regulatory T cells is attributed to their high levels of homeostatic proliferation. Through a series of experiments, we demonstrate that alterations in the proportion of regulatory to effector T cells are not due to a direct effect on regulatory T cells themselves, as regulatory T cells appear to be under the control of a homeostatic niche. Instead perturbations of the TRP-1 CD4+ T cell population are attributable to changes in the proliferation of the effector T cells, which can be modulated by antigen level. We also confirm that the TRP-1 specific regulatory T cells possess suppressive functions through a combination of *in vivo* and *ex vivo* assays. However, attempts to deplete or indirectly target the Foxp3+ TRP-1 CD4+ T cells to demonstrate a regulatory T cell dependent impairment of anti-tumor efficacy were unsuccessful. The development of a genetically modified TRP-1 specific DTR depleter mouse strain will allow for the future investigation of this subject.

Results and Discussion

6.1. At a supra-physiologic precursor frequency the proportion of regulatory T cells is elevated and sustained through robust proliferation

During the characterization of the tumor infiltrating lymphocytes we observed an increase in the percentage of regulatory T cells within the tumor in the lower precursor frequency group. We were interested in whether the higher composition of regulatory T

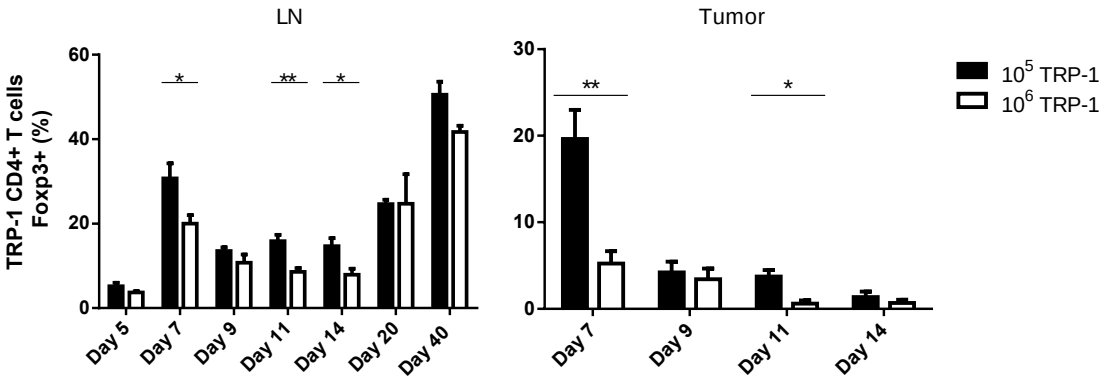

Figure 6.1. Throughout the anti-tumor immune response a lower clonal abundance favors the accumulation of TRP-1 specific CD4+ regulatory T cells.
C57BL/6J mice were implanted with 1 x 10^5 B16 tumor cells. Eighteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 10^5 or 10^6 TRP-1 CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. On the indicated days lymphocytes were isolated from lymph node and tumor and the percentage of Foxp3+ regulatory T cells within the TRP-1 CD4+ T cell population was evaluated (n=5 mice/group). Data are represented as mean ± SEM.

cells was unique to the tumor and the time points examined. When we extended the characterization of the regulatory T cell subset to a timecourse experiment, an increased percentage of regulatory T cells within the less clonally abundant TRP-1 CD4+ T cell population was observed throughout the anti-tumor immune response both in the periphery and in the tumor (Figure 6.1). The largest proportions of regulatory T cells were observed in the lymph node and the tumor on day 7 during the anti-tumor response and following the contraction phase high levels of Tregs were maintained in the lymph node (Figure 6.1). To determine if the increased proportion of regulatory T cells observed at lower clonal abundances was due to altered proliferation of the regulatory T cell subset, we compared Ki67 staining of the TRP-1 regulatory T cells to the effectors. We found that proliferation of Tregs within the group receiving 10^5 compared to 10^6 TRP-1 CD4+ T cells was elevated throughout the response (Figure 6.2B). Additionally, comparable or elevated overall levels of proliferation were observed in the regulatory

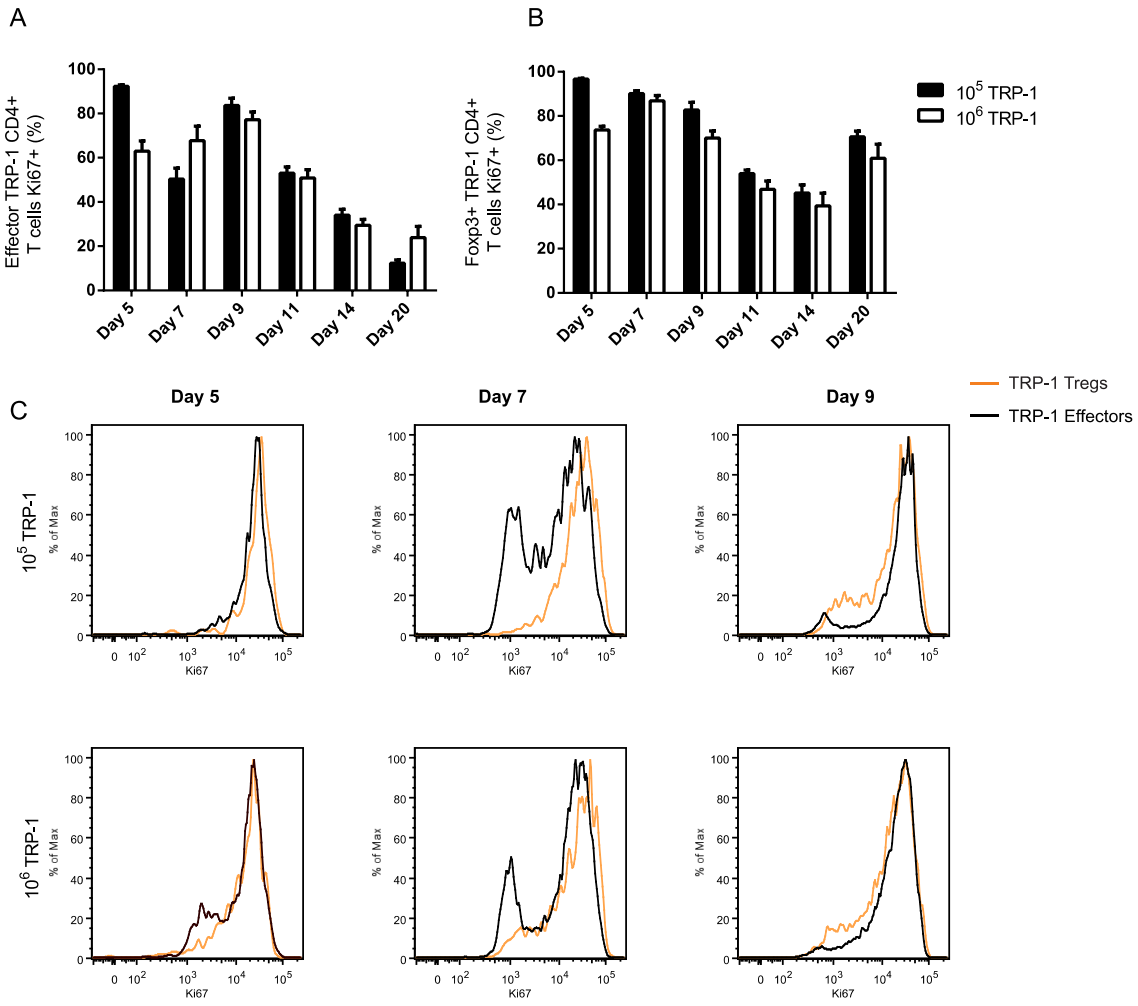

Figure 6.2. A higher degree of proliferation is observed in the regulatory compared to the effector TRP-1 specific CD4+ T cells.

C57BL/6J mice were implanted with 1 x 10^5 B16 tumor cells. Eighteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 10^5 or 10^6 TRP-1 CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. On the indicated days lymphocytes were isolated from lymph node and tumor characterized for proliferation by Ki67 staining. (A) Summary of percentage of Ki67+ effector TRP-1 CD4+ T cell population. (B) Summary of percentage of Ki67+ regulatory TRP-1 CD4+ T cell population. (C) Representative histogram plots of Ki67 staining separated by subset in lymph node (n=5 mice/group). Data are represented as mean ± SEM.

TRP-1 CD4+ T cells compared to the effector population (Figure 6.2A,B). Upon examination of the histogram plots for Ki67 staining it appears that the vast majority of regulatory T cells are undergoing division or have previously divided regardless of clonal abundance, whereas the percentage of effectors dividing changes over time and is impacted by T cell precursor frequency.

6.2. Balance between effector and regulatory TRP-1 CD4+ T cells is mediated by antigen and homeostatic mechanisms

We observed robust proliferation of TRP-1 specific regulatory T cells upon adoptive transfer into lymphopenic tumor bearing mice and performed a series of experiments to investigate the factors regulating their expansion within the TRP-1 CD4+ T cell population. We questioned whether the observed proliferation was dependent on the expression of ubiquitous self-antigen, the presence of tumor, or merely a reflection of the normal immune homeostasis of regulatory T cells. To address the contribution of ubiquitous self-antigen to the expansion of the TRP-1 specific regulatory T cell population, we adoptively transferred TRP-1 CD4+ T cells of varied precursor frequencies (10^6 and 10^5) into non-tumor bearing mice sufficient or deficient in the expression of the antigen TRP-1. High levels of proliferation, as measured by Ki67 expression, occurred in both regulatory and effector TRP-1 CD4+ T cell populations after transfer into mice deficient in TRP-1 (Figure 6.3B,C). When the regulatory T cell composition of the TRP-1 specific CD4+ T cell population was compared in the wildtype and TRP-1 deficient mice no clear differences emerged (Figure 6.3A). However, when the absolute numbers of total TRP-1 specific CD4+ T cells and those expressing Foxp3 were compared in cognate antigen sufficient and deficient hosts, decreased T cell accumulation was observed in the TRP-1 deficient hosts (Figure6.3D,E). These results demonstrate that TRP-1 expression does not affect the relative composition of the TRP-1

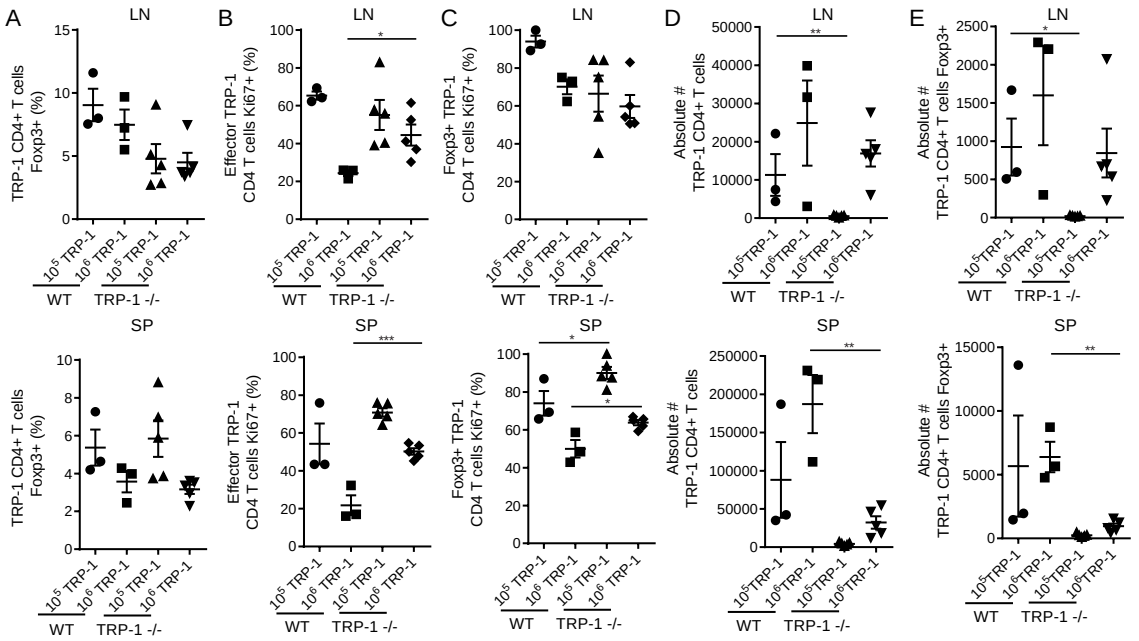

Figure 6.3. The proportion of TRP-1 specific regulatory T cells undergoing proliferation not impaired by absence of endogenous antigen.

Naïve wildtype C57BL/6J mice and TRP-1 deficient mice received 600 cGy of irradiation, followed by tail vein injection of 10^5 or 10^6 TRP-1 CD4+ T cells co-transferred with WT or TRP-1 deficient splenocytes for a total transfer quantity of 30 x 10^6 cells. On day 7 mice were euthanized and TRP-1 CD4+ T cells in the lymph node and spleens were evaluated. (A) Percentage of regulatory T cells within the TRP-1 CD4+ T cell population (B) Percentage of effector TRP-1 CD4+ T cells undergoing proliferation (C) Percentage of regulatory TRP-1 CD4+ T cells undergoing proliferation (D) Absolute number of total TRP-1 CD4+ T cells (E) Absolute number of Foxp3+ TRP-1 CD4+ T cells. *Top* lymph node, *bottom* spleen (n= 3,5 mice/group).

CD4+ T cell population, but does impact the extent to which the population expands and accumulates within the host. This indicates that differences in antigen level during an ongoing response may tune the amplitude of T cell accumulation, likely through the scaling of IL-2 (Tkach et al. 2014).

