**OREGON HEALTH & SCIENCE UNIVERSITY**
**SCHOOL OF MEDICINE – GRADUATE STUDIES**

# The Role of $CD4^+$ T cells During Adoptive and Active-specific Cancer Immunotherapy

By

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A DISSERTATION

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CERTIFICATE OF APPROVAL

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This is to certify that the PhD dissertation thesis of

Sarah Elizabeth Church

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Dr Andrew Weinberg

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Abbreviations Used

Ag: antigen

AIT: adoptive immunotherapy

APC: antigen presenting cell

CAR: chimeric antigen recepter

CM: central memory

CXCR: CXC-chemokine receptor

CTLA4: cytotoxic T lymphocyte antigen 4

DC: dendritic cell

dLN: draining lymph node

ELISA: enzyme-linked immunosorbent assay

EM: effector memory

FOXP3: forkhead box P3

GM-CSF: granulocyte-macrophage colony-stimulating factor

GrB: granzyme B

ICOS: inducible T cell costimulator

IFNγ: interferon gamma

IL-2: interleukin-2

i.p.: intraperitoneal

i.v.: intravenous

LAK: lymphokine-activated killer cells

MCA: 3-methylcholanthrene

MDSC: myeloid derived suppressor cell

MHCI/II: major histocompatibility complex I/II

NK: natural killer

PD-1: program death receptor 1

Pmel T cells: Pmel 17/gp100-specific $CD8^+$ Tg T cells

RAG: recombination activating gene

RLM: reconstituted lymphopenic mice

s.c.: subcutaneous

SCM: stem cell memory

STAT: signal transducer and activator of transcription

TCR: T cell receptor

Tg: transgenic

TGF$\beta$: transforming growth factor beta

Th: T helper cell

TIL: tumor infiltrating lymphocytes

TLR: toll-like receptor

TM: transitional memory

TNF$\alpha$: tumor necrosis factor alpha

TRAIL: tumor necrosis factor-related apoptosis inducing ligand

Treg: regulatory T cell

Tyrp: tyrosinase-related protein

TRP-1 T cells: tyrosinase-related protein 1 specific $CD4^+$ Tg T cells

WT: wild type

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Dedication

This thesis is dedicated to my parents, Dr. William and Patricia Church. You have always supported me in all of my pursuits, no matter where they have taken me. For that I cannot express my appreciation enough.

THESIS ABSTRACT

The role of CD4 T cells in cancer immunotherapy has been debated due to the multifaceted and diverse functions of CD4 T cell lineages. Pathogen-derived models have shown that CD4 T cells have an important role in helping to prime and maintain effective long-term immunity. However, there are multiple types of CD4 T cells such as Foxp3+ CD4 T cells called regulatory T cells (Tregs), which have an important role in resolving immune activation during infection and suppressing the immune response to self-antigens. Since tumor-associated antigens are often overexpressed or mutated self-antigens, Tregs are known to reduce anti-tumor immune responses. Chapter 2 will discuss the advantages of tumor-specific CD4 T cells during adoptive immunotherapy of melanoma in a lymphopenic model. We observed superior therapeutic efficacy of adoptive immunotherapy with tumor-specific CD4 and CD8 T cells in tumor-bearing RAG-deficient lymphopenic mice compared to treatment with CD8 T cells alone. Mice treated with CD4 and CD8 T cells had an increased number of tumor-reactive CD8 T cells and removal of CD4 cells early after adoptive immunotherapy reduced therapeutic efficacy and increased expression of the exhaustion marker PD-1 on CD8 T cells. Tumor-specific CD4 T cells were able to maintain effector phenotype cells and reduced the expression of the apoptosis inducing factor TRAIL on CD8 T cells. Together our findings indicate the advantage of using tumor-specific CD4 T cells in adoptive immunotherapy trials for cancer.

Chapter 3 will describe the elimination of Tregs in a multiple vaccination model, where anti-tumor immunity is induced in the same challenged or tumor-bearing mice (active-specific). Previously our group showed three vaccinations with a whole-tumor vaccine transduced to secrete GM-CSF produced fewer therapeutic T cells than a single vaccination. This loss in efficacy correlated with increased Tregs numbers. Our previous report used a model of T cell adoptive immunotherapy with splenocytes from vaccinated animals. It is possible that this model did not account for migration of tumor-specific T cells to resident tissues. Here we show that during active-specific immunotherapy partial depletion of CD4 T cells skewed homeostatic proliferation toward a non-Treg phenotype and enhanced protection to a large dose tumor challenge (20x $TD_{100}$) compared to non-depleted mice. We also examined whether route of vaccination altered Treg numbers or ability to protect against tumor challenge and found there was no difference in protection, using three different vaccination administration methods. Together these studies indicate the importance of CD4 T cells during priming and maintenance of anti-tumor responses. However, these studies also exemplify the paradox that CD4 T cells can be both positive and detrimental for anti-tumor immunity.

# CHAPTER 1

# Introduction

## ***Adaptive immunity and T lymphocytes***

### *Adaptive immunity*

The adaptive immune system is composed of B and T lymphocytes that originate in the bone marrow. B lymphocytes (B cells) develop in the bone marrow and precursor T lymphocytes (T cells) migrate to the thymus where they develop and mature, described further in the following section. B and T cells express unique receptors, B cell receptors (BCR) or immunoglobulins (Ig) for B cells and T cell receptors (TCR) for T cells. In order to develop a repertoire (approx. $3\times10^{11}$ combinations) of antigen (Ag) specificities, B and T cells utilize the DNA rearrangement system among variable (V), diversity (D) and joining (J) gene segments, called V(D)J recombination [1]. During this process DNA coding for the receptor binding-site is cut and rearranged using enzymes encoded by the recombination activation genes (RAG) 1 and 2 [2]. For this reason, RAG-deficient mice are unable to develop mature B or T cells [3]. This method of DNA recombination allows for incredible diversity of Igs and TCRs to recognize a wide variety of self and pathogen antigens (Ags).

B cells can also further increase Ig diversity by somatic hypermutation and class switching [4]. B cells can eventually develop into plasma cells that secrete Ig called antibodies which have the ability to neutralize, opsonize or target cells to destroy pathogens [5,6]. The remainder of this section will focus on development and maturation of conventional T cells.

### *T cell development*

T cell receptors (TCRs) on $CD8^+$ and $CD4^+$ T cells recognize antigen in the context of peptides bound to major histocompatibility complex class I (MHCI) and major histocompatibility complex class II (MHCII) molecules presented on antigen presenting cells (APCs), respectively [7,8]. When T cells initially migrate to the thymus they are double-negative $CD4^-CD8^-$ cells and the TCR has not developed. Once the TCR is expressed, cells become double-positive $CD4^+CD8^+$ (DP). At this point T cells encounter self-antigens, presented on MHCI and MHCII molecules by the thymic epithelium and thymic-resident immune cells, resulting in positive and negative selection [9,10]. During maturation DP cells encounter either peptide/MHCI or MHCII complexes driving them to become single positive (SP) $CD8^+CD4^-$ or $CD4^+CD8^-$ cells, respectively. During positive selection, some $CD4^+CD8^-$ cells with high-affinity for self-peptides upregulate the transcription factor Forkhead box P3 (Foxp3) to become regulatory T cells (Tregs) [11]. These are often referred to as natural Tregs. At the same time DP and SP cells undergo negative selection, where T cells with high to medium affinity TCR to self-antigen are induced to undergo apoptosis leaving T cells with low affinity TCR reactivity to self-antigens to survive [10]. Negative selection is essential to eliminate self-reactive T cells that could attack normal tissue and result in autoimmunity.

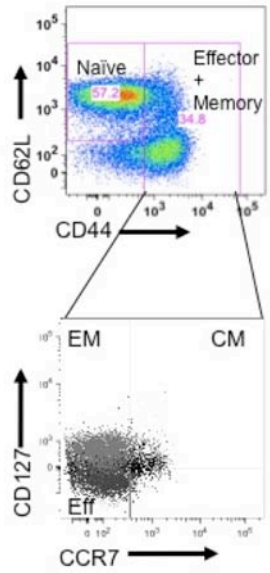
### *T cell maturation*

After development, SP naïve $CD4^+$ or $CD8^+$ T cells, migrate from the thymus to peripheral lymphoid organs, such as lymph nodes and the spleen. In

the periphery, T cells encounter APCs and pro-inflammatory signals that can induce maturation into effector and memory cells [12].

Three signals are associated with optimal stimulation of mature T cells, however only signal 1 and 2 are required for activation. Signal 1 is the antigen-specific interaction between MHC-peptide complexes on APC and TCR on T cells. Signal 2, known as co-stimulation, is a non-antigen specific interaction of molecules on the APC surface (CD80/86, CD40) and T cell surface (CD28, CD40L) [13,14]. Signal 3 are secreted or co-stimulator factors, such as cytokines (IL-12) or tumor necrosis factor receptor (TNFR) family member co-stimulatory molecules (OX40, 4-1BB) [15,16]. These 3 signals determine the fate of T cell maturation into effector and memory cells. During a typical adaptive immune response, an initial expansion of pathogen-specific effector T cells occurs with T cell numbers contracting once the pathogen is cleared, ultimately leaving an antigen-specific memory T cell population to monitor and prevent future infections [17]. A number of surface proteins are modulated on effector and memory T cells during these immune expansion and contraction phase, including CD44, CD62L, CCR7, KLRG1 and CD127. C-C chemokine receptor type 7 (CCR7) and L-selectin (CD62L) are involved with homing of T cells to the lymph node and both are present on naïve T cells [18]. Upon TCR stimulation, CD44 is upregulated making it an early indicator of activation [19]. CD44 is the receptor for hyaluronate on epithelial cells and has been shown to aid in early extravasation [19]. CD44 is expressed on both effector and memory T cells. The two markers, interleukin-7 receptor $\alpha$ (CD127) and killer-like lectin receptor G1 (KLRG1), can

distinguish between short lived effector T cells, $CD127^{lo}KLRG1^{hi}$, and early-memory T cells, $CD127^{hi}KLRG1^{lo}$ [20]. Effector T cells are distinguished by a number of cytotoxic molecules and cytokines, they produce including granzyme B (GrB), interferon-γ (IFNγ) and tumor-necrosis factor-α (TNFα), which are involved in anti-tumor responses and the elimination of pathogens [21-24]. Program death receptor 1 (PD-1) is another marker that is upregulated upon T cell activation [25]. However PD-1 has also been associated with chronic and tumor-stimulated T cells that lose function and become exhausted. There are several phenotypes of memory cells with distinct functions for monitoring and activation during pathogen reinfection. Effector memory ($T_{EM}$) $CD127^{hi}CCR7^{lo}CD62L^{lo}$ T cells reside in tissues and circulate in the periphery to monitor for reinfections [26]. $T_{EM}$ cells are functional within 1-2 hours after antigen restimulation, producing IFNγ, interleukin-4 and interleukin-5 [26]. Central memory ($T_{CM}$) $CD127^{hi}CCR7^{hi}CD62L^{hi}$ T cells traffic to lymphoid tissues and function to




| Subset | Phenotype |
|---|---|
| naïve | $CD44^{-}CD62L^{+}CCR7^{+}$ |
| effector | $CD44^{+}CD62L^{-}CCR7^{-}$ $KLRG1^{+}CD127^{-}$ |
| $T_{EM}$ | $CD44^{+}CD62L^{-}CCR7^{-}$ $KLRG1^{-}CD127^{+}$ |
| $T_{CM}$ | $CD44^{+}CD62L^{+}CCR7^{+}$ $KLRG1^{-}CD127^{+}$ |
| $T_{SCM}$ | $CD44^{-}CD62L^{+}CCR7^{+}$ $CD127^{+}CD122^{+}sca\text{-}1^{+}$ |

**Figure 1-1. Overview of murine T cells subsets.** A representative gating strategy for distinguishing between naïve, effector, $T_{EM}$, and $T_{CM}$ cells and a description of phenotype.

generate more $T_{CM}$ and $T_{EM}$, by producing interleukin-2 (IL-2), upon secondary infection [26,27]. Although $T_{CM}$ share a number of molecules with naïve cells, $T_{CM}$ are activated much faster than naïve cells upon antigen encounter. Recently a new memory population, stem cell memory ($T_{SCM}$), has been shown to respond to antigen challenge similar to $T_{CM}$ and $T_{EM}$, but retain a naïve-like phenotype $CD44^{lo}CD62L^{hi}CCR7^{hi}$ with the addition of sca-$1^{hi}$ and $CD122^{hi}$ [28,29]. They are also different from $T_{CM}$ and $T_{EM}$ because $T_{SCM}$ retain the ability to differentiate into multiple memory phenotypes [29]. Chapter 2 will utilize many of these surface and functional molecules to analyze anti-tumor responses (Figure 1-1).

***CD4 T cell lineages***

$CD4^+$ helper T cells (Th) are one of the primary sources of IL-2 in the immune system. They have a wide range of functions and encompass an ever-growing number of subsets including Th1, Th2, Th17, Tfh and regulatory T cells (Tregs). Th1 lineage commitment is induced by IL-12 produced by APCs and characterized by expression of the major transcription factor T-bet [30]. IL-12 signals via the signal transducer and activator of transcription (STAT) 4 and eventually T-bet [30]. T-bet activation regulates the production of interferon-γ (IFNγ) and suppression of Th2 cytokines. The transcription factor GATA binding protein 3 (GATA-3) characterizes the Th2 lineage [31]. IL-4 produced by activated Th2 cells signals through STAT6 leading to activation of GATA-3 [31,32]. Th2 cells have been most commonly associated with allergic inflammation and are often associated with an ineffective anti-tumor response [33,34].

| Table 1. Subsets of T helper cells [29-43] | | | |
|---|---|---|---|
| **Subset** | **Transcription Factor** | **Cytokines produced** | **Function** |
| Th1 | T-bet | IFNγ, TNFα, IL-2 | Pathogen and anti-tumor defense and augment CD8 T cell function |
| Th2 | GATA3 | IL-4, IL-5 | Parasite defense and are associated with asthma and allergic inflammation |
| Th17 | RORγt | IL-17, IL-23, G-CSF | Involved in autoimmunity and defense against bacterial infection |
| Tfh | Bcl6 | IL-21, IL-10 | Provide help to B cells, class switching |
| Treg | Foxp3 | TGFβ, IL-10 | Regulation, immunosuppression and are associated with poor tumor prognosis |

Th17 cells were first identified to be associated with tissue inflammation in autoimmune disease [35]. Th17 cells produce interleukin-17 (IL-17) and granulocyte-colony stimulating factor and are controlled by the master transcription factor retinoic orphan receptor gamma t (RORγt) [36]. Studies have shown that *in vitro* Th17 polarized cells (treated with TGF-β, IL-6 and anti-IFNγ) reprogram during lymphopenia-induced homeostasis to become potent tumor-eliminating Th1-like cells [37]. Another lineage of Th are the T follicular help (Tfh) cells, which have an important role for helping B cells produce antibodies and aid in class switching [38]. They are characterized by expression of CXC-chemokine receptor 5 (CXCR5) and the master transcription factor Bcl6 [39]. Tfh cells produce IL-21, which has been shown to have a number of anti-tumor effects [40-42]. Tregs express the forkhead box P3 (Foxp3) master regulator [43]. Discussion of Tregs will be expanded later in this section. Table 1 shows a summary of Th subsets.

### *Mechanisms of CD4 T cell help*

Early studies using several microbe models showed that CD4 T cell help aids both priming and maintenance of naïve and memory CD8 T cells [44,45]. During priming, CD4 T cell help makes use of the co-stimulatory molecule CD40 on APCs [45]. CD40 on APC interacts with CD40 ligand (CD40L) on T cells enhancing presentation of peptide MHC complexes, producing cytokines and increasing expression of other co-stimulatory molecules (CD80/86) [14]. CD4 T cell help has also been shown to decrease expression of the apoptosis inducing factor TNF-related apoptosis inducing ligand (TRAIL) and increase the expression of the survival factor Bcl-2, thereby enhancing CD8 T cell survival [46]. IL-2 produced from CD4 T cells has long been hypothesized as a mechanism for CD4 T cells to enhance CD8 T cell function, mainly because administering IL-2 in the absence of CD4 help can restore dysfunctional CD8 T cells that are primed in the absence of CD4 T cell help (helpless) [47]. Although paracrine IL-2 from CD4 T cells is likely important, recently it was shown that CD4 T cell help actually induces autocrine production of IL-2 from CD8 T cells [48]. Thus, further indicating CD4 T cells are helping CD8 T cells through multifaceted mechanisms.

### *Regulatory T cells*

Regulatory T cells (Tregs), characterized by the expression of the transcription factor Foxp3, suppress the immune response to self-Ags and prevent autoimmunity [49,50]. Tregs can be categorized as either natural or induced, by whether they are generated in the thymus or in the periphery,

respectively [51]. Tregs produce the immunosuppressive cytokines transforming growth factor β (TGFβ) and interleukin 10 (IL-10), and can reduce T cell and APC activation and promote tolerance [52]. The accumulation of Ag-specific induced Tregs is thought to diminish anti-tumor immune responses [53]. Therefore, distinguishing between induced and natural Tregs could help to better understand the role of Tregs in tumor immunity. A number of markers have been associated with natural Treg function. Here we will discuss two markers, which will be utilized in Chapter 3, inducible T cell co-stimulator (ICOS) and Helios. ICOS is a member of the CD28 co-stimulatory pathway that is associated with activation of both effector T cells and Tregs. ICOS expression on natural Tregs has been associated with both TGF-β and IL-10 production and increased suppressive ability, compared to ICOS-negative Tregs [54]. Helios is a transcription factor in the IKAROS family, which has been shown to be associated with recent thymic emigrants [55]. Although Helios was thought to distinguish natural from induced Tregs recent literature has refuted this showing expression of Helios in Tregs delineates a highly suppressive Treg subset [56,57]. Elimination of tumor-induced Tregs will be further discussed in the context of vaccine immunotherapy later in this section.

## ***History of tumor immunology and immunotherapy***

### ***The immune response to tumors***

As early as the 1900's Paul Ehrlich suggested the immune system decreased the prevalence of cancer in aged organisms [58]. While Ehrlich was first

to describe intrinsic immunity to cancer, William Coley, sometimes referred to as the father of immunotherapy, injected inactivated erysipelas toxin (*streptococcus pyogenes*) directly into unresectable tumors, therefore being the first to elicit anti-tumor immunity using pathogens [59]. This method eliminated a number of tumors long-term leading to the treatment now known as Coley's toxin. Following Ehrlich and Coley's work, many scientists tried to confirm the role of the immune system in tumor prevention with varying degrees of success. Not until the seminal work of Prehn and Main demonstrated tumor-specific immunity to 3-methylcholanthrene (MCA)-induced murine tumors did cancer immunity become believable to modern immunologists [60,61]. The key to the success of these studies was the availability of syngeneic mice, and the use of syngeneic tumors or skin grafts to determine that immunity was against the tumor, not normal tissue. Furthermore, they showed that immunity to each MCA-induced sarcoma was specific because tumor vaccinated mice did not develop tumors upon challenge with the homologous tumor, but did grow tumor when challenged with a sarcoma derived from another syngeneic mouse. This suggested antigen diversity among the different chemically induced tumors.

### *Cancer Immunosurveillance*

Macfarland Burnet and Lewis Thomas described their observations that patients had increased incidence of neoplasia when they received lymphodepleting regimens either during organ transplantation or during the course of cancer treatment [62,63]. Based on Coley, Prehn and Main's previous

studies and their own observations, they hypothesized that lymphocytes were responsible for eliminating continuously arising nascent cancer cells. Burnett termed this, immunosurveillance [62]. This hypothesis was not confirmed until many years later with the use of inbred immune-deficient RAG-/- mice [64]. These studies showed that lymphocytes and IFNγ coordinate to protect against MCA-induced tumors. Cancer immunosurveillance is now well accepted as dogma within the field of tumor immunology. Moreover Robert Schreiber and colleagues have expanded upon immunosurveillance and hypothesized that the immune system has a broader effect on tumor growth called immunoediting [65]. They hypothesized that immunoediting happens in three phases: elimination, equilibrium and escape [65]. The elimination phase is synonymous with immunosurveillance. During the elimination phase the endogenous innate and adaptive immune system removes malignant cells before they are grossly visible. This phase is most similar to the immune system's recognition of foreign antigens. During the equilibrium phase the immune system can recognize the tumor, but the tumor is able to limit the effects of the endogenous immune response and establish homeostasis. At the equilibrium stage tumor cells may be present, however not progressing at a measurable rate. During the escape phase tumor has evaded the immune response and is actively progressing. Often during the escape phase tumors will lose immunogenic Ags and acquire new mutations. Most recently Schreiber's group showed that rejection of tumors expressing highly immunogenic Ags was dependent on T cells and that escape mutants did not elicit a potent anti-tumor T cell response [66].

## ***Adoptive immunotherapy***

### *Melanoma as a tumor immunity model*

Metastatic melanoma is a devastating disease with a 10-year survival rate of less than 15% [67] and is becoming more prevalent in young adults, from 18-39 years of age [68]. Melanoma is an excellent model for studying tumor immunity and immunotherapy because many T cell specific melanoma Ags have been identified (MART-1, TRP1/2, pmel/gp100) and it is thought to be an immunologically responsive tumor [69-72]. Early after tumor immunology was described, the search for tumor Ags began, and researchers sought ways to elicit an immune response in order to eliminate tumors without targeting normal tissue. Optimal tumor-associated Ags would be unique to, or overexpressed on, tumor cells with little or no expression on normal tissue. Furthermore, targeting tumor Ags that are required for maintenance of the malignant phenotype would ultimately reduce the generation of immune escape variants. Melanoma-associated Ags were some of the first tumor Ags discovered [73] by isolating tumor-infiltrating T lymphocytes (TIL) and identifying tumor targets to which they reacted [72,74]. Some of the early melanoma Ags discovered include melanoma antigen recognized by T cells (MART1) (MHCI-restricted), gp100 (MHCI-restricted) and tyrosinase (MHCI + MHCII-restricted) [69,70,75]. One reason for these early discoveries was due to the success in growing TILs from melanomas compared to other types of cancer [76-78]. Another reason melanoma is considered immunologically responsive is that patients with a strong anti-melanoma response often develop vitiligo, an autoimmune disease characterized by

immune cells targeted to the melanocyte antigen tyrosinase-related protein 1 (Tyrp1) resulting in skin depigmentation [79]. For these reasons melanoma is considered to be an excellent model for studying the immune response to cancer and a number of tools are available including transgenic mice with MHCI-restricted (pmel/gp100) and MHCII-restricted (TRP1) T cells that are specific for melanoma-associated antigens [37,80].

