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Enhancing adoptive T cell transfer therapies:

Novel treatments through IL-12 CD8 T cell priming and adjunctive use of a histone deacetylase inhibitor

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Molecular & Medical Pharmacology

by

Dominique Naomi Lisiero

2012

ABSTRACT OF THE DISSERTATION

Enhancing adoptive T cell transfer therapies:

Novel treatments through IL-12 CD8 T cell priming and adjunctive use of an HDAC inhibitor

by

Dominique Naomi Lisiero

Doctor of Philosophy in Molecular & Medical Pharmacology

UNIVERSITY OF CALIFORNIA, Los Angeles, 2012

Professor Robert M Prins and Professor Caius Gabriel Radu, Co-chairs

Adoptive cell transfer (ACT) immunotherapy is the infusion of a large number of autologous tumor specific immune cells that have the ability to proliferate, traffic and mediate tumor eradication. Tumor specific cells are obtained by expansion of endogenous tumor specific lymphocytes ex vivo, or by the in vitro redirection of lymphocyte specificity through genetic engineering. The addition of a preparative lymphodepletion prior to ACT has dramatically improved the efficacy and responsiveness of adoptively transferred cells through increased access to homeostatic cytokines and elimination of suppressive T regulatory cell populations. Although associated with serious toxicities, administration of high doses of the T cell growth factor, IL-2, has further expanded the ability of adoptively transferred cells to proliferate and persist in vivo. However, despite the initial proliferation and effector function of these tumor specific T cells, a majority of patients fail to achieve complete remission. This can occur as a result of multiple mechanisms including immunoediting of the tumor, the initial differentiation state of transferred cells or an eventual induction of T cell tolerance.

This thesis describes the utilization of two different strategies in order to prolong the proliferative and functional anti-tumor activity of adoptively transferred tumor specific T cells. The first strategy addresses whether the in vitro expansion and priming of tumor specific T cells with the pro-inflammatory cytokine IL-12 could enhance ACT in an in vivo melanoma model. Previous research in our group indicated that administration of a Toll like receptor (TLR)-7 agonist, imiquimod, enhanced dendritic cell priming of tumor specific T cells. We hypothesized that pro-inflammatory cytokine signals as a result of TLR7 administration, such as IL-12, could have a direct effect on adoptively transferred T cells without the systemic toxicities seen by imiquimod administration. We were able to demonstrate that in vitro IL-12 priming and expansion of tumor specific T cells prior to adoptive transfer dramatically improved polyfunctional cytokine secretion and anti-tumor activity without severe systemic toxicities in comparison to priming with only IL-2. Furthermore, we were able to show that this increased anti-tumor activity relied upon enhanced IL-2 signaling, and abrogated the need for high doses of systemically administered IL-2.

The second strategy we employed utilized the administration of a histone deacetylase (HDAC) inhibitor, LBH589, in combination with ACT. HDAC inhibitors have been shown to increase the expression of TNF superfamily associated death receptors, MHC molecules and costimulatory ligands in multiple cell lines. The ability to pharmacologically immunomodulate a tumor with LBH589 provided a strong rationale for its adjunctive use with adoptively transferred tumor specific T cells. In an in vivo subcutaneous melanoma model, we were able to demonstrate that LBH589 mediated enhanced tumor regression. However, we were surprised to learn that LBH589 was modulating T cell function, even in the absence of tumor. LBH589 treated groups had significantly decreased T regulatory cell populations and significantly increased the proliferation and polyfunctional phenotype of tumor specific T cells over time. Furthermore, the potent effector phenotype of these T cells was characterized by an enhanced expression of the IL-2 receptor, CD25, and the TNF superfamily co-stimulatory ligand, OX-40.

In conclusion, this thesis demonstrates two distinct strategies to enhance adoptive cell transfer in a pre-clinical model. These strategies clearly highlight the necessity of highly functional and proliferative T cells with an ability to continuously traffic and secrete multiple effector cytokines. Our findings also reinforce that responsiveness to the growth factor IL-2 through continued and enhanced expression of the IL-2 receptor, CD25, provides a critical determinant of antitumor activity. Both strategies enhanced ACT by two distinct mechanisms and highlights the need to further investigate the molecular basis of HDAC activity in activated effector T cells during an antitumor response and whether this is compatible with pro-inflammatory signals such as IL-12.

The dissertation of Dominique Naomi Lisiero is approved.

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UNIVERSITY OF CALIFORNIA, Los Angeles

2012

To the real women of science

Table of contents

		Page
Abstract of the dissertation		ii-iv
Committee Page		v
Dedication		vi
Table of Contents		vii
Acknowledgements		viii
Vita		x
Chapter 1:	Introduction to adoptive cell transfer therapy – from pre-clinical models to patients	1
	References	28
Chapter 2:	Enhanced sensitivity to IL-2 signaling regulates the clinical responsiveness of IL-12 primed CD8+ T cells in a melanoma model.	49
	References	79
Chapter 3:	Histone deacetylase inhibitor, LBH589, enhances the proliferative and polyfunctional capacity of adoptively transferred tumor specific T cells.	88
	References	114

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Chapter 2 in this thesis is a version of (Lisiero et al. 2011).

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Chapter 1:

Introduction to adoptive cell transfer therapy – from pre-clinical models to patients

Introduction

In its beginnings, tumor immunotherapy strategies relied heavily upon anecdotal evidence of the immune system's ability to control or prevent tumor growth. However, advances in basic immunological mechanisms dramatically impacted the field of tumor immunotherapy, allowing for the creation of highly targeted therapies. This introduction covers the major discoveries, clinical trials, and preclinical findings, that led to the development of modern T cell transfer therapies. This includes the discovery of IL-2, lymphocyte activated killer (LAK) cells, adoptive cell transfer of tumor infiltrating lymphocytes (TIL), and the genetic engineering of T lymphocytes.

Immune surveillance and antitumor immunity

The immune system is a key component involved in the control and progression of tumor growth. In 1909, Paul Ehrlich posited that the immune system recognizes and protects the host against the outgrowth of cancerous cells even in the absence of therapeutic intervention. Almost fifty years later, Thomas and Burnett would ascribe a surveillance function to the immune system and its relationship with pre-malignant cells (1). Although the mechanisms by which a tumor escapes the surveillance of an intact immune system are continuing to be defined, the ability to harness the specificity and potency of tumor specific lymphocytes through active and passive vaccinations has captivated many as an attractive cancer therapy (2).

Intratumoral bacillus Calmette-Guerin (BCG)

The first widely tested immunotherapy for the treatment of cancer was the intratumoral injection of BCG. BCG is an attenuated strain of Mycobacterium bovis and is still the only prophylaxis available for tuberculosis (3). First used in 1921, anecdotal evidence accumulated that patients with tuberculosis exhibited lower frequencies of cancer. Small animal studies demonstrated antitumor activity of BCG as a prophylaxis and as a treatment for previously

established tumors (4). Although the exact mechanisms by which BCG exerts its effects are still unknown, its thought that vaccination at the tumor site creates an inflammatory reaction and attracts antigen presenting cells (APCs) to dying tumor cells where a predominant Th1 response ensues (5). Despite some successes, excitement of BCG waned as clinical studies failed to show antitumor activity in a broad spectrum of human tumors. With the exception of superficial bladder cancer, BCG is no longer used as a single agent therapeutic. Despite this, BCG was able to provide evidence that a non-specific immunotherapy could cause tumor regression and provided strong rationale for the utilization of immune modulating agents in the treatment of cancer.

Early beginnings of adoptive immunotherapy

Adoptive immunotherapy is the infusion of a large number of autologous lymphocytes with anti-tumor activity into a tumor bearing host. This therapy does not rely upon the stimulation of an endogenous response (active immunotherapy), but allows for the recovery and/or ex vivo generation of tumor reactive cells that can exhibit antitumor activity in vivo (6). In 1955, Mitchison conducted one of the first studies that demonstrated the feasibility of cellular mediated tissue rejection. In this study, cells from the tumor draining lymph node were adoptively transferred to tumor bearing hosts and were shown to have reactivity (7). Further studies in animals began to clarify the potency of immune cells from syngeneic hosts immunized with irradiated tumor. In the 1960s, a syngeneic rat model showed declined growth rates of chemically induced fibrosarcomas when treated with syngeneic lymphocytes obtained following donor immunization (8). By the late 1960s, it was demonstrated that the transfer of lymphocytes from immunized donors could cause regression of virally or chemically induced tumors and this effect could be effectively inhibited by cyclophosphamide, an agent with immunosuppressive activity (9, 10). Soon after, it was demonstrated that these same tumor reactive lymphocytes could be propagated in vitro by their continuous sensitization to irradiated tumor cells and that

these lymphocytes retained their anti-tumor properties both in vitro and when adoptively transferred in vivo (11). Although these proof of principle studies demonstrated the ability of transferred cells to control tumor growth, the reliance of these early studies on continuously immunized hosts for tumor reactive lymphocytes were clearly not clinically translatable treatments.

In humans, early experiments in the 1960s by Chester Southam and colleagues utilized highly questionable methods to explore the effects of lymphocytes on the kinetics of tumor cell growth in cancer patients (12). In a series of experiments, tumor cells were harvested from advanced cancer patients and then mixed with autologous lymphocytes collected from peripheral blood. Mixtures containing tumors cells by themselves or with autologous lymphocytes were then reimplanted subcutaneously as human tumor autografts and monitored for growth. Growth was significantly inhibited in half of patients when autografts contained the patient's own lymphocytes. This provided a basis for inhibitory activity by lymphocytes that could be utilized to control tumor growth in humans. However, ex vivo expansion and reinfusion of tumor reactive lymphocytes was still not feasible.

The impact of T cell growth factor (TCGF, Interleukin-2)

The inability to culture T cells and stimulate their proliferation in vitro was a major limitation that was finally addressed by the identification and molecular cloning of IL-2. Up until this point, the predominant mitogenic stimulus for lymphocytes was considered to be an antigenic stimulus. The dogma that only a specific antigen was necessary for stimulating T cell growth minimized the impact that a growth factor could lend to T cell proliferation (13). Studies in the 1960s began to describe soluble factors present in media conditioned by allogeneic mixed lymphocyte cultures that were able to stimulate the proliferation of lymphocytes (14, 15). It would be another ten years until TCGF (later renamed IL-2) would be fully identified as a factor specific for T cells with the ability to expand and maintain large populations of T cells in vitro.

The discovery of TCGF by Ruscetti et al, though by chance, completely revolutionized T cell biology (16, 17). Within the span of a few years, a monoclonal antibody specific for IL-2 led to its purification, the cloning of its cDNA, and further production of its recombinant form in *E. coli* (18, 19).

Single agent high dose Interleukin-2

With the ability to induce the in vivo expansion of lymphocytes, IL-2 was immediately considered as a potential antitumor therapeutic (20). It was hypothesized that a small percentage of circulating lymphocytes was tumor reactive and could be forcibly stimulated to expand and achieve effector activity with IL-2. Administered as a single agent, IL-2 was shown to induce 5-10% complete responses that remained durable in metastatic melanoma and renal cell carcinoma patients. High doses can induce toxic side effects such as capillary leak syndrome and are often administered until a maximally tolerated dose is reached (21). Despite these toxicities, high dose IL-2 was FDA approved as a treatment for metastatic renal cell carcinoma in 1992 and for metastatic melanoma in 1998. The utilization of high dose IL-2 for the last 20 years has provided the ability to retrospectively assess the success of this therapy. For melanoma, cutaneous localization of disease and the absence of multiple metastases, and for metastatic renal cell carcinoma, a “favorable” histological assessment and carbonic anhydrase IX expression are positive prognostic factors for this treatment (22–26). However, definitive molecular or immunological prognostic factors are severely lacking and would greatly enhance the selection of responsive patient populations for an otherwise toxic and non-specific treatment.

Lymphokine activated killer (LAK) cells

The discovery of IL-2 and its ability to potently stimulate the growth of activated T cells in vivo led to the characterization of lymphokine activated killer (LAK) cells and their utilization as one of the first cellular therapeutics. LAK cell activity was first observed by the culture of

peripheral blood lymphocytes in high concentrations of IL-2 and were hypothesized to arise through the in vivo injection of high doses of IL-2 (27). These cytolytic cells were not antigen specific, but showed anti-tumor activity specifically towards autologous cell lines previously resistant to NK cell killing (28, 29). In small animal studies, LAK cells or LAK cells supported by systemically administered IL-2 significantly reduced established tumor masses and metastases in distinct anatomical sites in multiple strains of mice (30, 31). Supported by these animal studies, LAK cells in combination with IL-2 were utilized in patients with advanced metastatic disease and were able to demonstrate a reduction in tumor volume in more than 40% of patients (32). LAK cells alone could not demonstrate any objective responses. The only toxicities present with this treatment were associated with the administration of IL-2, which was discontinued once the maximum tolerable dose was reached. Although the utilization of LAK cells in combination with IL-2 showed early promising results, a prospective trial in patients with metastatic renal cell carcinoma or melanoma was unable to show a significant benefit of LAK cells and systemic IL-2 in comparison to IL-2 therapy alone (33). The non-specific nature of LAK cells allowed for a treatment modality that could theoretically provide anti-tumor activity in patients where tumor specific T cells were unable to harvested or produced. However, without tumor specificity the ability to achieve long lasting immunological memory against tumor recurrence was lost. Instead, the field turned toward more specific immunological approaches to target and eradicate large tumor masses.