We directly assessed whether T cell abundance was affected by antigen dose and how changes in the quantity of antigen would impact the regulatory T cell composition of the TRP-1 CD4+ T cell population. Naïve wildtype hosts received adoptive transfer of TRP-1 CD4+ T cells at varied precursor frequencies (10^6 and 10^5) and were co-transferred with splenocytes that had been pulsed with titrated quantities of TRP-1 peptide. The TRP-1 specific T cell response to the peptide was evaluated in the spleen 7 days following adoptive transfer, due the diffuse administration of antigen resulting in a lack of draining lymph node. Consistent with the observations made in the TRP-1 deficient mice, increased antigen lead to an increase in the absolute number of total TRP-1 CD4+ T cells, including TRP-1 specific regulatory T cells (Figure 6.4B,C). Interestingly, the group that had received no antigen possessed the largest percentage of regulatory T cells within the TRP-1 CD4+ T cell population; when the quantity of antigen was increased, the percentage of regulatory T cells fell, regardless of the initial precursor frequency (Figure 6.4A). The decreased percentage of regulatory T cells observed at high antigen concentrations was not associated with decreased proliferation within the regulatory T cell population. In fact, the percentage of regulatory T cells undergoing proliferation remained fairly constant across the range of antigen administered, with exception. However, differences in the proliferation within the effector TRP-1 population were observed, with higher levels of antigen resulting in a decrease in the percentage of the population undergoing proliferation (Figure 6.4D). At first glance this seems paradoxical, as decreased proliferation of effector TRP-1 CD4+ T cells would result in an increased percentage of regulatory T cells. However, when proliferation is examined as a function of the absolute number of T cells undergoing

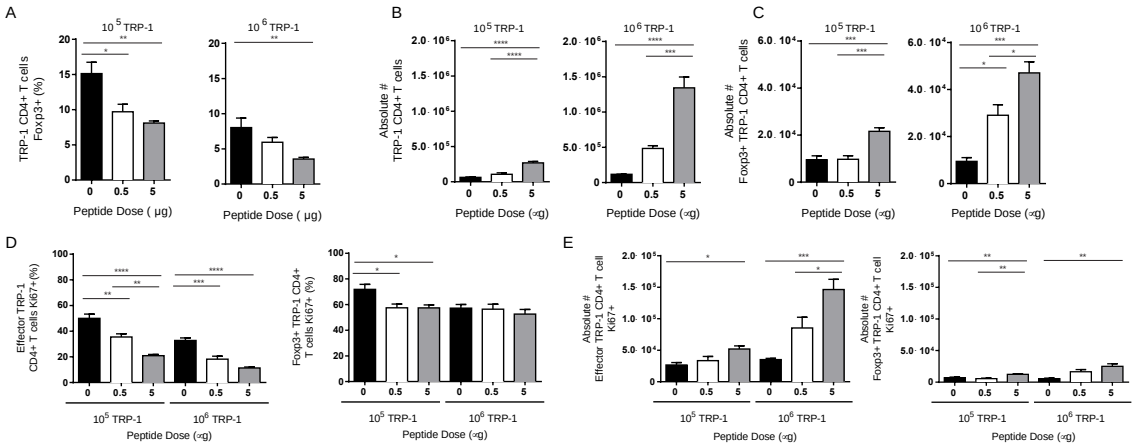

Figure 6.4. Antigen dose modulates the absolute number and composition of the TRP-1 CD4+ T cell population through effector T cell proliferation.
Naïve C57BL/6J mice received 600 cGy of irradiation, followed by tail vein injection of 10^5 or 10^6 TRP-1 CD4+ T cells. T cells were co-transferred with WT splenocytes that had been incubated for 2 hours with the indicated dose of peptide, washed, and then used to bring the total transfer quantity to 30 x 10^6 cells. On day 7 mice were euthanized and TRP-1 CD4+ T cells in the spleen were evaluated. (A) Percentage of regulatory T cells within the TRP-1 CD4+ T cell population. (B) Absolute number of total TRP-1 CD4+ T cells. (C) Absolute number of Foxp3+ TRP-1 CD4+ T cells. (D) Percentage of effector and regulatory TRP-1 CD4+ T cells undergoing proliferation. (E) Absolute number of effector and regulatory TRP-1 CD4+ T cells undergoing proliferation (n= 5 mice/group). Data are represented as mean ± SEM.

proliferation, significantly more effector T cells are proliferating at high antigen concentrations, which could account for the decrease observed in the proportion of regulatory T cells (Figure 6.4E). These results confirm that antigen level does dictate the accumulation of TRP-1 CD4+ T cells, and that increased clonal abundance favors greater T cell accumulation at equivalent antigen doses. Antigen appears to affect the proliferation of the effector T cell population more acutely than regulatory T cells, which displayed a relatively consistent level of proliferation. This finding suggests that changes to the percentage of regulatory T cells within the TRP-1 CD4+ T cell population, may not be driven by any alteration in the expansion of regulatory T cells, but instead by the proliferation of the effectors (Almeida, Zaragoza, and Freitas 2006).

After we had determined that expression of endogenous and exogenous cognate antigen was unnecessary to observe proliferation of regulatory T cells within the TRP-1 CD4+ T cell population, we wanted to address the contribution of the tumor itself. Immunosuppressive elements within the tumor microenvironment, such as cytokines like TGF-β, have been implicated in the accumulation and peripheral conversion of regulatory T cells (Liu et al. 2007). To examine if tumor is influencing expansion of regulatory T cells we adoptively transferred TRP-1 CD4+ T cells at different clonal abundances (10^6 and 10^5) into naive hosts, B16 tumor bearing hosts, or hosts bearing B78H1 tumors, which lack expression of the antigen TRP-1 and components of the antigen presentation pathway. B78H1 tumors were used to dissect the relative contribution of tumor itself versus the confounding effects of increased antigen load.

In previous experiments assessing the regulatory T cell subset, a greater percentage of regulatory T cells were found in mice possessing a lower clonal abundance of TRP-1 CD4+ T cells. In this experiment, however, different clonal abundances of TRP-1 CD4+ T cells gave rise to a similar composition of regulatory T cells in mice bearing B16 tumors (Figure 6.5A). Upon re-examination of previous data (Figure 4.2) it is clear that the composition of the regulatory T cell subset is often similar between the two clonal abundances being examined; however the range extends much higher in the group with a lower clonal abundance. The dynamic range of regulatory T cell frequency within the population provides further support for the possible contribution of regulatory T cells to the bi-modal distribution of anti-tumor responses observed at lower clonal abundance, with a higher percentage of regulatory T cells hypothetically favoring continued tumor growth.

Conversely, TRP-1 specific regulatory cells composed a greater percentage of the population at in groups of naïve mice or mice bearing B78H1 tumor when TRP-1 CD4+ T cells were at the lower clonal abundance (Figure 6.5A). This is a crucial observation, as it re-enforced the importance of antigen as a modifier of the balance between

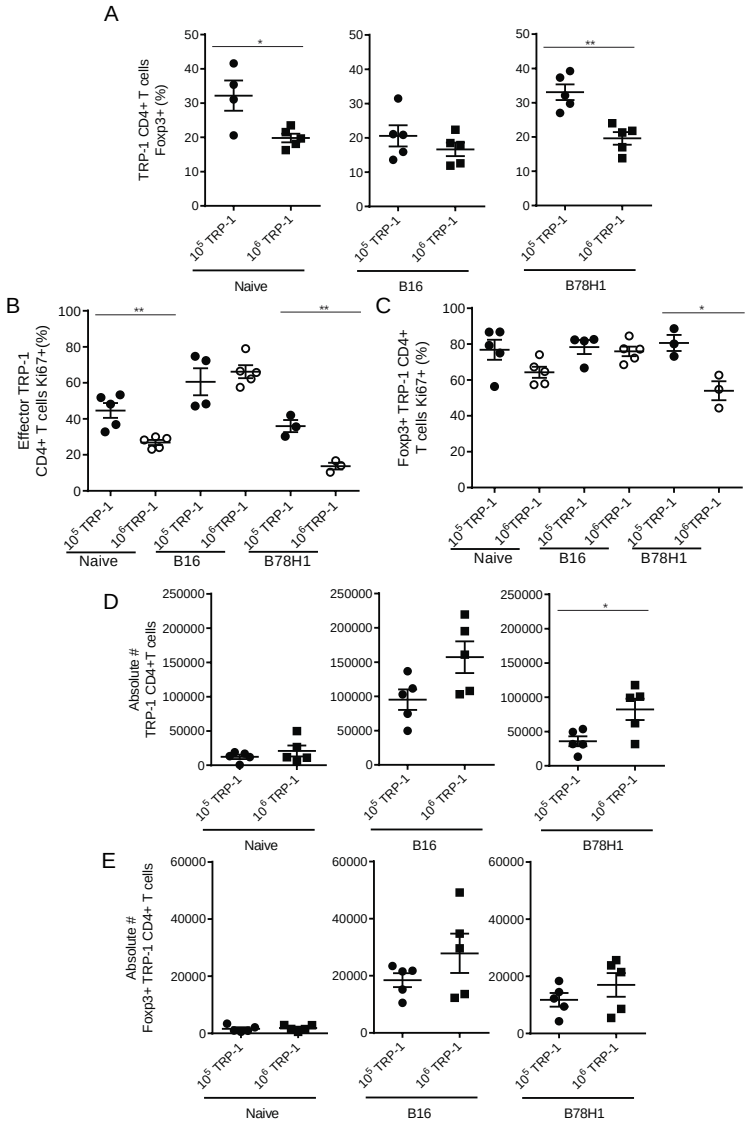

Figure 6.5. The effect of tumor on the TRP-1 CD4+ T cell population is largely driven by the expression of TRP-1 antigen.

C57BL/6J mice were implanted with 1 x 10^5 B16 tumor cells, 2.5 x 10^5 B78H1 tumor cells, or remained naive. Fourteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 10^5 or 10^6 TRP-1 CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. On day 7 mice were euthanized and TRP-1 CD4+ T cells in the lymph node were evaluated. (A) Percentage of regulatory T cells within the TRP-1 CD4+ T cell population. (B) Percentage of effector TRP-1 CD4+ T cells undergoing proliferation (C) Percentage of regulatory TRP-1 CD4+ T cells undergoing proliferation (D) Absolute number of total TRP-1 CD4+ T cells (E) Absolute number of Foxp3+ TRP-1 CD4+ T cells. Representative data from at least 3 independent experiments (n= 4-5 mice/group). Data are represented as mean ± SEM.

regulatory and effector T cells with the same antigen specificity. Similar to what had been previously observed in mice receiving antigen, this time in the form of a B16 tumor, it is the proliferation of the effector CD4+ T cell compartment that is primarily altered. In this experiment, the percentage of effectors undergoing proliferation increased in the presence of tumor bearing cognate antigen, while the proliferation of regulatory TRP-1 CD4+ T cells remained unchanged among the three groups (Figure 6.5B,C). This finding, taken together with the previous experiment examining peptide antigen, provides evidence for a homeostatic default within the TRP-1 CD4+ T cell population that favors a higher percentage of regulatory T cells in the absence of antigen. This implies that mice experiencing higher exposure to tumor antigen would possess a lower regulatory T cell frequency due to the expansion of effector TRP-1 CD4+ T cells. Additionally, any alteration in the proliferation of regulatory T cells is likely dependent on disrupting homeostasis, such as through the manipulation of IL-2 production. The concept of a homeostatic default favoring increased immune regulation is evolutionarily sensible for the prevention of autoimmunity and ensures that equilibrium is only perturbed in situations of high antigenic insult. Additional empirical evidence for a regulatory T cell homeostatic niche derived from the TRP-1 CD4+ T cell model is the observation that the absolute numbers of regulatory T cells are consistent in the peripheral lymphoid organs independent of clonal abundance (Figure 6.6).

B16 tumor also caused increases in the absolute number of TRP-1 CD4+ T cells in the lymph node, which supported previous observations about high levels of antigen favoring increased T cell accumulation. Interestingly, B78H1 tumor lacking antigen also led to a greater accumulation of T cells compared to naïve mice (Figure 6.5D,E). This suggested that the high accumulation of TRP-1 specific CD4+ T cells in B16 tumor bearing mice is not only dependent on antigen, but is also partly regulated by the tumor itself. Likely, this increase was due to the physiological changes that accompany tumor growth, such as lymphadenopathy and splenomegaly, which would expand the niche

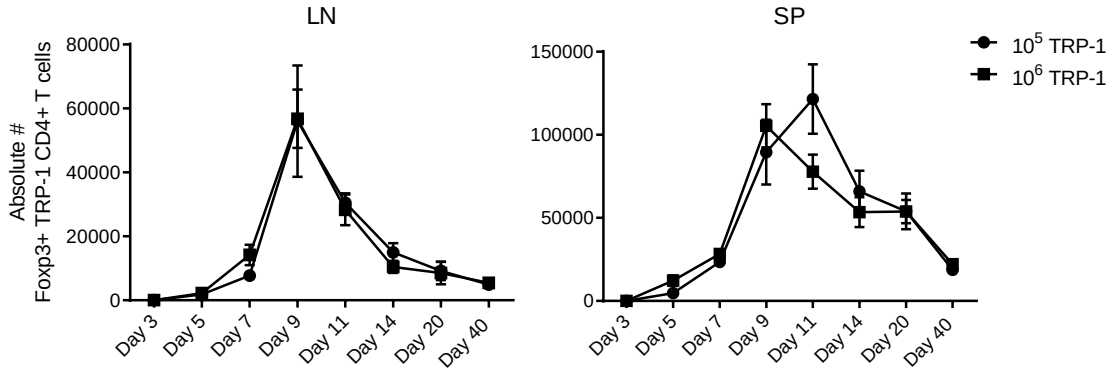

Figure 6.6. The absolute number of TRP-1 specific regulatory CD4+ T cells does not fluctuate in peripheral lymphoid organs due to alterations in clonal abundance. C57BL/6J mice were implanted with 1 x 10^5 B16 tumor cells. Eighteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 10^5 or 10^6 TRP-1 CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. On the indicated days lymphocytes were isolated from lymph node and spleen and the absolute number of Foxp3+ regulatory T cells within the TRP-1 CD4+ T cell population was evaluated (n=5 mice/group). Data are represented as mean ± SEM.

available for TRP-1 CD4+ T cells occupy. In support of this hypothesis, the absolute number of regulatory T cells in the spleen is higher at later time points in the 10^5 group (Figure 6.6). The kinetics of tumor regression are slightly delayed at lower precursor frequencies resulting in a higher tumor burden at later stages of the response, which sustains the larger T cell niche.

6.3. TRP-1 specific regulatory T cells display suppressive function in vivo and in vitro

In order to better understand whether the regulatory T cells within the total TRP-1 CD4+ T cell population have the capacity to attenuate the immune response, we conducted a number of assays to evaluate their suppressive function. To accomplish this we utilized a TRP-1 TCR transgenic mouse possessing a knock-in allele at the Foxp3+ site enabling production of a Foxp3-GFP protein chimera (Fontenot et al. 2005). This allowed for Foxp3+GFP+ regulatory T cells to be sorted from the TRP-1 CD4+ T cell population and facilitated transfer of a pure TRP-1 specific effector population.

Additionally, the effector T cells could be reconstituted with the sorted Foxp3+GFP+ regulatory T cells to restore the normal effector to regulatory T cell balance within the population. The abundance of Foxp3+ regulatory T cells was significantly lower in the reporter mice, comprising only 3-5% of the TRP-1 CD4+ T cell population, compared to the 8-10% observed in the unaltered TRP-1 TCR transgenic population. This posed some technical limitations regarding the quantity of TRP-1 specific regulatory T cells that could be isolated and required control groups to be reconstituted populations, rather than an unsorted control, to faithfully recapitulate the immune composition of previous experiments.