### *Adoptive immunotherapy and Interleukin-2*

Interleukin-2 (IL-2) was first named T cell growth factor due to its ability to induce lymphocyte proliferation and stimulation [81]. IL-2 stimulates proliferation of T cells, cytotoxic activity of natural killer (NK) cells and antibody production from B cells [33]. T cells express the receptor for IL-2, which is composed of 3 subunits, $\alpha$, $\beta$ and $\gamma$ (CD25, CD122 and CD132). IL-2R$\alpha$ (CD25) is the high-affinity IL-2 receptor and is associated with activated, memory and regulatory T cells [11,82-84]. IL-2R$\gamma$ (CD132) is the common receptor for the gamma chain cytokine family, which includes IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 [33]. Deficiency in the $\gamma$-subunit results in X-linked severe combined immunodeficiency with patients lacking T, B and NK cells [85].

Due to the immuostimulatory potential of IL-2, Rosenberg and colleagues hypothesized that it could be used to generate tumor reactive lymphocytes. Early studies found that when lymphocytes were isolated from tumors and cultured with IL-2, it generated effector cells, termed lymphokine-activated killer cells (LAK), that specifically lysed tumor cells [86-88]. Murine studies found that adoptive

transfer of these LAK cells could substantially reduce established pulmonary metastases [89]. Therefore, Rosenberg and colleagues quickly proposed to use LAK cells for adoptive immunotherapy of patients with cancer. However, generation of LAK cells from patients can be technically challenging. An alternate way to generate LAK was the generation *in vivo* by administering recombinant IL-2 (rIL-2) directly to patients. Administration of rIL-2 to mice with established pulmonary metastases decreased tumor burden, however when adoptive immunotherapy with LAK cells and rIL-2 were combined, they observed the largest decrease in tumor burden [90]. Soon after, clinical trials were started using rIL-2 as a single agent therapy, as well as in combination with LAK cells [91-93]. The use of rIL-2 is now FDA approved for treatment of patients with melanoma and renal cell carcinoma [91]. Since these initial observations there have been continuous studies that include modifying the tumor milieu and enhancing co-stimulation using artificial APCs in order to achieve durable anti-tumor responses for adoptive immunotherapy with TIL [94-97]. One of the most influential discoveries for adoptive immunotherapy (AIT) of cancer was that inducing lymphopenia prior to AIT enhanced therapeutic efficacy and persistence of anti-tumor immune responses [98].

***Lymphopenia-induced proliferation in adoptive immunotherapy***

Lymphopenia, the substantial reduction of lymphocytes in the blood, can be induced by a number of methods including radiation, chemotherapy or genetic mutation (RAG-deficient mice) [3,99,100]. Each method of lymphodepletion provides

unique advantages, which include induction of inflammation, increased access to APCs and MHC molecules, reduction of cytokine consuming cells and tolerogenic cells (Tregs), and induction of co-stimulatory molecules [101,102]. Furthermore, it is well known that transferring naïve cells into lymphopenic mice enhances proliferation and activation of T cells with an effector and memory-like phenotype [103-105]. In addition it has been recently shown that induction of lymphopenia reprograms T cells to be less tolerogenic [106]. Pre-clinical models combining induction of lymphopenia with AIT have consistently shown increased therapeutic efficacy and long-term tumor-specific T cell persistence [107-109]. These observations in murine models have been directly translated to clinical studies with adoptive immunotherapy. In a pivotal clinical trial 46% (6/13) of patients had objective clinical responses when treated with a combination of nonmyeloablative chemotherapy, prior to AIT [98]. More recently, a study utilizing a myeloablative regimen of radiation (12 Gy) and chemotherapy (cyclophosphamide and fludarabine), in combination with TIL, hematopoetic stem cell reinfusion, and IL-2, resulted in 72% objective response rates with 22% (20/93) of treated patients experiencing complete tumor regression [110]. These beneficial results warrant further study into the benefits of AIT in a lymphopenic environment.

***The role of CD4 T cell help in adoptive immunotherapy***

The role of CD4 T cell help in tumor immunity has been controversial, particularly in combination with lymphodepletion, since lymphopenia-induced proliferation is thought to stimulate mechanisms that abrogate the need for CD4

T cell-induced help [111]. As discussed earlier many tumor-associated antigens are self-Ags, which promote Treg development and immune tolerance. However, there are a growing number of papers indicating CD4 T cells enhance the anti-tumor response [112-114]. Chapter 2 will describe the benefits of using tumor-specific CD4 T cell help for adoptive immunotherapy of melanoma in a lymphopenic environment.

## ***Multiple vaccinations, whole tumor vaccines and granulocyte macrophage colony-stimulating factor as an adjuvant***

### *History of GM-CSF as an adjuvant*

Early during the study of whole tumor vaccines there was a push toward finding an appropriate vaccine adjuvant. In a seminal study by Dranoff and colleagues, they compared a panel of irradiated whole murine melanoma tumor vaccines transduced to secrete IL-2, IL-4, IL-5, IL-6, TNF$\alpha$, IFN$\gamma$ or granulocyte macrophage colony-stimulating factor (GM-CSF) for capacity to provide protective immunity [115]. In this study, mice were immunized with irradiated B16-F10 murine melanoma cells transduced to secrete a single cytokine or a single cytokine and IL-2. The same mice were then challenged with non-transduced B16-F10 cells. Only mice immunized with the irradiated GM-CSF or GM-CSF and IL-2 transduced tumors had significantly enhanced long-term protection and a tumor-specific immune response. Furthermore, since IL-2 transduced irradiated whole tumor immunization did not improve protection on its own, the improved protection was GM-CSF mediated. GM-CSF activates, differentiates and

increases migration of macrophages, granulocytes and dendritic cells, subsequently enhancing cross-presentation of tumor-antigens to T cells [116]. This is exemplified in pre-clinical studies where a poorly immunogenic B16BL6-D5 melanoma becomes highly-immunogenic when transduced to secrete GM-CSF [117].

***Do multiple vaccinations improve therapeutic efficacy?***

Even though many tumor-associated Ags have been identified, no consensus has been reached on the optimal Ags to target for therapeutic immunization against tumors. Furthermore, targeting one or two dominant Ags can result in tumor escape variants [66]. Classic tumor immunotherapy studies frequently start with a single immunization with irradiated immunogenic tumor cells, followed by serial immunization with live tumor cells to generate ‘‘immune’’ mice with a tumor-specific immune repertoire [60,118-120]. Immune responses in mice that reject tumor challenges are likely to be substantially different from mice receiving repetitive vaccinations with a vaccine that does not contain viable tumor cells. Recently, our group reported that T cells from thrice-vaccinated mice were significantly less effective in adoptive transfer studies than T cells from mice receiving a single vaccination [121]. A striking difference observed in multiply vaccinated animals was an increase in the number of CD4 regulatory T cells (Tregs). Elimination of these regulatory cells during the second and third vaccinations resulted in a recovery of therapeutic efficacy. At the same time, a number of large phase II/III clinical trials found that patients receiving multiple

vaccines had significantly worse outcomes than control arms [122,123]. This included 2 adjuvant studies where patients were randomized to receive a vaccine composed of 3 allogeneic melanoma cell lines [124]. One study included 1166 patients with stage III melanoma and a second study included 496 patients with stage IV melanoma [122-124]. At the interim analysis, both studies were halted because of significantly worse outcomes in the tumor vaccine arms [123]. These results prompted our group as well as many in the field to evaluate the rationale for repetitive vaccinations [121,123,125] (this paragraph is modified from Church et al. [126]).

***Autologous whole-tumor GM-CSF producing vaccines***

The findings of Dranoff and colleagues [115], that GM-CSF adjuvant improves tumor-specific immunity, prompted an initial clinical trial with 29 metastatic melanoma patients treated with irradiated autologous melanoma cells transduced with GM-CSF (median of GM-CSF secretion, 534 ng/$10^6$ per 24 hours) [127]. One complete response (CR), 1 partial response (PR), and 1 mixed response were observed at 36 months after vaccination, 29% (10/35) of patients were alive, and 4 had no evidence of progressive disease. All patients had a substantial number of dendritic cells (DCs), macrophages, eosinophils, and B and T cells at the site of vaccination, which correlated with tumor destruction.

In a second clinical trial, a GM-CSF construct was transduced into autologous non-small cell lung cancer (NSCLC) cells for use as a vaccine [128]. In this study, 3 CRs were reported for 33 patients vaccinated [128]. There was a

significant positive correlation ($p < 0.03$) between the amount of GM-CSF produced (40 ng/$10^6$ cells per 24 hours) and increased patient survival [128]. The 9% CR rate in this small group of patients provided some measure of enthusiasm for this approach. However, generating autologous vaccines is technically difficult and although within the scope of some academic medical centers, is not an easily commercialized product. For these reasons, allogeneic GM-CSF-secreting vaccines were considered an attractive alternative for treatment because of standardized transduction efficacy and off-the-shelf availability.

***Allogeneic whole-tumor GM-CSF producing vaccines***

Based on the complete remissions observed after vaccination with an autologous NSCLC vaccine, a second NSCLC study using an allogeneic leukemia K562 cell line genetically engineered to secrete GM-CSF was begun [129]. The vaccine comprised a mixture of isolated autologous tumor cells and allogeneic GM-CSF-secreting bystander cells (K562); the idea being that the bystander cells would produce GM-CSF at the site of the vaccine without having to gene-modify the autologous tumor. There were no objective clinical responses in 49 vaccinated patients. Besides the addition of K562 bystander cells, another difference in this trial and the autologous NSCLC vaccine trial was that the amount of GM-CSF secreted, which, on average, was 25 times higher with the K562 bystander cells than in the original NSCLC trial that resulted in 3 complete responders [129].

An alternative to mixing bystander cells that secrete GM-CSF with

autologous tumor cells to generate a vaccine is to transduce GM-CSF expression vectors into allogeneic tumor cells of the same histology as the tumor to be treated. These cells would presumably share antigens with the patient's tumor cells but could be used where it is difficult or impossible to obtain autologous tumor. Prostate cancer is a good candidate for this off-the-shelf approach as it metastasizes to the bone, and it is virtually impossible to isolate sufficient tumor cells for autologous vaccine production. Prostate GVAX vaccine is composed of two allogeneic tumor cell lines, PC3 and LNCAP, both of which are transduced to secrete GM-CSF [129]. Phase I/II trials in patients with advanced disease were performed, with prostate GVAX vaccine administered at a low (100 x $10^6$ cells 28 days x 6), medium (200 x $10^6$ 14 days x 12), or high (300 x $10^6$ 14 days x 12 and 500 x $10^6$ x 1) dose with corresponding survival of 23, 20, or 34.9 months, respectively [130]. In another trial, patients were treated with a 500 x $10^6$ cell priming dose and 12 booster vaccinations with 100 x $10^6$ or 300 x $10^6$ GVAX cells, biweekly for 6 months (129). Progression-free survival assessed by bone scans was 2.8 and 5 months with low- and high-dose vaccines, respectively [131]. Although phase II results were promising, phase III trials comparing GVAX to chemotherapy were terminated because the vaccine group was worse, but some late analysis showed improved survival with vaccine [132]. One explanation for this is that booster vaccinations are not improving therapeutic efficacy. A clinical trial using a single prostate GVAX immunization is now being initiated (personal communication with Dr deGruijl) (this paragraph is modified from Church et al. [126]).

### *GM-CSF also has negative anti-tumor effects*

As described above whole tumor vaccines secreting GM-CSF have had highly variable results in clinical trials [126]. One possible explanation for this is that GM-CSF has considerably different effects on the immune response depending on the route of administration. For instance, in pre-clinical models, continuous low-dose secretion of GM-CSF, such as with whole tumor cells transduced to secrete GM-CSF, has been shown to be considerably more effective than single high-dose injections of GM-CSF [133,134]. However, GM-CSF produced by tumor cells *in vivo* can induce suppressor cells [135,136]. Another reason for variability in GM-CSF adjuvant vaccine trials could be due to the dose and route of GM-CSF used as an adjuvant: low doses of GM-CSF given once per day do not facilitate cross-presentation of antigens and activation of APCs, and high doses of GM-CSF can induce immunosuppression by myeloid-derived suppressor cells (MDSCs) and Tregs [137,138]. The following section will address the role of multiple vaccinations and possible combination therapies that can attenuate negative immunomodulatory effects, thereby enhancing the therapeutic efficacy of vaccination.

## ***Improving the therapeutic efficacy of multiple vaccinations***

### *Vaccine route and frequency affects therapeutic efficacy*

A number of pre-clinical and clinical studies have demonstrated the importance of route and frequency of vaccine and adjuvant delivery [126]. Studies in rats combining 5 injections of GM-CSF with a peptide vaccine either

intradermally (i.d.) or subcutaneously (s.c.) showed that i.d. injections result in a larger number of MHCII+ APCs in the vaccine-draining LN compared to s.c., suggesting that i.d. administration better activates the adaptive immune system [139]. Another study showed that immunization with a whole tumor vaccine transduced to secrete GM-CSF improved efficacy when given at the same site as tumor challenge, however efficacy was decreased when the vaccine was administered to multiple distant sites [140]. The importance of location of vaccine administration was exemplified in a therapeutic cervical cancer vaccine trial where two tumor-associated peptide pools were delivered to one limb or two separate limbs, 4-times every 3-weeks [141]. Patients treated at one site did not increase their anti-tumor response with booster vaccination; however, if the peptides were delivered to separate sites anti-tumor responses progressively increased after each booster vaccination [141]. Chapter 3 will examine how changing dose and route of vaccine affects protective ability and induction of immunosuppression.

### *Epitope spreading and heterologous prime boost to enhance vaccine efficacy*

Epitope spreading is described as immunization with a peptide or pool of peptides that results in an immune response either to epitopes within a protein that were not included in the vaccine or epitopes of a different protein not within the vaccine. In a number of clinical trials, Disis and colleagues have evaluated epitope spreading in the context of tumor vaccines and showed that it correlated

with an increase in a vaccine-specific Th1 response and a decrease in serum TGF-β [142-144]. Multiple trials have reported a relationship between humoral or T cell tumor antigen epitope spreading and improved clinical outcome [145-150]. These studies have reported both epitope spreading within the immunogen and intermolecular spreading to peptides from entirely different proteins.

One method to maximize and focus the immune response to the tumor-associated Ag of choice while avoiding the deleterious effects of vector-specific immunity is through heterologous prime-boost strategies. Heterologous prime boost provides a priming vaccination with an Ag delivered in one vector and comes back with a second type of vector that shares the same target antigen. The augmented immune response to the target antigen and the inflammatory response would also support the development of intraepitope spreading.

One approach to increase the magnitude of the tumor-specific T cell response is to use a microbial-based vaccine vector expressing one or more tumor-associated antigens contained within the irradiated tumor cell vaccine. Priming with a whole-cell vaccine can elicit T cell responses to multiple tumor Ags of limited magnitude, which when boosted by a microbial-based vaccine expressing a defined tumor-associated Ag can promote exceptional expansion of T cells specific for the shared Ag [151].

Live-attenuated vectors based on the intracellular bacterium *Listeria monocytogenes* have performed remarkably in this capacity by introducing the inflammatory environment to induce memory T cell persistence [152]. In preclinical studies, mice immunized with a whole-cell vaccine exhibited primed vaccine-

specific CD8 T cells. However, this CD8 T cell response typically comprised only a fraction of a percent of the total CD8 T cells (0.1%-0.3%). Boosting this response with a live-attenuated *L. monocytogenes* vaccine can greatly expand the Ag-specific CD8 T-cell population to 17% to 21% of the total CD8 T cell population (unpublished data courtesy of Dr Bahjat). One potential caveat of this approach is that T cells specific for epitopes encoded by the boosting vaccine may expand at the expense of T cells recognizing other tumor-associated Ags [153]. Similar improvements have been observed in heterologous prime-boost immunization regimens using viral vectors expressing tumor-associated Ags [154,155] ([156]. Heterologous prime boost with the proper adjuvants may expand an existing immune response and potentially generate a de novo response; however, determining timing, location, and type of prime-boost vaccination may be important for inducing persistent anti-tumor immunity (this paragraph is modified from Church et al. [126])

***Myeloid derived suppressor cells during vaccine immunotherapy***

Human and murine vaccine studies have shown that high-dose (>1500 ng per 24 hours) GM-CSF secreting whole tumor vaccines are less effective than lower doses (30-300 ng per 24 hours) of GM-CSF [137,157]. Pivotal studies by Borrello and colleagues, examined the effect of high-dose GM-CSF on the anti-tumor immune response [137]. In these studies tumor-bearing mice were immunized s.c. with whole tumor cells and bystander cells that secreted varying amounts of GM-CSF (30, 300, 1500, 3000, and 6000 ng per 24 hours). Only

vaccination with irradiated whole tumor plus 30 or 300 ng per 24 hours bystander cells significantly improved survival compared to vaccination without GM-CSF. Moreover, reduced therapeutic efficacy observed with the high-dose (≥1500 ng per 24 hour) vaccination corresponded to an increased number of CD11b+Gr-1+ MDSC. These MDSCs inhibited Ag-specific CD4 T cell responses [137]. MDSCs secrete inducible nitric oxide synthase (iNOS) and arginase and upregulate inhibitory ligands such as program death ligand 1 (PD-L1), which all contribute to T cell suppression [138]. Therefore it is possible that high-doses of local (s.c.) GM-CSF cause MDSC to be recruited to the vaccine-draining lymph node (dLN), where they decrease tumor-specific T cells priming. A number of drugs have been identified that inhibit or decrease induction of MDSCs [158]. Drugs that inhibit phosphodiesterase 5 (PDE5) reduce tumor-induced MDSC by down-regulating iNOS and arginase [159]. A clinical trial is currently in progress combining the PDE5 inhibitor tadalafil with vaccination and GM-CSF adjuvant [159,160] Chapter 3 will address the role of vaccine route and dose on MDSC accumulation in an active-specific immunotherapy of melanoma.

***Eliminating regulatory T cells during vaccine immunotherapy***

A high frequency of circulating Tregs has been observed in patients with lung, ovarian, breast, colorectal, esophageal, gastric, hepatocellular, leukemia, lymphoma, melanoma, and pancreatic cancers (reviewed in Zou [161]). This increase in Tregs, particularly within the tumor, has been associated with poor prognosis [162]. Preclinical and clinical trials have tried to eliminate Tregs in

combination with vaccination. In a pre-clinical study, 3 vaccinations with a whole-cell vaccine secreting GM-CSF decreased therapeutic efficacy and increased Treg numbers [121]. When CD4 cells were partially depleted before the second and third vaccinations, anti-tumor immunity was restored, identifying CD4 depletion as a method to decrease Tregs. This approach could be rapidly translated to clinical trials, as there is a humanized CD4-depleting antibody zanolimumab (Hu-max-CD4), which has been used to treat cutaneous T-cell lymphoma [163]. This antibody is being used in combination with IL-2 in a phase II clinical trial to reduce Tregs [160,163]. Although, CD4-depletion in pre-clinical models has increased anti-tumor immune responses, some level of CD4 T-cell help is likely critical for priming and maintenance of memory CD8 T cells, and depletion of the beneficial CD4 T cells could be detrimental to long-term immunity [46,164]. Therefore, other methods have been used to target Tregs, including CD25 blockade or depletion and small molecule inhibitors of TGF-β. Human Tregs express high levels of IL-2Rα (CD25). Two types of CD25-targeted antibodies have been used to reduce Treg numbers in cancer patients [165]. The humanized CD25-blocking monoclonal antibody, daclizumab, has been used to reduce Treg numbers in multiple clinical trials. The best immune stimulatory results were observed in a trial where patients received daclizumab 1 week before 5 vaccinations with hTert or survivin peptides plus GM-CSF, which resulted in an increased antigen-specific CTL response [165]. Another CD25-targeted therapy, the immunotoxin denileukin diftitox (ONTAK), which is a fusion protein of IL-2 coupled with the active enzyme of diphtheria toxin. ONTAK was originally

developed for treating T cell lymphoma; however, it is currently being used in combination with vaccination as a method to reduce Tregs in a number of cancers, including renal carcinoma [166-168]. Renal cell carcinoma patients who were pretreated with ONTAK followed by vaccination with DCs transfected with tumor RNA exhibited a 7.2- and 7.9-fold median increase in CD4 and CD8 T cell responses against RNA-transfected DCs, respectively [167]. There are a number of pitfalls to CD25-depletion strategies. First, CD25 is expressed on activated CD4 and CD8 effector T cells, and eliminating these cells can reduce important tumor-reactive T cells; second, these CD25-depletion strategies do not always significantly reduce Treg numbers when confirmed by Foxp3 expression [82,169-171]. Finally, CD25 is expressed on APCs involved in IL-2 signaling to T cells, and depletion/blocking of these APCs decreases T-cell activation [172].