Autologous tumor vaccination for generation of vaccine primed lymphocytes

In an attempt to harvest higher quality tumor specific lymphocytes, autologous tumor vaccinations were tested in clinical trials for a number of cancers including head and neck carcinoma, renal cell carcinoma, and gliomas (34–36). This therapy utilized the vaccination of patients with irradiated autologous tumor cells (admixed with the cytokine GM-CSF or adjuvant BCG), followed by recovery of vaccine primed lymphocytes from tumor draining lymph nodes.

This was followed by in vitro lymphocyte expansion with IL-2, and the eventual reinfusion of tumor reactive cells back into patients. The most favorable clinical responses were for vaccinations against renal cell carcinoma with an overall response rate of more than 25% and almost half of these patients experiencing complete responses (34). These responses were positively correlated with the ratio of IFN-γ:IL-10 secretion by vaccine primed lymphocytes in response to autologous tumor. Although this treatment generated a certain proportion of clinical responses, the labor intensive procedure was not a feasible protocol for the generation of purified tumor reactive cells. Instead, the isolation of tumor reactive cells from within the tumor itself could provide a more feasible alternative.

Tumor infiltrating lymphocyte (TIL) therapy

Although high dose IL-2 is able to mediate complete, durable regressions in a small population of metastatic melanoma and renal cell carcinoma patients, this therapy is not well tolerated for many cancer patients. In an attempt to generate a more tumor specific response, a new therapeutic modality involving the recovery, isolation of, and ex vivo expansion of tumor infiltrating lymphocytes (TILs) was tested. It was hypothesized that the specific localization of lymphocytes in the tumor was indicative of tumor specific reactivity. By removing these lymphocytes from a presumably suppressive tumor environment that lacked proper costimulation, these cells could be properly stimulated, expanded, and regain antitumor effector function. One of the first studies describing the utilization of TILs demonstrated regression of a virally induced sarcoma by the ex vivo restimulation of tumor infiltrating lymphocytes. This demonstrated that lymphocytes could recognize specific antigens in the context of MHC, and more importantly, that these cells were not of a LAK cell origin (37).

Almost immediately following the identification and isolation of IL-2, the ability to culture and expand TILs became feasible in both pre-clinical and clinical settings (38, 39). For pre-clinical

studies, TILs retained their tumor reactivity in vitro and were able to mediate the in vivo regression of a wide variety of established syngeneic tumors, both solid and metastatic. TILs alone were more significantly effective in mediating antitumor activity than LAK cells given with IL-2. Even more promising, TILs in combination with IL-2 were able to demonstrate the most potent response of any of these treatments explored previously (40). These pre-clinical studies were extended to human clinical trials, and TILs were, again, found to be far superior to LAK cells in vitro. Autologous TILs from human metastatic melanoma were expanded in vitro in the presence of IL-2 and lysed autologous tumor cells in vitro despite being in culture for more than 9 weeks. Furthermore, expansion and cytotoxicity of these TILs was dependent upon the presence of the T cell receptor, CD3, and IL-2. These distinctions further confirmed the identity of these cells as non-LAK T lymphocytes with true recognition of a specific antigen (41). However, these in vitro assays did not answer whether these cells had antitumor activity in human patients.

One of the first clinical trials utilizing TILs with IL-2 was able to show measureable antitumor responses in 40% of metastatic melanoma patients that lasted from 2-13 months (42). In addition to metastatic melanoma, the efficacy of TILs with IL-2 for metastatic disease other than melanoma were tested and shown to produce partial regressions (43). In the case of a small trial involving glioma patients, TILs combined with IL-2 was able to demonstrate feasibility without severe intracranial toxicities and produced one complete responder (44). By 1994, the results of a comprehensive study with more than 80 metastatic melanoma patients utilizing TILs with IL-2 revealed a 30% measureable response, with 5 patients demonstrating a complete response (45). This study produced a more extensive characterization of the critical parameters necessary for a successful antitumor response in patients. One of the major limitations encountered involved the expansion of a sufficient number of TILs for infusion. Animal studies had indicated that a minimum of 10^{10} TILs were necessary in order to produce any therapeutic

benefit (43). 69% of samples from this 1994 study were successfully grown out to 10^{10} TILs, and 60% were grown out to the target for this therapy, 10^{11} TILs. Although the majority of tumors were able to provide an adequate number of TILs for expansion, a substantial number of patients were ineligible for treatment. These results demonstrated that improved methods to recover and expand TILs were needed in order to improve efficacy. Presently, metastatic melanoma and renal cell carcinoma are in the minority of tumors that can generate the required number of TILs for infusion, making this a limited treatment for solid tumors. However, the absolute number of cells was not the only significant parameter, the functional quality of TILs generated from expansion also appeared to distinguish patient responsiveness. Prognostic factors associated with objective clinical responses included younger cultures and increased T cell doubling time. Other studies suggested that analysis of resulting T cell subsets from TILs cultures, cytolytic effector function, and cytokine secretion were critical parameters necessary to generate a more efficacious treatment (46). Even 20 years later, these issues are still being addressed for current adoptive cell transfer treatments.

Identification of the first tumor associated antigen

As the field of immunology began to further describe the intricate cellular and molecular components involved in an immune response, the field of tumor immunology worked just as tirelessly to understand the mechanisms of tumor recognition and T lymphocyte effector function. In 1974, Zinkernagel and Doherty explained the ability of MHC to determine cytotoxic effector function in T lymphocytes (47). By 1987, the genes for T cell receptors had been identified, and the crystal structure of peptide/antigen bound to MHC had been elucidated. However, the mechanism by tumor specific T lymphocytes recognized autologous tumors was still not completely clear. Antibody blockade of TCR structures and MHC addressed these mechanisms more approachable but were not entirely convincing (48, 49). Important questions still remained as to whether CTL were recognizing specific antigens only present on tumors, whether these

antigens were restricted by MHC, and whether antigens were shared with normal tissue. These questions, and the ultimate identity of these antigens, would be absolutely critical in pushing the development of more specific cancer vaccines forward.

In 1991, the first tumor associated antigen was identified through a laborious screening process. DNA generated from a cosmid library of a melanoma cell line was transfected into the same melanoma cell line that had been separately and painstakingly selected as an antigen loss variant. Recognition of a 12 kb piece of DNA would eventually lead to the identification of MAGE (for melanoma antigen) in the context of HLA-A1, as the first tumor rejection antigen (50). This would be followed by the identification of several other melanoma associated antigens, which included, Melan A/MART-1, tyrosinase, and gp100 (49, 51–55). Many other tumor associated antigens, not just those originating from melanoma, have been identified by a number of different methods. They are still being analyzed in a number of vaccination protocols with varying degrees of success (56). The relative characteristics of these antigens reveals a full diversity of peptides derived from aberrantly expressed intronic sequences, tumor specific mutations, normal nonmutated genes present in only the tumor and other specific tissues, or normal genes present during a differentiation process but re-expressed during tumorigenesis. Most importantly, these tumor antigens are largely non-patient specific and can be shared across tumors of multiple origins, presenting a valuable opportunity for broad applicability of tumor vaccines (57, 58).

With a greater understanding of the identity of tumor rejections antigens, the field of tumor immunology began to enter into a molecular era with clearer molecular targets set forth. The days of non-specific cellular responses resulting from large bolus administrations of IL-2 and the subsequent production of LAK cells would now give way to a new understanding of T cell biology and T cell derived therapeutics. However, the fact that many of these antigens turned

out to be self proteins also raised new questions of strategies for breaking this immune self tolerance and suppression.

In vivo trafficking of tumor specific lymphocytes

Although the adoptive transfer of a large number of TILs (~10^{11} cells) was able to demonstrate clinical responses, why this treatment was not more consistently effective was still a persistent concern. Questions turned to focus on the fate of the TILs being adoptively transferred, and the rates at which they could be trafficking to the tumor (if they were even trafficking preferentially into the tumor) and how long they were being retained systemically. In order to answer these questions, several methods were employed to track T cell trafficking *in vivo*. The first utilized the labeling of TILs or autologous PBMCs with radioactive indium-oxine (^{111}In). This labeling allowed the visualization of lymphocytes *in vivo* and demonstrated that 13 out of 18 patients had tumor specific localization of lymphocytes at the tumor site. When tumors could be resected and sampled alongside normal tissue, a much higher uptake ratio was recorded in ^{111}-In labeled TIL in comparison to ^{111}-In labeled autologous PBMCs. Localization of labeled TIL in the tumor increased over time, however clinical efficacy could not be correlated to this increase (59). ^{111}-Indium labeling demonstrated specific localization of TILs following infusion, but its utilization was associated with numerous drawbacks, which included, loss of cell viability through autoradiation, high spontaneous release of ^{111}-In from labeled cells, and a short half life of approximately 2.8 days.

In order to more accurately and consistently represent the distribution of adoptively transferred lymphocytes, TILs were retrovirally transduced with a gene encoding neomycin resistance (60). Introduction of this specific gene was able to specifically and sensitively differentiate adoptively transferred TILs from endogenous lymphocytes in peripheral blood by PCR or Southern blot analysis. Neomycin resistance also allowed for the ex vivo selection, expansion, and analysis of

TILs that had been shown to have enhanced persistence in patients. Following transduction, results from these studies indicated that the inserted gene retained its biological activity in vivo, did not lead to the malignant transformation of TILs, and did not affect the T cell proliferation or cytolytic activity in vitro. Subsequent analysis of peripheral blood showed that the incidence of transduced cells ranged from 1 in 3,000 after 6 days to 1 in 16,000 after 4 days in two different patients.

More importantly, this study marked the first time that the National Institutes of Health had approved the transfer of a foreign gene into humans by utilization of retroviral mediated transduction of lymphocytes. Demonstration of both the feasibility and safety of gene therapy for humans not only opened a potential new path for the creation of more efficacious tumor infiltrating lymphocytes, but also suggested that other genetic deficiencies such as hemophilia might be corrected through the same means.

Lymphodepletion prior to adoptive cell transfer

The frequency of circulating TILs amongst peripheral lymphocytes only days following infusion revealed that they constituted approximately 0.01% of peripheral lymphocytes (60). Such a low percentage was speculated to result from a number of endogenous mechanisms that may exist normally to regulate the pool of peripheral T lymphocytes. Consistent with this idea, mouse models demonstrated that lymphodepletion prior to ACT treatment with tumor specific T cells showed enhanced antitumor immunity (61–63). Such results might be due to multiple mechanisms, such as the elimination of immunosuppressive lymphocyte populations or an alteration of lymphocyte homeostasis. In order to test whether a regimen of lymphodepletion prior to adoptive cell transfer could augment clinical responses, metastatic melanoma patients were given a combination of chemotherapeutic drugs known to cause non-myeloablative lymphodepletion, cyclophosphamide and fludaribine, for 7 days prior to adoptive transfer (64). A

large number of highly avid in vitro selected tumor reactive TIL clones (avg. of 10^{10} cells) and a high dose of IL-2 (avg. of 9 doses) were administered into patients treated with the cyclophosphamide and fludarabine regimen. Half of the 13 patients treated showed objective clinical responses (at least 50% regression of sum of tumor lesions). When peripheral blood was examined after ACT, some patients demonstrated an extremely high absolute lymphocyte count, with the majority of peripheral blood skewed towards a CD8+ T cell phenotype. Two patients exhibited significant expansion of individual T cell receptor beta chain variable regions 7 and 12, which were later shown to have specificity to the self tumor associated antigen, MART-1. Presumably because of the high preponderance of MART-1 specific cells, 5 patients also demonstrated destruction of normal melanocytes (vitiligo and anterior uveitis) during the course of treatment. This manageable autoimmunity highlighted the successful *in vivo* expansion of highly avid and functional tumor/self reactive lymphocytes specific for MART-1. These cells also appeared to retain the ability to secrete IFN-γ within the tumor site due to the fact that post-treatment biopsies showed an overwhelming increase in the expression of two targets of IFN-γ, MHC class I and II. Addition of a lymphodepletion regimen was able to fulfill some critical requisites of adoptive immunotherapy – the ability to transfer tumor specific cells that could mediate tumor regression by maintaining effector function, expanding in vivo, and persisting systemically for long periods of time.

Generation of antigen specific T cell clones

TIL therapy relies upon the expansion of a bulk population of lymphocytes that demonstrate in vitro target recognition and the ability to mediate tumor regression. However, within these TILs, cells with irrelevant specificity or suppressive function are more than likely to be present. With the identification of tumor associated antigens, the ability to expand and infuse a homogenous population of antigen specific T cells was achievable. One method utilized a peptide specific vaccination to the tumor associated antigen, gp100, with incomplete Freund's

adjuvant in order to generate gp100 specific T lymphocytes (65). Lymphocytes collected from peripheral blood and TILs were collected and stimulated in vitro with gp100 peptide and IL-2. Through multiple rounds of screening and expansion of limited dilution clones, cells with the highest IFN-γ secretion were selected for administration to patients. Another method utilized a lengthy restimulation of peripheral blood with gp100 or MART-1 peptide pulsed dendritic cells (66). These cells would later be screened for cytolytic activity against tumor targets and expanded for infusion. These two trials demonstrated the safety and efficacy of the adoptive transfer of a large number of tumor specific T cells targeting self antigens. In both studies, no complete responses were reported, antigen specific T cell frequencies remained low, and post-infusion biopsies also revealed tumors that had subsequently lost target antigen expression. This data revealed that the targeting of single antigens could create a situation in which the remaining tumor cells were antigen loss variants and no longer feasible targets for repeated T cell infusions. Furthermore, without epitope spreading and high T cell frequencies (as seen with host lymphodepletion), a successful antitumor immune response would not be able to provide meaningful clinical responses.