First, we examined the ability of TRP-1 specific regulatory T cells to modulate the development of cytotoxicity and effector function within the CD4+ T cell subset. Foxp3+GFP+ regulatory TRP-1 CD4+ T cells were sorted from GFP- effector TRP-1 CD4+ T cells; tumor-bearing hosts received either a pure effector population of 90,000 GFP- TRP-1 CD4+ T cells or 90,000 effectors supplemented with 10,000 GFP+Foxp3+ regulatory T cells and both groups received co-transfer of open repertoire splenocytes. On day 6 post-transfer, we conducted an *in vivo* killing assay, in which splenocytes were divided into two populations and labeled CellTrace Violet $(CTV)^{hi}$ or CTV^{lo}. The CTV^{hi} population was loaded with the MHC-II restricted peptide recognized by the TRP-1 CD4+ T cells and the CTV^{lo} cell population was used as control. Both populations were transferred intravenously and killing was evaluated by comparing the ratio of peptide-loaded targets to unloaded controls on the following day. In addition to this we examined other correlates of immunity, such as cytokine production upon restimulation, within the total CD4+ T cell population due to an inexplicable loss of congenic marker expression in the TRP-1 specific population. A striking decrease in target cell killing was observed in the group that had received adoptive transfer of regulatory T cells (Figure 6.7A). This decrease was correlated with a similar decline in effector function within the CD4+ T cell compartment. In the group that had received regulatory T cells, fewer CD4+ T cells

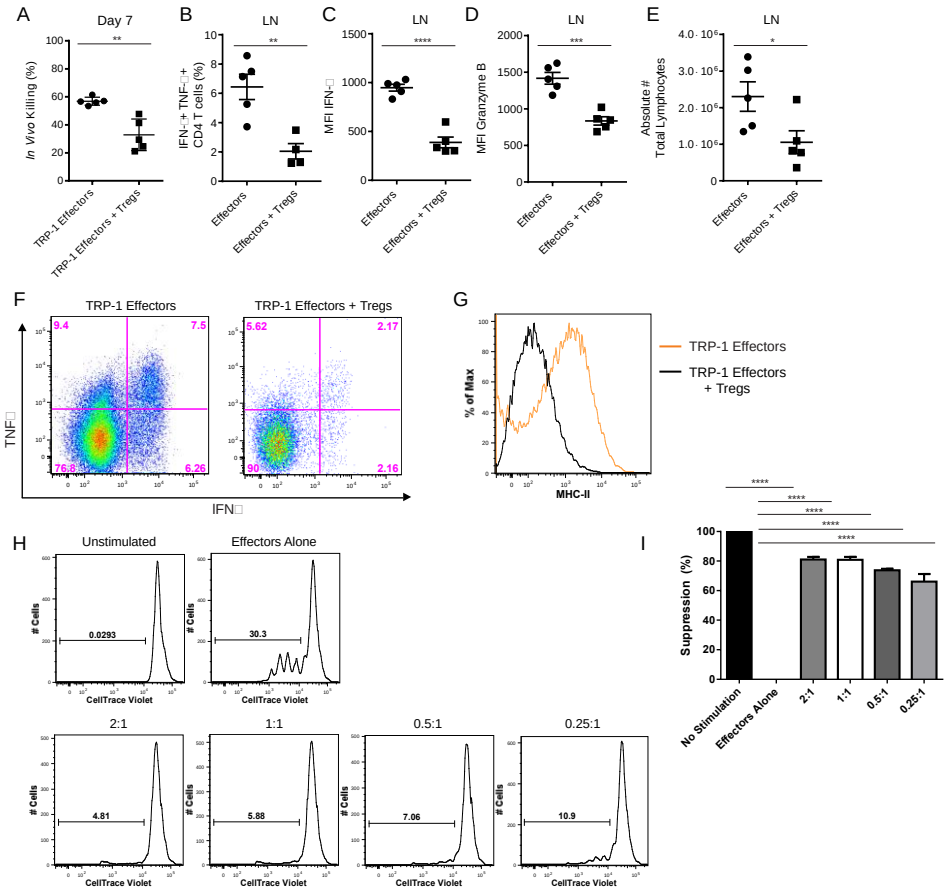

Figure 6.7. TRP-1 specific regulatory T cells display suppressive function *in vivo* and *in vitro*.

C57BL/6J mice were implanted with 1 x 10^5 B16 tumor cells and fourteen days post tumor challenge hosts were pre-conditioned with 600 cGy of irradiation. Mice received tail vein injection of 90,000 GFP- effector TRP-1 CD4+ T cells, sorted from a Foxp3-GFP reporter mouse, or 90,0000 GFP- TRP-1 CD4+ T cells supplemented with 10,000 GFP+ TRP-1 specific regulatory T cells. These cells were co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. On day 6, mice were transferred with CellTraceViolet (CTV) labeled *in vivo* killing assay targets. (A) On day 7 killing of labeled targets in the spleen was assessed. (B) Function of the entire CD4+ T cell compartment was evaluated after re-stimulation by ICS for IFNγ+ TNFα+ subsets (C) IFNγ production. (D) Granzyme B expression. (E) Total cellularity of the LN was evaluated. (F) Representative flow plots of IFNγ+ TNFα+ subsets. (G) MHC-II expression in CD45- tumor subset of recipient animals (n=5 mice/group). Sorted GFP+ TRP-1 regulatory T cells were co-incubated with stimulated or unstimulated CTV labeled effector TRP-1 CD4+ T cells at indicated regulatory to effector ratios. (H) Representative histogram plots of effector TRP-1 CD4+ T cell proliferation. (I) Percent suppression of proliferation. Representative data of at least 3 independent experiments. Data are represented as mean ± SEM.

showed compound production of IFNγ and TNFα (Figure 6.7B,F); total expression of IFNγ and granzyme B was also decreased (Figure 6.7C,D). The decline in effector function of the CD4+ T cells negatively impacted positive feedback in the tumor and resulted in a decrease in MHC-II expression in mice receiving regulatory T cells (Figure 6.7G). Additionally, the total cellularity of the draining lymph node was reduced by around 50% in the group receiving TRP-1 specific regulatory T cells, suggesting that regulatory T cells restrained lymphocyte proliferation or inhibited reconstitution of the irradiated lymph node (Figure 6.7E). The ability of regulatory T cells to alter the expansion of effector CD4+ T cell populations has been previously described (Almeida et al. 2002; Almeida et al. 2012).

To directly assess the capacity of TRP-1 specific regulatory CD4+ T cells to suppress proliferation of target cells we performed a classic *in vitro* suppression assay. Target effector TRP-1 CD4+ T cells were labeled with CellTraceViolet and stimulated to proliferate in the absence or presence of varying ratios of TRP-1 regulatory T cells. Suppression of proliferation was observed in all conditions in which regulatory T cells were present, with the highest level of suppression (80%) observed at a 2:1 ratio of regulatory T cells to effectors (Figure 6.7H,I). These results demonstrate that TRP-1 regulatory T cells are capable of functioning as suppressor cells and could be partly responsible for the attenuated anti-tumor responses observed at lower clonal abundances.

6.4. Depletion of TRP-1 specific regulatory T cells by targeting CD25 was unsuccessful

While we have demonstrated that these regulatory T cells display suppressive function both *in vivo* and in *in vitro* suppression assays, we have yet to clearly establish whether eliminating these cells could alter the efficacy of the anti-tumor response. Other groups have successfully depleted regulatory T cells in a non-specific manner by targeting the CD25 component of the IL-2 receptor with the antibody PC61 (Setiady, Coccia, and Park 2010). This strategy exploits the high CD25 expression observed on

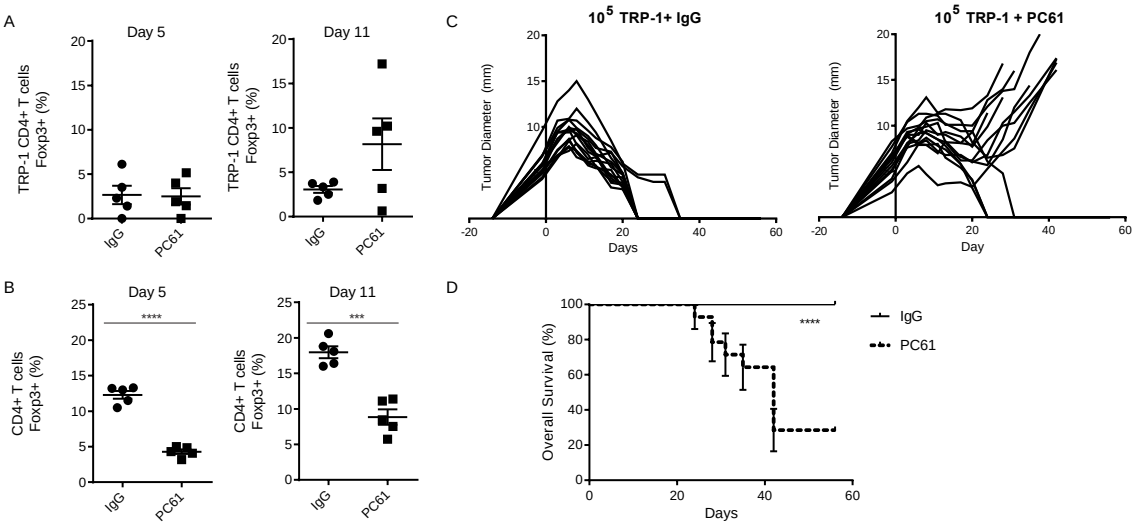

Figure 6.8. Depletion of TRP-1 specific regulatory T cells by CD25 targeting antibody PC61 was unsuccessful.

C57BL/6J mice were implanted with 1 x 10^5 B16 tumor cells. Fourteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 10^5 TRP-1 CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. Mice received intra-peritoneal injection of 200 μg of an antibody targeting CD25 (PC61) or an IgG control beginning on the day prior to adoptive transfer and every other day until 21 days post-transfer. (A) Mice were bled on the indicated days and TRP-1 specific regulatory T cell depletion and (B) total CD4+ T cell regulatory T cell depletion were confirmed by flow cytometry (n=5 mice/group). (C) Tumor diameter was measured every 3-5 days by caliper and is represented on the graph by individual lines. (D) The overall survival of each group was plotted. Mice were euthanized when tumor diameter reached 2 cm (n=14-15 mice/group). Data are represented as mean ± SEM.

Foxp3+ regulatory T cells and their dependence on IL-2 consumption. However, one caveat to this approach is that it also non-specifically targets other cell populations expressing high levels of CD25, such as activated T cells. To determine if this approach could be used to elucidate the function of the TRP-1 specific regulatory T cells *in vivo,* hosts bearing established melanoma received TRP-1 CD4+ T cells at a precursor frequency of 10^5 and were treated with PC61 or an isotype control. On days 5 and 11 after adoptive transfer regulatory T cell depletion was confirmed in the peripheral blood. When the total CD4+ T cell population was examined PC61 treatment had reduced the percentage of regulatory T cells at both time points (Figure 6.8B). However, the proportion of TRP-1 specific regulatory T cells in the population was either unchanged or even insignificantly increased (Figure 6.8A). Antibody depletion targeting CD25 facilitated impaired tumor growth relative to the control (Figure 6.8C). This resulted in a dramatic reduction in overall survival from 100% in the isotype control treated animals to just over 20% survival in the animals receiving CD25 depleting antibody (Figure 6.8D).

These results were unexpected, however the most likely explanation is that both activated effector and regulatory TRP-1 CD4+ T cells were depleted by PC61 due to high expression of CD25. This would have resulted in a failure to reach the critical concentration of T cells in the tumor necessary for regression. Using the blood to validate this hypothesis would be inconclusive and these observations were not investigated further due to the lack of efficacy of this approach. In order to specifically target the Foxp3+ TRP-1 regulatory CD4+ T cells, the ideal approach would be to cross the TRP-1 TCR transgenic mice with a Foxp3-DTR mouse strain. In the resulting strain, the Foxp3+ TRP-1 CD4+ DTR+ T cells would be depleted following adoptive transfer through the injection of diphtheria toxin. However, because the TRP-1 CD4+ T cell TCR transgenic line was maintained on a TRP-1 and RAG deficient background, generation of this strain was not a trivial endeavor. At the time of the writing of this thesis, the strain has been successfully established in our lab and others and provides a means to further

investigate the role of TRP-1 specific regulatory T cells in the modulation of effector TRP-1 CD4+ T cell responses and homeostasis.

6.5 The proportion of regulatory T cells is under similar homeostatic control in RAG deficient hosts

We were unsuccessful in demonstrating a direct role for the TRP-1 specific regulatory T cells in suppression of the anti-tumor response in tumor bearing wildtype hosts due to the failure of CD25 targeted deletion and unavailability of Foxp3-DTR mice. In another attempt to establish function of the TRP-1 regulatory T cells *in vivo* we exploited the RAG knock out model, in which TRP-1 CD4+ T cells can be manipulated without the alteration of bystander populations. This would allow us to correlate any differences in anti-tumor efficacy to perturbations of the regulatory T cell compartment. We first characterized the regulatory T cell composition within the TRP-1 specific population upon adoptive transfer into tumor bearing wildtype and RAG deficient hosts. The percentage of Foxp3+ TRP-1 CD4+ T cells was reduced in RAG knockout hosts compared to wildtype animals, despite a significant increase in the absolute number of TRP-1 CD4+ T cells (Figure 6.9A,B). Although we had previously hypothesized that the increased anti-tumor efficacy observed in RAG deficient mice was partly due to a reduction in regulatory T cells, we had primarily been considering the contribution of the endogenous co-transferred regulatory T cell compartment. The observed reduction In TRP-1 specific regulatory T cells provided additional support for the hypothesis of reduced regulatory T cell suppression in RAG deficient hosts.

We have established that alterations in the percentage of TRP-1 specific regulatory T cells are due to variations in the relative expansion of the effectors in wildtype mice. To determine if this tenet extends to RAG deficient hosts we compared the population composition and absolute numbers in wildtype and RAG deficient tumor bearing mice in the presence and absence of co-transferred splenocytes. The co-transfer

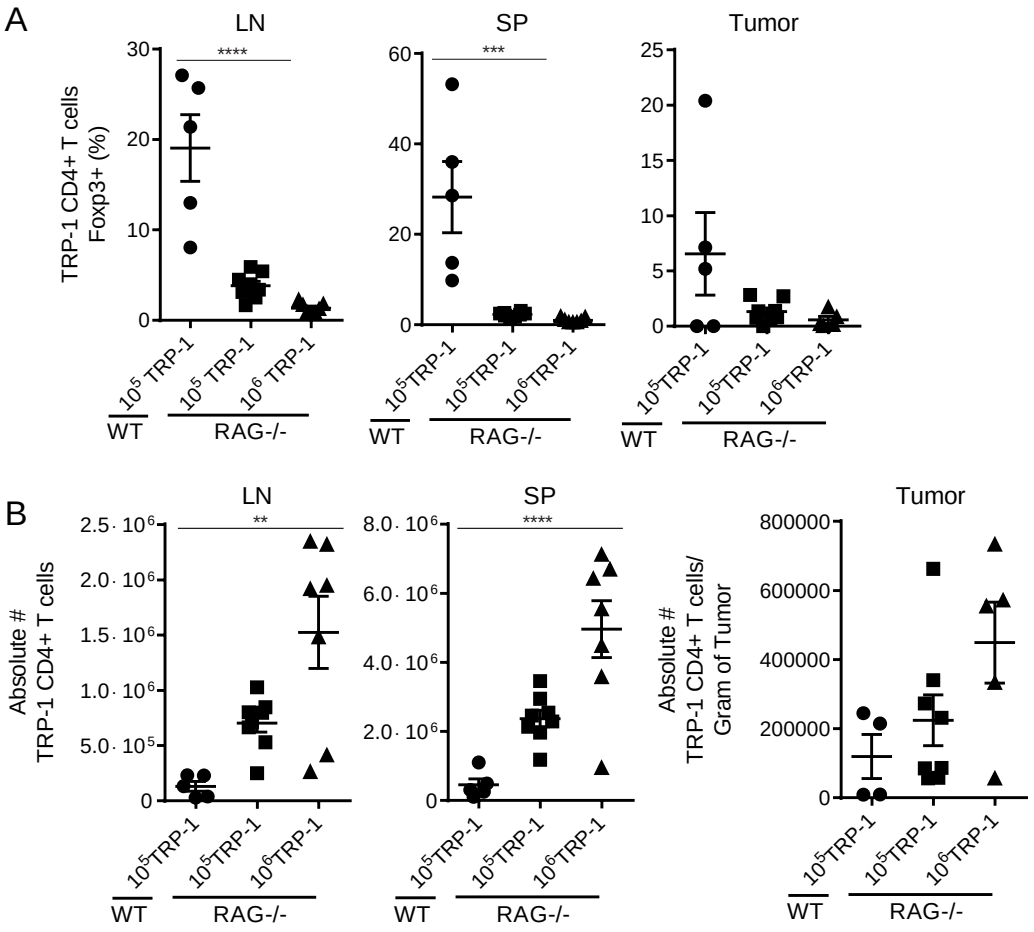

Figure 6.9. TRP-1 CD4+ T cells accumulate at high numbers in RAG deficient mice and exhibit decreased regulatory T cell composition.