Another method to reduce Treg tolerance is to limit the induction of new Tregs. TGF-β, which is secreted by many tumors, suppresses effector T cells and induces Tregs and tumor-associated macrophages [173]. SM16, a small-molecule inhibitor of TGF-β type 1 receptor (ALK5) kinase, has been shown to decrease mesothelioma recurrence and metastatic breast cancer progression by altering anti-tumor immunity [174-176]. These studies suggest that systemic TGF-β inhibition may be a promising addition to combination immunotherapy strategies (modified from Church et al.[126]). Furthermore, the use of immunogenic chemotherapies, cytokine administration, toll-like receptor (TLR) agonists and immune stimulatory antibodies are promising options for combination therapy

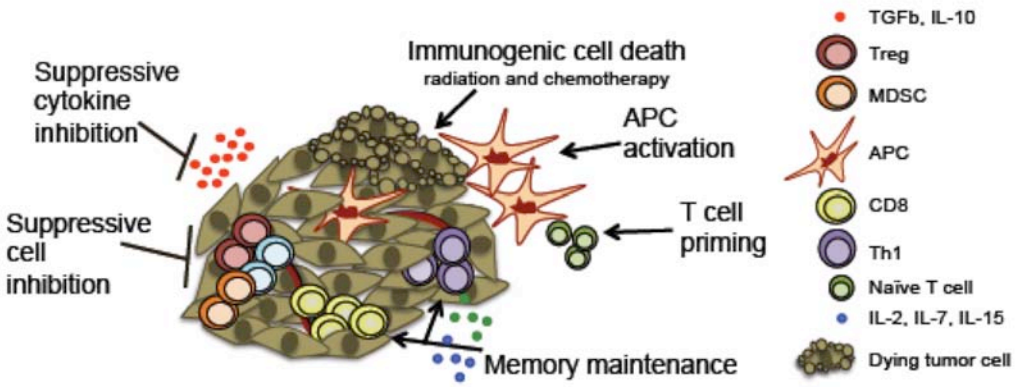



**Figure 1-2. Mechanisms to enhance anti-tumor immunity**. Immune stimulatory and immunosuppressive mechanisms that can be targeted to improve anti-tumor immunity. Anti-tumor immunity can be stimulated by: inducing APC activation to aid in priming of naïve T cells, which would be optimal in the presence of Th1 CD4 T-cell help, increasing access to cytokines involved in memory T cell homeostasis (IL-2, IL-7, IL-15) and eliciting immunogenic tumor cell death by radiation or chemotherapy. Inhibiting immunosuppressive cells (Tregs and MDSC) and cytokines (TGFβ and IL-10) can also augment the anti-tumor immune response.

with multiple vaccinations, but these are not within the scope of this dissertation.

Figure 1-2 provides an overview of possible targets for combination therapy.

# CHAPTER 2

## Tumor-specific CD4 T cells Enhance the Therapeutic Efficacy of CD8 T Cells in a Lymphopenic Environment

**Abstract**

Cancer immunotherapy that combines the induction of lymphopenia with adoptive transfer of T cells genetically engineered to express receptors specific for tumor specific/associated antigens has shown promise in clinical trials. However, little is known regarding the role for tumor-specific CD4 T-cell help in this setting. Since CD4 T cells have the ability to aid priming and maintain effector and memory CD8 T cells, we examined whether tumor-specific CD4 T cells enhance CD8 T-cell adoptive immunotherapy in a lymphopenic environment. Our model employed doses of TRP1 CD4 T cells and Pmel CD8 T cells that when transferred individually were subtherapeutic; however, when transferred together they provided significant ($p \leq 0.001$) therapeutic efficacy with no visible tumor growth over 90 days. Therapeutic efficacy correlated with the increased number of effector and memory CD8 T cells with tumor-specific cytokine expression. When combined with CD4 T cells, transfer of total (naïve and effector) or effector CD8 T cells were equally effective, suggesting CD4 T cells can help mediate therapeutic effects by maintaining endurance and function of activated CD8 T cells. The CD4 T cells appear to be required early, as their elimination 10 days after transfer failed to reduce therapeutic efficacy. The CD8 T cells recovered from mice treated with both CD8 and CD4 T cells had decreased expression of TRAIL and PD-1 compared to CD8 T cells from animals receiving only CD8 T cells suggesting that CD4 T cells help reduce apoptotic death of CD8 T cells. These data support combining tumor-specific CD4 and CD8 T cells for the adoptive immunotherapy of patients with cancer.

## Introduction

Metastatic melanoma is a devastating disease with a 10-year survival rate of 10-15% [67]. A number of immune therapies, including high-dose interleukin-2 and ipilimumab, have yielded promising results for treatment of melanoma [177,178]. Significant success in treatment of melanoma has also been achieved with combination therapy using adoptive T cell immunotherapy (AIT), nonmyeloablative chemotherapy and high doses of IL-2 [96,98,179]. The majority of these studies utilize T cells expanded from tumor infiltrating lymphocyte (TIL) cultures, however the use of human T cells transduced to express tumor-reactive T-cell receptors (TCR) or chimeric antigen receptors (CARs) is a growing area of research [179,180]. Therapy utilizing genetically engineered TCR T cells and CARs has the potential to treat a broader patient base since it may not require the generation of large numbers of tumor-specific T cells from the patient, which remains a limiting and time-consuming factor. Furthermore studies with CARs have shown long-term tumor regression and tumor-specific T cell persistence for over 6 months [181].

Both clinical trials and murine models, studying adoptive immunotherapy using TIL or TCR transgenic (Tg) T cells against melanoma have shown AIT is more effective in a lymphopenic than in a lymphoreplete environment [98,108]. A lymphopenic environment can be in established a number of ways including chemotherapy (cyclophosphamide), radiation, a combination of both or by the simulated use of genetic models of mice that lack endogenous T and B cells (recombinase activating gene (RAG) deficient mice) [3,99]. Each lymphopenia

inducing method provides a different combination of mechanisms that enhances AIT including elimination/absence of suppressive cells and non-specific cells that consume cytokines, less competitive access to antigen-presenting cells (APC), induce inflammation, killing tumor cells, activating APCs or overcoming T cell exhaustion [100,106,109]. Additionally, combination adoptive immunotherapy with induction of lymphopenia has been shown to induce memory and circulating CD8 T cell persistence, which has correlated with successful clinical outcome [182-184]. This is likely because CD8 T cells are a major and perhaps the dominant mechanism to eliminate tumor via cytokines or by directly killing tumor cells and thus combinations that maintain and or increase tumor-specific CD8 T cells would be expected to improve therapeutic efficacy [23,185,186].

The importance of CD4 T-cell help for both priming and maintenance of memory CD8 T-cell immunity has long been appreciated [45,164]. Our lab and others have shown that AIT with CD8 T cells in CD4-depleted or MHCII-deficient (that lack conventional CD4 T cells) mice initially eliminated tumor, but did not result in long-term anti-tumor immunity and tumors eventually recurred [187,188]. In contrast, multiple studies have shown that partial or transient CD4-depletion can enhance anti-tumor responses, but since these models do not eliminate CD4 T cells completely there may be a small population of CD4 T cells programming or maintaining CD8 T cell function [121,189]. Moreover induction of lymphopenia is thought to abrogate the need for CD4 help since it increases CD8 T cell exposure to homeostatic cytokines, IL-7 and IL-15, driving memory T cell formation and enhancing anti-tumor immune responses [111,190-192].

Although it has not been directly examined, it is possible that some of the CD8 T cell TCR gene transfer studies had decreased objective response rates due to a lack of tumor-specific CD4 T cells. Two clinical studies utilizing MART-1 and/or gp100-specific HLA class I restricted TCR gene transfer for treatment of metastatic melanoma resulted in decreased objective clinical response rates, 13% (2/15) and 30% (6/20), when compared to therapy using TIL (51%-71%) [98,179,193]. While these are small studies, one possible explanation for this lower response rate is the absence of tumor-specific CD4 T cells in the TCR gene therapy studies suggesting CD4 T cells may be playing a role. However, attempts to identify tumor-specific CD4 T cells in the peripheral blood of patients following adoptive immunotherapy with TIL has been largely unsuccessful and expanding tumor-specific CD4 T cells from cancer patients has been difficult [194]. In this chapter we investigated the importance of tumor-specific CD4 T-cell help for long-term CD8 T cell anti-tumor immunity during lymphopenia-driven homeostasis.

## Materials and Methods

*Tumor cell lines and Metastases*

We used the poorly immunogenic subclone, D5, isolated from the spontaneously arisen B16BL6 melanoma [117]. T-cell stimulation assays were done using D5 CIITA and the unrelated syngeneic sarcoma MCA-310 CIITA; both were modified to express the human class II major histocompatibility complex transactivator [107,108]. D5, D5 CIITA and MCA-310 CTIIA were propagated using 10% FBS RPMI 1640 supplemented with 2 mmol/L L-glutamine, 0.1 mmol/L non-essential amino acids, 1 mmol/L sodium pyruvate, 5 μg/ml gentamicin sulfate (Lonza) and 50 μM/L β-mercaptoethanol (Sigma). All tumor cell lines were propagated for less than 6-weeks.Three-day established pulmonary metastases were generated by injecting 2 x $10^5$ D5 cells intravenously (i.v.). Rechallenge experiments used 2 x $10^4$ D5 cells injected subcutaneously (s.c.). Tumors were measured in perpendicular directions every 2-4 days.

*Mice and Adoptive Immunotherapy*

TRP1 TCR x tyrp-$1^{bw}$Rag1-/- (RAG1-/- tyrp1 protein-deficient MHC Class II restricted TCR Tg) male mice were used to isolate tumor-specific TRP1 CD4 splenocytes (gift from Dr. Nicholas Restifo and The Jackson Laboratory). TRP1 CD4 T cells are specific for the murine tyrosinase-related protein 1 peptide [37], which is expressed on melanomas. Female RAG1-/- tyrp1-protein-deficient littermates, which lack the TCR transgene were used as hosts. RAG1-/- pmel-1 (MHC Class I restricted TCR Tg) mice were generated by breeding RAG1-/- (The

Jackson Laboratory) with Tg pmel-1 mice (gift of Dr. Nicholas Restifo), and used to isolate pmel tumor-specific CD8 T cells from male mice.

Recognized principles of laboratory animal care were followed (Guide for the Care and Use of Laboratory Animals, National Research Council, 1996) and all animal protocols were approved by the Earle A. Chiles Research Institute animal care and use committee.

Single cell suspensions of pmel splenocytes were incubated for 2 days on 5 μg/ml anti-CD3 (2C11) in a 24-well plate followed by 3 days with 60 International Units (IU)/ml IL-2 (Chiron) in a lifecell tissue culture bag (Baxter) (referred to as αCD3-IL-2 expansion). TRP1 CD4 splenocyte suspensions were enriched using a pan T cell isolation kit (Miltenyi). Intravenous injections used 1 x $10^6$ αCD3/IL-2 expanded pmel cells and/or 1000 enriched TRP1 cells, unless otherwise noted. Mice also received intraperitoneal (i.p.) injections of 90,000 IU IL-2 (Chiron) given daily for 3 days. Mouse lungs were resected and stored in Feketes solution. Metastases were enumerated by counting black nodules on the lung surface. Maximum tumor burden was recorded as 200 metastases.

*Flow Cytometry*

Spleens were disrupted using a 3 ml syringe in a 6-well plate and filtered to single cell suspensions. Red blood cells were lysed using ACK buffer (Lonza). Cells were stained for phenotyping and sorting with combinations of the following antibodies CD4 Qdot605 (Invitrogen), CD8-PE-Cy7, CD3-Percp-eFluor710, CD62L-Pacific blue/eFluor450, CD127-PE, CD127-APC-eFluor780, PD-1-FITC,

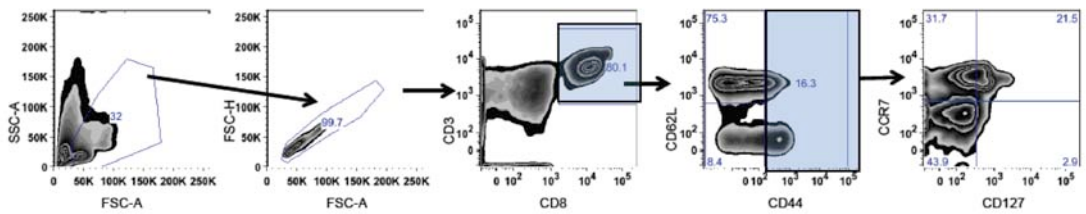



**Figure 2-1: Gating strategy for flow cytometry phenotyping.** T cells were gated on live/singlet/CD3/CD8/CD44 by CD62L, all CD44-positive were further gated on CCR7 and CD127.

CCR7-APC, CD44-AF700, PD-1-PE, TRAIL-PE, Bcl-2-FITC, FOXP3-eFluor450 (eBioscience), VB14-PE, VB13-APC, CD4-APC-Cy7, CD4-APC-H7, LAG-3-PE, CD95-APC (Becton Dickinson). Intracellular staining was performed using the eBioscience fix-perm kit. The gating strategy for memory T cells is shown in Figure 2-1. Briefly, cells were gated on live/singlet/CD3/CD8 or CD4/CD44 and/or CD62L, all CD44-positive cells were further gated on CCR7 and/or CD127. Blood counts were calculated using Flow-Count Fluorospheres (Beckman Coulter). For intracellular cytokine staining (ICS) splenocytes were incubated 18-24 hours adding 5 μg/ml Brefeldin A (Sigma) after two hours. Cells were stained with LIVE/DEAD fixable yellow stain (Invitrogen-Molecular Probes), CD8-V500 and CD4-APC-H7 (Becton Dickinson). Cells were fixed and permeablized (Becton Dickinson) then stained with IFN-γ-PE (Becton Dickinson), TNF-α-FITC, Granzyme B-PE-Cy7, IL-17-FITC and/or IL-2-eFluor450 (eBioscience) or IL-2-Brilliant Violet 421 (Biolegend). ICS cells were gated on live-singlet lymphocytes negative for live-dead dye, followed by CD4 or CD8 and individual cytokines. Proliferation was detected using CFSE (Invitrogen-Molecular Probes) as previously reported [195]. All samples were run on a BD LSRII or BD Aria and

analyzed using FlowJo (Treestar), Pestle and SPICE (Courtesy of Mario Roederer at the NIAID Vaccine Research Center).

*Depletion and blocking antibodies*

CD4 and CD8 depleting antibodies were made from 2.43 or GK1.5 hybridomas (ATCC), respectively by purifying ascites using Biosephra MEP Hypercel (Ciphergen) as described previously [196] or purchased from BioXcell. CD40L-blocking antibody (MR1) and hamster IgG were purchased from BioXcell. Rat IgG control antibody was purchased from Sigma. All antibodies were administered i.p.

*Statistics*

Unpaired or paired student t tests were done for analysis of cell numbers and phenotype using Prism (Graphpad). Mantel-Cox log rank tests were used to analyze survival curves (Prism, Graphpad). A p value of $<0.05$ was considered significant.

## Results

*Adoptive immunotherapy with both pmel and TRP1 Tg cells augments therapeutic efficacy*

Large numbers ($5 \times 10^5 - 2 \times 10^6$) of TRP1 Tg cells have been shown to eradicate moderately immunogenic melanomas [197,198]. Therefore, to generate a lymphopenic model in which therapeutic efficacy was dependent on both CD8 and CD4 T cells, we needed to identify a dose of TRP1 CD4 Tg T cells that was not therapeutic on their own. To do this three-day experimental pulmonary metastases were established by intravenous injection of the poorly immunogenic B16BL6 clone, D5 in tyrp-1$^{bw}$Rag-1-/- (RAG1-/- tyrp1 protein-deficient [37]) female mice, which lack endogenous T and B cells. Mice received adoptive transfer of CD4-enriched TRP1 Tg cells at doses ranging from $1 \times 10^6$ to 50 cells. TRP1 CD4 T cells failed to fully eliminate metastases regardless of their number, but animals treated with 5000 or fewer CD4 T cells had greater than 50 metastases (Figure 2-2). Consequently a dose of 1000 CD4-

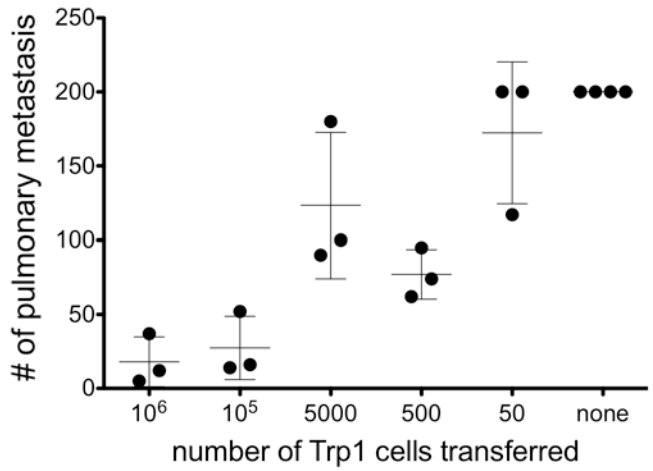



**Figure 2-2: Determining the number of TRP1 transgenic cells that eliminates 3-day pulmonary metastases.** Mice with 3-day established D5 pulmonary metastases were treated with $1 \times 10^6$, $1 \times 10^5$, 5000, 500 or 50 CD4-enriched splenocytes from TRP1 transgenic mice. Results are presented as the number of pulmonary metastases 22 days after adoptive transfer.

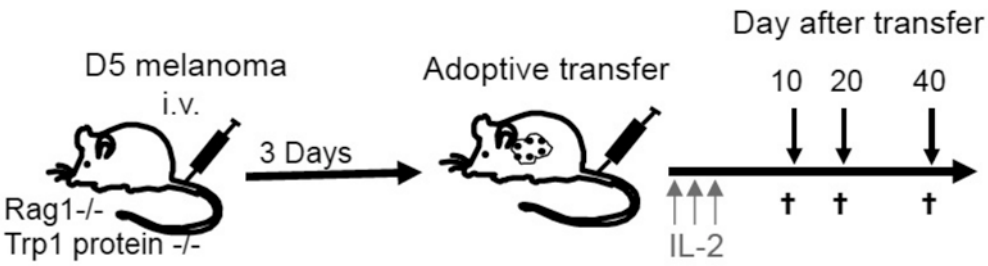



**Figure 2-3. Overview of RAG1-/- lymphopenic experimental pulmonary metastasis model.** RAG1-/- lymphopenic trp1 protein-deficient mice were treated with 2 x $10^5$ D5 melanoma cells i.v. After 3 days the same mice were treated with 1 x $10^6$ anti-CD3-stimulated and IL-2 expanded pmel-CD8 Tg cells and 1000 CD4-enriched TRP1 Tg cells (pmel + TRP1), pmel alone (pmel), TRP1 alone (TRP1) or no cells (IL-2 only). All mice also received 3 doses of 90,000 IU IL-2 i.p. daily. Mice were euthanized for analysis 10, 20 and 40 days after adoptive immunotherapy.

enriched TRP1 cells were used for all subsequent experiments. For all experiments, pmel CD8 T cells were activated for 2 days with 5 μg/ml anti-CD3 followed by expansion with low-dose IL-2 (60 IU/ml) for 3-days, which will be referred to as αCD3/IL-2 expansion. RAG1-/- tyrp1 protein deficient lymphopenic mice with 3-day experimental D5 pulmonary metastases were treated with $10^6$ αCD3/IL-2 expanded pmel and 1000 naïve CD4-enriched TRP1 (pmel + TRP1), $10^6$ αCD3/IL-2 expanded pmel alone, 1000 TRP1 alone, or no treatment (Figure 2-3). All mice received 90,000 IU IL-2 i.p. daily for 3 days. Ten and 20 days after adoptive transfer mice treated with both pmel and TRP1 cells had significantly less tumor burden than mice treated with either pmel or TRP1 alone (Figure 2-4A). Mice treated with both pmel and TRP1 showed no evidence of tumor after 40 days, while pmel or TRP1 alone groups succumbed to tumor burden before 27 days (Figure 2-4A).

Treatment with both pmel and TRP1 significantly increased the number of CD8 T cells in the blood and the spleen 10 and 20 days following adoptive

transfer, compared to treatment with pmel alone (Figure 2-3B). This is notable because persistence of anti-tumor CD8 T cell has been correlated with long-term

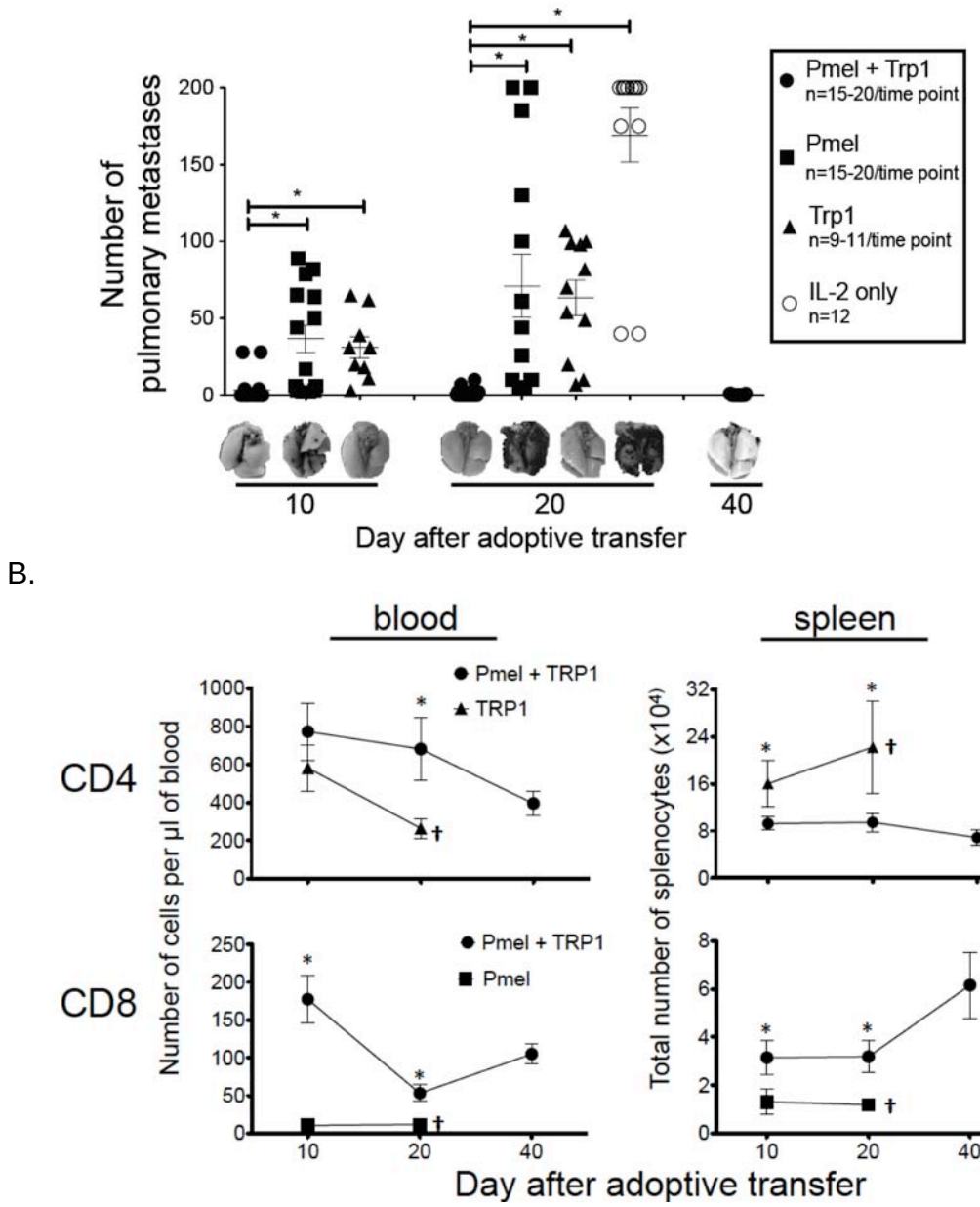



**Figure 2-4 Treatment with tumor specific pmel and TRP1 T cells in the lymphopenic environment eliminates tumor and increases pmel T cells in the blood and spleen.**

A) Number of pulmonary metastases at each time point with representative resected lungs. B) Total number of CD3+CD4+ or CD3+CD8+ cells in the blood or spleen at day 10, 20 and 40 following adoptive immunotherapy. Combination of 4 experiments, n=9-20 per time point, as indicated. *p<0.001

tumor control [199]. Treatment with pmel and TRP1 also resulted in increased CD4 T cells in the blood compared to TRP1 treatment alone, however there were decreased CD4 T cells in the spleen (Figure 2-4B). This suggests that even though there are more CD4 T cells in the spleen, with TRP1 treatment alone, the CD4 cells are significantly less ($p<0.05$) effective at eliminating tumor metastases alone than the combination of TRP1 and pmel CD8 T cells.