Genetic engineering of lymphocytes

With the identification of specific tumor associated antigens and the ability to genetically engineer lymphocytes, the adoptive transfer of autologous, antigen specific, tumor specific, lymphocytes became feasible (51, 53, 55, 60). Furthermore, the reliance on a minimum/sufficient number of tumor infiltrating lymphocytes or vaccine induced tumor specific T lymphocytes for adoptive cell transfer therapy was no longer an impediment. In 1986, the first example of the redirection of T cell specificity through the transfection of lymphocytes with a new TCR was achieved in mice with the TCR specific for the fluorescein hapten (67). This study demonstrated the possibility that the expression of new TCR α and β chains was sufficient to redirect the specificity and function of a T cell towards a completely new antigen. The TCR

recognizing MART-1 was the first human tumor associated antigen TCR to be successfully cloned and expressed in another cell line (68). The expression of this TCR was achieved by transient transfection of MART-1 TCR α and β chain cDNA. While this TCR efficiently recognized peptide pulsed targets, it was only partially able to recognize melanoma cell lines that expressed MART-1.

Retroviral transduction of T cells with the human MART-1 TCR was first reported in 1999 and was able to demonstrate in vitro recognition and lysis of HLA matched melanoma cell lines (69). The ability to transduce T lymphocytes with tumor/antigen specific TCRs that retain effector function has now been shown for diverse antigens including MART-1, NY-ESO, gp100, MDM2, and MAGE-A1 allowing for the effective targeting of numerous tumors (69–73). Furthermore, the ability for transduced lymphocytes to retain efficacy, persist, and promote antitumor activity in preclinical studies has also been demonstrated numerous times (74–77). The next challenge remained to bring these genetically engineered lymphocytes into clinical trials.

The first clinical trial reporting the infusion of autologous lymphocytes transduced with the TCR specific for MART-1 (clone DMF4) demonstrated the successful transduction of human lymphocytes that retained reactivity against MART-1 peptide and MART-1 expressing tumors *in vitro* (78). Unfortunately, only 13% of patients (2/15) demonstrated an objective clinical response. Although these transduced cells were safely retained for prolonged periods of time, many questions were raised regarding whether these cells retained a functional TCR. The authors hypothesized that mispairing of transduced TCR chains with endogenous TCR chains may necessitate in the TCR design. Further, efficacy would be addressed with a more highly avid TCR and an increased amount of lymphodepletion prior to infusion. The second clinical trial reporting infusion of autologous lymphocytes transduced either with a new highly reactive MART-1 (clone DMF5) or the gp100 reactive TCR demonstrated higher objective clinical

responses, with a 30% response for patients receiving the MART-1 TCR and 19% for the gp100 TCR (79). Although these two clinical trials were able to demonstrate the safety and efficacy of genetically modified lymphocytes for use as therapeutics, their response rates did not match those seen for lymphodepletion in combination with ACT TIL therapy (~50% response rate) (80). Still, many questions regarding adoptive immunotherapy with genetically engineered T cells remain, particularly concerning the phenotype of cells transferred, and how to generate the most potent T cells with the ability to persist, expand, and mediate tumor regression.

Mouse models of adoptive cell transfer

In order to address the prerequisites for a productive antitumor response and the resulting responses generated by the adoptive transfer of tumor specific T cells, pre-clinical models are used to refine and generate more effective immunotherapy protocols in a mechanistic fashion. T cell receptor transgenic mouse models specific for tumor associated antigens such as gp100, TRP1, TRP2, and SV40 large T antigen have been developed (81–86). The Pmel-1 TCR transgenic mouse model allows for the generation of a large number of $gp100_{23\text{-}33}$ specific $H\text{-}2D^b$ restricted CD8+ T cells (82). These T cells are able to recognize the syngeneic B16 melanoma cell line and other tumors of neuroectodermal lineage, and when adoptively transferred into mice can demonstrate regression of established tumors (87). In vivo efficacy of Pmel T cells was also shown to be completely dependent on concomitant administration of a peptide vaccination and systemic IL-2.

Lymphodepletion and the creation of cytokine sinks

With the ability to more closely examine the adoptive transfer of tumor specific T cells in a pre-clinical model, the immunological parameters necessary to support an antitumor immune response have now been examined more carefully. In the case of lymphodepletion in combination with TIL, therapy in patients demonstrated remarkable clinical responses, but the

mechanisms behind this increased functionality were not well understood. In a study utilizing the Pmel adoptive transfer model with established B16 tumors, Restifo and colleagues were able to attribute enhanced tumor regression to the increased availability of the homeostatic cytokines, IL-7 and IL-15, following nonmyeloablative lymphodepletion (5 Gy) (88). Lymphodepletion did not increase the proliferation of adoptively transferred cells, but instead enhanced the release of inflammatory cytokines such as IFN-γ and TNF. A follow up study which compared nonmyeloablative lymphodepletion (5 Gy) with a myeloablative dose (9 Gy) in combination with a hematopoietic stem cell transplant resulted in increased tumor regression (89). This myeloablative lymphodepletion increased serum levels of IL-7 and IL-15, increased the absolute number of Pmel T cells, and significantly increased the ratio of adoptively transferred Pmel T cells to T regulatory cells and NK cells. An even more intense lymphodepletion regimen which included twice daily irradiations (24 Gy max), resulted in the most effective conditioning prior to adoptive cell transfer, while managing radiation derived toxicities (90). In addition, elevated systemic levels of IL-6 and LPS were observed as doses of irradiation were increased, suggesting a previously known role for innate immunity in enhancing antitumor immunity. The knowledge that more intense doses of radiation enhanced the efficacy of adoptive cell transfer was then tested in the clinic. Cohorts receiving an increased dose of radiation (12 Gy) in comparison to those receiving (2 Gy) prior to TIL infusion resulted in a maximal 72% response rate in comparison to 51% (80, 91). These results provide a good example of how appropriately designed pre-clinical models can inform and potentially improve clinical studies in patients.

Cytokine priming of T cells

The generation of TILs and tumor specific T cells in vitro was radically changed by the introduction of recombinant IL-2. However, since the discovery and cloning of IL-2, additional cytokines relevant to T cell homeostasis had been identified. IL-2 is a member of the common gamma (γ) – chain cytokine family. IL-2 enhances and stimulates the initial proliferative burst

and survival of T cells, but also induces activation induced cell death. All members of this family share this common-γ chain in their receptors (CD132). Two more members of this family, IL-7 and IL-15, regulate the homeostatic proliferation and survival of memory CD8+ T cells. While the functional receptor for IL-7 is composed of the IL-7 receptor α chain (CD127) with the common-γ chain, the receptor for IL-15 is composed of the IL-15 receptor α chain in addition to a β-chain (CD122) that it shares with IL-2 and the common-γ chain. Finally, the receptor for IL-21 is composed of the IL-21 receptor α chain and the common-γ chain. While IL-2, IL-7, and IL-15 activate STAT5A and STAT5B signaling, IL-21 mainly activates STAT1, STAT3, and to a lesser extent, STAT5A/B (92). Though IL-12 exerts many of its effects on B-cells, it has also been shown to be a critical component of CD4 help for CD8 T cell memory formation (93). Whether these cytokines could differentially program adoptively transferred cells prior to adoptive transfer remained unknown.

IL-15

IL-15 priming of adoptively transferred tumor specific T cells dramatically increased antitumor activity (94). Phenotypically these cells resembled central memory – like T cells while, not surprisingly, their IL-2 counterparts resembled effector memory T cells. An additional study revealed that these T cells retained a lymph node homing phenotype, as characterized by the expression of multiple integrins and homing markers, which was responsible for the therapeutic efficacy of IL-15 primed T cells (95). These studies suggested that the inability of IL-2 generated cells to persist in vivo could be due to an inability to traffic to lymph nodes and receive proper antigen presentation in vivo. A lack of proper costimulation and survival signals could be remedied by the addition of IL-15 either in vitro during the priming and activation of these cells, or by exogenous administration following adoptive transfer. Further, these studies suggested that specific cytokines could radically change the phenotype and function adoptively transferred cells making them more suited for an antitumor response.

IL-7

IL-7 is another member of the γ – chain cytokine family that is involved in the homeostatic maintenance of naïve and memory T cells. However, whether the survival signals IL-7 could impart to CD8+ T cells could increase fitness and antitumor activity following adoptive transfer was still unknown. These questions were addressed in a tumor model expressing a viral peptide from lymphocytic choriomeningitis virus, gp33 (96). Administration of IL-7 with a vaccine or an adoptive transfer therapy increased the ex vivo survival and cytolytic activity of tumor specific T cells and controlled tumor growth in vivo. This enhanced immune response was characterized by the downregulation of multiple negative regulators, such as TGF-β receptor II and Cbl-b, as well as a decrease in serum TGF- β and an increase in the inflammatory cytokines, IL-12p70 and TNF. IL-7 also enhanced T cell survival and function in a mouse model of chronic viral infection and a humanized mouse model of HIV (97, 98). Similar to the administration of IL-7, the cellular availability of IL-7 following increasing doses of lymphodepletion increases T cell survival and antitumor reactivity. However, toxicities and effects on long term immune function related to high doses of lymphodepleting chemotherapies and irradiation makes IL-7 a more suitable adjuvant. In order to mimic the biological effects of lymphodepletion, IL-7 and T regulatory cell depletion (anti-CD25 antibody) were combined with the adoptive transfer of Pmel T cells and showed superior tumor rejection in comparison to irradiation induced lymphopenic hosts (99). Initial dose escalation trials with IL-7 in humans has confirmed it is well tolerated and can significantly increase absolute lymphocyte counts and broaden T cell repertoire diversity (100). This preliminary study does not provide any indications that IL-7 has antitumor activity as a single agent, but shows indications that it may enhance other immunotherapy strategies.

IL-21

IL-21, another member of the γ – chain cytokine family, is generally produced by CD4+ T cells and its receptor is found on T cells, B cells and NK cells. It has a profound effect on B cells and antibody production, but its role in the effector function of CD8+ T cells during an antitumor immune response was not clear (92). In order to address this, Pmel T cells were cultured in the presence of IL-21 and analyzed for cytolytic activity in vitro and in vivo (101). In vitro, IL-21 primed cells secreted low levels of IFN-γ and IL-2, and were unable to lyse peptide loaded cells. However, following in vivo expansion, IL-21 cells retained significant proliferative advantage with enhanced tumor destruction in comparison to IL-15 and IL-2 primed cells. IL-21 primed cells differentially expressed stem-cell like transcription factors and significantly downregulated the expression of a transcription factor associated with effector T cell fate, Eomesodermin. In addition to conclusions drawn from the priming of IL-15 cells, differentiation of CD8+ T cells into an effector fate with IL-2 imparts a phenotype associated with poor antitumor activity in vivo. Cytokines associated with a central memory like fate, IL-7, IL-15 and IL-21, all effectively retain the proliferative potential and eventual effector function of T cells and warrant further investigation.

IL-12

IL-12 is an inflammatory cytokine secreted by innate immune cells such as dendritic cells, macrophages, and neutrophils and can enhance the effector function of CD8+ T cells. Early studies implicated enhanced IFN-γ secretion by a combination of T and NK cells as a major component of IL-12 mediated antitumor activity. However, in a phase II clinical trial for the treatment of renal cell carcinoma, the hospitalization of 12 patients and the subsequent deaths of two patients following IL-12 administration cast a dark shadow upon the utilization of systemic IL-12 (102). In order to circumvent systemic toxicities, while still taking advantage of the potent antitumor properties of IL-12, the in vitro priming of T cells with IL-12 and the generation of genetically modified IL-12 secreting tumor specific cells were utilized successfully in preclinical

models. The in vitro priming of IL-12 Pmel T cells and mechanisms responsible for increased antitumor efficacy will be discussed in Chapter 2 of this dissertation. The genetic modification of tumor specific T cells to secrete IL-12 has been utilized in diverse tumor models with observed systemic changes in cytokines such as IFN-γ. Pegram et al achieved superior antitumor efficacy without the requirement for lymphodepletion prior to adoptive cell transfer (103). IL-12 secreting specific cells also became resistant to T regulatory cell mediated suppression and had enhanced lytic activity. In a separate study utilizing IL-12 secreting Pmel T cells, IL-12 altered the function of tumor infiltrating antigen presenting cells and allowed for increased cross presentation of cognate antigen (104). These findings demonstrate that the local delivery of IL-12 is safe, efficacious and able to modulate the function of tumor specific T cells and antigen presenting cells in a preclinical tumor model. Interestingly, studies of CD8+ T cell priming with IL-15 and IL-21 suggested that a central memory, stem cell like state was advantageous for antitumor immunity. However, IL-12 priming produces characteristically robust effector T cell phenotypes together with hallmarks of central memory, and challenges the conclusions derived from γ-chain cytokine studies. Whether priming with IL-12 or priming with a γ-chain cytokine is more efficacious has yet to be addressed.

T cell aging

Telomeres are long nucleotide repeats located on the ends of chromosomes. They function to prevent abnormal fusions, recombinations, or degradation of chromosomes undergoing replication. Upon each cell division, the length of a telomere becomes progressively shorter and, in humans, ultimately leads to cellular senescence (105). This is not the case in rodents, which use telomere independent mechanisms to determine senescence. Stem cells, germ cells, and some activated lymphocytes do not undergo this progressive shortening. In humans, naïve lymphocytes typically have the longest telomeres; however subsets of CD8+ effector T cells in the periphery appear to have longer telomeres than naïve CD8+ T cells (106).