WT C57BL/6J or RAG deficient mice were implanted with 1 x 10^5 B16 tumor cells. Fourteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 10^5 or 10^6 TRP-1 CD4+ T cells with the absence of co-transfer in RAG deficient hosts. On day 7 mice were euthanized and TRP-1 CD4+ T cells in the lymph node, spleen, and tumor were evaluated. (A) Percentage of regulatory T cells within the TRP-1 CD4+ T cell population. (B) Absolute number of total TRP-1 CD4+ T cells (n= 4-5 mice/group). Data are represented as mean ± SEM.

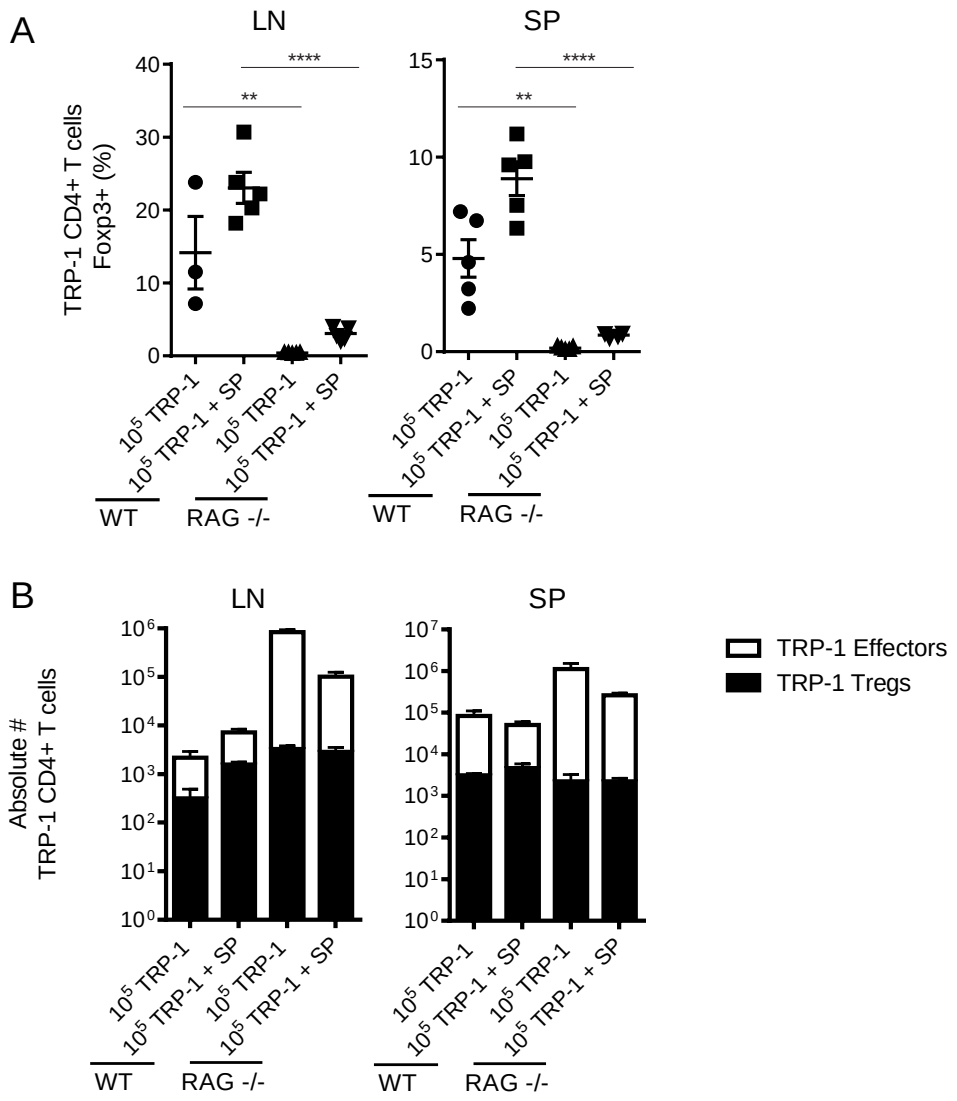

Figure 6.10. The absolute number of TRP-1 specific regulatory T cells shows little variation compared to the effector T cell compartment.

WT C57BL/6J or RAG deficient mice were implanted with 1 x 10^5 B16 tumor cells. Fourteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 10^5 TRP-1 CD4+ T cells with or without co-transfer of naïve splenocytes to bring total transfer quantity to 30 x 10^6 cells. On day 7 mice were euthanized and TRP-1 CD4+ T cells in the lymph node and spleen were evaluated. (A) Percentage of regulatory T cells within the TRP-1 CD4+ T cell population. (B) Absolute number of total regulatory and effector TRP-1 CD4+ T cells on log scale (n= 5 mice/group). Data are represented as mean ± SEM.

condition was used to determine if bystander T cells were capable of restoring the TRP-1 regulatory T cell compartment in RAG deficient hosts to wild type levels, theoretically via population derived IL-2. We did observe a modest increase in the percentage of regulatory T cells in both wildtype and RAG deficient hosts receiving transfer of bystander splenocytes, however this enhancement in RAG knockout animals did not approach restoration of wildtype levels (Figure 6.10A). This modest increase of regulatory T cell frequency in the conditions receiving splenocytes was once again not due to any measurable alteration in regulatory T cell accumulation, as the absolute number of regulatory T cells in the lymph node and spleen were comparable especially in the RAG knockout hosts. Instead, increased regulatory T cells were observed due to restricted accumulation of TRP-1 specific effectors in the presence of co-transferred splenocytes (Figure 6.10B.). This is likely due to the bystander cells filling the homeostatic niche that the TRP-1 CD4+ T cells would have occupied in their absence. This finding confirms that modulation of regulatory T cell composition, even in chronically lymphopenic hosts, is primarily shaped by the proliferative potential of the effector T cells relative to the size of a static regulatory T cell niche. It also raises the possibility that bystander cell transfer can be used to limit effector expansion within a clonal population, which has implications for the use of adoptive cell therapies in autoimmune disorders.

6.6. Manipulation of IL-2 modifies the magnitude and composition of the TRP-1 CD4+ T cell response

To determine if the regulatory T cell population could be directly altered in RAG deficient mice through therapeutic intervention we explored the use of cytokine blocking antibodies. We believed this approach would have a greater probability for success in lymphopenic animals due to the absence of bystander cells, which may act as cytokine sinks. Tumor bearing RAG knockout mice received adoptive transfer of 10^5 TRP-1

CD4+ T cells. One day prior to transfer and every other day following, mice received IL-2 blocking antibody or antibody targeting IL-7R. IL-2 blocking antibody was intended to directly suppress regulatory T cells proliferation, while we believed IL-7R blocking antibody would constrain the lymphopenia-induced proliferation of the effector TRP-1 CD4+ T cells. Contrary to this, IL-2 blockade was found to lead to an overall increase in the percentage of regulatory T cells due to the inhibition of effector TRP-1 proliferation (Figure 6.11A,B,C). In addition to this, the absolute number of the total TRP-1 population exhibited a sharp decline (Figure 6.11.D,E). IL-7 receptor blockade resulted in a similar, yet more modest increase in regulatory T cell percentage and decrease in overall TRP-1 CD4+ T cell absolute number. This experiment demonstrated that IL-2 and IL-7R blockade would be an ineffective approach for a tumor regression experiment; it would be unclear whether a decline in efficacy were attributable to the increase in regulatory T cell percentage or reduction of the total TRP-1 T cell population. A result such as this would have phenocopied the CD25 targeting by PC61 experiment conducted in wildtype mice.

In the converse experiment we endeavored to modulate the expansion of TRP-1 specific CD4+ T cells by providing exogenous IL-2 in the form of IL-2 complexes. IL-2 complexes increase the half-life of IL-2 and are used to target specific T cell subsets, which is dependent on the antibody clone employed (Boyman et al. 2006). We used IL-2 in complex with JES6-1, which has been demonstrated to preferentially enhance regulatory T cell expansion. Naïve and tumor bearing recipients of TRP-1 CD4+ T cell adoptive transfer were treated with IL-2 complexes or a PBS control. We observed an increase in the percentage of regulatory T cells in the lymph node of naïve mice receiving IL-2 complexes. The regulatory T cell subset in the lymph node and spleen of tumor bearing mice treated with IL-2 complexes was slightly increased compared to control, but the difference was not significant (Figure 6.12A). The composition of the TRP-1 CD4+ T cell tumor infiltrate remained unchanged. Modest increases in the absolute number of

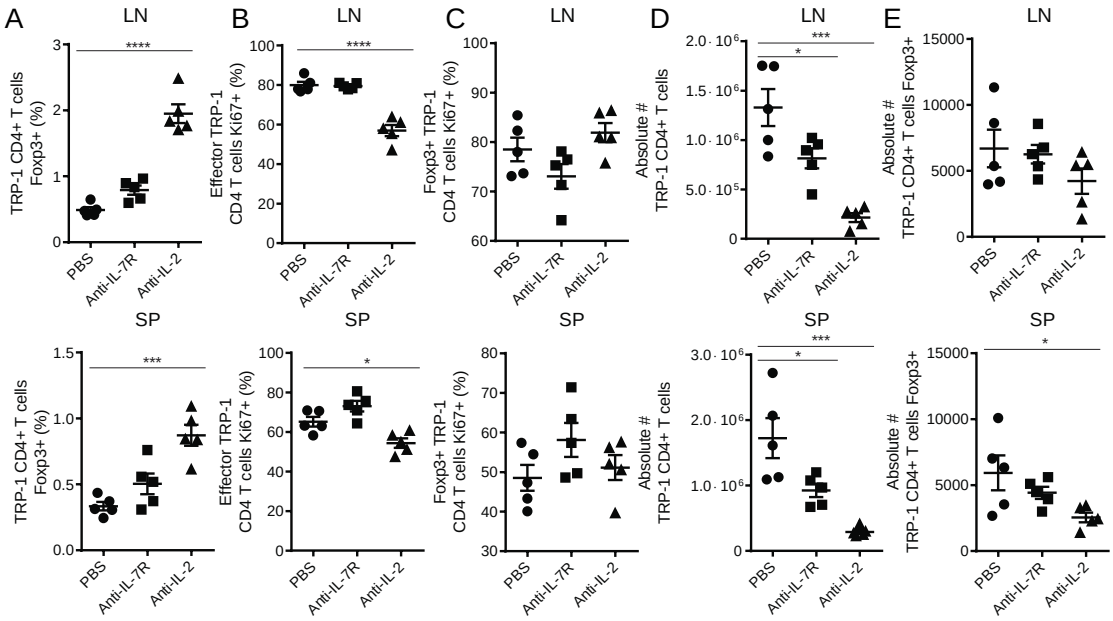

Figure 6.11. IL-2 blockade resulted in a decrease of TRP-1 CD4+ T cell absolute number and an enhanced regulatory T cell population.

RAG deficient mice were implanted with 1 x 10^5 B16 tumor cells. Fourteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 10^5 TRP-1 CD4+ T cells in the absence of co-transfer. Mice received intra-peritoneal injection of 200 μg of an antibody targeting IL-2 or IL-7R or PBS control beginning on the day prior to adoptive transfer and continuing every other day until mice were euthanized on day 7 to characterize the TRP-1 CD4+ T cells in lymph node and spleen. (A) Percentage of regulatory T cells within the TRP-1 CD4+ T cell population. (B) Percentage of effector TRP-1 CD4+ T cells undergoing proliferation (C) Percentage of regulatory TRP-1 CD4+ T cells undergoing proliferation (D) Absolute number of total TRP-1 CD4+ T cells (E) Absolute number of Foxp3+ TRP-1 CD4+ T cells (n= 4-5 mice/group). Data are represented as mean ± SEM.

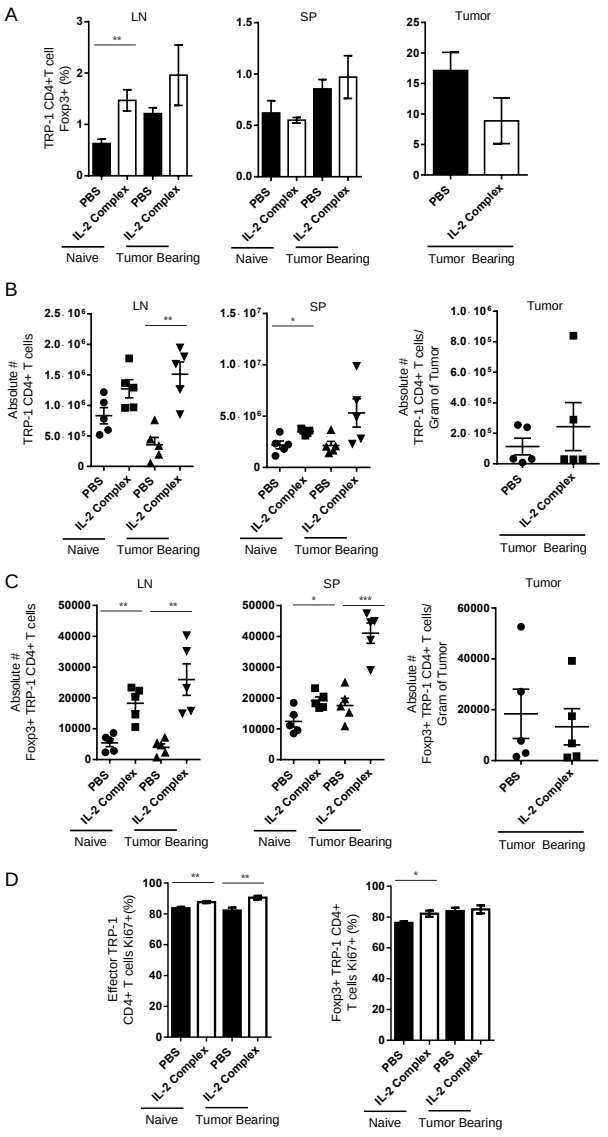

Figure 6.12. IL-2 blockade resulted in a decrease of TRP-1 CD4+ T cell absolute number and an enhanced regulatory T cell population.