*The addition of TRP1 T cells enhances the number and function of pmel CD8 T cells*

CD4 T cells are known to be important for maintenance of effector and memory CD8 T cells [164,200], therefore we determined whether adoptive transfer of both pmel and TRP1 T cells increased the number and frequency of memory and effector pmel CD8 T cells compared to adoptive transfer with pmel alone. Mice receiving combined therapy exhibited an increased number of effector (CD44+CD62L-CD127-) pmel CD8 T cells in the blood and spleen (Figure 2-5A). There was also an increase in total memory (CD44+CD127+) pmel CD8 T cells in the blood and effector memory (CD44+CD127+CCR7-) pmel CD8 T cells in the spleen.

The composition of naïve (CD62L+CD44-), effector (CD44+CD127-CCR7-), effector memory (CD44+CD127+CCR7-) and central memory (CD44-CD127+CCR7+) pmel CD8 T cells 10 and 20 days after adoptive transfer indicates a much smaller proportion of effector memory phenotype pmel CD8 T cells in mice treated with pmel T cells alone (Figure 2-5B).

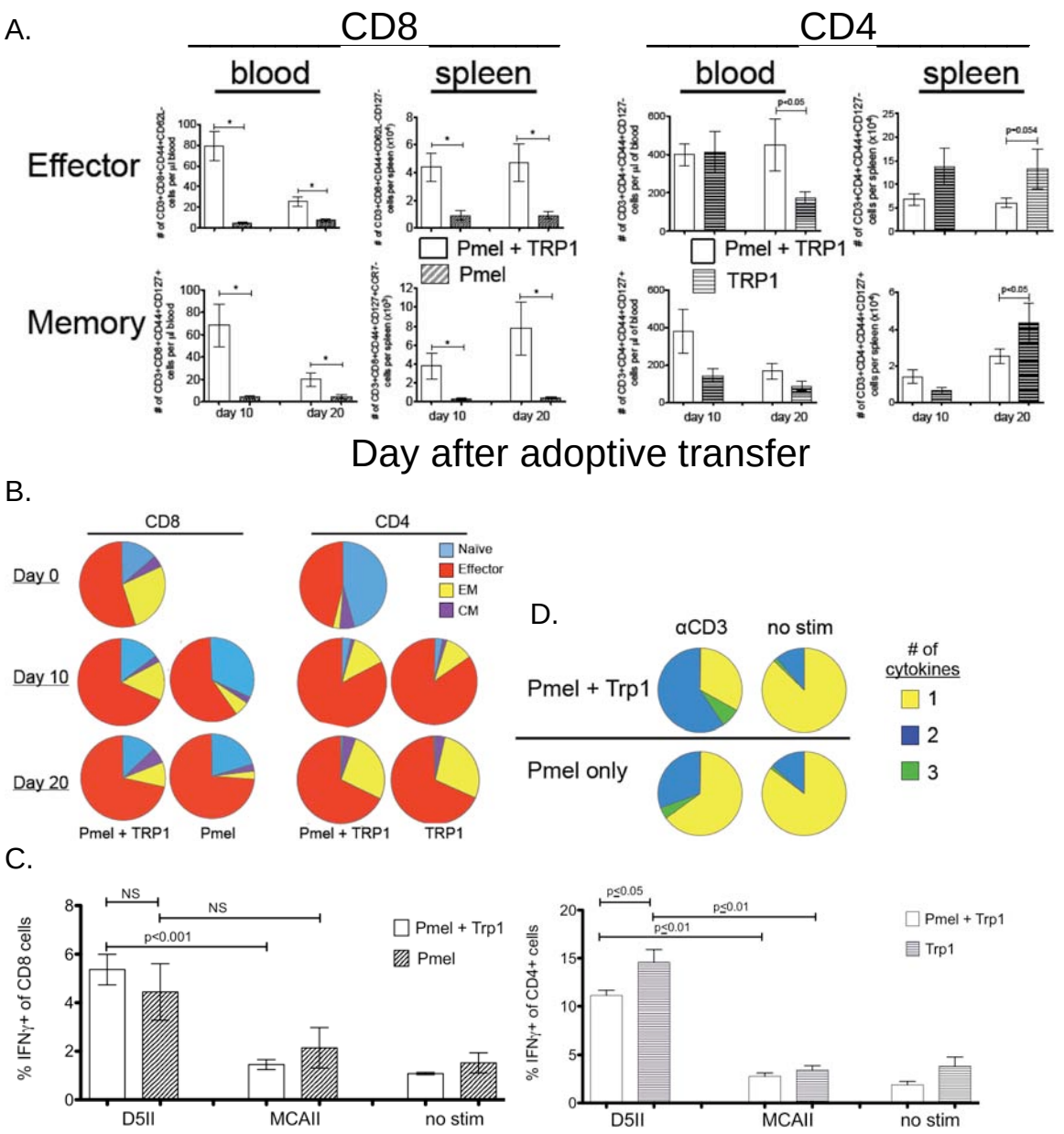



**Figure 2-5. Treatment with tumor-specific pmel and TRP1 T cells in the lymphopenic environment increases survival and function of effector and memory pmel T cells.** A) Effector (CD44+CD62L-CD127-) and memory (CD44+CD127+) CD8 or CD4 T cells in the blood and effector and effector memory (CD44+CD62L-CD127+CCR7-) in the spleen 10 and 20 days after adoptive immunotherapy with both pmel and TRP1 compared to treatment with pmel alone or TRP1 alone. B) Distribution of naïve, effector, effector memory (EM) and central memory (CM) phenotype CD8 or CD4 T cells in the spleen at the day of transfer (day 0), 10 or 20 days after adoptive transfer. C) Percent of tumor-specific IFNγ expressing CD8 or CD4 T cells stimulated with specific D5 CIITA (D5II), non-specific syngeneic MCA-310 CIITA (MCAII) or unstimulated (no stim) 20 days after adoptive immunotherapy. A-C) Combination of 4 experiments, n=9-20 per time point. D) ICS of CD8+ gated cells using splenocytes from pmel + TRP1 or pmel treated mice stimulated with αCD3 or unstimulated. Representative pies from one experiment. *p<0.001

The presence of tumor-specific TRP1 CD4 T cells increased the number and frequency of pmel effector CD8 T cells that eliminated the tumor and also increased the number of long-lived memory T cells (Figure 2-5B and data not shown). We next evaluated the function of pmel CD8 T cells from pmel and TRP1 treated mice and those treated with pmel alone. Intracellular cytokine staining of splenocytes 10 days after transfer, showed that pmel CD8 T cells from pmel and TRP1 treated mice had a significantly ($p \leq 0.001$) higher frequency of D5-specific IFNγ production compared to their response following stimulation with the syngeneic but unrelated MCA-310 sarcoma. In contrast, pmel CD8 T cells from pmel only treated mice exhibited an increase percent of IFNγ positive pmel CD8 T cells, but this difference did not reach statistical significance (Figure 2-5C). Pmel and TRP1 treated mice also had a higher frequency of CD8 T cells exhibiting polyfunctional cytokine expression (TNFα, INFγ, Granzyme B and IL-2), which has been associated with an enhanced vaccine response and long-lived T cells (Figure 2-5D) [24,201]. We analyzed the phenotype of TRP1 CD4 T cells in blood and spleen and found increased numbers of effector and effector memory in TRP1 only treated mice compared to pmel and TRP1 treated mice (Figure 2-5A). There were significantly more effector TRP1 CD4 T cells in the blood of pmel and TRP1 treated mice, however this was only at the day 20 time point and did not translate to a proportional difference in the entire population (naïve, effector, EM, CM) as we observed in the CD8 T cells (Figure 2-5A-B). Mice treated with either pmel and TRP1 or TRP1 only had an equal percentage of tumor-specific IFNγ producing cells (Figure 2-5C).

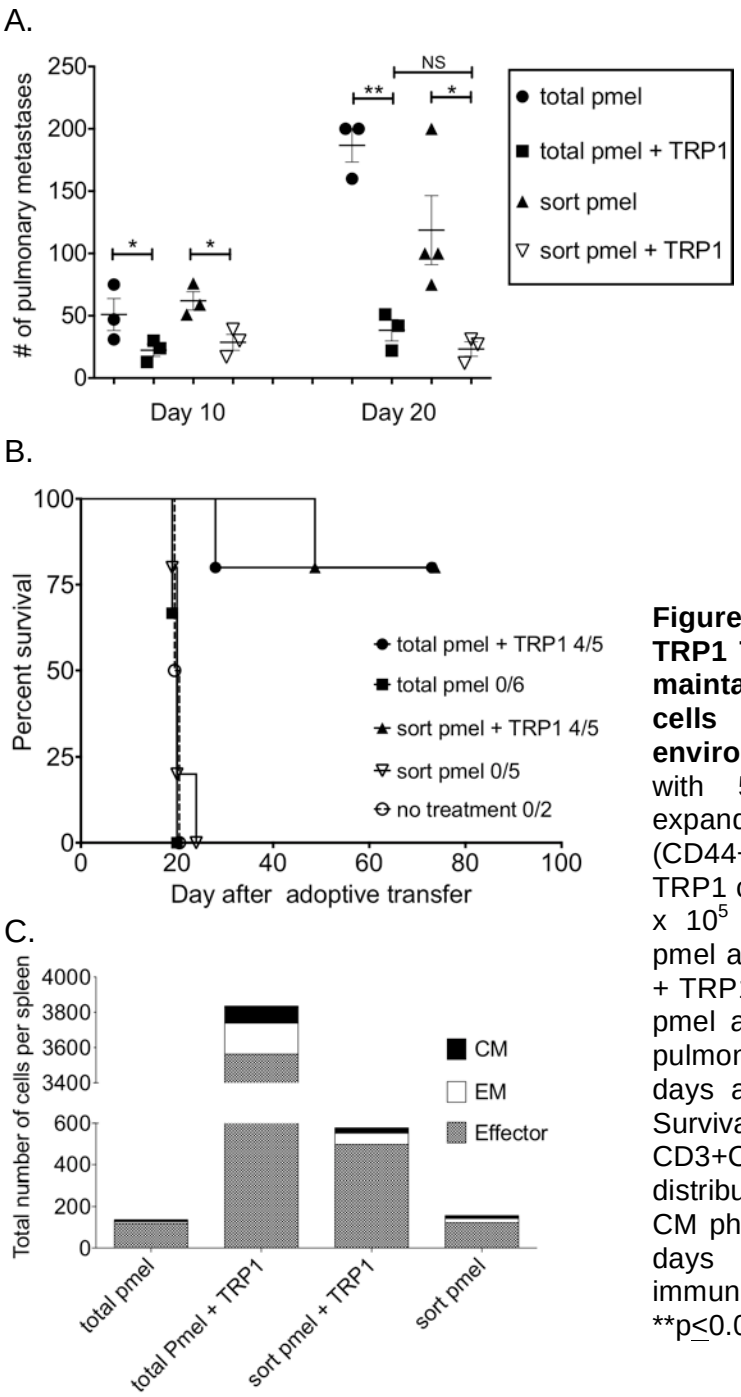



**Figure 2-6 Tumor-specific TRP1 T cells are important for maintaining activated pmel cells in the lymphopenic environment.** Mice were treated with 5 x $10^5$ anti-CD3-IL-2 expanded sorted effector (CD44+CD62L-) pmel and 1000 TRP1 cells (sort pmel + TRP1), 5 x $10^5$ total CD3-IL-2 stimulated pmel and 1000 TRP1 (total pmel + TRP1), sort pmel alone or total pmel alone. A) Total number of pulmonary metastases 10 and 20 days after adoptive transfer, B) Survival. C) Total number of CD3+CD8+ T cells with distribution of effector, EM and CM phenotypes in the spleen 10 days after adoptive immunotherapy. *$p \leq 0.05$, **$p \leq 0.001$

Furthermore previous studies using this D5 experimental metastases model suggest CD8 T cells are the predominant mechanism for eliminating tumor [196].

For these reasons the remaining studies focused on the evaluation of CD8 T cells.

*TRP1 T cells help maintain pmel CD8 T cells*

We found that following adoptive transfer of tumor-specific Tg CD4 and CD8 T cells, tumor had not recurred by 40 days and most animals were apparently cured of their disease (Figure 2-4A and data not shown). We hypothesized that CD4 T cells could be helping to prime a small number of naïve CD8 T cells that still remain after αCD3/IL-2 expansion. Therefore, we phenotyped αCD3/IL-2 expanded pmel cells at the time of adoptive immunotherapy (Day 0). This analysis revealed a large population (15-20%) of CD44-CD62L+ CD8 T cells (referred to as αCD3/IL-2 expanded phenotypically naïve (CD44-CD62L+) (Figure 2-5B). This observation surprised us, so we examined whether CD4 T cells needed this αCD3/IL-2-expanded phenotypically naïve (CD44-CD62L+) population to help prime CD8 T cells or whether they were maintaining effector phenotype CD8 T cells. We eliminated these αCD3/IL-2 expanded phenotypically naïve (CD44-CD62L+) CD8 T cells by sorting on effector phenotype pmel cells as CD44+ and CD62L-. We then compared treatment with 5 x $10^5$ sorted effector CD44+CD62L- pmel and 1000 TRP1 T cells (sort pmel+ TRP1), 5 x $10^5$ sorted effector CD44+CD62L- pmel alone (sort pmel), 5 x $10^5$ total CD3/IL-2 stimulated pmel and TRP1 (total pmel + TRP1) or 5 x $10^5$ total pmel alone (total pmel) (Figure 2-6). Adoptive immunotherapy with sorted effector pmel or total pmel, combined with TRP1 T cells had significantly

less tumor growth at 10 and 20 days following treatment compared to mice treated with either pmel cell population alone (Figure 2-6A). The majority of mice treated with both pmel and TRP1, either sorted or total, survived longer than 40 days with no symptoms of tumor progression (Figure 2-6A-B). Furthermore, while mice treated with sorted pmel and TRP1 had fewer splenic CD8 T cells than mice receiving total pmel and TRP1, their numbers were still increased compared to mice treated with only total or sorted pmel T cells 10 days after transfer (Figure 2-6C). Since elimination of the αCD3/IL-2 expanded phenotypically naïve (CD44-CD62L+) did not diminish efficacy it suggests that tumor-specific TRP1 CD4 T cells are able to enhance the function of activated pmel CD8 T cells.

We also wanted to determine whether αCD3/IL-2 expanded phenotypically naïve (CD44-CD62L+) pmel CD8 T cells truly were naïve or exhibited more of a stem-cell memory phenotype T cell. Therefore we compared *ex vivo* αCD3/IL-2 expanded phenotypically naïve (CD44-CD62L+) cells, αCD3/IL-2 expanded effector phenotype cells (CD44+CD62L-) and unstimulated pmel splenocytes. We first evaluated whether αCD3/IL-2 expanded phenotypically naïve (CD44-CD62L+) pmel cells were proliferating. Splenocytes were labeled with CFSE prior to αCD3/IL-2 expansion and evaluated on the $5^{th}$ day of culture. Interestingly, pmel cells that retained a naïve (CD44-CD62L+) phenotype following stimulation had proliferated (>5x) extensively like pmel cells with an effector phenotype (CD44+CD62L-CD127-CCR7-) (Figure 2-7A). In contrast, pmel cells that were not stimulated had divided only once or twice. Functionally, αCD3/IL-2 expanded

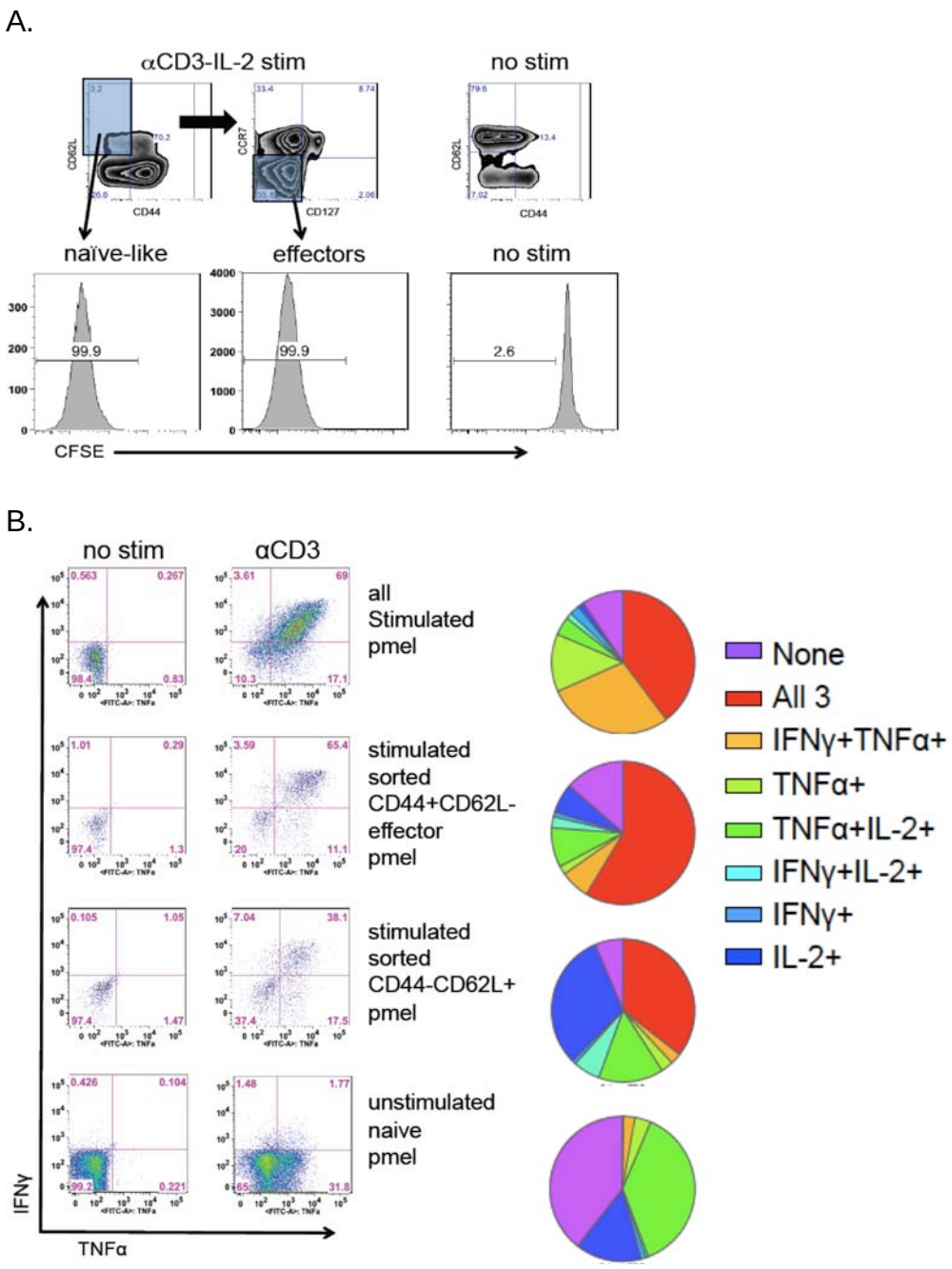



**Figure 2-7 Expanded effector and CD44+CD62L+ cells are functionally distinct from unstimulated pmel cells**.

Comparison of αCD3-IL-2 expanded total, naïve (CD44-CD62L+) phenotype, effector (CD44+CD62L-) phenotype or unstimulated pmel cells after 5 days in culture A) CFSE proliferation and B) TNFα, IFNγ and IL-2 cytokine expression. Pies represent distribution of cytokine expression after 24 hours with a second αCD3 stimulation.

phenotypically naïve (CD44-CD62L+) CD8 T cells expressed lower levels of TNFα and IFNγ compared to αCD3/IL-2 expanded effector phenotype (CD44+CD62L-) or total expanded pmel, however cells with the αCD3/IL-2 expanded phenotypically naïve (CD44-CD62L+) cells expressed higher levels of double positive TNFα and IFNγ than unstimulated pmel cells (Figure 2-7B). These αCD3/IL-2 expanded phenotypically naïve (CD44-CD62L+) cells have a distinct cytokine profile that seems to be in between activated and naïve cells. One possibility is that these αCD3/IL-2 expanded phenotypically naïve (CD44-CD62L+) cells are stem-cell memory (SCM) cells, however they do not express higher levels of CD122, sca-1 or CCR7, which are associated with SCM cells (data not shown). These data infer that tumor-specific CD4 T cells can act in conjunction with effector CD8 T cells to increase their numbers, tumor-specific function and efficacy, even in the absence of naïve T cell priming.