Furthermore, CD28 costimulation can maintain telomere lengths in T cells, demonstrating a crucial role for the proper costimulation of T cells and enhanced in vivo persistence and survival (105). The homeostatic cytokines, IL-7 and IL-15, can also prevent shortening of telomere length (107). Such findings warrant future studies to test whether the enhanced antitumor activity of cytokine primed, adoptively transferred CD8+ T cells is dependent strictly on telomere length. Clinically, melanoma patients receiving TILs with longer telomere lengths were significantly more likely to have an objective clinical response. Expression of CD28 and in vivo persistence was also associated with longer telomere lengths (108). In addition, the short-term culture of TILs, for the generation of "young" TILs with presumably longer telomeres, was also associated with an increase in objective responses (109). Whether costimulation through receptors other than CD28, such as OX-40, 4-1BB, or CD27, have the same ability to influence the telomere length and survival of adoptively transferred tumor specific CD8+ T cells has yet to be defined.

Regulators of T cell costimulation

The three signal model dictates that three separate components are necessary for the proper activation of T cells; 1) T cell receptor triggering 2) costimulation provided by an antigen presenting cell and 3) cytokine polarization. However, peripheral tolerance or the termination of a T cell response depends upon signaling through negative regulators of costimulation in order to prevent unwanted autoimmunity and associated tissue damage. Tumors, as well as viral infections, take advantage of these immune checkpoints in order to evade and incapacitate immune effector function (110). Antibody blockade of cytotoxic T lymphocyte associated antigen-4 (CTLA-4) and programmed death-1 (PD-1), has become a viable therapeutic option to reverse T cell exhaustion and impaired immune functions. Expressed on activated T cells, CTLA-4 binds its ligands CD80 and CD86 with a 100 fold higher affinity than CD28. A single dose administration of anti-CTLA-4 in preclinical studies increased CD8+ T cell effector function,

memory formation, and reversed tolerance (111, 112). A blocking antibody to CTLA-4 demonstrated a 4 month increase in the median overall survival of metastatic melanoma patients and received FDA approval (113). Provided in combination with adoptive cell transfer, this antibody has the potential to prolong the function and proliferation of tumor specific T cells and even further increase survival. Results from clinical trials for an antibody blocking the negative regulator PD-1 have also reported an objective response rate of 28% in metastatic melanoma patients when used as a single agent (114). Preclinical studies have indicated that blockade of PD-1's ligand, PD-L1, in combination with the adoptive transfer of Pmel T cells can be highly efficacious (115). Groups treated with adoptive cell transfer and anti-PD-L1 demonstrated enhanced antitumor activity and an increase in the activation and number of tumor infiltrating lymhocytes. Taken together, these results indicate that the ability to overcome immune dysfunction with antibody therapies can enhance T cell based therapies.

On the opposite side of negative costimulation, agonistic antibodies specific for costimulatory receptors involved in proliferation, memory formation and effector function have provided excellent targets to enhance tumor specific T cell function. OX40 and 4-1BB are TNF superfamily receptors that are expressed on T cells following activation. Signaling through these receptors can provide robust survival signals through Bcl-2 family members and can induce inflammatory cytokine secretion (116). In vivo triggering of OX40 with an anti-OX40 antibody treatment prolongs proliferation, enhances IL-2R (CD25) expression, and increases cytolytic activity of adoptively transferred CD8+ T cells. More importantly, anti-OX40 augments tumor regression by tumor specific CD8+ T cells in an in vivo tumor model (117). In Chapter 3 of this dissertation, we hypothesize that treatment with a histone deacetylase inhibitor in combination with an adoptive cell transfer therapy enhances anti-tumor activity through the upregulation of OX-40. Similar to OX40, 4-1BB provides costimulation to CD8+ T cells and enhances IFN-γ production and proliferation in an IL-2 independent manner (118). Utilization of an agonistic antibody targeting 4-1BB can augment anti-tumor activity of adoptively transferred CD8+ T cells

by enhancing their survival in a CD4+ T cell independent fashion (119). Furthermore, combined blockade of a negative regulator and stimulation through a costimulatory receptor has demonstrated enhanced tumor rejection than either therapeutic alone. Agonistic 4-1BB in combination with a CTLA-4 blockade in a Flt3L based melanoma tumor vaccine increases tumor infiltration by lymphocytes and shifts the balance of these cells to produce high effector to T regulatory cell ratios. These cells are highly functional, produce both IFN-γ and TNF, and are mostly Ki-67+ (120). Other mechanisms to promote proper costimulation in tumor specific T cells include the genetic engineering of lymphocytes to redirect T cell specificity and co-express 4-1BB with a tumor specific TCR (121). Regulating the coinhibitory and costimulatory molecules on antigen specific T cells raise another possibility to enhance adoptive cell transfer therapies in an adjuvant setting or by enhancing the quality of transferred cells through genetic engineering.

T effector to T regulatory cell ratio

Numerous studies have demonstrated that the ratio of effector T cells to T regulatory cells in the tumor can influence the clinical outcome. Furthermore, the ability to modulate this ratio therapeutically determines the quality of the antitumor response and subsequent tumor regression. In an example of this, Bui et al characterize the ensuing immune response in a syngeneic tumor model and the role of tumor infiltrating T regulatory cells within this model (122). Groups receiving tumors that were not rejected in syngeneic hosts (progressors) had a higher overall percentage of T regulatory cells in comparison to groups receiving tumors that were rejected in syngeneic hosts (regressors). Transient depletion of T regulatory cells resulted in decreased progressor type tumor growth and led to an increase in actively dividing CD4+ and CD8 T+ cells within the tumor. This data demonstrates the ability to modulate intratumoral lymphocyte populations as a potential mechanism to enhance an active vaccination. This hypothesis was explored in a model examining the therapeutic efficacy of a GM-CSF tumor vaccine (Gvax) in combination with CTLA-4 blockade (123). In this case, treatment with Gvax

and CTLA-4 blockade increased CD4+ and CD8+ T cell effector populations while reducing T regulatory cells in an established B16 melanoma tumor. These results directly correlated with tumor rejection. In vitro, T regulatory cells inhibited tumor specific IFN-γ secretion from CD8+ T cells isolated from Gvax/anti-CTLA-4 treated mice, demonstrating that CTLA-4 blockade may provide the therapeutic means necessary to counter T regulatory cell suppression. However, it remained unclear whether CTLA-4 blockade was targeting effector cells or T regulatory cells. Subsequent studies were able to elegantly demonstrate that blockade of both compartments was necessary for maximal tumor rejection (124). Furthermore, the ability of T regulatory cells to mediate suppressive activity through downregulation of CD80 and CD86 on dendritic cells illustrates the utility of CTLA-4 blockade in an active antitumor response (125). Modulation of T regulatory cell activity and/or number in vivo through lymphodepletion also represents a potent avenue to increase antitumor reactivity, which is seen in the case of 900 cGy irradiation prior to Pmel T cell adoptive transfer (89). We discuss the ability of an HDAC inhibitor in modulating T effector to T regulatory cell ratios in the periphery and intratumorally in an adoptive cell transfer model in Chapter 3.

Polyfunctional T cells

Tumor specific T cells can be generated under a number of different priming conditions. Their relative function and efficacy in vitro and in vivo has been typically assessed by IFN-γ secretion, proliferation, and cytolytic killing. In addition, the quality of tumor specific T cell responses can be quantified in terms of simultaneous production of numerous cytokines, also known as a polyfunctional response. This is especially important in a vaccination protocol, where the quality of the antitumor response can only be measured by patient survival. We and others have found that the generation of polyfunctional T cells is predictive of a more efficacious immune response. In the case of patients receiving an anti-CTLA-4 antibody, generation of tumor associated antigen (NY-ESO-1) specific T cells that could simultaneously secrete IFN-γ,

TNF, and MIP-1β could be measured in clinical responders, while nonresponders failed to mount a polyfunctional response (126). Pre-clinically, tumor eradication with Pmel T cells was enhanced when the generated cells were able to simultaneously secrete IFN-γ and TNF and undergo degranulation (CD107a+) (127). In the case of IL-12 primed tumor specific T cells, higher absolute numbers of polyfunctional T cells are generated following vaccination and this correlates with enhanced tumor control (Chapter 2). Similarly, treatment with an HDACi enhances the percentage and absolute number of tumor specific T cells following vaccination (Chapter 3). This suggests that a vaccination protocol must generate an adequate number of polyfunctional T cells through an enhanced cytokine environment or pharmacological intervention in order to successfully mediate tumor regression.

Conclusions

From the earliest studies examining the immunological mediators of tumor growth to the genetic engineering of tumor specific lymphocytes, the field of tumor immunotherapy has transitioned from observations and anecdotes to fully personalized medicine. In 2010, the approval of Sipuleucel-T/Provenge, an antigen specific dendritic cell vaccine for prostate cancer, opened the door for cellular based immunotherapeutics (128). In order for adoptive cell transfer therapies to become feasible, there are still many areas of research to be addressed. For example, the availability of tumor specific antigens is still problematic and highlights the need for further antigen discovery. Although some tumor associated antigens are immunogenic and shared across various malignancies, some of the most common shared tumor antigens are still self antigens and can result in autoimmunity and/or tolerance. The need to define broadly applicable immunogenic targets remains crucial, without which new vaccines cannot be designed and tested. Furthermore, the issue of narrow vaccines that target single tumor antigens have not shown the same efficacy as a broadly reactive TILs therapy. However, generation of a sufficient number of TILs is largely restricted for the treatment of melanoma and

renal cell carcinoma. To maximize individual responses, identifying and targeting multiple antigenic targets from a wide variety of tumors is necessary to make this therapy successful.

The ability to assess the relative responsiveness or health of an individual's immune system has remained an active area of interest. Using mass cytometry, thousands of signaling events in response to cytokine stimulation on a diverse range of immune cell subsets can be monitored (129). Utilizing information from this type of assessment will allow for a true measure of polyfunctional T cell status prior to and following adoptive cell transfer. From this data, patient populations that are more likely to respond to a specific therapy can be characterized through the identification of relevant biomarkers. Furthermore, the possibility to tailor the correct adjuvant or cytokine environment (IL-7 T cell priming vs. IL-21 T cell priming) through these biomarkers may increase the low rates of efficacy seen for the adoptive transfer of antigen specific lymphocytes.

The field of tumor immunotherapy has grown immensely through an understanding of basic immunology and human biology. Adoptive cell transfer therapies have demonstrated the ability to eradicate large tumors in multiple anatomical sites. By continuing to understand the antitumor immune response through preclinical and clinical studies, adoptive cell transfer therapies have demonstrated clear successes and a path towards promising improvements.

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Chapter 2:

Enhanced sensitivity to IL-2 signaling regulates the clinical responsiveness of IL-12 primed CD8+ T-cells in a melanoma model.

Introduction:

The adoptive transfer of high numbers of tumor reactive T-cells has matured into a feasible and promising anti-tumor therapy in patients with cancer (1). However, generation of T-cells with the ability to persist, traffic and maintain effector function following adoptive transfer has limited the effectiveness of this treatment strategy. *In vitro* cytokine priming allows for the programming of differentiated CD8+ T-cells with distinct phenotypes and *in vivo* characteristics (2-5). Thus, understanding the programs imparted to T-cells by diverse cytokine families has become an integral part in understanding and employing successful T-cell based therapies. Interleukin-2 was the first cytokine used to generate activated effector cells capable of lysing tumor cells *in vitro* and mediating tumor regression *in vivo* (6). However, such terminally differentiated effector cells failed to persist *in vivo* and control tumor growth in mouse models (7). Recent work in our own group demonstrated the ability of a Toll-like receptor 7 (TLR7) agonist, imiquimod, in combination with a dendritic cell vaccine to enhance priming of tumor specific CD8+ T-cells (8). We hypothesized that imiquimod augmented anti-tumor immunity by inducing proinflammatory cytokines such as type I interferons, TNFα, and IL-12 (9-12). However, the mechanism by which these proinflammatory cytokines induced superior priming of tumor specific CD8+ T-cells remained unclear.

Cytokines released by appropriately activated dendritic cells serve as the final requirement for acquisition of CD8+ T-cell functionality. In particular, the cytokine, IL-12, has been shown to be a potent third signal (13-16). CD8+ T-cells lacking this third signal become tolerant and fail to attain cytolytic effector function (17). This becomes an important consideration in designing tumor immunotherapy protocols, if one accounts for a tumor environment that fosters a lack of pro-inflammatory signals, poor antigen presentation and an overwhelmingly anti-inflammatory milieu (18-21). IL-12 has been shown to be a potent mediator of anti-tumor immunity by influencing the function of innate and adaptive immune responses and demonstrating anti-

angiogenic properties (22-24). However, high systemic doses of IL-12 with the ability to reach therapeutic levels in the tumor site are not very well tolerated (25, 26). In order to circumvent this obstacle, we directly primed tumor antigen specific CD8+ T-cells with IL-12 *in vitro*. This allowed us to dissect the phenotypic changes by which IL-12 could augment anti-tumor immunity induced by CD8+ T-cells.