A cohort of RAG deficient mice was implanted with 1 x 10^5 B16 tumor cells while another was left naive. Fourteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 10^5 TRP-1 CD4+ T cells in the absence of co-transfer. Mice received intra-peritoneal injection of 200 μg of antibody-IL-2 complex or PBS control beginning on the day of adoptive transfer and continuing every other day until mice were euthanized on day 7 to characterize the TRP-1 CD4+ T cells in lymph node, spleen, and tumor. (A) Percentage of regulatory T cells within the TRP-1 CD4+ T cell population. (B) Absolute number of total TRP-1 CD4+ T cells (C) Absolute number of Foxp3+ TRP-1 CD4+ T cells. (D) Percentage of effector and regulatory TRP-1 CD4+ T cells undergoing proliferation (n= 5 mice/group). Data are represented as mean ± SEM.

TRP-1 specific CD4+ T cells were observed in IL-2 complex treated animals, but the most notable difference was the large increase in the absolute number of TRP-1 specific regulatory T cells (Figure 6.12.B,C). Characterization of Ki67+ staining demonstrated increased proliferation of both effector and regulatory T cells in naïve IL-2 complex treated animals, but only the proliferation of effectors increased in tumor bearing mice (Figure 6.12D). We chose not to further pursue this approach in tumor regression experiments due to the inability of IL-2 complexes to demonstrate alterations in the tumor infiltrating TRP-1 population and the less dramatic phenotype observed in tumor bearing hosts. However, this may be interesting to pursue if further optimized to elicit more substantial changes. The TRP-1 specific regulatory T cells proved difficult to modulate, even in the absence of bystander cells, and we were unable to directly or indirectly validate the suppressive function of the regulatory T cells during an anti-tumor response.

CHAPTER 7: CHARACTERIZATION OF TRP-1 SPECIFIC CD4+CD8+ T CELLS

Introduction

Over the course of our investigations we focused solely on the activity of the TRP-1 specific CD4+ T cells and disregarded the contributions of a secondary population of TRP-1 specific T cells that expressed both CD4 and CD8 T cell co-receptors. While double positive T cells have been traditionally recognized as a differentiation state during thymic development, mature CD4+CD8+ extra-thymic T cells have begun to be acknowledged as a novel subset or state of differentiation warranting further study. While we and others describe this cell type in mice, CD4+CD8+ T cells have also been demonstrated in humans, primates, rats, chickens, and pigs (Overgaard et al. 2014). In humans and primates their numbers have been shown to increase with age and thymic involution (Laux et al. 2000; Lee et al. 2003). Additionally, this subset of cells has been described to expand within the context of many human diseases, including melanoma and other chronic conditions, such as viral infections and autoimmunity (Figure 7.1). However, the function of CD4+CD8+ T cells remains controversial and may vary depending on the disease context.

During HIV infection, CD4+CD8+ T cells have been shown to be highly proliferative, responsive to virus, and are polyfunctional effectors (Frahm et al. 2012). However, the role of this subset within human cancer remains largely undefined. In a study characterizing the CD4+CD8+ double positive subset in human melanoma, the authors observed a significant double positive T cell population in about 60% of cases investigated (Desfrancois et al. 2010). When the lymphocytes from tumor invaded lymph node, solid tumor metastases, and peripheral blood were compared, it was found that the CD4+CD8+ subset was enriched at the site of lymph node and tumor, but found at very low levels in the blood. Vβ usage analysis of the populations revealed that the TCR repertoire was somewhat diverse, despite evidence of a dominant clonal response in some

TABLE 1. CD4/CD8 DP T cells have been described in multiple human disease settings

Disease condition	Isolation site	References
Infectious disease		
HIV	Blood	[20, 21]
Chronic hepatitis B	Blood, liver	[22]
Chronic hepatitis C	Blood, liver	[22]
CMV infection	Blood	[23, 24]
Lepromatous leprosy	Blood	[25]
Chagas disease	Blood	[26, 27]
Cancer		
Cutaneous T cell lymphoma	Cutaneous tumor	[28]
Melanoma	Tumors, lymph nodes	[29]
Nodular lymphocyte predominant Hodgkin lymphoma	Lymph nodes	[30]
Colorectal cancer	Colonic mucosa	[31]
Human T cell leukemia virus type 1-associated leukemia	Blood	[32]
Breast cancer	Tumors, pleural effusions	[33]
Autoimmune disease		
Atopic dermatitis (eczema)	Skin, blood	[34]
Rheumatoid arthritis	Blood, synovium	[35]
Inflammatory bowel disease	Blood, intestinal mucosa	[36]
Cutaneous systemic sclerosis	Skin	[1]
Other		
GVHD	Skin	[37]

CD4/CD8 DP T cells have been described in various diseases, including infectious disease, cancer, autoimmune disease, and GVHD.

Figure 7.1. CD4+CD8+ double positive T cells have been detected during chronic viral infections, cancer, and autoimmunity.

Although CD4+CD8+ double positive T cells are recognized primarily as a pivotal state of differentiation during thymic development extra-thymic T cells expressing both co-receptors have been observed in humans and in mice in the disease setting. Adapted from (Overgaard et al., 20014).

of the patients examined. Using a CD4+CD8+ T cell clone derived from a patient demonstrating a dominant clonal response, it was revealed that the clone was HLA-I restricted and broadly reactive against patient matched tumor, as well as allogeneic tumor and healthy tissues, suggesting recognition of a ubiquitous self antigen. Upon restimulation of a polyclonal double positive population, via anti-CD3, the CD4+CD8+ T cell subset was found to produce IFN-γ, TNF-α, and IL-2, in combination with IL-4, IL-5, IL-13, and GM-CSF. This cytokine profile was similar to what had been observed during a prior study by the same group examining the double positive subset in the pleural effusions of breast cancer patients (Desfrancois et al. 2009). IL-4, IL-5, and IL-13 are more closely related to the Th2 lineage, are involved in the engagement of innate immunity, but have also been implicated in immune suppression. The study of double positive T cells within colitis has revealed possible suppressive functions due to observations of reduced disease severity when these T cells dominated the CD4+ T cell response (Reis et al. 2013). Impaired Th17 polarization, increased IL-10 and TGF-β production, and loss of CD40L expression have all been implicated as possible drivers of the decreased colitis observed upon transfer of CD4+CD8+ T cells (Reis et al. 2013; Das et al. 2003; Vacchio et al. 2014). Despite these observations, the true function of these cells is still ambiguous, as is why they preferentially accumulate in the disease setting.

The T cell co-receptors CD4 and CD8 are used to classify the two conventional αβ T cell lineages. Both co-receptors contain Ig-like domains, bind to MHC outside of the peptide-binding groove during antigen recognition, and signal through the tyrosine kinase Itk (Turner et al. 1990). During T cell development in the thymus and throughout T cell maturation, expression of these co-receptors is tightly regulated by a set of lineage specific transcription factors. When co-receptor double negative progenitor cells migrate from the bone marrow and into the thymus, the progenitor cells begin to re-arrange their TCR. After both subunits of a complete TCR have completed re-arrangement and are expressed on the T cell surface, the T cells transition to the double positive thymocyte

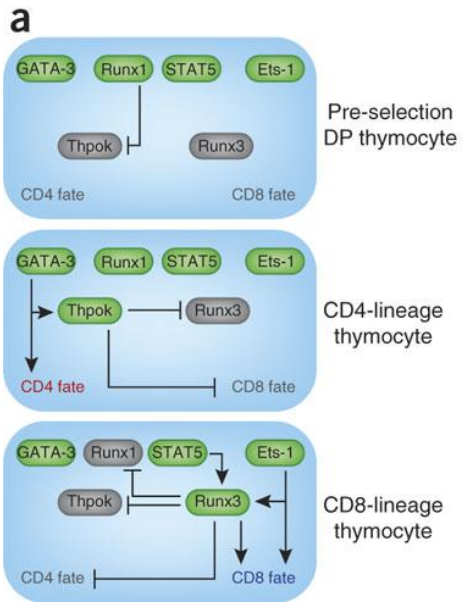

Figure 7.2. Regulation of T cell lineage commitment is dependent on the master regulators ThPOK and Runx3.
In the transition from the double positive thymocyte stage to individual single positive T cell subsets, the master regulators are induced by upstream factors and initiate the transcription of lineage specific markers while suppressing the alternate lineage. Adapted from Carpenter and Bosselut 2010.

stage and concurrently express both CD4 and CD8 co receptors. During positive selection in the thymic cortex, T cells that positively react with selecting peptide-MHC of an appropriate affinity survive to become single positive T cells (Starr, Jameson, and Hogquist 2003). This process is mediated by the exclusive expression of lineage specific master regulators. ThPOK is the master regulator expressed in the CD4+ T cell lineage and Runx3 is responsible for driving the CD8+ T cell lineage (Figure 7.2)(Carpenter and Bosselut 2010). While each transcription factor is responsible for directing lineage commitment, they are also involved in the active suppression of the alternate lineage fate. Runx3 binds to both the silencer region within the distal regulatory element of *ThPOK* and redundantly with Runx1 to the silencer of *CD4* to suppress their transcription (He et al. 2008; Sato et al. 2005). Conversely, ThPOK suppresses Runx3 expression and directly represses transcription of *Cd8a* and cytotoxic effector genes (Wang et al. 2008;

Egawa and Littman 2008). ThPOK has also been shown to bind to its own silencer to prevent de-repression by Runx3 (Mucida et al. 2013).

Although lineage commitment has long been regarded as non-plastic, recent work profiling the transcriptional regulation of CD4+CD8+ double positive T cells and cytolytic CD4+ T cells has shown that T cells can be post-thymically reprogrammed (Mucida et al. 2013). Using ThPOK reporter mice and ThPOK fate mapping mice it was shown that a subset of mature CD4+ T cells lose ThPOK expression and gain cytolytic potential due to the de-repression of the ThPOK silencer. These T cells were particularly enriched in the intestinal epithelium and regained expression of CD8a, which manifested itself on the cell surface in the form of CD8αα homodimer (Mucida et al. 2013). A subsequent study of these intraepithelial lymphocytes revealed that some intraepithelial CD4+ T cells expressed ThPOK concurrently with Runx3. Using dual reporter mice, the authors demonstrated the absence of $Runx3^{lo}ThPOK^{lo}$ cells, implicating that elevation of Runx3 precedes ThPOK loss. However, this remains unresolved as the same study also shows efficient ThPOK downregulation in the absence of Runx3, likely due to MAZR (Reis et al. 2013). Heterogeneity exists in this population, as it has also been demonstrated that double positive T cells can arise from activation of naive CD8+ T cell (Flamand et al. 1998); little is known about how this may occur.

Here we provide a model in which the CD4+CD8+ T cell population can be investigated within the context of tumor immunity. This chapter details our characterization of the development and phenotype of the CD4+CD8+ TRP-1 specific T cell population. We describe the expansion of the CD4+CD8+ TRP-1 specific T cell population at high clonal abundances during the effector phase of the anti-tumor response. During *in vitro* cell culture we find that propagation of this population is supported by cytokines known to signal through STAT5. When the memory phenotype of the double positive TRP-1 specific population was examined we found that it most closely resembled an effector memory phenotype accompanied by the uncharacteristic

upregulation of CD25. Additionally, the effector profile of these cells was found to skew towards a cytolytic phenotype, with high granzyme B expression, which further supports an effector memory type function. We believe that this enhanced effector profile is due to the downregulation of ThPOK in the CD4+CD8+ T cells, which allows the de-repression of Runx3 resulting in the acquisition of CD8 T cell lineage features.

Results and Discussion

7.1. TRP-1 specific CD4+CD8+ T cells are observed over the course of the anti-tumor immune response

During the height of the immune response, which occurs between days 7 and 11, we consistently observed the emergence of a TRP-1 specific CD4+CD8+ double positive population within hosts containing a high clonal abundance of TRP-1 specific T cells (Figure 7.3D). To initially characterize this unique subset of cells we re-analyzed previously conducted experiments with a focus on this new population of interest. In tumor bearing mice with a high clonal abundance of TRP-1 T cells, CD4+CD8+ T cells form within the CD3+ population of the lymph node and tumor on day 7 (Figure 7.3A), but can also be observed in the spleen and peripheral blood (data not shown). In the tumor especially, the formation of this population is frequently accompanied by the down regulation of CD4 expression (Figure 7.3A). Additional characterization of this subset revealed that these T cells express the CD8αβ heterodimer, as opposed to the CD8αα homodimer commonly found on intraepithelial lymphocytes (data not shown).

When TRP-1 specific T cells were present at a high clonal abundance a significant double positive T cell population originated only in mice bearing B16 tumors; naïve mice and mice bearing B78H1 tumors deficient in TRP-1 expression failed to elicit this response (Figure 7.3B). This finding suggests that this population is not regulated by homeostatic mechanisms similar to regulatory T cells, but is instead dependent on antigen encounter, which has been implicated in the generation of this subset

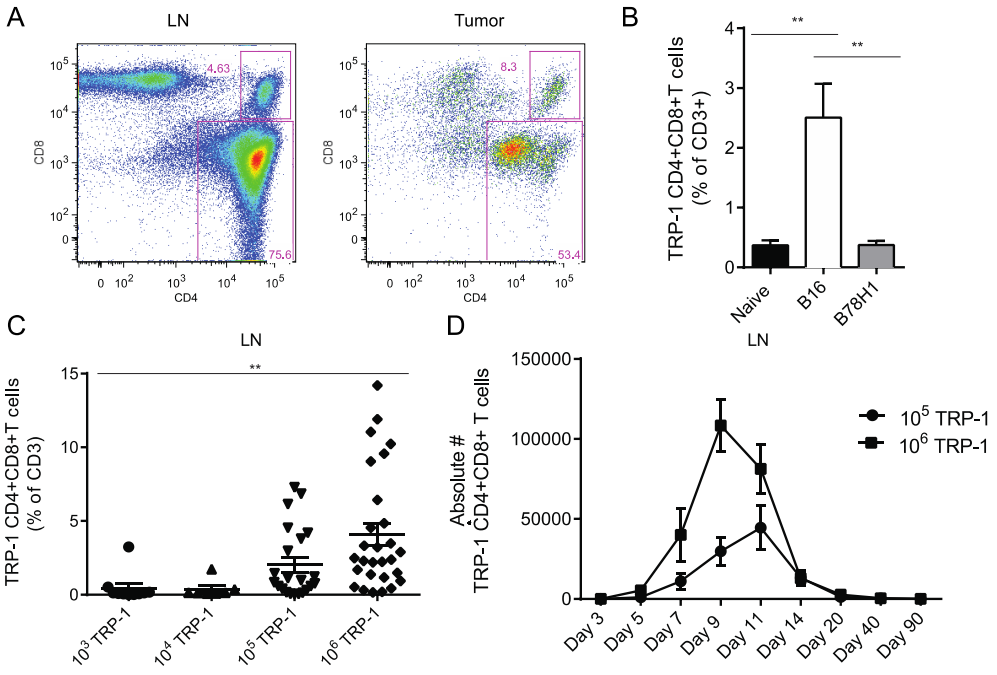

Figure 7.3. TRP-1 specific CD4+CD8+ T cells are observed over the course of the anti-tumor immune response.