*TRP1 help occurs early after adoptive immunotherapy*

We attempted to determine when, in relation to adoptive transfer, tumor-specific CD4 T cells were needed to maintain anti-tumor immunity. According to the model described in Figure 2-3, CD4 cells were depleted one-day prior, 3 and 10 days following AIT with pmel and TRP1 cells. Anti-CD4 antibody was administered one-day prior to adoptive transfer even though RAG1-/- mice have no T cells to ensure CD4 cells were immediately eliminated upon transfer. We expected this to replicate adoptive transfer with pmel alone. Indeed, depletion of CD4 T cells one-day prior to adoptive immunotherapy resulted in a large tumor

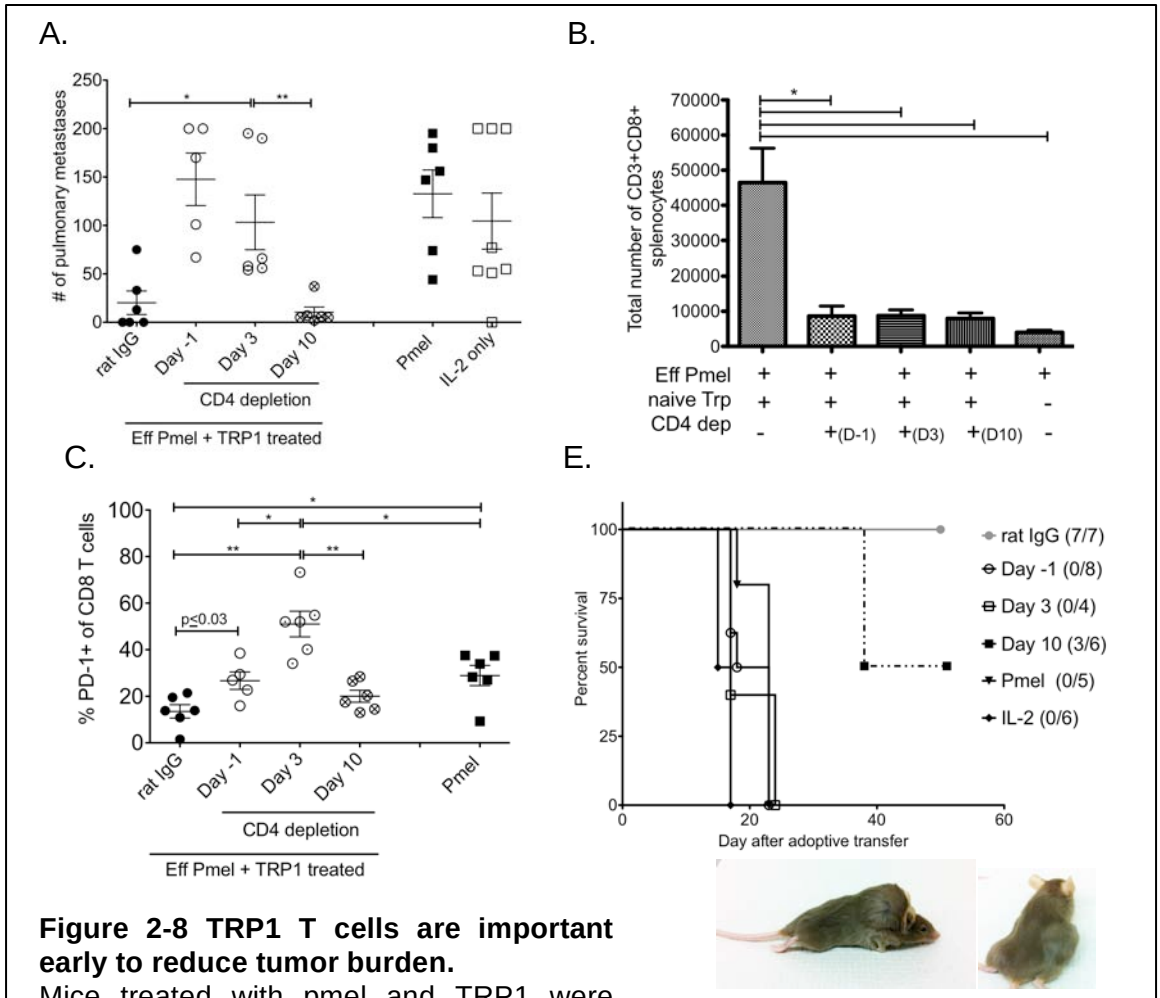



**Figure 2-8 TRP1 T cells are important early to reduce tumor burden.**
Mice treated with pmel and TRP1 were depleted of CD4 cells one-day prior, 3 or 10 days following AIT and compared to pmel alone or no treatment. All analysis was done 17 or 18 days following adoptive immunotherapy. Data are a combination of 2 experiments 5-7 mice per group A) Total number of pulmonary metastases 17 or 18 days after adoptive transfer and survival (representative experiment). B) Total number of CD3+CD8+ T cells in the spleen. C) Frequency of PD-1 expressing CD3+CD8+ T cells 17-18 days after transfer. D) Summary of the number of intracellular cytokines expressed by CD8+ cells (IL-2, TNFα, IFNγ and Granzyme B) with 24 hours αCD3 stimulation or no stimulation. E) Survival curve and representative of distant skin metastases that occurred 38 days after transfer in mice depleted of CD4 T cells 10 days after transfer.

burden that was similar to pmel treatment alone. CD4-depletion 10 days after transfer resulted in smaller tumor burden, similar to undepleted mice, depletion at day 3 gave results that were intermediate (Figure 2-8A). This suggests that tumor-specific CD4 T cells exert their effects sometime during the first 10 days following adoptive transfer. We examined whether CD4-depletion changed the number of CD8 T cells in the blood and spleen 17 or 18 days after adoptive immunotherapy, CD4-depleted groups showed a substantial decrease in the number of pmel CD8 T cells (Figure 2-8B and data not shown). We also examined the expression of the exhaustion marker, program cell death-1 (PD-1). PD-1 expression on pmel CD8 T cells was significantly higher among CD8 cells in all CD4-depleted groups (Day -1, 3 and pmel alone) than in CD8 T cells from undepleted mice (Figure 2-8C). Depletion of CD4 cells one-day before or 3 days after adoptive immunotherapy also correlated with decreased expression of IFN$\gamma$, IL-2, TNF$\alpha$ and Granzyme B compared to deletion at day 10 or undepleted mice (Figure 2-8D). However, long-term anti-tumor immunity was compromised in mice depleted of CD4 T cells 10 days after transfer compared to undepleted mice (Figure 2-8E). Interestingly, mice depleted of CD4 cells 10 days after transfer often developed

tumors at metastatic sites, such as the skin (Figure 2-8E). This suggests CD4 T cells maintain pmel CD8 T cells or potentially act to support trafficking of CD8 T cells to metastatic sites of tumor beyond 10 days.

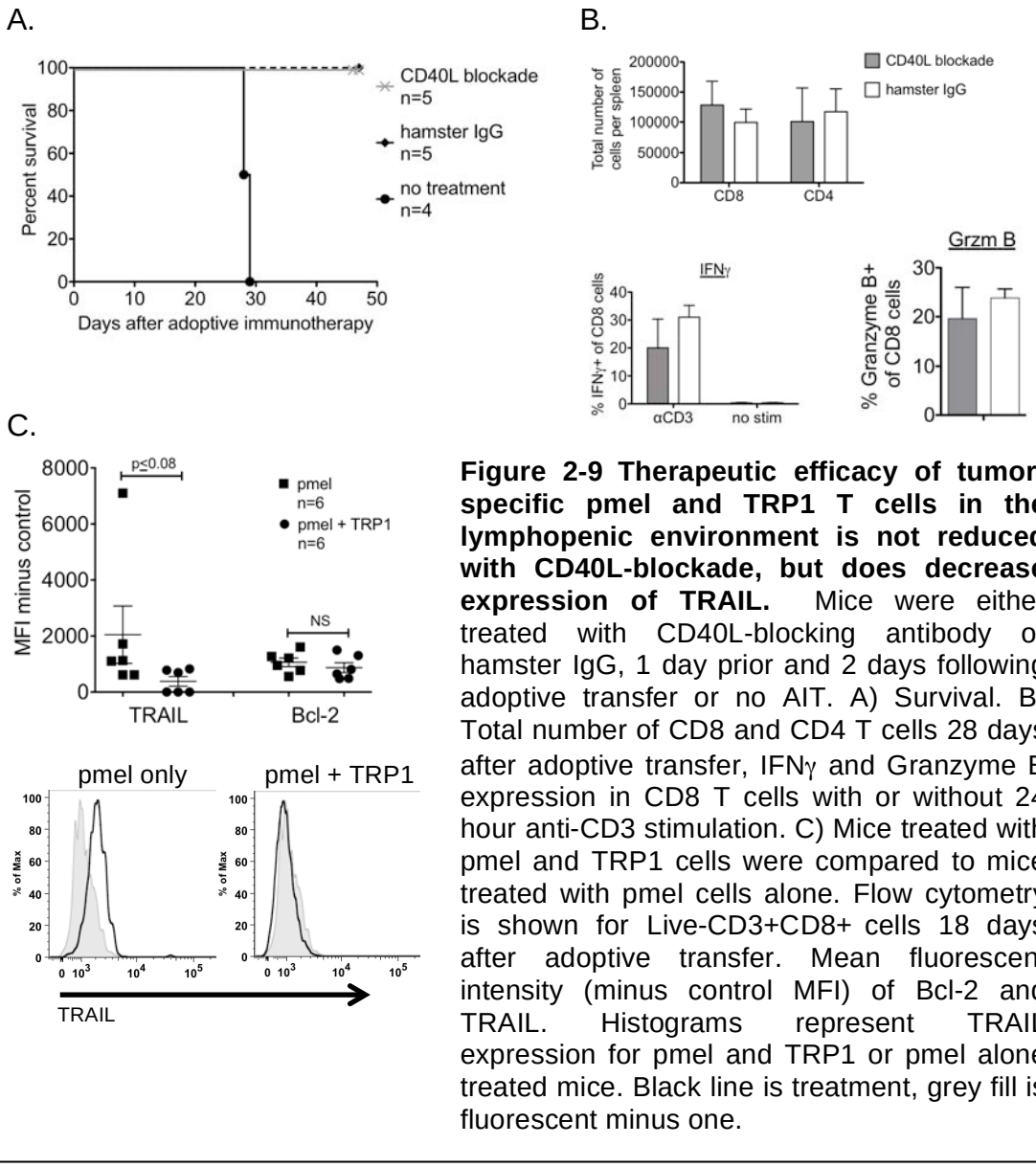



**Figure 2-9 Therapeutic efficacy of tumor-specific pmel and TRP1 T cells in the lymphopenic environment is not reduced with CD40L-blockade, but does decrease expression of TRAIL.** Mice were either treated with CD40L-blocking antibody or hamster IgG, 1 day prior and 2 days following adoptive transfer or no AIT. A) Survival. B) Total number of CD8 and CD4 T cells 28 days after adoptive transfer, IFNγ and Granzyme B expression in CD8 T cells with or without 24 hour anti-CD3 stimulation. C) Mice treated with pmel and TRP1 cells were compared to mice treated with pmel cells alone. Flow cytometry is shown for Live-CD3+CD8+ cells 18 days after adoptive transfer. Mean fluorescent intensity (minus control MFI) of Bcl-2 and TRAIL. Histograms represent TRAIL expression for pmel and TRP1 or pmel alone treated mice. Black line is treatment, grey fill is fluorescent minus one.

*Treatment with pmel and TRP1 decreases expression of TRAIL on pmel CD8 T cells*

In some models, CD4 T-cell help enhances CD8 T cell function and survival by CD40-CD40L interactions with APCs [202,203]. Therefore, we examined whether the augmented therapeutic effect seen here was due to CD40-CD40L

interactions. CD40L was blocked one day before and 2 days after adoptive immunotherapy using the anti-CD40L (MR1) antibody. There was no difference in overall survival or in the total number and function of T cells (Figure 2-9A + B) suggesting that in our model enhancement of CD8 T cells was not dependent on CD40-CD40L interactions. Another mechanism by which CD4 T cells help CD8 T cells is by increasing survival or decreasing apoptosis [46,47,204]. We examined Bcl-2 expression and the apoptotic factor TNF-related apoptosis inducing ligand (TRAIL) in CD8 T cells from pmel and TRP1 or pmel alone treated mice by flow cytometry. There was no difference in Bcl-2 expression; however, there was a substantial decrease in TRAIL expression when mice were treated with both pmel and TRP1 T cells (Figure 5C), implying the addition of tumor-specific CD4 T cells increases CD8 T cell persistence by reducing TRAIL induced apoptosis.

## Discussion

Previous studies suggested CD4 T-cell help must recognize antigen on the same APC as CD8 T cells to generate an effective CD8 T cell response [205]. We previously found that indeed tumor-vaccine specific CD4 T cells augmented therapeutic efficacy of adoptive immunotherapy with tumor-specific CD8 T cells in the RAG1-/- lymphopenic environment (manuscript in prep) [188]. Here we take advantage of the tumor-specific TRP1 MHC class II-restricted TCR transgenic CD4 T cells to examine the role of tumor-specific CD4 T cells in the lymphopenic environment. Our results suggest that tumor-specific CD4 T cells in combination with tumor-specific CD8 T cells augment therapeutic efficacy, maintain long-term tumor control and increase total survival and function of CD8 T cells. Additionally, we show that using a subtherapeutic dose of tumor-specific CD4 T cells could significantly ($p<0.001$) augment therapeutic efficacy of adoptive immunotherapy with tumor-specific CD8 T cells (Figure 2-4A) [197,198].

Our studies also suggest that tumor-specific CD4 T cells can support therapeutic efficacy by maintaining effector CD8 T cells, as we found that sorted αCD3/IL-2 expanded effector (CD44+CD62L-) phenotype pmel CD8 T cells combined with tumor-specific TRP1 CD4 T cells, and this resulted in long-term elimination of tumor. This ability of CD4 and CD8 T cells to cure mice of systemic tumor burden in the absence of a source of naïve CD8 T cells is strong evidence that CD4 T cells are maintaining CD8 effector T cells. Characterizing αCD3/IL-2 *ex vivo* expanded pmel showed that αCD3/IL-2 expanded phenotypically naïve (CD44-CD62L+) cells were actually expressing some TNFα and IFNγ and

proliferating similarly to the effector phenotype cells. One explanation for this apparent disconnect between a naïve phenotype and production of effector cytokines could be that CD62L and CD44 are being upregulated and downregulated very quickly during the αCD3/IL-2 stimulation causing them to display an atypical phenotype, including a population of CD44-CD62L- cells that are neither phenotypically naïve (CD44-CD62L+) or effector (CD44+CD62L-) phenotype (data not shown) [206]. If this is the case it further supports the idea that tumor-specific TRP1 CD4 T cells are acting to maintain activated CD8 T cells in the lymphopenic environment. Our data suggest tumor-specific TRP1 CD4 T cells maintain pmel CD8 T cells in the lymphopenic environment by decreasing TRAIL expression and not by CD40-CD40L interactions with APCs. This independence of CD40-CD40L interactions has been described in a number of other tumor models including a closely related model using vaccination with D5 transduced to secrete GM-CSF [187,207]. Recently, CD4 T-cell help was shown to stimulate autocrine IL-2 production by CD8 T cells [48]. Although administration of exogenous IL-2 clearly does not replace CD4 help in this model, we would not expect this because production of IL-2 by CD8 and CD4 T cells is continuous, has a increased half-life and is site-specific [92]. However, administration of exogenous IL-2 might stimulate autocrine CD8 and/or paracrine CD4 IL-2 production [47]. We did observe that AIT without administration of exogenous IL-2 reduced expansion of TRP1 CD4 T cells, suggesting exogenous IL-2 administration may be playing a role in CD4 T homeostasis and/or IL-2 production [208] (data not shown).

Tumor-specific TRP1 CD4 T cells were particularly important early following adoptive transfer, as elimination within 3 days, but not 10 days (Figure 2-8A) resulted in partial loss of therapeutic efficacy and correlated with an increase in the exhaustion marker PD-1 on CD8 T cells at day 18. The increase of PD-1 was most significant for the group depleted of CD4 cells 3 days after transfer (Figure 2-8C), suggesting that this time point may be particularly important for CD4 help. These findings are consistent with reports showing that antigen-specific CD4 T-cell help can decrease PD-1 expression on CD8 T cells in viral models [209,210]. Elimination of CD4 cells 10 days after adoptive transfer did not reduce therapeutic efficacy, measured by enumeration of pulmonary metastases at day 18 or result in increased PD-1 expression on CD8 T cells compared to undepleted mice. We did see a decrease in total number of pmel CD8 T cells in the day 10 depleted group, most likely due to CD4 T cells that are removed the majority of tumor has been eliminated and there is less antigen-driven proliferation of CD8 T cell, suggesting that increased CD8 T cell numbers are most important early when the majority of tumor is present. Both undepleted mice and day 10 depleted mice also had polyfunctional (IFN$\gamma$, TNF$\alpha$, Granzyme B, IL-2) CD8 T cells, 17-18 days after adoptive transfer, which are likely responsible for enhanced anti-tumor efficacy at this time point (Figure 2-8D). Interestingly, eliminating CD4 T cells 10 days after adoptive transfer resulted in late onset distant metastases (skin, ovaries) 40 days after transfer (Figure 2-8E). This development of distant metastases is consistent with previous studies using adoptive immunotherapy with CD8 T cells in MHCII-deficient mice [187]. Distant

tumor metastases were not observed in mice that received both pmel and TRP1 (undepleted), even 200 days after adoptive immunotherapy and might be explained by the role of CD4 T-cell help in CD8 T cell trafficking, which has been observed in other models [113,211] or that CD4 help is maintaining memory CD8 T cells, which are important for tumor immune surveillance [64]. Together these data show that tumor-specific CD4 T cells are important to reduce PD-1 mediated exhaustion during initial tumor elimination and for trafficking and/or maintenance of memory CD8 T cells when tumor-antigen has been reduced.

We propose this could translate to the therapeutic potential of tumor-specific HLA Class II-restricted TCR gene transduction, which has been reported to exhibit helper and cytotoxic capacities for use in combination with CD8 T cells [212]. The ability to generate tumor-specific CD4 T cells by gene transfer of TCR or CAR may relieve the burden of expanding large numbers of naturally generated tumor-specific human CD4 T cells *ex vivo* from TIL [212,213]. The data showing that murine tumor-specific CD4 T cells are beneficial early during adoptive immunotherapy could have important implications for how human CD4 T cells are applied during clinical trials. This could potentially reduce the risk for immune escape, which has been correlated with increased tumor-specific CD4 regulatory T cells [214]. In this model, we observed that ~30% (5/15 mice) of all pmel and TRP1 treated mice did develop amelanotic tumors, 100-200 days after adoptive transfer, at the primary metastatic site in the pleural cavity (data not shown). Mice that had recurrent amelanotic tumor had decreased total number and function of CD4 and CD8 T cells and tumor cells had reduced expression of gp100 and tyrp-

1 protein (data not shown). This indicates antigen loss is a potential problem and antigen-specific CD4 and CD8 T cells may need additional combination therapy to prevent tumor recurrence or target antigens that are critical to tumors survival. On the other hand, tumor-specific CD4 T cells could be important for continuously maintaining endogenous tumor-specific CD8 T cells or CD8 T cell trafficking during immunotherapy as indicated when CD4 cells were depleted 10 days after transfer (Figure 2-8E).

Alternatively, combining CD8 T cell AIT with vaccination that includes targets with CD4 epitopes may be a good way to induce endogenous tumor-specific CD4 helper T cell responses. This approach has the advantage of developing a broad range of CD4 T cells either by targeting multiple tumor-antigens or by eliciting epitope-spreading of endogenous CD4 T cells [149,215]. This broad CD4 T cell repertoire might reduce the significance of tumor antigen loss variants, which are seen in multiple preclinical models where a single antigen is targeted [66,214]. Here, the recent work of Twitty and colleagues reporting a novel vaccine strategy may be particularly helpful as their vaccine appears to generate immune responses against a range of common shared antigens [216].

Combination therapy with PD-1 blockade may also provide additional benefit since we observed increased PD-1 expression on tumor-specific CD8 T cells from mice depleted of CD4 T cells 3 days after transfer. Anti-PD-1 antibodies have had promising results, as single agents, in early clinical clinical trials and would be an excellent candidate for combination with adoptive immunotherapy trials [217]. The timing of anti-PD-1 administration would be

important both to help prevent CD8 T cell exhaustion and to eliminate exhaustion programming of T cells that can result during lymphopenia-induced proliferation [106].

While it has been difficult to identify tumor-specific CD4 T cells in several clinical trials of adoptive immunotherapy, it does not eliminate the possibility that such cells exist and express receptors against unknown antigens or mutations present in the tumor. Alternatively, these tumor-specific CD4 T cells may reside in peripheral tissues and be difficult to assess. Even a recent study that adoptively transferred effector CD8 T cells into nonmyeloablated patients excluded a role for CD4 T cells [96]. For many years investigators accepted transient CD4 depletion with monoclonal antibodies as evidence that CD4 T-cell help did not play a critical role in CD8 T cell adoptive immunotherapy of cancer. It is only with the advent of MHCII-deficient and reconstituted RAG-deficient mice that the role for tumor-specific CD4 T cells is being elucidated [187]. Together our data strongly argue that tumor-specific CD4 T cells play an important role in maintaining long-term systemic anti-tumor immunity and suggest that investigators should consider the benefits of including or promoting tumor-specific CD4 T cells, whether via vaccine or transduction of specific TCR or CAR constructs.