Utilization of the Pmel-1 T-cell receptor transgenic mouse has previously allowed us to model adoptive transfer immunotherapy in an established subcutaneous and intracranial tumor model (27, 28). Pmel-1 T-cells express a single transgenic TCR (T-cell receptor) that recognizes the $gp100_{25–33}$ H-$2D^b$–restricted epitope of gp100 (29). This endogenous tumor associated antigen is expressed by murine B16 melanomas, making this a clinically relevant model to study novel adoptive transfer therapies (30). Malignant melanoma is one of the most common solid tumor types to metastasize to the brain, and results in significantly decreased survival and exclusion from most clinical trials (31). Clinically, little advancement has been made in effective treatment courses for tumors developing within the confines of the immune privileged CNS. Although the standard of care consists of surgical resection, chemotherapy and radiation, these options often do very little to prolong survival. However, recent work has demonstrated the efficacy of immune based therapies as a new option for clinicians (32-35). The specificity and durability of tumor immunotherapy can theoretically provide a new avenue to target tumor cells while sparing normal brain tissue. Even more promising, many pre-clinical models and successful clinical trials have demonstrated meaningful anti-tumor immune responses (36-39).

Given that immune based therapies are beginning to grow into a feasible treatment modality for metastatic melanoma, combined with the importance of refining therapeutic strategies in humans, we have addressed the functional importance of priming CD8+ T-cells with IL-12 prior to adoptive cell transfer. We used the Pmel-1 TCR transgenic model to establish the *in vitro* and *in vivo* effects of priming tumor specific CD8+ T-cells with IL-12. However, we expanded

our findings to show similar phenotypic and functional changes in human PBMCs transduced with a tumor specific TCR and primed with IL-12. Our studies reveal what we believe are the critical determinants of therapeutic efficacy for enhanced anti-tumor T-cell responses and directly applies to the design of future clinical immunotherapy strategies.

Results:

Priming with antigen and IL-12 imparts a distinct lymph node homing phenotype and functionally enhances CD8+ T-cells.

Administration of the TLR7 agonist, imiquimod, as an adjuvant for immunotherapy in the CNS tumor setting previously demonstrated enhanced DC trafficking and priming of adoptively transferred CD8+ T-cells (8). We hypothesized that cytokine secretion induced by TLR activation played a significant role in functionally enhancing tumor specific CD8+ T-cell priming. To investigate these issues in greater depth, we primed naïve, gp100 specific splenocytes *in vitro* with hgp100 peptide in the presence of either IL-2 or IL-12. In comparison to priming CD8+ T-cells in the presence of IL-2, priming with IL-12 significantly increased expression of L-selectin (CD62L) and the integrin α_4 chain (Fig. 2.1A). No change was observed in the expression levels of the memory marker CD44.

In order to more fully characterize the gene expression patterns of IL-12 and IL-2 primed T-cells, we obtained RNA from IL-2 and IL-12 antigen primed Pmel-1 T-cells rested in their respective cytokine and examined global gene expression levels by cDNA microarray analysis (Fig. 2.1B). This analysis revealed a striking difference between cells primed in the presence of IL-2 or IL-12. Primarily, IL-12 imparts a strong effector program by upregulating the high affinity IL-2 receptor α-chain (*Il2ra)*. Furthermore, genes associated with cytotoxic effector function such as Interferon-γ (*Ifng)*, Perforin (*Prf1)*, and Granzyme K (*Gzmk)* were upregulated at least 2-fold ($p<0.05$) in relation to IL-2 primed T-cells. IL-12 priming also increased the expression of Killer

cell lectin-like receptor G1 (*Klrg1*), and the transcriptional repressor B-lymphocyte induced maturation protein-1 (Blimp-1, *Prdm1*) while downregulating the expression of the chemokine CCR7 (*Ccr7*) and the transcription factor Eomesodermin (*Eomes*) ($p<0.05$). This differential regulation of KLRG1, Blimp-1, CCR7 and Eomes suggests that IL-12 priming induces a distinct effector program that has been shown to be characteristic of short lived effector T-cells (46). Furthermore, loss of the WNT pathway transcription factor T-cell factor 1 (*Tcf7*) is highly indicative of antigen experienced mature CD8+ T-cells (47). This gene expression pattern would classically align IL-12 primed T-cells to a more highly differentiated effector phenotype (T_{em}) than those primed with IL-2. However, this differentiation program is surprisingly in disagreement with the high expression of L-selectin and CD49d (Fig. 2.1A), markers commonly reserved for naïve or quiescent central memory T-cells.

In addition to phenotypic changes conferred by priming in the presence of IL-12, we also examined functional characteristics in IL-12 primed CD8+ T-cells. We assessed *in vitro* effector function by characterizing the ability of IL-2 and IL-12 primed T-cells to retain a 'polyfunctional' phenotype. Recent publications have demonstrated a positive correlation between clinical outcome and the presence of polyfunctional T-cells (48, 49). These cells retain the ability to simultaneously secrete a diverse number of cytokines and cytolytic activities in response to antigen encounter. To test this, we re-stimulated T-cells for varying lengths of time in the presence of hgp100 peptide pulsed antigen presenting cells. CD8+ T-cells primed with IL-12 mobilized a greater number of cells to simultaneously express the cytokines IFN-γ, TNF-α, and the degranulation marker, LAMP-1 (CD107a) (Fig. 2.1C and 2.7). Furthermore, IL-12 primed cells also mobilized this polyfunctional trait sooner than their IL-2 primed counterparts.

The ability to mediate potent effector responses is one hallmark of CD8+ T-cells. However, the ability to mediate long-term protective immunity also governs the efficacy of a T-cell response. Previous studies have shown the role of IL-12 responsiveness to be critical in the maintenance

of antigen specific T-cells (50). Herein, we investigated the role of IL-12 priming in the formation of memory self-antigen specific CD8+ T-cells. Equal numbers of IL-2 or IL-12 primed CD8+ T-cells were adoptively transferred into mice that were irradiated the day before. One week later, groups were re-vaccinated with dendritic cells pulsed with gp100 peptide. Recall responses were measured in mice receiving a dendritic cell vaccination 40 days later, and compared to mice not receiving a re-vaccination. Groups that received IL-12 primed T-cells showed a significantly larger number of T-cells remaining (Fig. 2.1D). Groups that received IL-12 primed T-cells in addition to a dendritic cell vaccination on day 47, also showed significantly higher numbers of antigen specific memory T-cells remaining. This clearly demonstrates the ability of IL-12 priming to confer a significant survival advantage *in vivo* by maintaining a large population of antigen specific cells long after initial expansion. More importantly, IL-12 priming plays a dual role in the differentiation of CD8+ T-cells. In comparison to IL-2, IL-12 signaling not only induces a strong recall to antigen, but also mediates the ability to acquire a protective and diverse effector response.

IL-12 potentiates the IL-2 signaling pathway in CD8+ T-cells

In addition to having a distinct memory phenotype, it is known that IL-12 primed OVA-specific CD8+ T-cells demonstrate an upregulation of CD25 during homeostatic proliferation (51). Similarly, in the case of activated effector cells, we have found that IL-12 priming of gp100 tumor-specific Pmel-1 T-cells results in an immediate upregulation of the high affinity IL-2 receptor (CD25) (mean MFI=1309, SEM 221.2) in comparison to IL-2 primed cells (mean MFI=195, SEM 38.4) (Fig. 2.2A). The presence of CD25 within the IL-2 receptor complex drastically lowers the concentration of IL-2 required to illicit downstream signaling events, which can be monitored by measuring the levels of phophorylated STAT5 (pSTAT5) in response to stimulation with exogenous IL-2. IL-12 primed T-cells stimulated with IL-2 show high levels of phosphorylated STAT5 when stimulated with as little as 500 pg/mL of IL-2 (Fig. 2.2B). These

high levels of phosphorylated STAT5 were not observed in IL-2 primed T-cells at any concentration level of IL-2. As such, IL-12 primed Pmel-1 T-cells show a higher level of STAT5 phosphorylation events in response to IL-2 stimulation across all concentration levels tested. In order to concretely assess whether the increased expression of CD25 confers enhanced responsiveness to exogenous IL-2, cells were treated with anti-CD25 blocking antibody (clone PC61.6.3) prior to IL-2 stimulation. CD25 antibody blockade of IL-12 primed Pmel-1 T-cells significantly dampened IL-2 dependent STAT5 phosphorylation events at all concentration levels (Fig. 2.2B). In addition to testing responsiveness to IL-2, we also tested the ability of IL-12 primed CD8+ T-cells to respond to other common γ-chain cytokines using STAT5 as a downstream transducer. Both IL-2 and IL-12 primed T-cells showed similar levels of phosphorylated STAT5 in response to IL-7 and IL-15 (Fig. 2.2B). Thus, IL-12 priming specifically enhances perception of the IL-2 signal and not that of other closely related cytokines. However, the expression of CD25 may not be the only important factor in determining enhanced responsiveness to IL-2. We also checked for total protein levels of STAT5 present in IL-2 and IL-12 primed T-cells by Western blotting. IL-12 primed T-cells show a 1.6 fold change in the overall expression of unphosphorylated STAT5 (Fig. 2.2C). This indicates an overwhelming enhancement in both the perception and transduction of IL-2 by IL-12 primed CD8+ T-cells.

IL-12 priming increases *in vivo* proliferation at low systemic levels of IL-2

IL-2 is a potent stimulator of T-cell clonal expansion *in vivo*. Because *in vitro* results demonstrated enhanced IL-2 signaling, we asked whether IL-12 primed CD8+ T-cells showed greater proliferation at lower concentrations of IL-2 after adoptive transfer *in vivo*. To test this question, we labeled IL-2 or IL-12 primed T-cells with CFSE and adoptively transferred an equal number of these cells into lymphodepleted mice. Lymphopenia was induced by 500 cGy of whole body irradiation. The recipient mice were then vaccinated with hgp100 peptide pulsed

dendritic cells and either supported with i.p. injections of IL-2 at a high dose ($5x10^5$ IU), low dose ($1x10^5$ IU), or without IL-2. Three days after adoptive transfer, spleens were analyzed *ex vivo* for CFSE dilution. At the high dose, low dose, and absence of IL-2, IL-2 primed T-cells exhibited lower CFSE dilution (MFI = 422.4 +/- 20.9, 966.9 +/- 52.7, 6991 +/- 278.1 respectively) compared with IL-12 primed Pmel-1 T cells (MFI = 164.9 +/- 3.3, 191.4 +/- 5.2, 1314 +/- 52.3 respectively). IL-12 primed cells displayed complete dilution of CFSE at high and low doses of systemic IL-2 and a moderate level of CFSE dilution without IL-2 support (Fig. 2.3A). When we evaluated the absolute number of T-cells that could be obtained ten days after adoptive transfer, the number of CD8+ T-cells was significantly increased in treatment groups receiving IL-12 primed cells versus IL-2 primed cells. Mice that received IL-12 primed T-cells supported with high dose IL-2 possessed, on average, a four fold higher number of adoptively transferred cells compared with groups receiving IL-2 primed T-cells ($p<0.01$) (Fig. 2.3B). Groups of mice that received IL-12 primed T-cells, supported with a low dose of systemic IL-2, had a fivefold higher number than groups receiving IL-2 primed T-cells ($p<0.05$). These results demonstrate the stark differences underlying utilization of exogenous IL-2 in the two priming conditions. Clearly, IL-12 primed T-cells have an enhanced ability to persist and respond to IL-2 in a lymphopenic environment.

IL-12 cytokine priming stimulates proliferation and trafficking into diverse anatomical regions

Our data suggested that treatment with IL-12 primed T-cells showed increased T-cell proliferation *in vivo.* However, we also wanted to explore the possibility of T-cell trafficking through other relevant anatomical locations at different times. In order to accomplish this, we engineered T-cells, primed in the presence of either IL-2 or IL-12, to express firefly luciferase (Fig. 2.8). This gave us the ability to non-invasively monitor trafficking of adoptively transferred T-cells *in vivo* in real time. Furthermore, *in vivo* bioluminescent imaging allowed us to quantify

the relative number of adoptively transferred T-cells at discrete anatomical locations. Transduced T-cells were adoptively transferred into mice with established subcutaneous B16-F10 tumors in the lower left flank (Fig. 2.4A). The results became striking when comparing groups receiving IL-2 and IL-12 primed T-cells. When IL-12 primed T-cells were transferred, total flux (photons/second) was nearly 1-log fold higher in the inguinal lymph nodes and at the tumor site compared with IL-2 primed T-cells (Fig. 2.4B). Furthermore, we observed the characteristic proliferative burst of T-cells shortly following a dendritic cell vaccination one week following adoptive transfer (27, 52). This secondary expansion of T-cells following vaccination was significantly more pronounced in groups receiving IL-12 primed T-cells in comparison to IL-2 primed T-cells. Shortly thereafter, imaging showed the eventual contraction of these cells to the tumor draining lymph node, the neighboring inguinal lymph node and the bone marrow. Ultimately, real time imaging demonstrated that although IL-2 and IL-12 primed T-cells displayed nearly the same kinetics of expansion and contraction, IL-12 significantly increased T-cell proliferation and trafficking.