(A) C57BL/6J mice were implanted with 1 x 10^5 B16 tumor cells. Fourteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 10^6 TRP-1 CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. On day 7 post-transfer CD8+CD4+ double positive cells are observed within the CD3+ population in lymph node and tumor. (B) C57BL/6J mice were implanted with 1 x 10^5 B16, 2.5 x 10^5 B78H1, tumor cells or remained naive. Fourteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 10^6 TRP-1 CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. On day 7, CD4+CD8+ TRP-1 T cells were characterized in LN as a percentage of the CD3+ population (n=5 mice/group). (C) C57BL/6J mice were implanted with 1 x 10^5 B16 tumor cells. Fourteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 10^3, 10^4, 10^5, or 10^6 TRP-1 CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. On day 7, CD4+CD8+ TRP-1 T cells were characterized in LN as a percentage of the CD3+ population. Composite data of four independent experiments. (D) C57BL/6J mice were implanted with 1 x 10^5 B16 tumor cells. Fourteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 10^5 or 10^6 TRP-1 CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. On days indicated, absolute numbers of CD4+CD8+ TRP-1 T cells were quantified in the lymph node (n=5 mice/group). Data are represented as mean ± SEM.

(Mucida et al. 2013). However, in experiments examining the effect of intravenously administered antigen on TRP-1 CD4+ T cells, this population was not observed (data not shown). This indicated that antigen recognition alone is likely not sufficient to give rise to CD4+CD8+ T cells and that some other stimulus provided during the anti-tumor response or tumor itself is also necessary.

When TRP-1 CD4+ T cells were present at varied precursor frequencies in tumor bearing hosts, the double positive subset of TRP-1 specific T cells emerged only at high clonal abundances. At high clonal abundances of TRP-1 CD4+ T cells tumor was destroyed, theoretically resulting in the uptake of antigen from dead and dying tumor cells by antigen presenting cells. This could potentially lead to a positive feedback loop, whereby initial tumor killing potentiates additional tumor killing through increased antigen presentation. Thus, it is possible that high levels of TRP-1 antigen generated from tumor killing facilitate the formation of this population. In addition to this, we have demonstrated high levels of cytokine accumulation in the lymph node and tumor when TRP-1 specific T cells were most clonally abundant. Although unclear which cytokine, or combination of cytokines, drives this population, if the cytokine is TRP -1 CD4+ T cell derived or induced in other cell types by inflammation, it is likely most plentiful when TRP-1 CD4+ T cells are at the greatest clonal abundance. A final factor that has been observed to increase at the highest clonal abundance is TRP-1 CD4+ T cell mediated dendritic cell maturation (Figure 4.10). If TRP-1 CD4+CD8+ T cell expansion relies upon co-stimulatory signals upregulated by dendritic cells upon maturation, these co-stimulatory ligands may be most plentiful when the clonal abundance of TRP-1 CD4+ T cells is greatest.

7.2. Cytokines that signal via STAT5 support the induction of the double positive phenotype

To identify cytokines that contribute to the generation of the CD4+CD8+ TRP-1 T cell subset we expanded TRP-1 CD4+ T cells in vitro in the presence of a combination of cytokines. We stimulated our T cells in two different ways, by non-specific T cell activation through the antibody crosslinking of CD3 and CD28 and through antigen pulsed irradiated antigen presenting cells. Restimulation of OT-II CD4+ T cells with antigen pulsed APCs in combination with TGF-β and retinoic acid has been previously described to give rise to CD4+CD8+ T cells in culture (Reis et al. 2013). In this pilot experiment, we instead chose to examine cytokines that are known to signal via STAT5. We focused on STAT5 due to its importance in the regulation of Runx3 expression via IL-7 receptor signaling during thymic development (Park et al. 2010). Runx3 upregulation has been described to precede ThPOK downregulation during the development of CD4+CD8+ T cells in mucosal immunity (Reis et al. 2013). In antibody stimulated T cells STAT5 cytokines, in combination with TGF-β, were sufficient to raise the ratio of CD4+CD8+ double positive TRP-1 T cells to CD4+ TRP-1 T cells (Figure 7.4A). Culture of TRP-1 CD4+ T cells in the presence of antigen presenting cells and STAT5 cytokines alone was sufficient to shift this ratio above the highest observed with antibody stimulation. The highest ratios were observed when homeostatic STAT5 cytokines, such as IL-2 and IL-7, were combined (Figure7.4B). Interestingly, in this condition, the lowest ratios of double positive to single positive T cells observed were in cultures containing TGF-β. This does not necessarily suggest that TGF-β is less important during culture with antigen presenting cells. It is possible that the soluble TGF-β used for culture is competing for TGF-β receptor with more relevant isoforms of TGF-β present on the APCs. The higher overall ratios observed following APC stimulation compared to antibody stimulation could be due to expression of TGF-β on the antigen presenting cells or alternatively the need for some other co-stimulatory receptor.

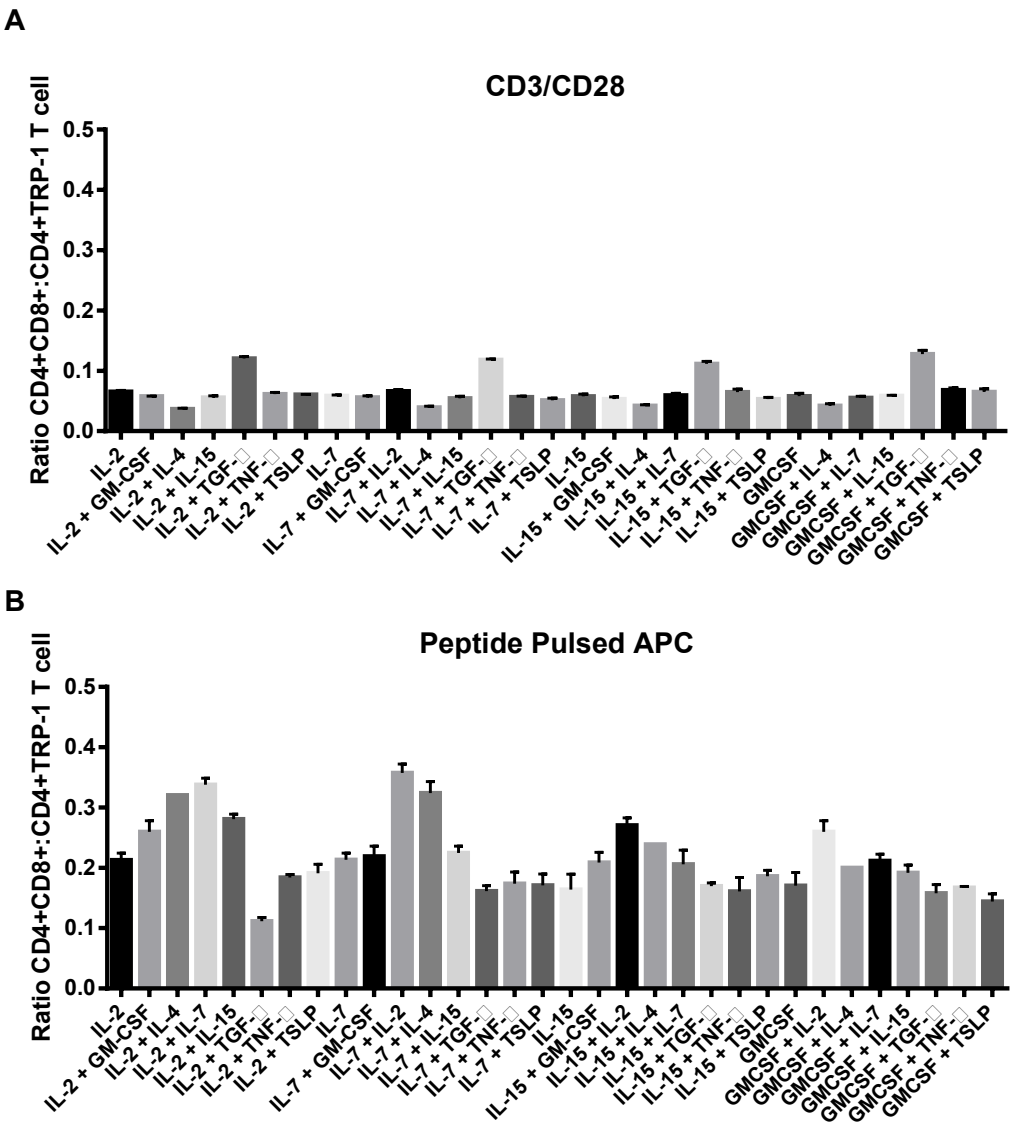

Figure 7.4. Cytokines that signal via STAT5 support the induction of the double positive phenotype.

MACS purified TRP-1 CD4+ cells were stimulated to expand in culture for 5 days in the presence of the indicated cytokines. The ratio of double positive CD4+CD8+ TRP-1 specific T cells to single positive CD4+ TRP-1 specific T cells was calculated. Each condition was replicated in duplicate. (A) Stimulation of T cells via anti-CD3 and anti-CD28 antibodies. (B) Stimulation of T cells with irradiated TRP-1 antigen pulsed APCs.

The studies implicating TGF-β as necessary for the genesis CD4+CD8+ double positive cells have all been conducted in the unique microenvironment of the intestine. It is therefore possible that this T cell subset is being induced during anti-tumor immunity through a distinct pathway.

7.3. TRP-1 specific CD4+CD8+ T cells resemble effector memory T cells

The successful *in vitro* induction of CD4+CD8+ T cells by the homeostatic cytokines IL-2 and IL-7 prompted us to characterize the activation status and memory phenotype of this T cell subset. We found that the CD4+CD8+ T cell population at high clonal abundance is 80% $CD44^{hi}CD62L^{lo}$, indicating that these cells were effector or effector memory T cells (Figure7.5A). When we examined expression of CD25 and CD127 in both the single positive CD4 population and CD4+CD8+ population there were clear differences. In the lymph node, the majority of both subsets expressed CD127 (Figure7.5B,C). However, in the tumor a large percentage of the double positive T cell subset expressed both CD127 and CD25 (Figure7.5B,D). Although the phenotype of the double positive T cells in the lymph node and tumor resembles effector memory based on CD127 expression and low levels of CD62L, the CD25 expression in the tumor is unusual. Others have suggested that sustained CD25 expression may occur in response to elevated IL-2 levels within an antigen rich niche, which would explain the tumor restricted expression; in CD8+ T cells, sustained CD25 expression has also been linked to increased granzyme expression and a terminally differentiated phenotype (Kalia et al. 2010). Future evaluation of IL-2 production and expression of terminal differentiation markers, such as KLRG1, within this subset is warranted. Additionally, examining the persistence of these cells at the site of the tumor lesion and their expression of CD103 would determine if the double positive T cells are differentiating into tissue resident memory cells.

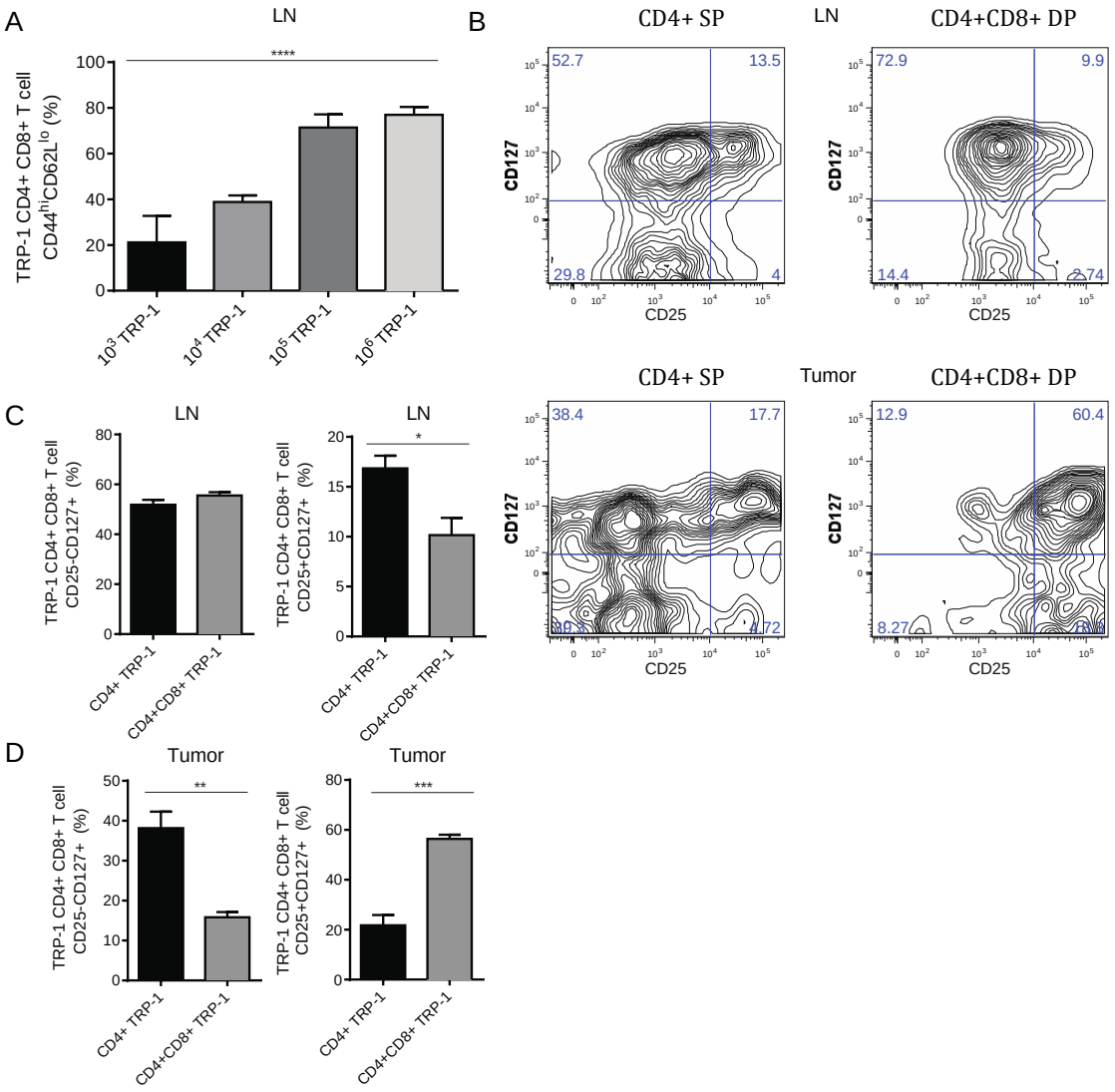

Figure 7.5. A unique phenotype most consistent with effector memory T cells is observed within the TRP-1 specific CD4+CD8+ T cell population.