# CHAPTER 3

## Partial Depletion of CD4 T cells Enhances the Protective Effect of Multiple Vaccinations in an Active-specific Immunotherapy Model of Melanoma

**Abstract**

Few immunotherapists would accept the concept of a single vaccination inducing a therapeutic anti-cancer immune response in a patient with advanced cancer. But what is the evidence to support the “more-is-better” approach of multiple vaccinations? Recently, our group reported that T cells from mice vaccinated three times with a GM-CSF-secreting whole tumor (D5-G6) were significantly less effective in adoptive transfer studies than T cells from mice receiving a single vaccination. A striking difference observed in multiply vaccinated animals was an increase in the number of regulatory T cells.

We hypothesized that in a protective (active-specific) vaccine model thrice-vaccinated mice would reject a tumor challenge due to resident effector cells that were not present in the spleen; the source of T cells adoptively transferred in the previous model. This was not the case. Multiple vaccinations protected 31% of mice from a minimal (2x $TD_{100}$) tumor challenge and none of the mice receiving a high-dose (20x $TD_{100}$) tumor challenge. As previously reported, regulatory T cell numbers increased with more vaccinations. Interestingly, partial depletion of CD4 T cells one-day prior to the $2^{nd}$ and $3^{rd}$ vaccination increased protection to the large tumor challenge (33% survival $p<0.006$), but did not improve protection from the minimal tumor dose (20% survival). Mice depleted of CD4 cells had an increased ratio of CD8+ to CD4+Foxp3+ cells with each subsequent vaccination, compared to non-depleted mice. Fourteen days after the third vaccination CD4-depleted mice had a larger

proportion of proliferating (Ki67+) Foxp3-negative CD4 T cells, in the blood and spleen, compared to non-depleted mice.

We also examined whether the location of immunization altered multiple vaccine efficacy. Previous studies have shown that immunizing patients at multiple-sites led to a more persistent anti-vaccine immune response during multiple vaccinations compared to patients vaccinated at one-site. We compared three strategies, however there were no significant differences between protection or frequency of Tregs, B cells, macrophages, myeloid derived suppressor cells or dendritic cells in the vaccine dLN with any of the immunization routes. Together these data suggest that multiple vaccinations with CD4-depletion could be beneficial for treatment of cancer, but would likely be more effective with additional combination immunotherapy.

## Introduction

An effective vaccine would be an attractive alternative for treatment of cancer because these can be easily manufactured and are “off-the-shelf” and less costly than currently approved therapies. Unfortunately, few therapeutic vaccine trials have shown significant clinical improvement [126,132,178,218,219]. Unlike prophylactic viral vaccines, which prime a naïve immune response, cancer vaccines are administered after cancer has progressed and immune tolerance has been established. The majority of therapeutic clinical trials utilize multiple booster vaccinations, however few investigators have examined whether more vaccinations actually improves therapeutic efficacy [126]. Four recent studies, one in mice and three in humans, observed that multiple vaccinations reduced the therapeutic efficacy of immunization [121,124,220,221]. Two of three clinical studies utilized granulocyte macrophage-colony stimulating factor (GM-CSF) as an adjuvant. GM-CSF acts to recruit, activate, and mature granulocytes, macrophages and dendritic cells and is able to enhance cross-presentation of antigens [116]. Seminal studies that transduced a plethora of single cytokines into irradiated whole tumor vaccines found that only GM-CSF improved protection compared to untransfected tumor cells [115]. Moreover studies show that local, continuous-release of GM-CSF is beneficial for anti-tumor immunity, but single high-dose administration of GM-CSF can induce immunosuppression [115,134,137,222]. Previously our lab reported that lymphocytes from mice vaccinated once with a melanoma cell line transduced to produce GM-CSF (D5-G6) were therapeutic, but lost therapeutic efficacy after three vaccinations [121]. It is possible

that in this model, which utilized *in vitro* expanded splenocytes from vaccinated mice for adoptive immunotherapy, tumor-specific immune cells trafficked to tissues and therefore were not present in the spleen at the time of harvest. In this study we examined whether one or three vaccinations protected during active-specific immunotherapy, such that the same mice that received vaccination were challenged with tumor. In our previous study we also observed an increase in regulatory T cells (Tregs) with each progressive vaccine, and that partial depletion of CD4 cells using an anti-CD4 antibody (GK1.5) one day prior to the 2$^{nd}$ and 3$^{rd}$ vaccine restored the therapeutic efficacy of the T cells [121]. Therefore we treated thrice-vaccinated mice with anti-CD4 antibody to determine if this enhanced protection during active-specific immunotherapy. We also examined the effect of multiple immunizations combined with CD4-depletion in a therapeutic model. Natural and peripheral tumor-induced Tregs have distinct roles in mitigating anti-tumor immunity [53,223]. A number of surface markers have been associated with distinguishing natural and peripheral induced Tregs, including ICOS and Helios [54,55,57]. Inducible T cell costimulator (ICOS) is reported to define natural Tregs that produce both TGFβ and IL-10 [54]. While heavily debated, Helios expression on Tregs, was originally described as a marker associated with natural T cells that had recently migrated from the thymus [55]. However, Helios expression on Tregs does correspond to a distinct subset of Tregs with enhanced suppressive capabilities [56,57]. Therefore we analyzed Tregs from multiply vaccinated mice with or without CD4-depletion to look for changes in ICOS and Helios.

Pre-clinical studies have reported that administering irradiated whole-tumor GM-CSF secreting vaccine at the same site as tumor challenge enhances protection [140]. However, clinical trials have reported increased anti-tumor responses to multiple vaccinations when vaccines are administered to separate limbs, while immune responses decreased after booster vaccinations when the immunization was administered to the same site on one limb [141]. These data suggest that multiple locations may be better to boost vaccine efficacy. Since there does not seem be a consensus on which vaccine route had the greatest efficacy, we performed three separate routes of vaccine administration in our multi-vaccine model and analyzed immune cell accumulation (vaccine-draining lymph node (dLN) and spleen), tumor-specificity, and protection against tumor challenge.

## Materials and Methods

*Cell lines and mice*

Tumor cell lines were propagated for less than 6-weeks in complete media consisting of 10% FBS RPMI 1640 (Lonza) supplemented with 2 mmol/L L-glutamine, 0.1mmol/L non-essential amino acids, 1 mmol/L sodium pyruvate (Lonza), 50 μM/L β-mercaptoethanol (Sigma) and 5 μg/ml gentamicin sulfate (Lonza). These studies utilized the poorly immunogenic subclone, D5 isolated from the spontaneously arisen B16BL6 melanoma [117]. Immunizations used D5-G6, a D5 clone stably transduced to secrete 60 ng/ml/$10^6$ cells/24 hours GM-CSF (Figure 3-1) [117]. Stimulation assays utilized the sarcoma MCA-310 and D5 modified to express the human class II major histocompatibility complex transactivator, MCA-310 CIITA and D5 CIITA respectively [107,108]. C57BL/6 mice (Charles River Laboratories) were maintained in compliance with recognized principles of laboratory animal care (Guide for the Care of Use of Laboratory Animals, National Research Council, 1996) and all protocols were approved by the Earle A Chiles Research Institute animal care and use committee.

*Reconstituted lymphopenic mice, immunizations and tumor challenge*

Reconstituted lymphopenic mice (RLM) were treated with 200 mg/kg cyclophosphamide (Baxter) once per day for two days. One day following C57BL/6 splenocytes (1 x $10^7$) were injected intravenously. D5-G6 immunization was given the same day as adoptive transfer. Mice receiving multiple vaccinations were immunized two more times, 2 weeks apart (day 0, 14, 28).

Single vaccine controls were given to RLM or intact mice at the 28 day time point. Immunization consisted of irradiated (10,000 RADS) 5 x $10^6$ D5-G6 cells administered subcutaneously (s.c.) to four or one location, as indicated. D5 tumor challenge was given 14 days following final vaccination. Mice were sacrificed for analysis 14 days following each vaccination. Tumor size was monitored using a caliper measuring bi-directionally every 2-3 days. Mice were sacrificed when tumors size reached 150 $mm^2$.

*Flow cytometry and depletion antibodies*

Intracellular staining was performed on LN and spleen cells using eBiosciences fix/perm buffers. Single cell suspensions were made by manually disrupting tissue using a 3-ml syringe in a 6-well plate, filtered and red blood cells were lysed using ACK buffer (Lonza). Cells were stained with the following antibodies: Foxp3-eFluor450, ICOS-FITC, CD3-Percp-eFluor780, CD44-PE-Cy7 (eBiosciences), Ki67-AF700, CD8-V500, CD25-APC, CD4-APC-H7 (Becton Dickinson) and Helios-PE (Biolegend). Lymphocytes in blood samples were enumerated using Beckman Coulter flow count beads. Surface staining for myeloid cells was performed using CD11b-eFluor605NC, CD11c-PE-Cy7, MHCII-APC-Cy7, Gr-1-FITC, F4/80-eFluor450 (eBiosciences), and B220-V500 (Becton Dickinson). All samples were run on a BD LSRII and analyzed using FlowJo (Treestar), Pestle and SPICE (Courtesy of Mario Roederer at the NIAID Vaccine Research Center). Anti-CD4 antibody GK1.5 was purified from ascites using Biosephra MEP Hypercel (Ciphergen) as described previously [196] or

purchased from BioXCell, was administered i.p. (200 μg). Control mice were administered 200 μg rat IgG2b antibody (Sigma).

*Cytokine release assay and ELISA*

For the IFNγ cytokine release assay, splenocytes from vaccinated animals were harvested, manually processed and suspended in single cell suspensions (as above). Splenocytes were activated by incubating $10^6$ cells/ml with 5 μg/ml of soluble αCD3 (2C11) for 2 days followed by expansion of $10^5$ cells/ml with 60 IU IL-2/ml for 3 days. Tumor cells, D5-CIITA, MCA-310-CIITA were plated in 24-well plates. Expanded T cells were added at a 10:1 ratio with tumor cells. Supernatants were collected after 20 hours and frozen at -20 $^oC$. Interferon-γ ELISA was run using the manufacturers protocol (Becton Dickinson). For analysis of GM-CSF production, 1 x $10^6$ live or irradiated (10,000 RADS) D5-G6 cells were incubated for 24-hours in a 6-well plate (8 ml). Supernatants were collected and GM-CSF concentration was analyzed by ELISA using the manufacturers protocol (Becton Dickinson). The concentration of IFNγ or GM-CSF was determined by regression analysis.

*Statistics*

Unpaired or paired student t tests were done for analysis of cell numbers and phenotype using Prism (Graphpad). Mantel-Cox log rank tests were used to analyze survival curves (Prism, Graphpad). A p value of less than 0.05 was considered significant.

## Results

Since GM-CSF can greatly affect the efficacy of tumor vaccination we measured the concentration being produced by D5-G6 cells. D5-G6 cells irradiated with 10,000 RADS, a level of irradiation capable of preventing cell division, had similar GM-CSF production (50-60 ng/ml/$10^6$ cells) as non-irradiated cells during the first 24-hours, (Figure 3-1). We examined whether D5-G6 vaccination would be more effective in reconstituted lymphopenic mice (RLM) versus lymphoreplete mice since our previous studies generated therapeutic cells in RLM [121]. In these studies a single vaccination with the "suboptimal" dose of D5-G6 ($5 \times 10^6$ cells) provided the same low level of protection (23% versus 17%) (Figure 3-2). This is in contrast to studies using an "optimal" dose of irradiated D5-G6 ($10^7$) cells, which provides 90-100% protection from a minimal tumor challenge [34]. We chose to use the "suboptimal" dose ($5 \times 10^6$

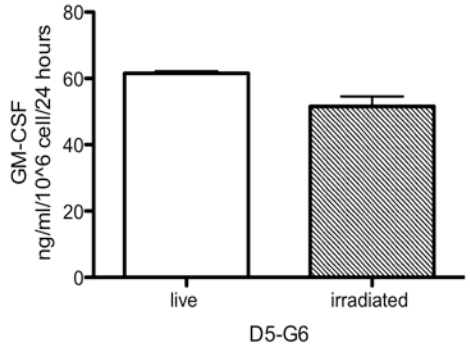



**Figure 3-1. GM-CSF-production by D5-G6 cells.** Live or irradiated D5-G6 cell supernatants were collected and analyzed by GM-CSF ELISA.

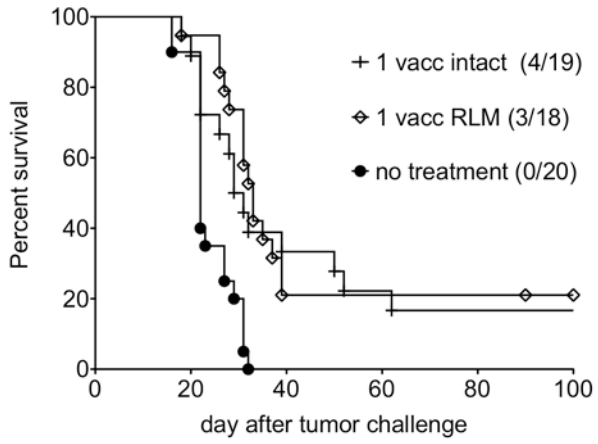



**Figure 3-2. One vaccination protects equally in reconstituted lymphopenic or lymphoreplete mice**. Survival curve of RLM or lymphoreplete mice vaccinated with a "suboptimal" dose of irradiated D5-G6 cells ($5 \times 10^6$). All mice were challenged with $2 \times 10^4$ D5 cells s.c. 14 days following vaccination or unvaccinated controls.

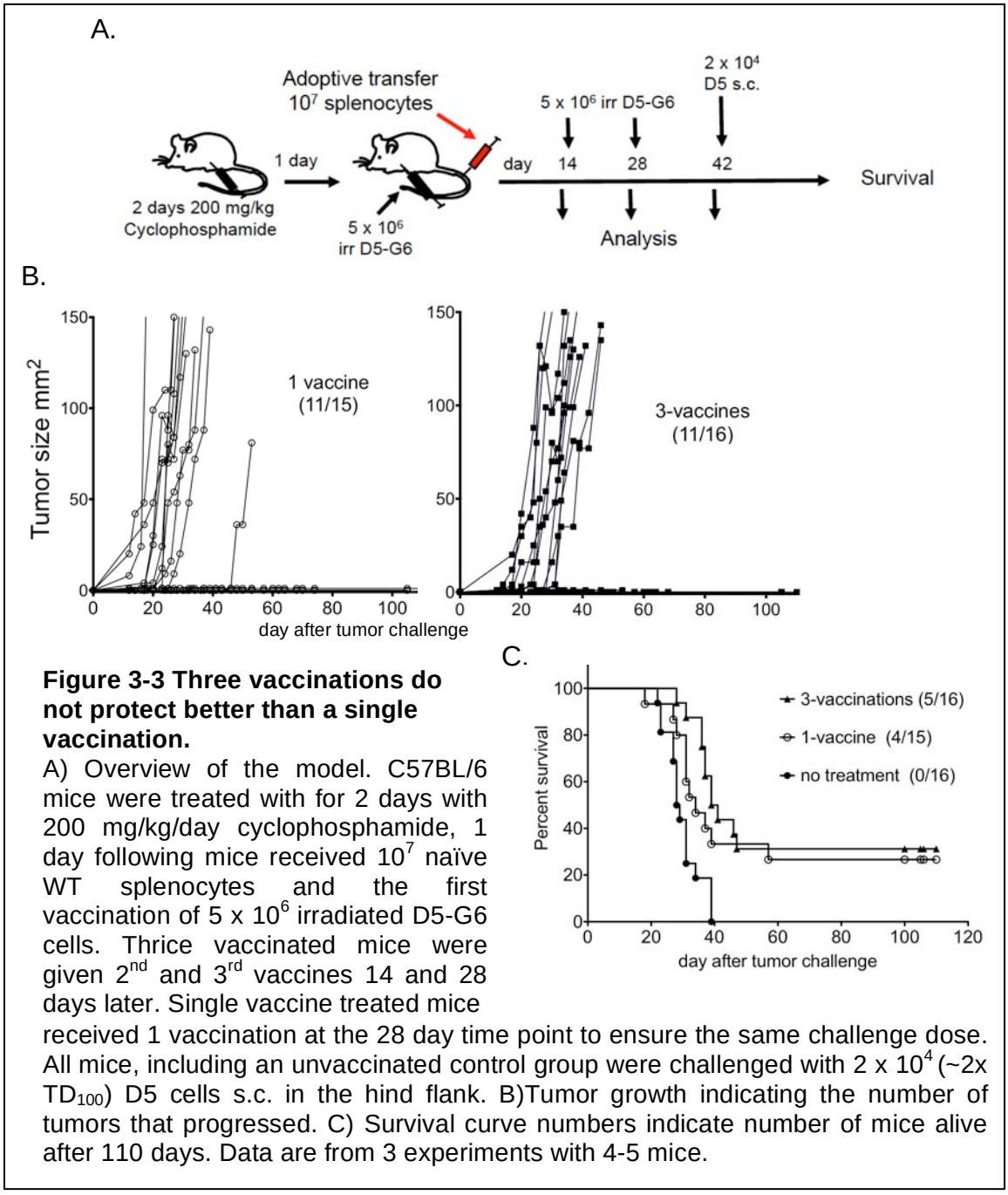



**Figure 3-3 Three vaccinations do not protect better than a single vaccination.**
A) Overview of the model. C57BL/6 mice were treated with for 2 days with 200 mg/kg/day cyclophosphamide, 1 day following mice received $10^7$ naïve WT splenocytes and the first vaccination of 5 x $10^6$ irradiated D5-G6 cells. Thrice vaccinated mice were given 2nd and 3rd vaccines 14 and 28 days later. Single vaccine treated mice received 1 vaccination at the 28 day time point to ensure the same challenge dose. All mice, including an unvaccinated control group were challenged with 2 x $10^4$ (~2x $TD_{100}$) D5 cells s.c. in the hind flank. B)Tumor growth indicating the number of tumors that progressed. C) Survival curve numbers indicate number of mice alive after 110 days. Data are from 3 experiments with 4-5 mice.

D5-G6 cells) of vaccine that does not protect with one vaccine, to see whether the administration of multiple vaccines at 2-week intervals (day 0, 14, 28) would improve vaccine efficacy. In this case, the total dose of irradiated cells administered 50% less then the dose administered with the “optimal” vaccine. We next examined if mice immunized with 3 vaccinations were better protected

from the minimal tumor challenge (2x $TD_{100}$). RLM were administered D5-G6 (5 x $10^6$) on the day of reconstitution and received two additional vaccines (5 x $10^6$) at 14 day intervals (day 0, 14 and 28). Fourteen days following the 3rd vaccination mice were challenged with D5 tumor (Figure 3-3A). Single vaccine RLM controls received vaccination at the day 28 time point so that they were challenged 14 days following their first vaccine. This insured that all doses were consistent. Survival was significantly ($p<0.001$) increased for both once and thrice vaccinated mice compared to no vaccination, however there was no significant difference in protection between one and three vaccines (Figure 3-3B).

We also examined whether location of immunization altered vaccine efficacy in a 3 vaccine setting. We compared three strategies, shown in Figure 3-5A: in one the total vaccine dose (5 x $10^6$ D5-G6) was administered at one site, which rotated to a different limb for each vaccination (1-site rotating). In the second, the total vaccine (5 x $10^6$ D5-G6) dose was administered to the same site on the opposite flank from challenge (1-site same). The third split the dose into 4 aliquots (4 x 1.25 x $10^6$ D5-G6), administered to each limb for each vaccine (4-site). It is worth noting that 4-site immunized mice received tumor challenge at one of the same sites as vaccination.