IL-12 primed CD8+ T-cells show superior anti-tumor activity in intracranial and subcutaneous tumor models

The ability of tumor associated antigen specific CD8+ T-cells to proliferate *in vivo* and retain effector function after adoptive transfer still remains a challenge in immunotherapy. Previously, we demonstrated that a tripartite treatment regimen, consisting of IL-2 primed T-cells, gp100 peptide pulsed dendritic cell vaccination, and a high dose of systemic IL-2, significantly prolonged survival in an intracranial tumor model (27). In order to evaluate whether IL-12 primed gp100 specific CD8+ T-cells were capable of exhibiting anti-tumor activity, we adoptively transferred IL-2 or IL-12 primed CD8+ T-cells into nonlethally irradiated mice bearing 7 day established intracranial B16-F10 melanoma tumors. When $2.5x10^5$ IU of systemic IL-2 was utilized with adoptive transfer and DC vaccination, the median survival was 30 and 45 days with

IL-2 and IL-12 primed cells, respectively ($p=0.0017$, Mantel-Cox). With a systemic dose of 1.0×10^5 IU IL-2, the median survival of groups was 26.5 and 44 days with IL-2 and IL-12 primed T-cells respectively ($p=0.0025$, Mantel-Cox) (Fig. 2.5A). In addition, we also utilized this tripartite treatment with 10-day established subcutaneous B16-F10 melanoma tumors. Groups that received IL-12 primed T-cells showed a significantly decreased tumor size in comparison to groups receiving IL-2 primed cells, or radiation alone (Fig. 2.5B). Administration of an anti-CD25 blocking antibody after adoptive transfer did not significantly alter the growth of tumors treated with IL-2 primed T-cells. However, the same anti-CD25 blocking antibody significantly reduced the anti-tumor activity of IL-12 primed T-cells (Fig. 2.5B).

As demonstrated previously in non-tumor bearing mice, groups receiving IL-12 primed T-cells possessed significantly increased absolute numbers of T-cells (1.8×10^6 cells) two weeks post-adoptive transfer in comparison to groups receiving IL-2 primed T-cells (6.8×10^4 cells, $p<0.01$) (Fig. 2.5C). Thus, IL-12 priming of CD8+ T-cells appears to impart an instructional program that allows for efficient utilization of IL-2. However, expansion of adoptively transferred cells does not necessarily correlate with the ability of these cells to display anti-tumor activity. In order to ascertain that these cells retained the ability to functionally respond to their cognate antigen, splenocytes from adoptively transferred mice were restimulated *ex vivo* with gp100 peptide and stained for the polyfunctional markers IFN-γ, TNF-α, and CD107a. Groups treated with IL-12 primed T-cells possessed a greater number of polyfunctional T-cells (1.8×10^6) compared to groups treated with IL-2 primed T-cells (6.8×10^4, $p<0.01$) (Fig. 2.5E). Thus, this significant expansion of tumor specific T-cells expands cells with diverse function. More importantly, we were interested to test whether an increase in functional IL-12 primed cells could show enhanced accumulation within tumors. Thus, tumor infiltrating lymphocytes (TIL) were stained and analyzed. The density of adoptively transferred TIL (Thy1.1+CD8+ TIL/milligram of tumor) was significantly greater in groups that received IL-12 primed T-cells (5.3 cells/mg of tumor) in

comparison to groups that received IL-2 primed T-cells (0.7 cells/mg of tumor, $p<0.05$) (Fig. 2.5E). This lends further support to the idea that the expansion and trafficking of highly functional T-cells is critical for producing effective anti-tumor immunity.

Priming of tumor specific human PBMCs with IL-12 produces potent effector cells

The efficacy of adoptive cell transfer relies upon the maintenance of T-cells with the ability to both rapidly proliferate *in vivo* and retain effector function. Although we have demonstrated these enhanced effector functions in a murine model, we wished to demonstrate the ability of IL-12 to mediate its protective effects in human tumor specific T-cells. Utilizing a clinically relevant retroviral transduction system, we expressed a gp100-specific T-cell receptor in normal human PBMCs (43, 53). Although this chimeric TCR contains constant regions of murine origin, the variable regions are of human origin and specifically recognize human $gp100_{154\text{-}162}$ in the context of HLA.A*0201 (54). PBMCs were initially activated utilizing a stimulatory anti-CD3 antibody (clone OKT3) in the presence of either IL-2 or IL-12. We then examined the resulting IL-2 and IL-12 primed TCR transduced PBMC for differences in their phenotype and their similarity to IL-12 primed antigen specific murine T-cells. Within the CD8+ T-cell population, approximately 80% of cells expressed the chimeric gp100 T-cell receptor as indicated by positive staining for mouse T-cell receptor-β (mTCRb) (Fig. 2.6A). Similar to results seen in the priming of mouse CD8+ T-cells, transduced human CD8+ T-cells primed in the presence of IL-12 showed an upregulation of both CD62L and CD25 (Fig. 2.6A). Furthermore, upon re-stimulation with antigen, cells primed in the presence of IL-12 showed a more diverse effector phenotype in comparison to cells primed in the presence of IL-2. This effector phenotype was derived from a significant increase in the simultaneous expression of IFN-γ and TNF-α in response to antigen (Fig. 2.6B). These results demonstrate the successful production of human tumor specific T-cells with an enhanced ability to mobilize effector cytokines when primed in the

presence of IL-12. In addition, these results highlight the ability of a prototypic inflammatory cytokine as an important component in mediating T-cell based anti-tumor immunity.

Discussion

Our present studies have demonstrated the superior activity of tumor-specific CD8+ T-cells primed in the presence of IL-12 for adoptive transfer immunotherapy. Use of IL-12 during *in vitro* priming significantly altered the phenotype, function, and memory formation of CD8+ T-cells. Increased expression of both the high affinity IL-2 receptor and its downstream transducer, STAT5, clearly potentiated the IL-2 signal. This efficient utilization of IL-2 translated into enhanced *in vivo* T-cell expansion and tumor infiltration. More importantly, administration of IL-12 primed T-cells led to decreased tumor burden in a subcutaneous tumor model, and increased survival in an intracranial tumor model compared with CD8+ T cells primed in IL-2. From these studies, we conclude that IL-12 priming imparts an instructional program to CD8+ T-cells that results in the development of potent effector cells with the ability to perceive proliferative signals *in vivo*, traffic, persist, and induce anti-tumor immunity.

Recent studies have demonstrated the importance of cytokines in programming the differentiation of naïve T-cells into functional effector cells (2, 3, 55, 56). Our use of IL-12 in these studies highlights the need for an instructive third signal in the expansion and use of tumor specific effector T-cells for adoptive immunotherapy (17, 20). Recent studies in the viral and tumor literature have also suggested an important link between ‘polyfunctional’ T-cell responses and control of disease progression (48, 49, 57, 58). These polyfunctional T-cells demonstrate the distinct ability to mobilize a diverse array of cytokines simultaneously in response to cognate antigen restimulation. Our results indicate that the IL-12 signal may potently diversify the functional repertoire. However, even more importantly, IL-12 signaling also potentiates the ability of T-cells to become more sensitive to cytokines such as IL-2, which

are important for expansion and survival *in vivo*. This combined phenotype may potentially be of significant use to the field of adoptive immunotherapy. Presently, high systemic doses of IL-2 are needed to support and maintain the *in vivo* proliferation of appropriately primed T-cells (59). Often, these maximally tolerated doses of IL-2 come at the risk of inducing vascular leak syndrome and other systemic side effects (60). As a result of IL-12 priming, adoptive cell transfer therapies could potentially avoid the side effects associated with high dose IL-2 and reduce the absolute number of T-cells required for potent anti-tumor activity.   As others have shown, production of IL-12 by a very low number of tumor specific T-cells increases their expansion even in the absence of IL-2 and vaccination (61). These characteristics could potentially enable adoptive immunotherapy approaches to overcome the technical and monetary hurdles of producing large numbers of functional tumor-specific T-cells for clinical use.

Although IL-2 remains a necessary component for adoptive immunotherapy protocols, the mechanisms by which STAT5 signaling promotes proliferation and possibly anti-tumor immunity have not been defined. Recent studies have shown enhanced memory formation in the presence of IL-12. Our own studies point to the rapid expansion of IL-12 primed antigen specific cells immediately following re-vaccination. However, such work has not distinguished whether IL-2 signaling influences *in vivo* T-cell persistence, memory formation, or effector function in IL-12 primed T-cells. Recent studies have implicated the strength of IL-2 signaling and the expression of CD25 as key determinants in formation of short-lived effector cells (62, 63). Nevertheless, we must take into account that CD8+ T-cell effector programming by an inflammatory cytokine, such as IL-12, may uncouple IL-2 signaling from directing formation of memory T-cells. Instead, enhanced IL-2 signaling may program highly proliferative IL-12 primed T-cells with the ability to traffic into the bone marrow and remain as long-lived memory cells.

Interestingly, our results have also indicated an increased expression of CD62L. Compounded with increased sensitivity to IL-2, CD62L expression has the capability to enhance lymphoid

organ trafficking and subsequent accessibility to antigen presenting cells. This marker is expressed immediately following IL-12 priming, but is slowly downregulated after adoptive transfer (data not shown) and *in vivo* expansion. This initial upregulation of CD62L confers a central memory-like phenotype to IL-12 primed CD8+ T-cells. Characteristically, long lived memory T-cells express a low level of differentiation which can be monitored by high levels of CD62L, CD127, and the transcription factors Eomes, and Tcf7. However, IL-12 priming imparts a more differentiated CD8+ T-cell phenotype, with high levels of the transcription factor Blimp-1 and the marker KLRG1. Furthermore, these T-cells express high levels of CD25, and quickly mobilize IFN-γ upon peptide re-stimulation. This phenotype is most commonly associated with effector memory T-cells (Tem), which more efficiently mobilize effector function than their quiescent Tcm counterparts. Thus, IL-12 programs a distinct phenotype to CD8+ T-cells that does not noticeably fit into traditional T-cell memory classification. Instead, our results suggest that the utilization of CD8+ T-cells for adoptive transfer with the ability to efficiently home to lymphoid organs and tumors sites, while remaining sensitive to homeostatic and proliferative cytokines, may be the critical determinants of therapeutic efficacy.

Materials and Methods:

Animals and cell lines

All mice were bred and kept under defined-flora pathogen-free conditions at the AALAC-approved Animal Facility of the Division of Experimental Radiation Oncology at UCLA. Mice were handled in accordance with the UCLA animal care policy and approved animal protocols. The B16-F10 murine melanoma cell line was obtained from the ATCC (Rockville, MD) and the Phoenix-ampho cell line was kindly provided by Caius Radu (University of California, Los Angeles, Dept. of Molecular and Medical Pharmacology).

Tumor implantation and irradiation

C57BL/6 mice (6–12 weeks of age) were implanted subcutaneously in the lower left flank with 1-2.5x10^5 B16-F10 melanoma cells per mouse. Intracranial implantations were done as previously described (30). Mice received 1 × 10^3 B16-F10 melanoma cells injected in a total volume of 2 ul PBS.

Prior to adoptive transfer, lymphopenia was induced by total body irradiation given at a nonmyeloablative dose (500 cGy).

In Vitro Activation of Pmel-1 T-cells

Lymph nodes and/or spleens were harvested from Pmel-1 mice and cultured with human IL-2 (100 U/ml, NCI Preclinical Repository, Developmental Therapeutics Program), or IL-12 (10 ng/ml, Peprotech, Rocky Hill, NJ), and hgp$100_{25–33}$ peptide (NH_2-KVPRNQDWL-OH, 1μg/ml, Biosynthesis, Inc., Lewisville, TX) in X-VIVO 15 (Lonza, Walkersville, MD) supplemented with 2% FBS. After 72 hours, cells were washed twice with PBS and re-cultured in IL-2 (100 U/ml) or IL-12 (10 ng/ml) at a concentration of 1x10^5 cells/ml for an additional 48 hours. Cells were washed twice with PBS and resuspended at 10 or 50x10^6 cells/ml and immediately injected i.v. via tail vein in 0.1 ml of PBS per mouse. All mice, with the exception of studies utilizing 5x10^6 CFSE labeled T-cells per mouse, were adoptively transferred with 1x10^6 T-cells each. T-cells were labeled with 5 uM CFSE according to manufacturer's specifications (Invitrogen). Prior to adoptive transfer, cells were analyzed by FACS in order to ensure that the MFI of each group was approximately equal and around 6000-8000 MFI.

Microarray analysis

Pmel-1 T-cells were stimulated as described above. Naïve Pmel-1 T-cells were obtained from CD8+ Thy1.1+ sorted Pmel-1 splenocytes. Shortly after the final 48 hour cytokine stimulation for IL-2 and IL-12 primed Pmel-1 CD8+ T-cells, RNA was obtained utilizing the RNeasy Mini kit (Qiagen). RNA from 2 replicate experiments from each group were labeled and hybridized to

Affymetrix Mouse Gene 1.0 ST arrays (UCLA DNA Microarray Core). Analysis was completed by calculating average intensities for each cytokine condition and determining differential gene expression by the Bioconductor R package, OneChannelGUI (40). Genes represented as differentially expressed show a fold change greater than 2 and a p-value < 0.05. All array data has been deposited into the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/ with the accession number GSE22443).

Retroviral Transduction of Pmel-1 T-cells and Human PBMCs

The Phoenix-ampho cell line was co-transfected with the retroviral packaging vector, pCL-Eco, and a retroviral vector containing firefly luciferase as previously published (27, 41, 42). Retroviral supernatants were obtained over a 12 hour period. After an initial 24 hours of activation, Pmel-1 T-cells were transduced by spin-fection with 1 mL of retroviral supernatant, 1.6 ug/mL polybrene (Sigma-Aldrich) and 2 ug/mL lipofectamine (Invitrogen) at 850 x g for 2 hours at 32°C. Spin-fection was repeated 24 hours later under the same conditions. Normal priming conditions were followed after transduction of Pmel-1 T-cells.