(A) C57BL/6J mice were implanted with 1 x 10^5 B16 tumor cells. Fourteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 10^3, 10^4, 10^5, or 10^6 TRP-1 CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. On day 7, lymph node was stained for expression of CD44 and CD62L. Composite data of $CD44^{hi}CD62L^{lo}$ percentage within CD4+CD8+ TRP-1 T cell population (n=3-5 mice/group). (B) Within the cohort of mice receiving 10^6 TRP-1 CD4+ T, lymph node and tumor were evaluated for expression of CD25 and CD127. Representative flow cytometry plots of CD25 and CD127 expression. (C) Composite data of *right* CD25-CD127+ and *left* CD25+CD127+ percentage within LN and (D) Tumor CD4+ single positive and CD4+CD8+ TRP-1 T cell population (n=5 mice/group). Data are represented as mean ± SEM.

7.4. A mixed cytotoxic phenotype distinguishes double positive TRP-1 specific T cells from the single positive compartment

We profiled the cytokine production and granzyme B expression of the double positive TRP-1 specific T cells to determine if they were performing as effector cells during the anti-tumor response. Similar to what had been observed in the single positive population, an increased clonal abundance of TRP-1 T cells potentiated a reciprocal increase in effector function in the double positive population as measured by granzyme B and IFN-γ production (Figure 7.6A,B). Although CD4+CD8+ TRP-1 specific T cells producing both IFN-γ and TNF-α were characterized, they did not comprise a substantial component of the population. When the effector function of the single positive and double positive TRP-1 populations was compared at high TRP-1 precursor frequencies, we found a significant decline in TNF-α production within the double positive population. This observation explained the absence of a CD4+CD8+ TRP-1 T cell population producing both IFN-γ and TNF-α. Conversely, an increase in the expression of granzyme B and IFN-γ in the CD4+CD8+ TRP-1 T cells was also observed (Figure 7.6C). The phenotype of elevated IFN-γ and granzyme B expression recapitulates what groups characterizing CD4+CD8+ intraepithelial T cells and loss of ThPOK expression had observed in their models (Mucida et al. 2013; Vacchio et al. 2014).

To assess if the high granzyme B expression resulted in greater cytotoxicity, the double positive TRP-1 T cells were sorted from the lymph nodes and tumors of tumor bearing hosts and embedded in a clonogenic killing assay. Killing mediated by the CD4+CD8+ TRP-1 specific T cells was slightly enriched in cells isolated from the lymph node compared toCD4 single positive TRP-1 specific T cells and comparable in the T cells isolated from the tumor. The potent killing ability of the double positive T cells indicated that they were possibly acting as effectors of tumor killing. However, only a limited number of effector cytokines were examined in this experiment and exploring the

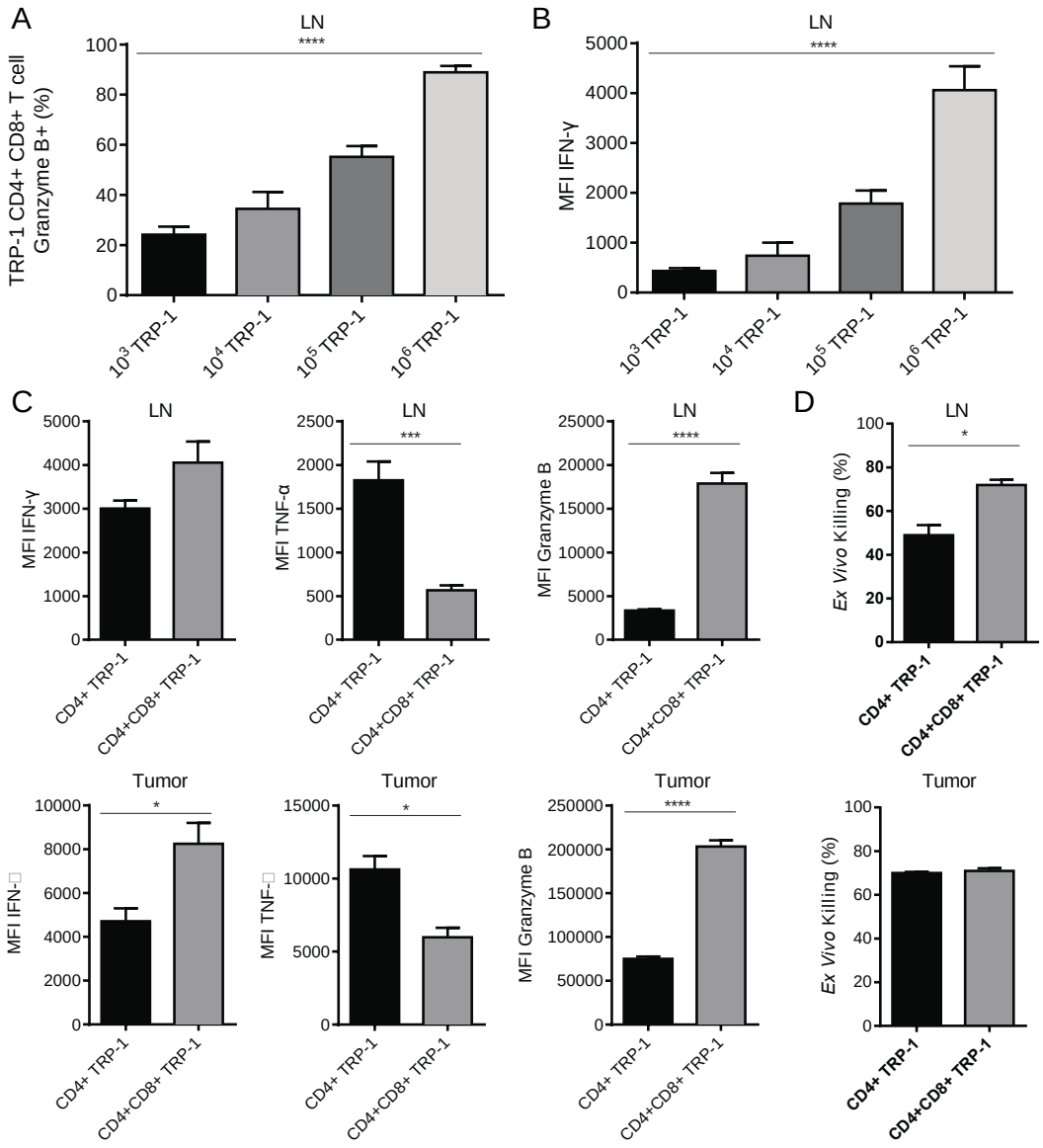

Figure 7.6. Relative to CD4+ TRP-1 specific T cells the double positive population displays higher granzyme B expression and lower production of TNF-α.
(A) C57BL/6J mice were implanted with 1 x 10^5 B16 tumor cells. Fourteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 10^3, 10^4, 10^5, or 10^6 TRP-1 CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. On day 7, function was evaluated after re-stimulation by ICS for percentage of CD4+CD8+ TRP-1 population granzyme B or (B) IFN-γ positive. (C) Within the 10^6 TRP-1 CD4+ cohort, IFN-γ, TNF-α, and granzyme B production in the CD4+ and CD4+CD8+ TRP-1 population was compared in *top* lymph node and *bottom* tumor (n=5 mice/group). (D) On day 7 TRP-1 CD4+ and CD4+CD8+ T cells were sorted from *top* LN or *bottom* tumor and co-embedded with B16 in an *ex vivo* killing assay, percentage of B16 killed. Data are represented as mean ± SEM.

full range of cytokine production within this subset would give better insight into their function.

To better address the role of these T cells in anti-tumor immunity *in vivo*, transfer experiments could be performed with the CD4+CD8+ TRP-1 specific T cells in tumor bearing mice. To begin to explore this, we adoptively transferred TRP-1 CD4+ T cells into tumor bearing RAG deficient hosts and treated them with CD8 depleting antibodies to remove the double positive T cells. The RAG deficient model was chosen so that depletion of endogenous CD8+ T cells would not confound interpretation of the results. No difference in the anti-tumor response was observed (data not shown). This finding was not very informative due to the different effector kinetics of TRP-1 CD4+ T cells and the reduced formation of the double positive population in RAG deficient hosts. In the future, the converse experiment should be performed, in which only the double positive TRP-1 specific population is transferred into RAG sufficient or deficient tumor bearing animals. However, a more elegant approach to address the function of this phenotype in tumor immunity would rely on genetic targeting of the transcription factors regulating this cell population in TRP-1 TCR transgenic mice.

7.5. Downregulation of ThPOK expression and reciprocal de-repression of Runx3 occur in the double positive TRP-1 specific T cell subset

When we examined the transcriptional regulation of TRP-1 specific CD4+CD8+ T cells we observed a dramatic reduction in the expression of ThPOK (Figure 7.7A-B). However, the levels of Runx3 expression were comparable in CD4+ and CD4+CD8+ TRP-1 specific T cells; this was not surprising as others have demonstrated that T-bet and Runx3 act cooperatively in Th1 cells to induce high levels of IFN-γ production (Djuretic et al. 2007). The combination of ThPOK downregulation with Runx3 expression explained the increased cytotoxic phenotype observed in the double positive T cells. We detected a subtle, but non-significant decrease in CD4 expression, likely due to the

downregulation of ThPOK. Additionally we investigated two signaling components involved in the thymic development of CD8+ T cells: STAT5b and SOCS1. Stat5b expression was examined due to its known role in Runx3 induction by IL-7 signaling. SOCS1 is a negative regulator of common gamma chain cytokine signal transduction and can specifically antagonize the induction of *Runx3* during thymic development (Park et al. 2010). More importantly, it has recently been demonstrated that ThPOK specifically targets members of the SOCS family to prevent expression of *Runx3* and acquisition of the CD8 lineage fate (Luckey et al. 2014). STAT5b signaling did not appear to be altered at the transcriptional level. However, SOCS1 expression was lower in the CD4+CD8+ TRP-1 specific T cells (Figure 7.7A-B). This implicates the decline of ThPOK mediated SOCS1 expression as a mechanism for the observed increase in CD8 lineage markers.

Although transcriptional levels of *Runx3* were comparable in the CD4+ single positive and CD4+CD8+ double positive TRP-1 subsets, protein expression of Runx3 is regulated by transcriptional promoter activity that was unable to be resolved in the previous experiment. Most Runx3 protein is derived from the long form transcript generated from distal promoter usage (Egawa and Littman 2008). To determine if protein expression of Runx3 differed in the single positive and double positive TRP-1 specific subset, despite comparable levels of transcription, we characterized Runx3 protein expression by flow cytometry on day 7 of the anti-tumor response. Runx3 protein was increased in the double positive TRP-1 specific T cells in both lymph node and tumor (Figure7.8A-B). When RT-PCR was performed to detect the distal promoter transcript of Runx3, we observed preferential enrichment of this transcript in CD4+CD8+ TRP-1 cells, despite some heterogeneity (Figure7.8C). Thus, we conclude that the emergence of the TRP-1 specific CD4+CD8+ T cell fate is well correlated with altered expression of transcription factors responsible for T cell lineage commitment.

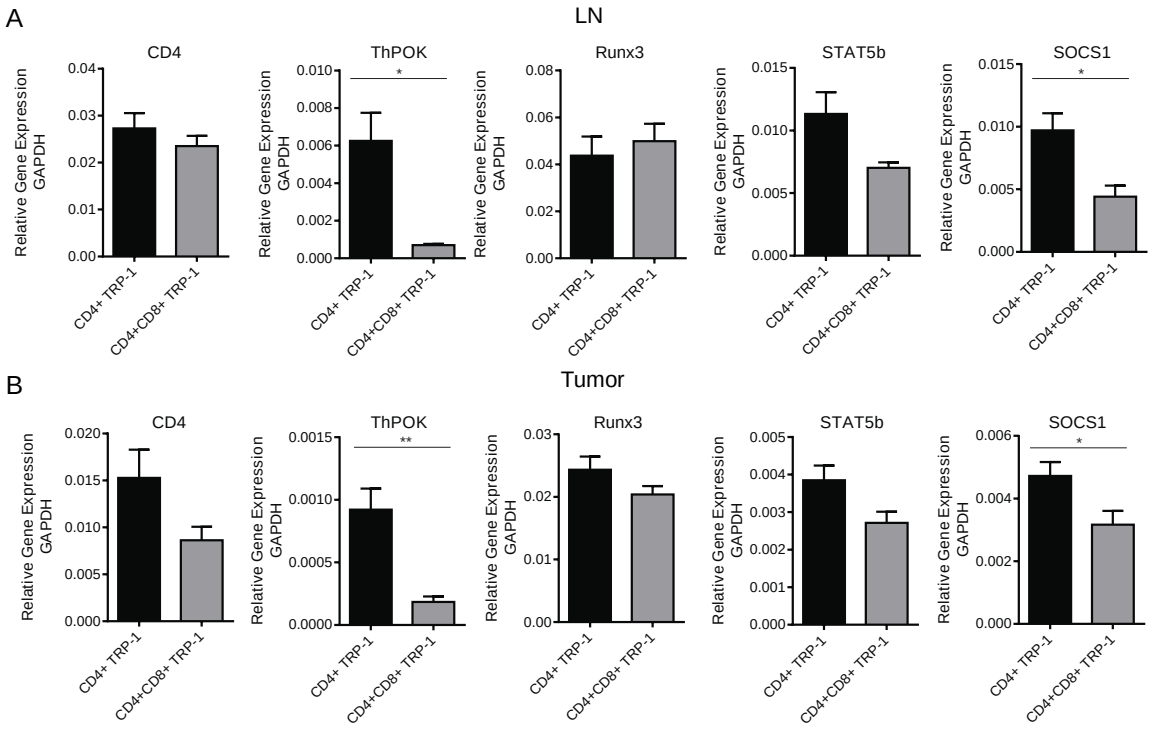

Figure 7.7. ThPOK expression is significantly downregulated in CD4+CD8+ TRP-1 T cells.

(A) C57BL/6J mice were implanted with 2.5 x 10^5 B16 tumor cells. Fourteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 10^6 TRP-1 CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. On day 7 TRP-1 CD4+ and CD4+CD8+ T cells were sorted from LN or (B) tumor directly into Trizol for RNA extraction. qRT-PCR was performed to profile gene expression of candidate transcription factors, as well as upstream and downstream targets. Each condition was repeated in triplicate. Data are represented as mean ± SEM.