Mice were sacrificed and we analyzed spleens, vaccine dLNs and blood from immunized mice 14 days following each vaccination, such that 1st vaccine was day 14, 2nd vaccine was day 28 and 3rd vaccine was day 42. Altering vaccination route did not change the total number or frequency of Tregs (CD3+CD4+Foxp3+) (Figure 3-4B), ratio of CD8+ to Tregs (Figure 3-4C),

MDSC (CD11b+Gr-1+), macrophage (F4/80+), DC (CD11c+MHCII+) or B cells (B220+) in the spleen or vaccine dLN (Figure 3-4D and data not shown). Interestingly, T cells isolated from mice where a single vaccine was administered to a different site every 2 weeks (1-site rotated) had a higher

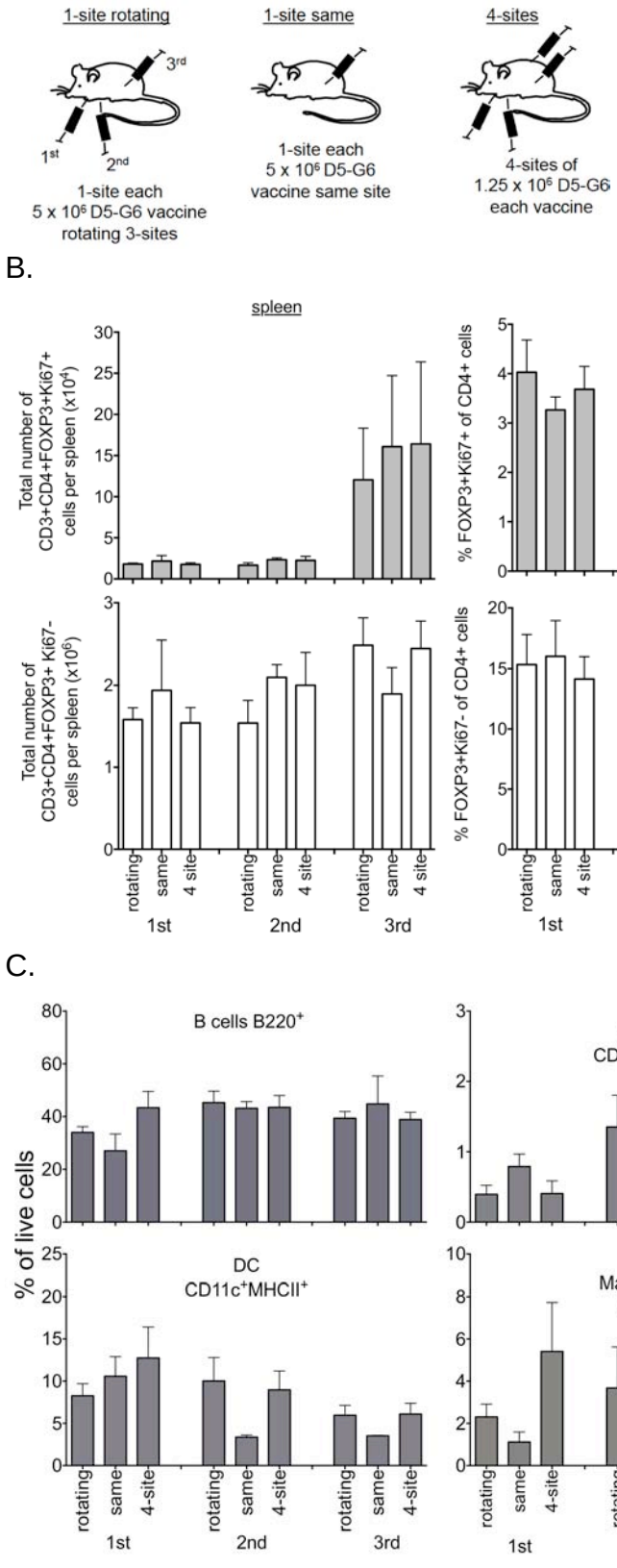



**Figure 3-4. Altering vaccination route does not increase protection against tumor challenge.**

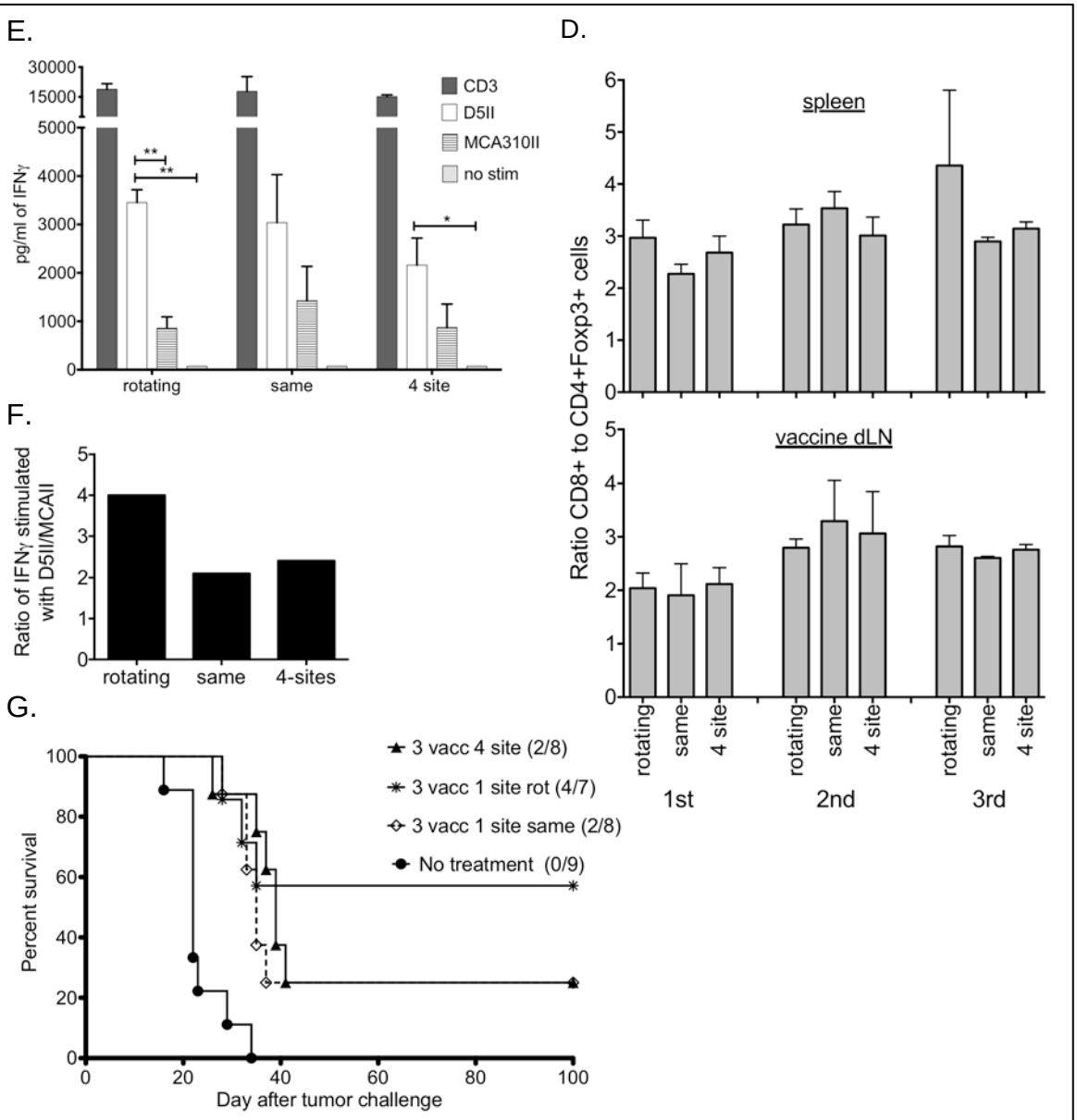



**Figure 3-4 cont. Altering vaccination route does not increase protection against tumor challenge.**

A) Description of vaccination route. Each vaccination was given in the same scheme as Fig 3-2A. B) Total number of proliferating, Ki67+ (gray) and non-proliferating Ki67- (white) CD3+CD4+Foxp3+ Tregs in the spleen and vaccine dLN 14 days after each vaccination. C) Percent of B cells (B220+), MDSC (Gr-1+CD11b+), DC (CD11c+MHCII+) and macrophage (F4/80+) in the dLN. D) Ratio of CD3+CD8+ to CD3+CD4+Foxp3+ in spleen and vaccine dLN 14 days after each vaccination. E) Tumor-specific IFNγ using splenocytes at day 42 stimulated with specific tumor D5II, syngeneic MCA-310II tumor, no stim or αCD3 for 24 hours. F) Ratio of D5II to MCA-310II stimulated IFNγ production. B-F) Data are a combination of 2-4 independent experiments using 2-3 mice per group. G) Survival plot comparing vaccination route. Numbers indicated mice that survived. Data are a combination of 2 experiments with 7-9 mice per group. **p<0.001, *p<0.05.

fold increase (4-fold) of tumor-specific IFNγ production (D5II/MCA-310II), compared to mice vaccinated at the same one (1-site same) or same four (4-site) locations (2.4 and 2.1-fold) (Figure 3-4E-F). The 1-site rotated vaccinated mice were also the only group where the increase in IFNγ between D5II and MCA-310II stimulated cells reached statistical significance ($p<0.001$) (Figure 3-4E). While rotating the vaccine increased the tumor-specific IFNγ responses this did not translate into increased vaccine potency as all vaccination routes protected equally against tumor challenge (Figure 3-4G). We did observe greater tumor free survival (57% versus 25%), however this was not significant (Figure 3-4G). Since depleting CD4 cells one day prior to the 2$^{nd}$ and 3$^{rd}$ vaccinations previously restored the therapeutic efficacy of T cells, we followed the same schedule of partial CD4-depletion for active-specific immunotherapy to determine whether it enhanced protection. The vaccination and analysis schedule are shown in Figure 3-5A. We observed an increase in total number of proliferating (Ki67+) and non-proliferating (Ki67-) Tregs after each subsequent vaccination in the spleen and blood, however this increase was not as substantial in the vaccine dLN (Figure 2-B-C and data not shown). Mice that received CD4-depletion had decreased total numbers of Tregs and increased ratio of CD8 to Tregs in the spleen and blood, but did not have a reduced number of Tregs in the vaccine dLN (Figure 3-5B-C and data not shown). CD4-depleted mice also had significantly more ($p<0.001$) tumor-specific IFNγ production (Figure 3-5D). CD4-depleted mice had 6-fold increase in D5-specific compared to non-specific MCA-310II stimulated IFNγ-production, while undepleted mice had only a 2.2-fold increase (Figure 3-5E).

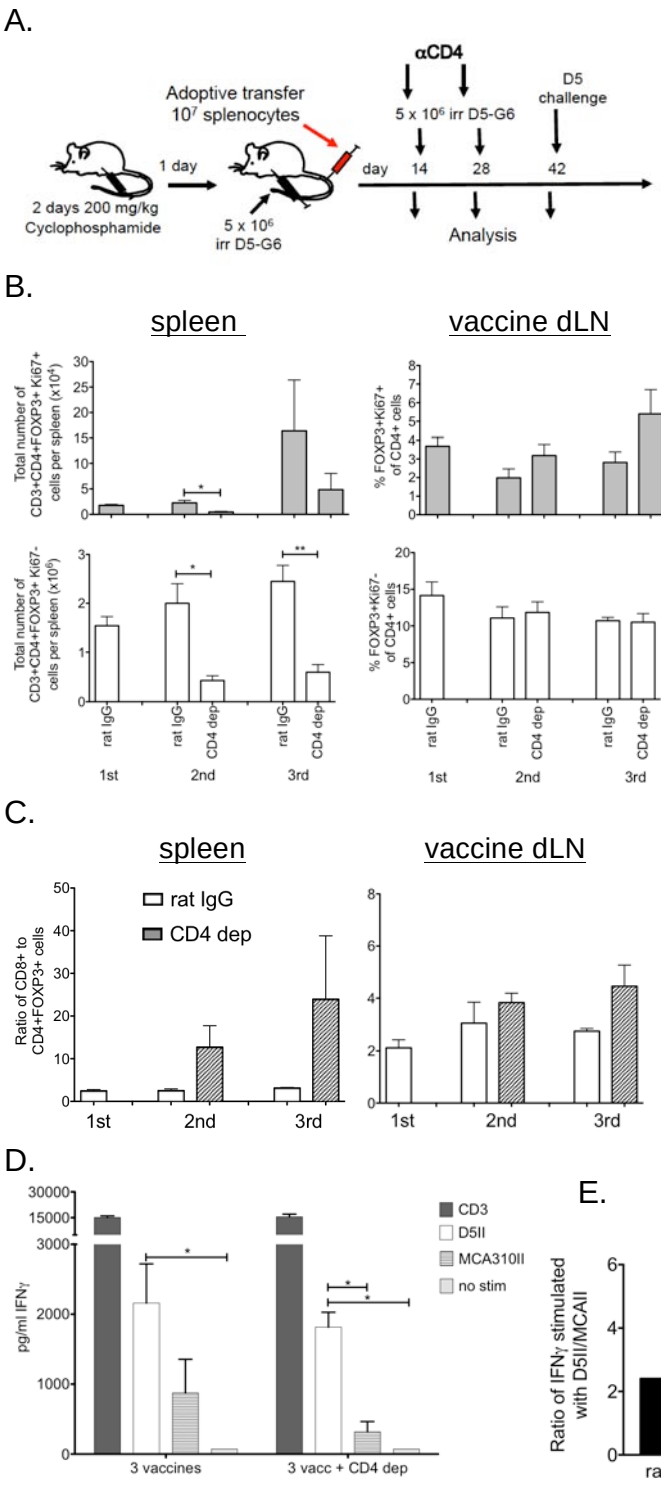



**Figure 3-5. Partial CD4-depletion decreases Tregs in the spleen and increases fold difference in tumor-specific IFNγ.** A) Overview of the multiple vaccination model. Mice were treated with 200 mg/kg cyclophosphamide for 2 days, one day following mice received $10^7$ naïve splenocytes and 5 x $10^6$ irradiated D5-G6 cells s.c. The same mice then received additional immunizations 14 and 28 days after adoptive transfer. One day prior to second and third vaccination mice were administered 200 μg CD4 or rat IgG i.p. The single vaccination group received vaccination at the 28 day time point. All mice were challenged with D5 cells and followed for survival. Additional mice were sacrificed 14 days after each vaccination for analysis. B) Total number or percent of proliferating (Ki67+) or non-proliferating (Ki67-) Tregs 14 days after each vaccination in the spleen and vaccine dLN. C) Ratio of CD8 to Tregs in the spleen and blood. D) IFNγ secretion after 20 hour stimulation with αCD3, specific-tumor D5II, non-specific tumor MCA-310II or no stimulation. E) Ratio of D5II to MCA-310II IFNγ production **p<0.001 *p<0.05

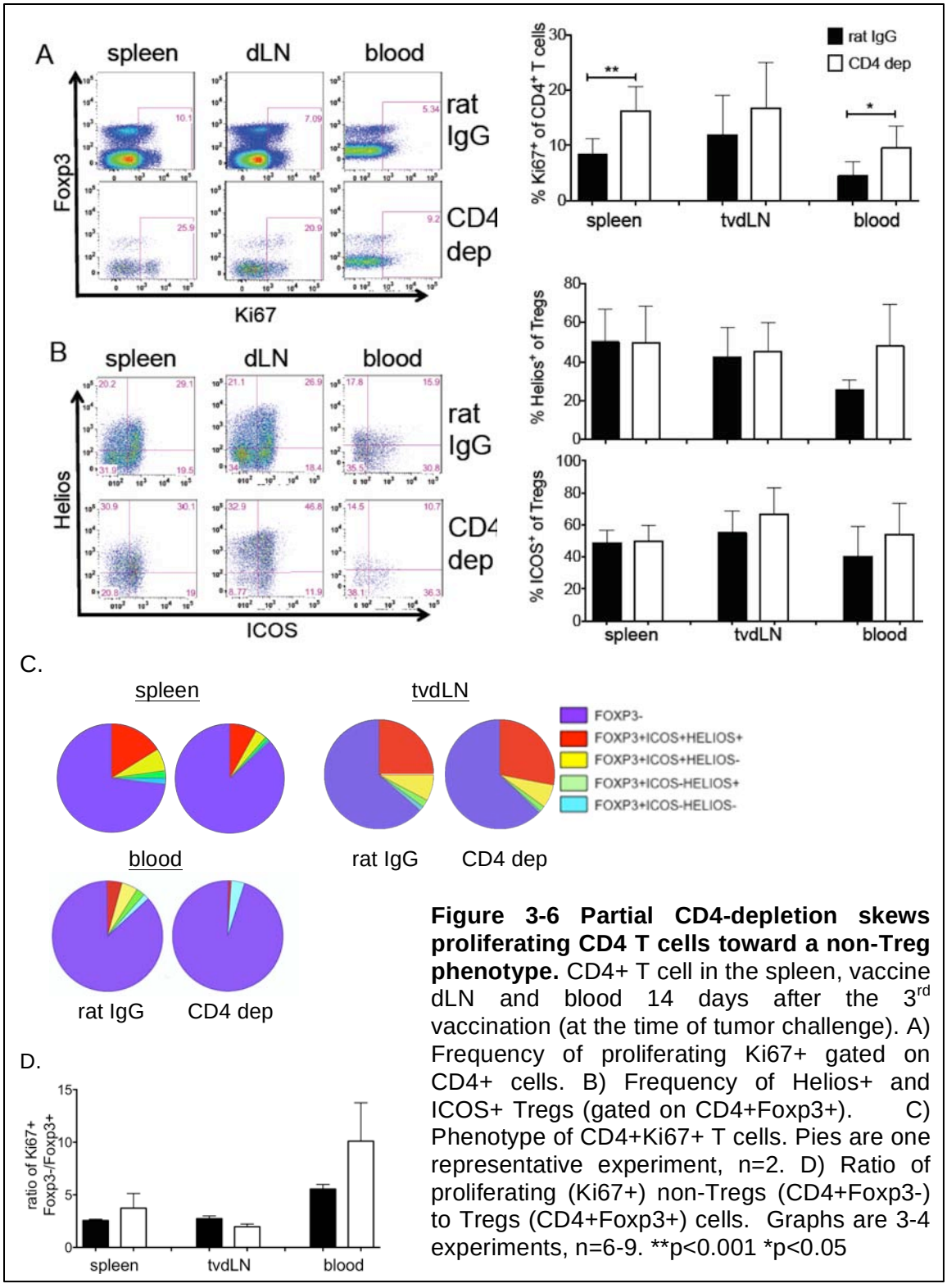



**Figure 3-6 Partial CD4-depletion skews proliferating CD4 T cells toward a non-Treg phenotype.** CD4+ T cell in the spleen, vaccine dLN and blood 14 days after the 3rd vaccination (at the time of tumor challenge). A) Frequency of proliferating Ki67+ gated on CD4+ cells. B) Frequency of Helios+ and ICOS+ Tregs (gated on CD4+Foxp3+). C) Phenotype of CD4+Ki67+ T cells. Pies are one representative experiment, n=2. D) Ratio of proliferating (Ki67+) non-Tregs (CD4+Foxp3-) to Tregs (CD4+Foxp3+) cells. Graphs are 3-4 experiments, n=6-9. **p<0.001 *p<0.05

We also determined the phenotype of proliferating (Ki67+) CD4 T cells using two markers for Treg function, ICOS and Helios at the time of tumor challenge [55,224]. There was a significantly larger frequency of proliferating CD4 T cells in the blood and spleen when mice received partial CD4-depletion compared to rat IgG thrice-vaccinated mice at day 42 (Figure 3-6A). However, there was no difference in frequency of proliferating ICOS or Helios expressing Tregs (Figure 3-6B-C). Proliferating CD4 T cells in mice partially depleted of CD4 cells were skewed toward a non-Treg phenotype (Foxp3-) in the spleen and blood compared to non-depleted mice (Figure 3-6C). Mice

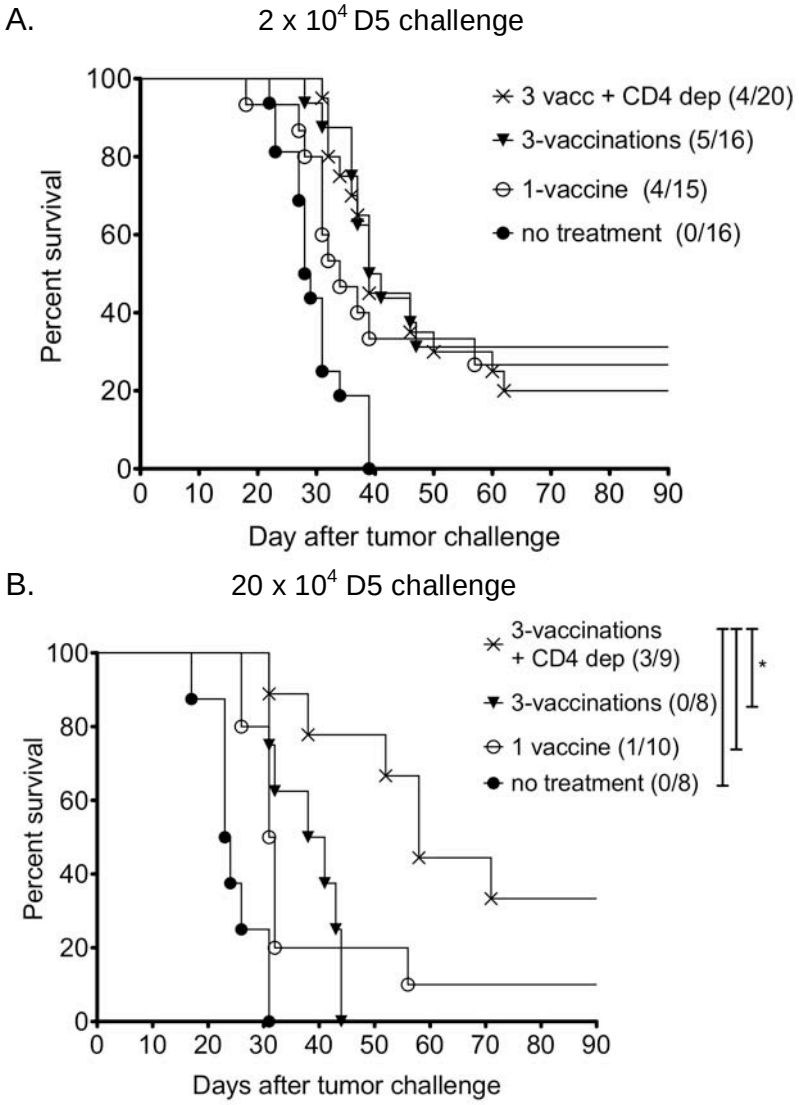



**Figure 3-7. Depleting CD4 T cell prior to 2nd and 3rd vaccination protects against a large dose tumor challenge.** Survival curves comparing thrice vaccinated mice with or without CD4 depletion. A) Low dose tumor challenge, 2 x$10^4$ D5. Combination of 3 experiments using 5-7 mice each. B) Large dose tumor challenge, 2 x $10^5$ D5. Combination of 2 experiments using 4-5 mice each.

receiving 3 vaccinations with or without CD4-depletion were subsequently challenged with either a minimal (2x $TD_{100}$) or high-dose (20x $TD_{100}$) of tumor. To our surprise, partial CD4-depletion did not protect against the low dose tumor challenge, but did significantly enhance protection against a high dose challenge (Figure 3-7A-B). Finally we wanted to determine whether one or three vaccinations augmented anti-tumor responses in a therapeutic model. Due to our observations in the high-dose tumor model, mice were given a high-dose of tumor, and were vaccinated with irradiated D5-G6 on day 3, 10 and 17 (Figure 3-8A). One group of vaccinated animals received anti-CD4 on days 9 and 16. Multiply vaccinated control mice were administered rat IgG. This was compared to mice that received either one vaccination on day 3 or no vaccination. There were no significant differences in tumor growth or survival for any of the treatments (Figure 3-8B).

## Discussion

Irradiated D5-G6 vaccine used in this study produced ~50 ng/ml/$10^6$ cells per 24 hours (Figure 3-1). This level of continuous GM-CSF-production is much lower than those previously described to induce immunosuppression [137]. We had expected that a single vaccination with a "suboptimal" (5 x $10^6$) vaccine would improve protection in RLM, because lymphopenia-induced proliferation, in combination with vaccination is known to enhance anti-tumor immunity [107,109]. However, we found that a single vaccine dose of 5 x $10^6$ protected equally in reconstituted RLM and lymphoreplete mice (Figure 3-2). Therefore, we did not consider lymphodepletion a factor in the multi-vaccine experiments.