Human PBMCs were stimulated with anti-CD3 antibody (clone OKT-3) and either IL-2 or IL-12 for two days. Activated PBMCs were then transferred to plates containing supernatant from a PG13-based gp100 TCR stable retroviral packaging cell line obtained from Dr. Paul Robbins (Surgery Branch, NCI/NIH) (43). On the following day, cells were transferred to a fresh plate containing viral supernatant. Cells were then removed from viral supernatant on day 5 and then allowed to rest until they were analyzed on day 8.

Bone marrow-derived DC and Vaccination

The generation of dendritic cells (DC) from murine bone marrow progenitor cells was performed as previously published (30, 44). Briefly, bone marrow cells were initially cultured overnight in a Petri dish. On day 1, nonadherent cells were collected and plated in 24-well plates with murine

interleukin-4 (IL-4, 500 U/ml, R&D Systems, Minneapolis, MN) and murine granulocyte-macrophage colony stimulating factor (GM-CSF, 100 ng/ml, R&D Systems). On day 4, 50% of the media was removed and adherent cells were re-fed with an addition of 1 ml per well of RPMI + 10% FBS plus the same concentration of cytokines. DCs were harvested as the loosely adherent cells from the day-8 cultures. DC were resuspended at $2x10^6$ cells/ml in PBS and pulsed with human $gp100_{25–33}$ peptide at a concentration of 10 μM for 90 minutes at room temperature. After two washes in PBS, $hgp100_{25–33}$ peptide pulsed DC ($hgp100_{25–33}$/DC) were immediately prepared for injection in 0.2 ml of PBS per mouse. Injections were given s.c. at 4 sites on the back.

Bioluminescent imaging

Bioluminescent imaging was performed as previously published (27, 45). Mice were initially anesthesized with isofluorane or ketamine/xylazine. In order to minimize light absorption by black fur, mice were shaved prior to imaging. Seven minutes following intraperitoneal injection of D-luciferin (30 mg/ml), mice were imaged in a Xenogen IVIS imaging system coupled to a CCD camera. Mice were imaged for 7-20 minutes with an acquisition time of 8-10 seconds. Analysis of bioluminescent image data was completed with Living Image software. Regions of interest were drawn around discrete anatomical areas and used to calculate bioluminescent signal. This signal was expressed and graphed as total flux (photos/second).

Pmel-1 T-cell stimulation and intracellular FACS staining

Approximately $1x10^6$ Pmel-1 T-cells were stimulated at a 1:10 ratio with naïve C57BL/6 splenocytes pulsed with $hgp100_{25–33}$ peptide. GolgiPlug protein transport inhibitor (BD Biosciences) and APC conjugated anti-CD107a antibody (2 ug, clone 1D4B, BD Biosciences) were added to each well containing T-cells and/or pulsed splenocytes. Cells were stimulated at 37°C for 0-300 minutes. After the appropriate incubation time, cells were placed on ice in the

dark until all cells could be stained at the same time. Cells were washed with PBS containing 2% FBS and subsequently stained with surface markers. Following extracellular staining, cells were fixed with Fixation Buffer (eBioscience) and permeabilized with 1X Permeabilization Buffer (eBioscience). Intracellular staining was completed in 1X permeabilization buffer on ice in the dark. Cells were stored in 4°C until analysis.

Flow cytometry and antibodies

Spleens, lymph nodes and tumors were harvested from mice following adoptive transfer. Spleens and lymph nodes were passed through 70 um cell strainers to generate single-cell suspensions. Lymphocytes were obtained after hypotonic lysis and enumerated using trypan blue exclusion. A total of 1-2x10^6 cells in PBS with 2% FBS were used for the staining procedure. To determine the number of tumor infiltrating lymphocytes (TILs), tumors were carefully weighed and subsequently minced with a scalpel. The tumor was then placed on a rotator in collagenase with DNase for 2-3 hours. Small mononuclear cells within the tumor were enumerated by trypan blue exclusion. Approximately 1x10^6 lymphocytes were used for staining. TILs were calculated by determining the total number of CD8+ Thy1.1+ cells per mg of tumor.

Fluorochrome conjugated antibodies to CD4 (clone RM4-5), CD8 (clones 5H10 and 53-6.7), CD25 (clone PC61), CD44 (clone IM7), CD62L, CD107a, IFN-γ (clone XMG1.2), and TNF-α (clone MP6-XT22) were obtained from BD Biosciences. PE-Cy7 conjugated Thy1.1 (clone HIS51) was obtained from eBioscience. The purified blocking anti-CD25 antibody (clone PC61.5.3) was obtained from Bio X Cell. All FACS analysis was performed with the use of an LSRII (BD Biosciences). Gates were set based on isotype specific control antibodies (data not shown). Data was analyzed using FlowJo software.

Western Blot

Total cell lysates from IL-2 and IL-12 primed Pmel-1 T-cells were obtained by using cell extraction buffer (Invitrogen) supplemented with 1mM PMSF (Sigma-Aldrich) and protease inhibitor cocktail (Sigma-Aldrich) used in accordance to manufacturer's instructions. SDS gel and immunoblot analysis were performed according to standard protocols. Antibodies used were STAT5 (9363, Cell Signaling Technology) and β-tubulin (2146, Cell Signaling Technology).

Statistics

Data is represented as the mean plus or minus standard error. Significance was determined using a paired Student's *t*-test. Generated *p*-values are two-tailed, and $p < 0.05$ was considered statistically significant. Survival curves were plotted using the product limit estimation test of Kaplan-Meier. Statistical differences in survival were calculated using the Wilcoxon log-rank test. All statistical analysis and graphs were constructed using GraphPad software.

Figure 2.1: Phenotypic and functional changes conferred by priming in the presence of IL-12.

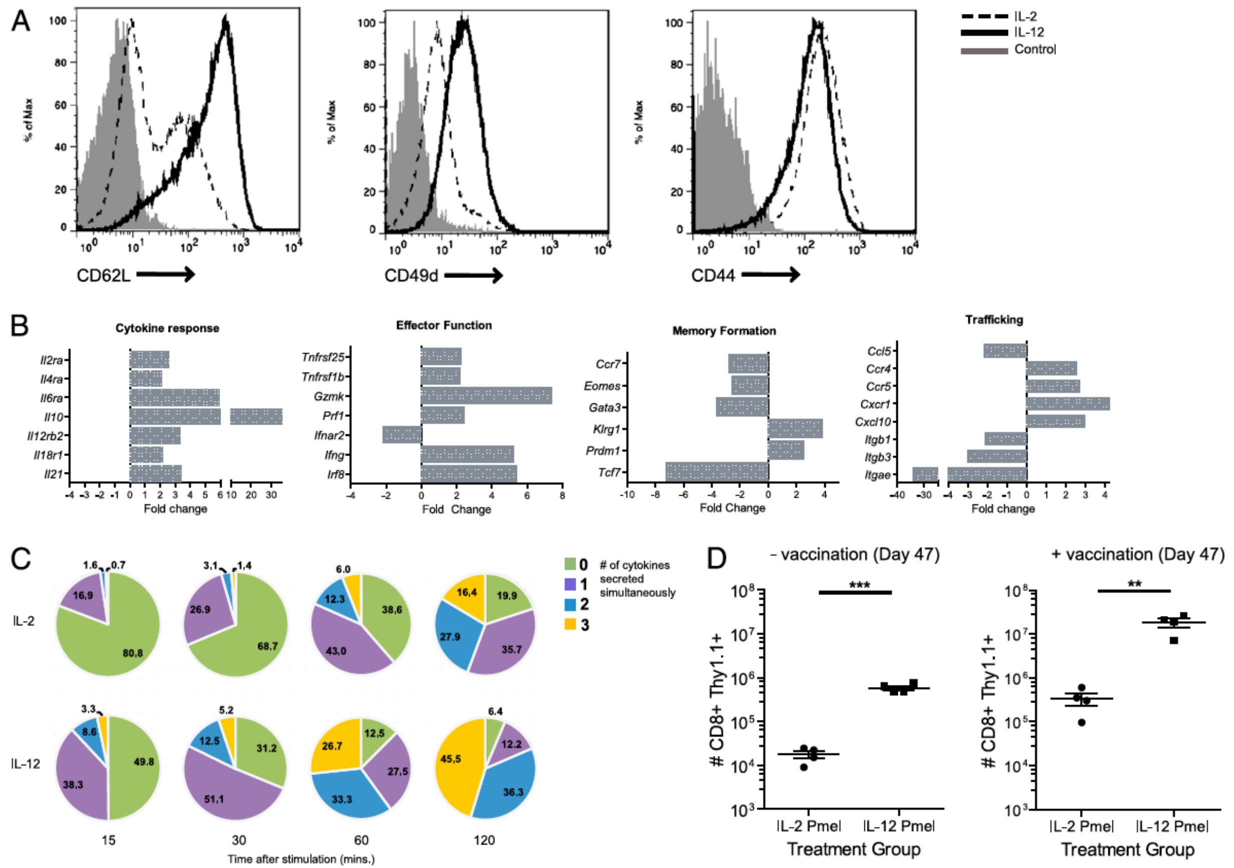

Figure 2.1: (A) Pmel-1 CD8+ T-cells were cultured in the presence of IL-2 or IL-12 and stained after 6 days of in vitro re-stimulation with the indicated antibody or an isotype control. Cells were gated on the CD8+ Thy1.1+ population and analyzed by FACS. (B) Microarray gene analysis was performed on Pmel-1 CD8+ T-cells primed in the presence of IL-2 and IL-12. The plots indicated show expression of selected genes associated with cytokine response, effector and memory function of CD8+ T-cells as well as lymphocyte trafficking. Expression levels of IL-12 primed T-cells relative to IL-2 primed T-cells denote fold change. All genes shown have at least a 2 fold change in expression with a p-value less than 0.05. (C) IL-2 or IL-12 primed Pmel-1 CD8+ T-cells were stimulated with hgp100 peptide pulsed Bl/6 splenocytes for the indicated

times. Percentages indicate the number of cytokines (IFNγ, TNFα, and CD107a) Pmel-1 T-cells were secreting simultaneously. (D) Lymphopaenic mice were adoptively transferred with $1x10^6$ IL-2 or IL-12 primed Pmel-1 T-cells. All groups were re-vaccinated on day 7 with DCs pulsed with gp100. Groups that indicate "(+) vaccination" were re-vaccinated on day 47. These findings are representative of one experiment that has been conducted at least three times with similar findings.

Figure 2.2: IL-12 priming enhances the IL-2 signaling pathway.

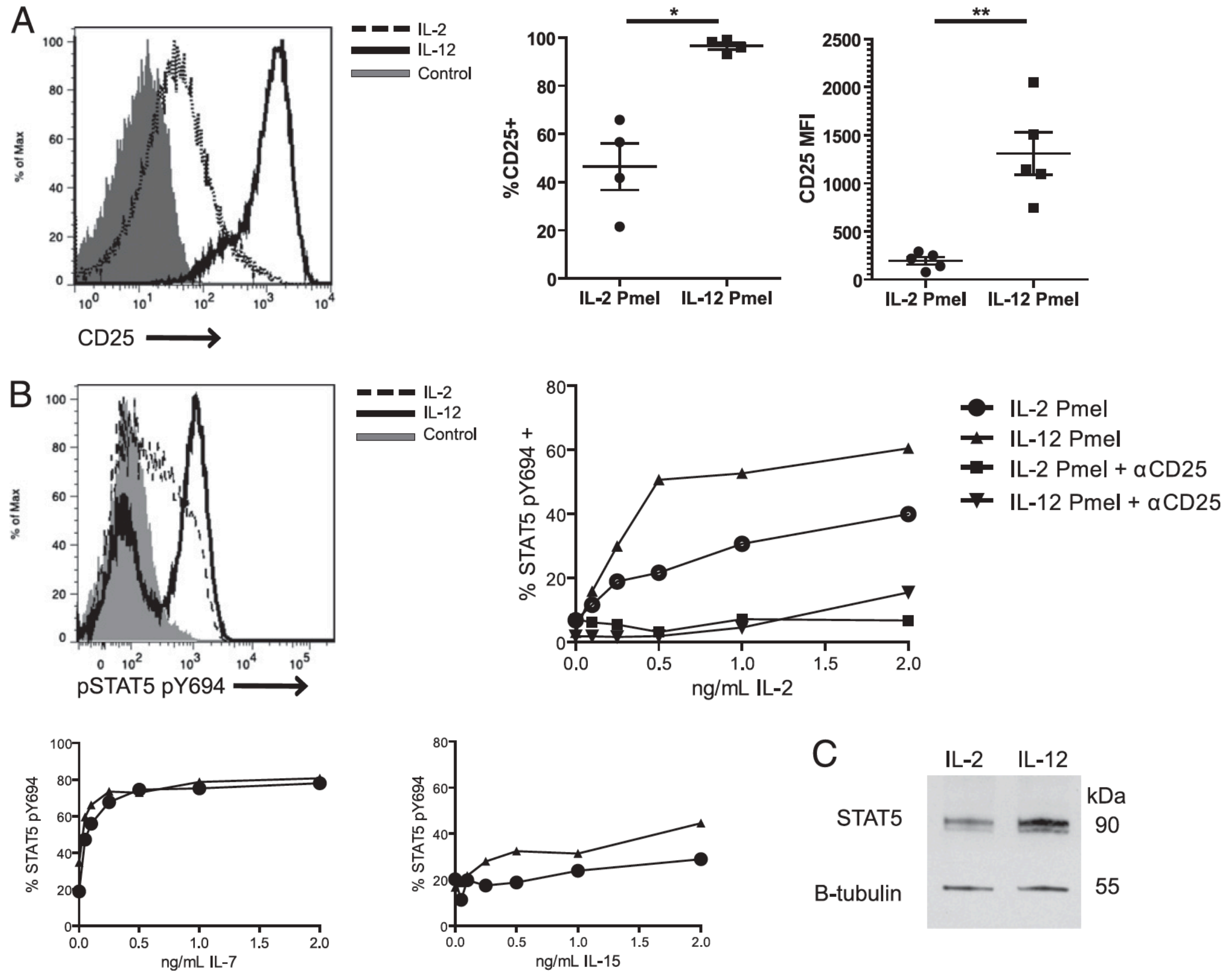

Figure 2.2: (A) Pmel-1 CD8+ T-cells primed in IL-2 or IL-12 were stained for CD25 6 days after in vitro re-stimulation. (B) IL-2 and IL-12 primed CD8+ T-cells were stimulated with varying concentrations of IL-2, IL-7 or IL-15 at 37°C and immediately fixed with paraformaldehyde and then stained intracellularly for the expression of activated STAT5 (pSTAT5) by FACS. Blockade of CD25 was performed by incubating cells with anti-CD25 antibody for 30 minutes on ice, stimulating with IL-2 and then immediately fixing prior to pSTAT5 mAb staining. A representative flow plot shows pSTAT5 expression when cells were unstimulated or stimulated with 2 ng/mL of IL-2. (C) STAT5a/b and β-tubulin expression were detected by Western blotting

using whole cell lysates from IL-2 and IL-12 primed CD8+ T-cells. Results are shown from one of three identical experiments.