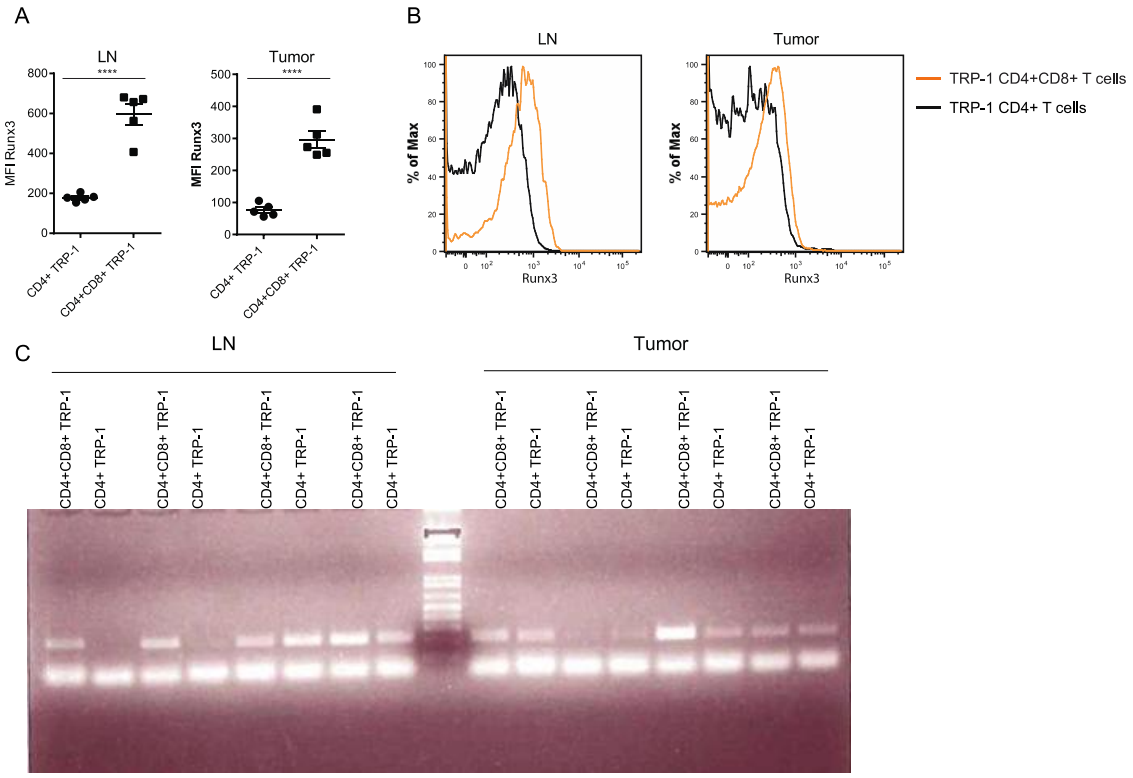

Figure 7.8. In CD4+CD8+ TRP-1 specific T cells expression of Runx3 protein and transcription of long form *Runx3* is increased.

(A) C57BL/6J mice were implanted with 2.5 x 10^5 B16 tumor cells. Fourteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 10^6 TRP-1 CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. On day 7, TRP-1 specific CD4+ and CD4+CD8+ T cells in the LN and tumor were evaluated for Runx3 expression (n=5 mice/group). Summary of Runx3 expression in lymph node and tumor (B) Representative histogram plots (C) C57BL/6J mice were implanted with 2.5 x 10^5 B16 tumor cells. Fourteen days post tumor challenge mice received 600 cGy of irradiation, followed by tail vein injection of 10^6 TRP-1 CD4+ T cells co-transferred with naïve splenocytes for a total transfer quantity of 30 x 10^6 cells. On day 7 TRP-1 CD4+ and CD4+CD8+ T cells were sorted from LN or tumor directly into Trizol for RNA extraction. RT-PCR was performed to profile gene expression of the long form of Runx3. Data are represented as mean ± SEM.

CHAPTER 8: PERSPECTIVE

This thesis describes how the precursory frequency of self-antigen specific CD4+ T cells affects a number of key aspects of the tumor directed immune response- the expansion of a clonal population, the differentiation of this population into effectors, the onset of T cell exhaustion, the balance of regulatory subsets, and induction of a novel CD4+CD8+ T cell phenotype. At its heart, this is a study of the population dynamics of a clonal population of T cells and while each of these individual observations has been elaborated on in isolation, they can only be fully appreciated when reflected on as a whole. We have found that at high clonal abundance T cell expansion is impaired, but the generation of effector function is vastly improved due to population sharing of IFN-γ and propose this as a mechanism of "clonal cooperation" akin to T cell quorum sensing. At the same time, we describe that the low clonal abundance of self-antigen specific T cells potentiates the development of T cell exhaustion. While we have not defined the mechanism behind this observation, we believe these two events are not happening in isolation. It is likely that the same clonal cooperation that occurs at high clonal abundance is enabling "protection" from T cell exhaustion through the induction of high levels of T-bet (Figure 8.1). Likewise, population feedbacks such as the increased maturation of antigen presenting cells also have a probable role in initiating the strong proliferation of effector T cells that allows them to overtake regulatory T cells. This shift in regulatory and effector T cell balance would further feedback on the development of T cell effector function, resulting in high levels of inflammation, which would propagate the emergence of novel differentiation states such as double positive T cells.

Although not already discussed at length or directly investigated in this work, an area of future importance is determining how much all of these observations depend on the affinity or functional avidity of the T cell clone investigated. While we have not empirically determined the affinity of the TRP-1 specific TCR, we have reason to believe

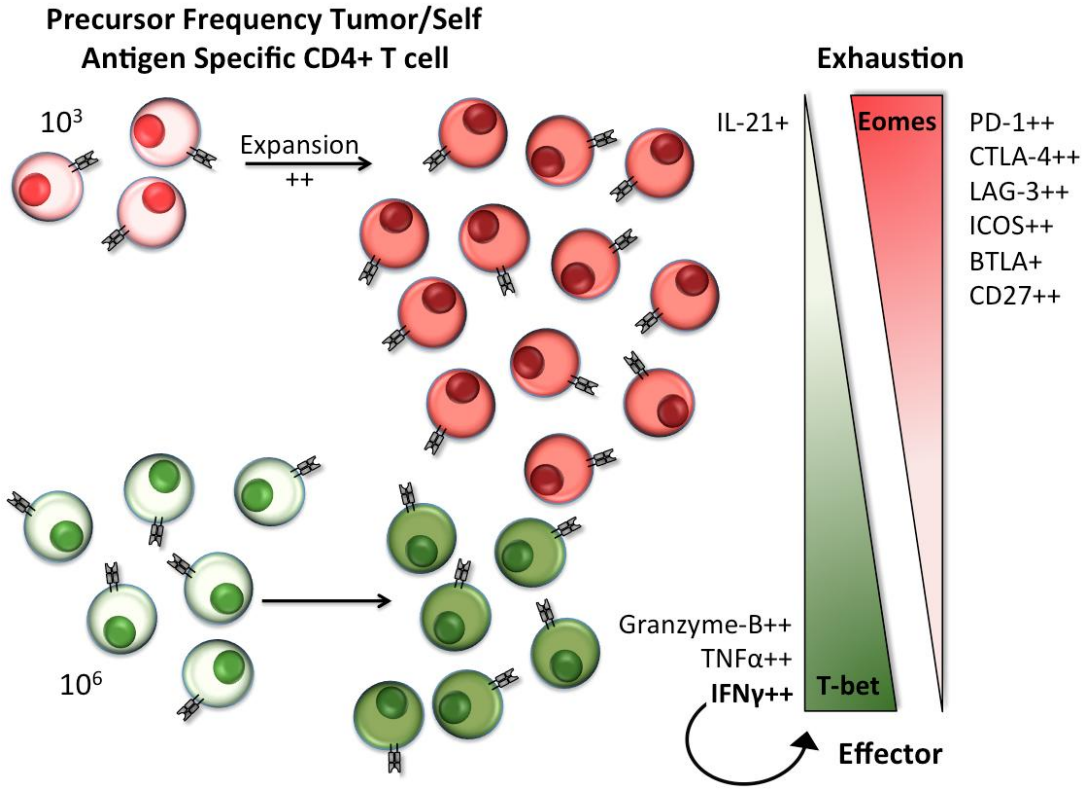

Figure 8.1. Precursor frequency potentiates anti-tumor efficacy and exhaustion. Low physiologic precursor frequencies of tumor-self antigen specific CD4+ T cells promote high levels of clonal expansion, but lead to functionally exhausted T cells that express high levels of exhaustion markers and the transcription factor Eomesodermin. High clonal abundance of self-antigen specific CD4+ T cells enables the generation of potent effector function through population sharing of IFN-γ, resulting in protectively high levels of T-bet expression.

it is relatively high. Firstly, this clone is a self-antigen specific T cell normally eliminated during negative selection, a process dependent on high affinity interactions with thymic epithelial cells. Secondarily, these T cells undergo rapid expansion upon adoptive transfer into lymphopenic hosts, which is correlated with high affinity TCR:pMHC interactions in the periphery. During lymphopenia induced proliferation, T cells of high affinity are also more likely to acquire a memory phenotype and show phosphorylation of their CD3e zeta-chains, which may contribute to the robust effector differentiation at high precursor frequencies and the over-activation resulting in T cell exhaustion at low clonal abundance. Additionally, the extensive proliferation of effector T cells is what allows for the shift in balance between the regulatory T cell subset and the effector T cell subset. One could hypothesize that this would happen more efficiently for T cell clones of a high affinity and that this decline in regulatory T cell frequency within the population would even further propel development of potent effector function. Likewise, these regulatory loops would play a role in the maintenance and emergence of immunodominant clones during a polyclonal T cell response against pathogens. This would also have profound implications for how the immune response may become dysregulated during the development of autoimmunity as a result of the expansion of high affinity self-reactive T cells.

It is surprising that CD4+ T cell mediated help does not impact the threshold of CD8+ T cell competition. We hypothesize that this might be due a lower sensitivity of CD8+ T cells to CD4+ T cell mediated positive feedback loops. Antigen is presented to CD8+ T cells on MHC-I, which is not only expressed by antigen presenting cells, but also by non-immune cells as well. If the tumor specific CD8+ T cells are primarily being primed by cells other than antigen presenting cells, it is possible they are not benefiting from the increased dendritic cell maturation observed following transfer of TRP-1 CD4+ T cells. Additionally, the expression of MHC-I is not as exquisitely controlled by IFNγ and shows different kinetics than MHC-II (Oyler-Yaniv, J., personal communication).

This might result in lower efficacy of IFNγ mediated positive feedbacks via increased antigen expression for CD8+ T cells. Although it is likely that factors such as these have little impact on increasing the factor of expansion, they have been known to raise the amplitude of the response, which would have theoretically shifted the thresholds at which anti-tumor activity is observed (Quiel et al. 2011). However, it is also possible that the mechanisms by which help is provided in this model are unable to overcome the program of competition and that regulation of alternative factors could improve outcome. CD8+ T cells show a defect in effector function formation that is not observed in CD4+ T cells. Through a better understanding of the mechanism behind this difference, it may be possible to directly target the symptom of poor effector function, rather than the intraclonal competition underlying it.

Another area of interest not addressed here is the development of diverse effector function within the TRP-1 CD4+ T cell population. These cells have been shown to produce Th1 cytokines such as IFNγ and TNFα, but also can make IL-4 and IL-17. While the *in vitro* culture experiment presented here does not address if the same cell can produce these cytokines, we know that this is possible from other observations. For the sake of simplicity we primarily focused on how the TRP-1 CD4+ T cells acquire Th1 type effector function and how this is affected by precursor frequency. However, it would be valuable to extend these observations to the development of the alternate helper fates such as the Th2 and Th17 lineage, as well as to the differentiation of cells capable of producing cytokines from mixed lineages. Normally, the production of cytokines from alternate lineages is under tight transcriptional regulation. However, the emergence of the double positive TRP-1 cell fate clearly illustrates that these cells are highly plastic. Moreover, it is already known that many of the transcription factors explored in double positive TRP-1 T cells, specifically ThPOK and Runx3, also regulate aspects of T cell helper differentiation. How these two observations possibly overlap at the transcriptional level is worth pursuing through an in depth characterization of the transcriptional and

epigenetic regulation of these cells. This work would contribute to a better understanding of the regulation of these networks in "normal" cells under steady state conditions and how they are altered in disease states. How high affinity TCR signaling can facilitate this alteration in phenotype warrants consideration.

It is still unclear whether the upregulation of inhibitory ligands on exhausted T cells are the consequence of exhaustion or one of the contributing causes. This model could be used to explore this question in CD4+ T cells within the context of anti-tumor immunity. Additionally, it is still unknown what happens to exhausted T cells after resolution of a chronic disease, such as following successful anti-tumor immunotherapy. It is possible that a state of former exhaustion will impair memory development and secondary responses should tumor begin to re-grow. The TRP-1 CD4+ T cell model has been successfully used in combination with antibody therapies targeting anti-CTLA4 and anti-OX40 to increase the anti-tumor efficacy of a sub-optimal number of cells. This would make TRP-1 CD4+ T cells an ideal model to address this question within the CD4+ T cell subset.

One aspect of TRP-1 specific regulatory T cell development we considered, but did not discuss is whether these T cells are converting from Foxp3- conventional CD4+ T cells into regulatory T cells in the periphery or if we are observing the expansion of a transferred Foxp3+ population. A similar question could also be asked of the TRP-1 specific CD4+CD8+ double positive T cells. We have performed experiments to explore these possibilities in the regulatory T cell population, however, obtained contradictory results. We believe that this is an important question to examine further because of the implications for adoptive cell therapy. T cells engineered to express chimeric antigen receptors are derived from patient peripheral blood and are not currently enriched for CD4 or CD8 T cells or depleted of regulatory T cells prior to transfer, depending on the institution, although this is an area of active investigation. If CARs are transduced into Foxp3+ T cells with a high homeostatic potential and transferred into patients that have

received lymphoablative pre-conditioning, these cells might undergo rapid homeostatic expansion and index themselves to responding effector cells, attenuating their response. Alternatively, if tumor antigen specific Foxp3+ cells accumulate due to peripheral conversion, re-emergence of regulatory T cells following lymphopenia would be an unavoidable consequence of any therapy utilizing adoptive cell transfer. If this were the case it would suggest that research should focus on slowing or preventing this process following transfer, rather than on the enrichment steps preceding transfer. One could imagine possibly engineering a non-suppressive cell to occupy this niche to prevent further accumulation of regulatory T cells.

CD4+CD8+ T cells have been found in the PBMCs of patients undergoing treatment for melanoma at Memorial Sloan-Kettering (data not shown). This model is of key importance to understanding how these CD4+CD8+ T cells function during the immune response to melanoma in both mice and patients. Considering the similarities between the TRP-1 specific CD4+CD8+ T cells and the double positive intraepithelial lymphocyte population, it is vital to investigate whether these cells are also present as an intraepithelial lymphocyte population in the skin. Runx3 is a known regulator of CD103, or so it is possible that these T cells express this epidermal-localizing molecule (Grueter et al. 2005). If this subset were found in the skin as persistent tissue resident memory cells, this would have important clinical implications. The combination of effector memory phenotype and elevated cytotoxic potential implicate these cells as a fast responding effector subset. Tumor antigen specific CD4+CD8+ T cells would be ideally adapted to provide a first line of defense *in situ* upon tumor recurrence and would likely be directly involved in immunosurveillance, specifically the equilibrium phase. Thus, it would be clinically beneficial for long-term maintenance of cancer for these cells to be abundant and specifically engaged. Conversely, if these cells were found to be deleterious to the immune response, they would represent a new suppressor subset to

target with immunotherapies. Understanding the factors involved in their generation and maintenance would allow immunologic manipulation of this population in favor of tumor eradication.

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