Contrary to our previous study where splenocytes from vaccinated mice were used for adoptive immunotherapy, we did not see decreased efficacy with more vaccinations during active-specific immunotherapy (Figure 3-3) [121]. This suggested tumor-specific T cells were not trafficking to tissue as we originally hypothesized. One possible reason for this is the original study administered vaccines to 4-sites in order to generate a large number of vaccine dLNs with tumor-specific cells, therefore we altered the immunization route to ensure we were eliciting an optimal anti-tumor response. In addition, other studies have shown that vaccine administration route can augment the anti-tumor response during booster immunizations [141]. Varying vaccine route and distribution did not influence accumulation of immune cells (Tregs, DCs, B cells, macrophage or MDSC) into the vaccine dLN or spleen and did not alter protection against tumor challenge (Figure 3-4B-D).

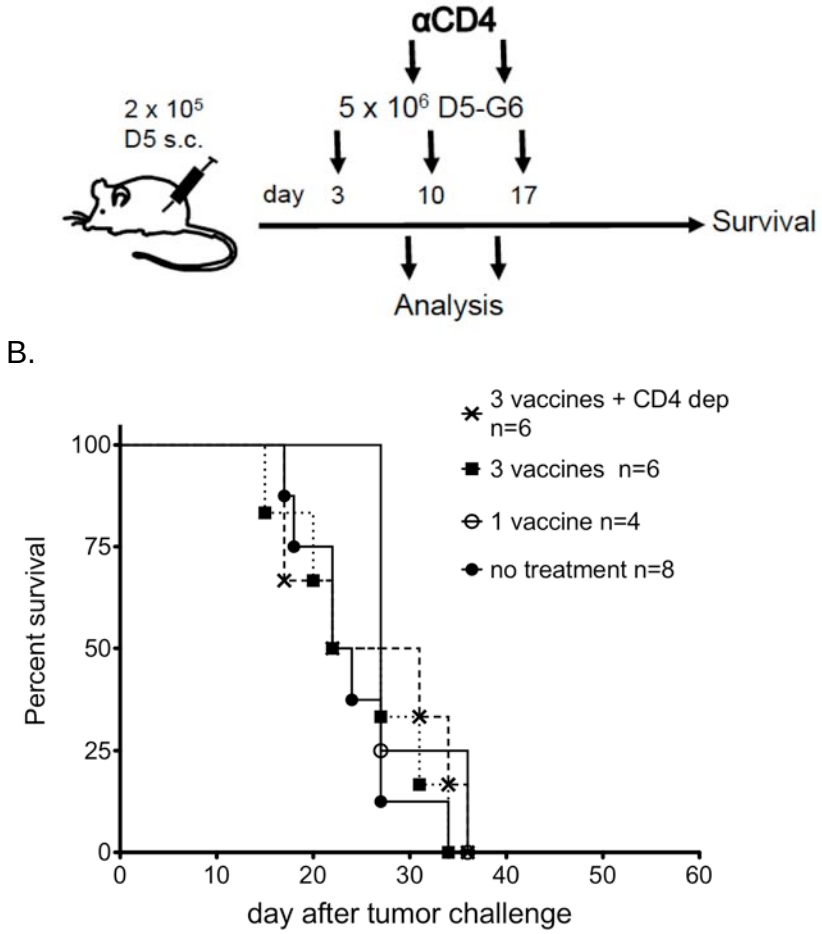



**Figure 3-8. CD4-depletion prior to 2nd and 3rd vaccine does not improve therapeutic efficacy of multiple vaccinations in a large tumor dose model.**
A) Overview of therapeutic model; mice are treated with a large dose of tumor (2 x $10^5$ D5) followed by D5-G6 vaccination 3, 10 and 17 days following tumor challenge. Mice were treated with 200 μg anti-CD4 or rat IgG 9 and 17 days after challenge and analyzed for survival. B) Kaplan-Meier curve representing one experiment with 4-8 mice per group.

These data were surprising, since a previous study with GM-CSF secreting whole tumor vaccination showed superior protection when animals were vaccinated at the same site as challenge versus a distant site [140]. We did observe an increase in tumor-specific IFNγ secretion from lymphocytes isolated from the 1-site rotating vaccination group (Figure 3-4E-F). This suggests that rotating vaccination strategy is priming more functional T cells, however this did not translate to increased protection (Figure 3-4G). One possibility is that because our 4-site, 1-site same and 1-site rotating (Figure 3-4A) vaccines were on the

main body of the mouse, GM-CSF induced APCs were able to migrate throughout the mouse skin, blood and lymphatics and therefore did not represent a localized vaccine response. Previous studies reporting increased anti-tumor response with separated vaccination were performed on human limbs, which would require APCs and T cells to migrate further [141]. This hypothesis is currently being tested using immunohistochemistry to examine vaccinated and non-vaccinated skin sections.

Enhanced protection with multiple vaccinations combined with CD4-depletion in the large dose tumor model, but not in the low dose tumor model, was perplexing. In both cases the cellular accumulation, CD4 T-cell proliferation, CD8:Treg ratios and 2.3-fold increase in tumor-specific IFN$\gamma$ production (D5II/MCA-310II), were the same during vaccination (Figure 3-5B-D and 3-6A-C). A possible explanation could be that the increase in tolerance promoting antigen with a large dose of poorly immunogenic D5 tumor causes the tumor to induce more antigen-specific suppressive Tregs and perhaps skewing the CD4 T cell population toward non-Tregs (CD4+Foxp3-) helps to improve the anti-tumor response. This is supported by previous studies, which have reported augmented suppressive capabilities of tumor-specific de novo Tregs compared to natural Tregs [53] and that eliminating Tregs enhances the therapeutic efficacy of D5 induced lymphocytes [225]. During our analysis Tregs were phenotyped using the markers ICOS and Helios, which are associated with Tregs with increased suppressive functions. We observed an increase in ICOS+Helios+ cells after 3 vaccinations suggesting multiple vaccinations is inducing more suppressive

Tregs (Figure 3-6B and data not shown). It is possible that the large dose tumor challenge acts to expand this suppressive Treg population.

Another possibility is that at the minimal dose tumor-growth is slower and the Treg population is able to recover before tumor progression, reestablishing a tolerized tumor environment. To address whether this is truly a Treg mediated phenomena, studies are being performed in DEREG mice, which express a diphtheria toxin receptor on Foxp3-expressing cells allowing for specific elimination of Tregs, and in TGFβRII-dominant negative mice, which lack TGFβ receptor signaling on CD4 T cells and therefore do not have induced Tregs [226,227]. Since tumor vaccine immunotherapy is generally administered therapeutically, we examined the affect of boosting vaccinations in a therapeutic model. We found that three vaccinations, with or without partial CD4 depletion prior to the 2$^{nd}$ and 3$^{rd}$ immunization were not therapeutic (Figure 3-8). D5, is a poorly immunogenic tumor and quite difficult to treat at this large dose (20x $TD_{100}$), it is possible that immunosuppression during priming of the immune system has already occurred before vaccines are administered, which is supported by previous studies showing T cells isolated from tumor-bearing mice are not therapeutic for adoptive immunotherapy [117]. The use of combination immunotherapies, such as vaccination plus ipilimumab, would likely enhance therapeutic efficacy of vaccination, but is beyond the scope of this project. Furthermore, the use of additional adjuvants or heterologous prime-boost strategies that utilize multiple vectors expressing the same tumor-associated antigens, might further improve therapeutic efficacy [228,229].

# Chapter 4

# Concluding remarks

The cure for cancer will likely involve collaboration of scientists from many disciplines. There are multiple ways to approach this, including early detection and targeting oncogenic gene signaling, which are very effective and not necessarily immune mediated [230,231]. As a cancer immunotherapist I believe that long-term cancer elimination can only be achieved by eliciting an anti-tumor immune response.

*Tumor-specific CD4 T cells enhance the therapeutic efficacy of CD8 T cells in a lymphopenic environment*

The studies in Chapter 2 show that the therapeutic efficacy of adoptive immunotherapy in a lymphopenic setting is augmented by the addition of tumor-specific CD4 T cells (Figure 2-4). This enhanced anti-tumor response corresponded to an increase in functional CD8 T cells (Figure 2-5). These tumor-specific CD4 T cells were able to maintain the anti-tumor function of effector CD8 T cells (Figure 2-6) even in the absence of a pool of naïve CD8 T cells to help prime into effectors. We also found that CD4 T-cell help was needed early to eliminate tumor and later to help prevent metastatic tumor growth (Figure 2-8). We observed that CD8 T cells from mice that were unable to eliminate tumor had higher expression of PD-1 than CD8 T cells from mice receiving therapeutic treatment (Figure 2-8C). Interestingly, we also observed the highest expression of PD-1 on CD8 T cells isolated at day 17 after CD4-depletion at day 3. This might be explained because these CD8 T cells will have proliferated in response to antigen upregulating PD-1 causing them to become further exhausted when

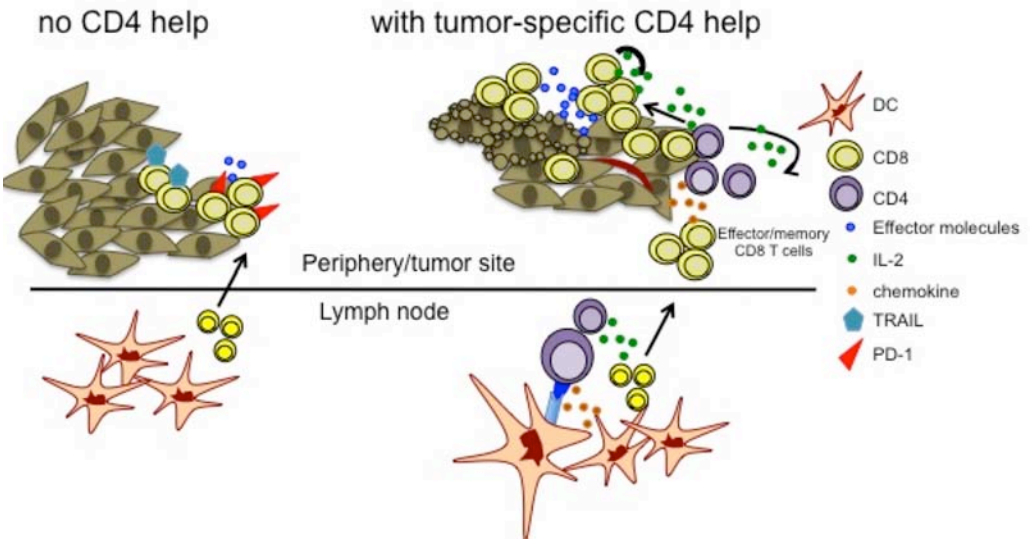



**Figure 4-1. Proposed model of tumor-specific CD4 T cell help during adoptive immunotherapy in a lymphopenic model.** In the absence of CD4 help CD8 T cells upregulate PD-1 become exhausted and upregulate TRAIL. In the presence of CD4 help; APCs express more co-stimulatory molecules to enhance activation of naïve CD8 T cells in the lymphoid organs. CD4 T cells in the periphery and at the tumor site maintain CD8 T cells by producing IL-2 and stimulating CD8 T cell autocrine IL-2 production. This increases proliferation of CD8 T cells and improves their effector function. CD4 T cells also induce production of chemokines at the tumor-site that improve trafficking of CD8 T cells.

CD4 T cells are deleted. We are currently breeding the Pmel Tg mice to PD-1 deficient mice and studies will be performed to determine whether elimination of PD-1 will improve the therapeutic efficacy of tumor-specific CD4 and CD8 T cells used for adoptive immunotherapy. Furthermore, constructs have been designed that are able to knockout the PD-1 gene in human T cells and are being tested in adoptive immunotherapy clinical trials [232].

The question that remains after these studies is whether CD4 T-cell help must be tumor-specific. Our lab and others have suggested that indeed CD4 T-cell help must be tumor-specific to have an effective CD8 T cell anti-tumor response (Friedman et al. manuscript in prep) [233].

We have recently performed adoptive immunotherapy studies combining polyclonally stimulated ovalbumin-specific CD4 T cells with tumor-specific ovalbumin or pmel CD8 T cells to treat D5 or ovalbumin (OVA) transduced D5 cells. These studies strongly suggest that CD4 T-cell help needs to be tumor-specific. However, ova-specific CD4 and ova-specific CD8 cells did not eliminate D5-Ova on their own and therefore we could not determine if T cells were functional (data not shown). Therefore future studies should be done to titer the number of ova-specific CD4 and CD8 T cells necessary for therapeutic efficacy and use this amount of cells to treat D5 and D5-ova tumors. Our data also suggest that tumor-specific CD4 T cells enhance survival of CD8 T cells by decreasing expression of TRAIL, thereby reducing CD8 T cell apoptosis, and not by CD40-CD40L interactions (Figure 2-9). Although this is consistent with previous observations in a closely related model, this will need to be verified with the use of TRAIL and CD40L deficient T cells [196]. Crossing the TRP1 Tg mice to TRAIL or CD40L-deficient mice would get at these mechanisms. Our observation that mice depleted of CD4 T cells 10 days after adoptive transfer developed distant metastases was interesting and consistent with previous results from studies with MHCII-deficient mice [187]. One possible explanation for this is that CD4 T cells are inducing chemokines that help CD8 T cells traffic to the tumor site. Another possibility is that CD4 T cells are helping to maintain long-term memory T cells. Future studies with chemokine deficient CD8 T cells could verify this mechanism. Figure 4-1 shows a proposed overview of tumor-specific CD4 T-

cell help during adoptive immunotherapy in the RAG-/- lymphopenic environment.

*Partial depletion of CD4 T cells enhances protection of multiple vaccinations during active-specific immunotherapy of melanoma*

Chapter 3 describes our studies during active-specific immunotherapy using multiple vaccination. Previously our lab found that T cells used for adoptive immunotherapy were therapeutic when isolated from mice receiving one vaccination ($1 \times 10^7$ D5-G6), but were not therapeutic when isolated from mice receiving 3 vaccinations ($1 \times 10^7$ D5-G6). We hypothesized that using a "suboptimal" dose would improve protection with subsequent vaccinations. This was not the case. We found an equally low number of mice were protected using one or three "suboptimal" vaccines ($5 \times 10^6$) (Figure 3-3). Future studies will examine whether higher doses ($1 \times 10^7$) of D5-G6 improve protection during active-specific immunotherapy. We know that a single high dose vaccination protects against a minimal tumor challenge (data not shown). Studies are ongoing in the lab to determine the effect of three immunizations with this vaccination dose ($1 \times 10^7$) during active-specific immunotherapy.

In the current study, we did observe an increase in the number of proliferating Tregs with more vaccinations (Figure 3-5). Surprisingly, partial depletion of CD4 T cells only enhanced protection against a large dose ($2 \times 10^5$), but not to a minimal tumor dose ($2 \times 10^4$) challenge (Figure 3-7). One possible explanation for this is that the response to minimal dose tumor challenge is not

decreased by induction of Tregs and therefore partially eliminating CD4 cells does not affect protection from tumor. Another possibility is that the large dose tumor challenge is boosting the anti-tumor response and increasing the frequency of tumor-specific CD8 T cells and the presence of proliferating CD4+Foxp3- T cells with partial CD4-depletion helps the CD8 T cell anti-tumor response. To further address this paradigm future studies will be done using the DEREG mice, which express the diphtheria toxin receptor on Foxp3+ cells, allowing for specific elimination of Tregs before the 2$^{nd}$ and 3$^{rd}$ vaccination [227]. Studies are also being done examining the role of natural Tregs using TGFβ-dominant negative receptor II (TGFβDNRII) mice. The TGFβDNRII mice have a dominant-negative TGFβ receptor on CD4 T cells and therefore are unable to respond to TGFβ [226]. TGFβ signaling is needed to induce Tregs in the periphery and thus these mice do not have induced Tregs [234]. The multiple vaccination studies in these mice will let us examine whether natural Tregs decrease the efficacy of multiple vaccinations.

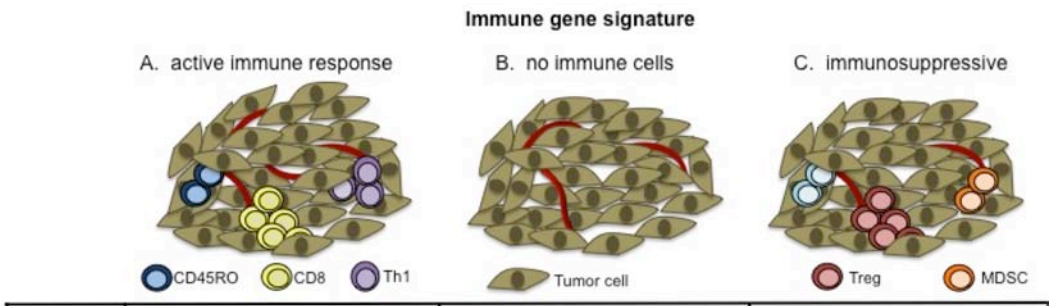
We did not observe large differences between the three vaccine administration routes we tried (Figure 3-4). One explanation is that since all the vaccines were given on the main body of the mouse there was no distinct separation of priming. Future studies looking at the effect of administration should choose sites that are further separated, such as the footpad. The most useful approach would be to analyze patient responses in a number of clinical trials that have used varied vaccine administration. Very few of these clinical studies have examined the difference in anti-tumor response in correlation with

vaccine administration and those that have reported positive results when vaccines were given on multiple limbs [126,141].

*Overall Summary*

In the studies presented here, we show that CD4 T cells are important for maintaining effector and memory CD8 T cells during adoptive immunotherapy in the lymphopenic setting (Chapter 2). These results provide significant incentive to include the use of CD4 T cells directed to tumor-associated targets in future clinical trials. Although these results implicate a role for CD4 T cells in supporting maintenance of CD8 T cells, tumor-specific CD4 T cells are also likely to be important for helping to prime an optimal anti-tumor CD8 T cell response [113]. We propose this priming response would require tumor-specific CD4 T cells [233]. Depleting the entire population of CD4 T cells in our multi-vaccine studies may seem to conflict with the results in Chapter 2, however in the CD4-depletion strategy, the CD4 T cell numbers are diminished for a short window of time only. Furthermore we see increased proliferation of non-Tregs ($CD4^+Foxp3^-$) (Figure 3-6). One hypothesis for this is that the CD4-depletion increases the number of tumor-specific CD4 T cells able to prime CD8 T cells. This is not only because CD4 T cells have a higher helper to Treg ratio, but also because elimination of the CD4 T cells gives room for CD8 T cell expansion.

The field of tumor immunology has placed emphasis on therapies that reprogram the anti-tumor immune response [178,217,235]. However, there still remains a cohort of patients that does not have preexisting anti-tumor immunity




| | | | |
|---|---|---|---|
| Dominant immune signature | CD3, CD8, Th1, CD45RO (memory) GZMB, PRF1 (effector function) CCL2-5, CXCL9, CXCL10 (chemokines) | Little or no immune infiltrate | FOXP3, TGFβ, IL-10 MDSC M2 macrophage |
| Response to vaccination | Quicker and stronger response to vaccination | Not responsive or less responsive to vaccination | No effect of vaccination |
| Potential treatment | Timing and heterologous prime-boost to optimize inflammatory environment and boost anti-tumor response. | Vaccine adjuvants (TLR agonists, GM-CSF) for optimal co-stimulation and priming. Inducing immunogenic tumor death. | Need to eliminate suppressive mechanisms by Treg depletion, TGFβ inhibition, chemotherapy or PDE5 inhibitors of MDSC. |
| Result of targeted vaccination | Boost existing immune response | Must prime a *de novo* anti-tumor response | Elimination of the immunosuppressive environment |

**Figure 4-2. Proposed model for how immune signature can be used to personalize vaccine strategies and improve patient outcomes.**
A) An active immune signature identified by phenotypic or genetic analysis of immune components within the tumor signals pre-existing immune response that is easier to augment by booster vaccines. This is the cohort of patients that respond to effective boosting vaccines. B) Tumors that lack an immune signature contain few immune cells, signaling the lack of an anti-tumor immune response. Vaccine strategies need to stress both priming of a *de novo* anti-tumor immune response as well as boosting of the immune response in order to be effective. C) Immunosuppressive gene signature including Tregs, MDSC and tolerogenic cytokines will block the immune response to vaccination and may have eliminated tumor-specific T cells. Vaccine strategies need to start with elimination of the suppressive environment/cells and subsequently prime and boost the anti-tumor immune response in order to develop a therapeutic immune response. All three scenarios are expected to benefit from agents that induce immunogenic death (modified from Church et al.[126]).

[236]. It is possible that these patients would not respond well to immune reprogramming treatments and would respond better to immunotherapies that prime a new tumor response. Identifying the immune phenotype of a patient's tumor prior to therapy could improve anti-tumor responses. We previously published a hypothesis of tumor immune phenotypes and potential approaches

for mediating an anti-tumor response for each phenotype, a) active immune cells, b) no immune cells and c) immunosuppressive (Figure 4-2) [126]. We hypothesized that patients with Th1 CD4 T cells in the tumor would fall in the active immune cell group, while patients with higher frequencies of Tregs in the tumor would be in the immunosuppressive group. It is most likely that individual tumors and separate metastases have a combination of these tumor immune signatures. Personalizing immunotherapies based on immune signature could be an excellent way to improve objective responses to immunotherapy [237].

In the scheme of these three scenarios, the no immune cell phenotype, where patients have little or no existing anti-tumor response, and require the generation of a de novo immune response, poses the most difficult challenge for the immunotherapy field. This is partially due to the evidence that many cancer patients have compromised immune systems, caused by chemotherapy and/or decreased thymic function with age [238]. The use of combination immunotherapies together with personalization of treatments provides promising hope for the future of cancer treatment and I propose that immunotherapists should not forget the importance of CD4 T-cell help when designing their treatment strategies.

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