Figure 2.3: IL-12 priming enhances the proliferation and sensitivity to low levels of IL-2.

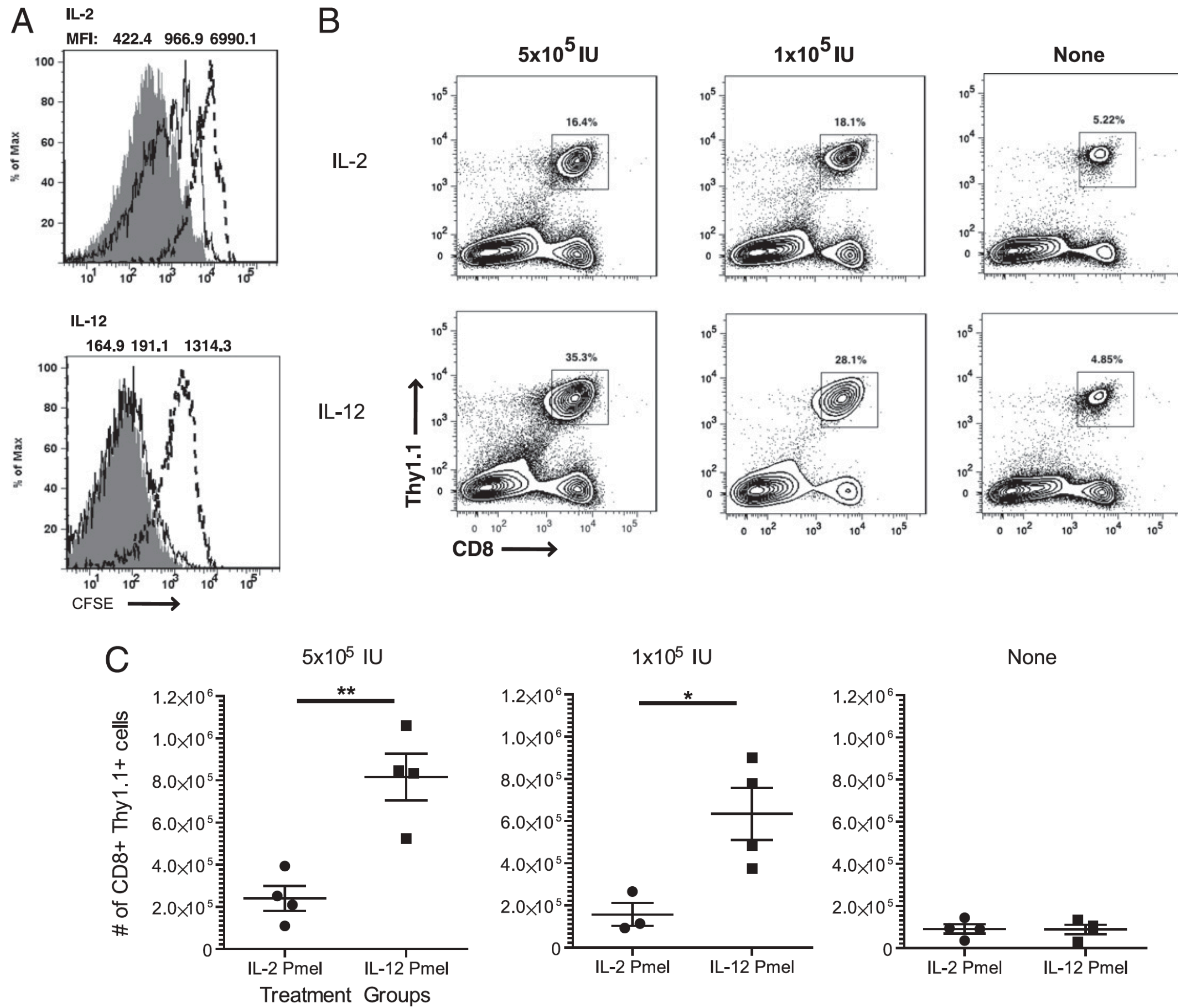

Figure 2.3: (A) Non-lethally irradiated Bl/6 mice were adoptively transferred with $5x10^6$ CFSE labeled IL-2 or IL-12 primed CD8+ T-cells. Recipient mice were then vaccinated with $hgp100_{25-33}$ peptide pulsed dendritic cells and supported with $5x10^5$ IU (grey), $1x10^5$ IU (solid), or no IL-2 (dashed). Three days after adoptive transfer, spleens were harvested and stained. MFIs are averages of four mice in each group (p<0.0001). (B and C) Adoptively transferred cells were recovered from spleens and stained for CD8 and Thy1.1. Plots show representative samples from each group of at least four mice. The scatter plot indicates mean +/- SEM. This experiment was conducted three times with similar results.

Figure 2.4: Bioluminescent imaging reveals extensive trafficking patterns of IL-12 primed T-cells.

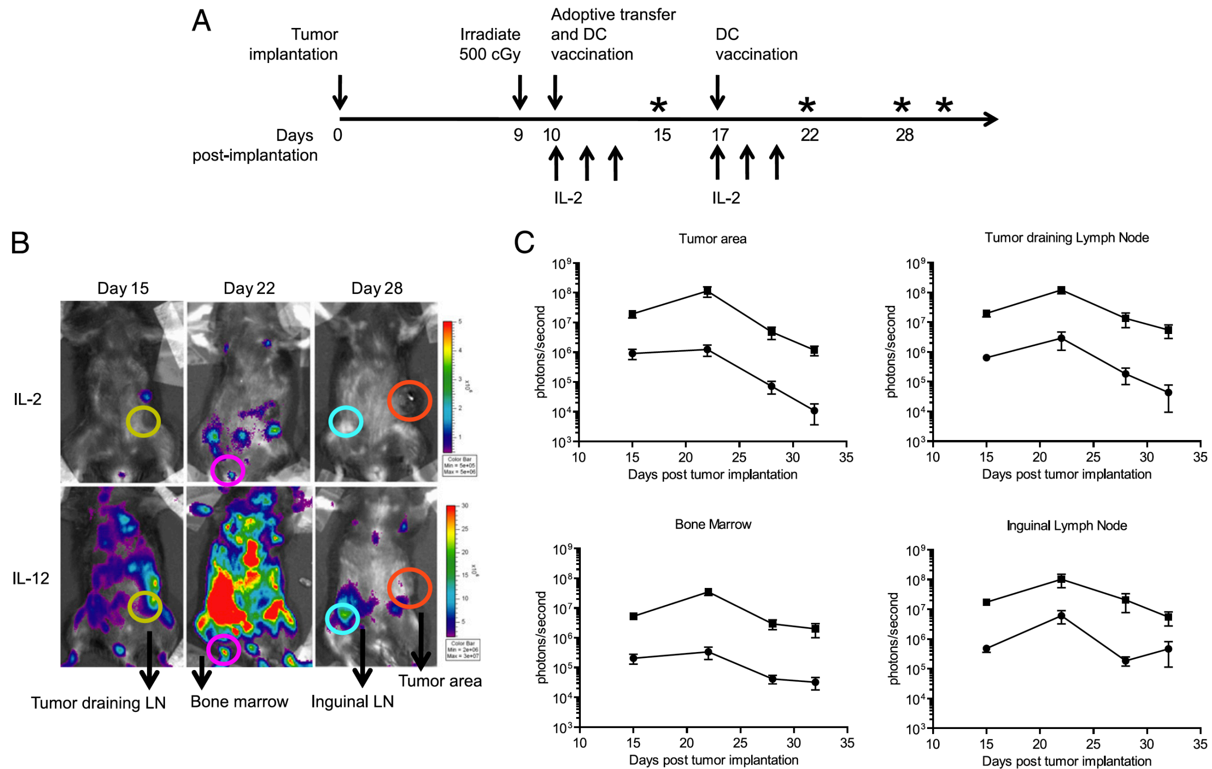

Figure 2.4: (A) Pmel-1 T-cells were transduced with a retroviral vector encoding firefly luciferase and $1x10^6$ cells were adoptively transferred into mice harboring subcutaneous B16-F10 tumors. For *in vivo* bioluminescent imaging, anesthetized mice were injected i.p. with 30 mg/ml D-luciferin and 10 second acquisitions were taken with Living Image software. Asterisks on the timeline indicate days in which images were acquired. (B) Regions of interest were drawn around the tumor (orange) draining lymph node (yellow), the bone marrow (pink), the inguinal lymph node (blue) and the surrounding tumor area. Total flux was analyzed and average +/- SEM was plotted. Results shown are representative of one of two identical experiments including at least four mice per group.

Figure 2.5: Treatment with IL-12 primed T-cells increases intratumor trafficking and decreases tumor burden.

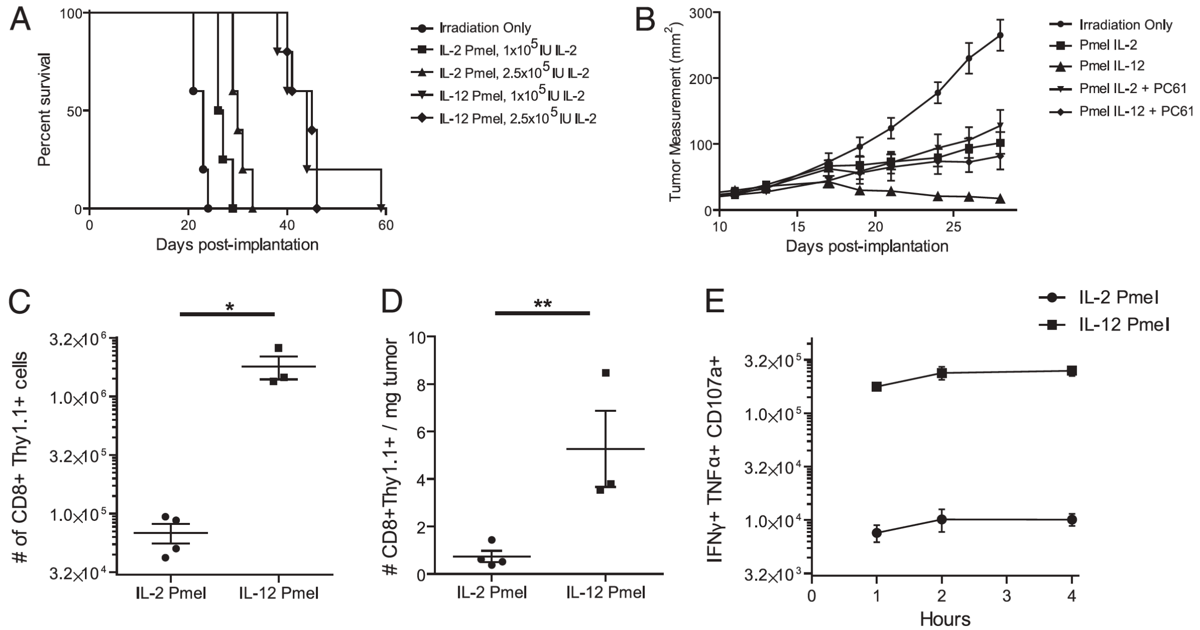

Figure 2.5: (A) B16-F10 melanoma cells were allowed to establish intracranially for 7 days. Mice were irradiated one day prior to adoptive transfer, and treated with $1x10^6$ T-cells, dendritic cell vaccine and the indicated amount of systemic IL-2. Mice were monitored for survival. (B) B16-F10 melanoma cells were allowed to establish subcutaneously for 10 days. Mice were irradiated one day prior to adoptive transfer. Groups received $1x10^6$ IL-2 or IL-12 primed T-cells. Groups receiving anti-CD25 antibody (clone PC61) received i.p. injections of PC61 in addition to the indicated amount of systemically administered IL-2. (C) Spleens were harvested 19 days post adoptive transfer and analyzed for CD8+ Thy1.1+ cells. (D) Tumors were also recovered 19 days post-adoptive transfer. Tumors were weighed and digested with collagenase. Small lymphocytes within the tumor were enumerated and utilized to calculate the total number of CD8+ Thy1.1+ cells within the tumor. (E) Splenocytes were stimulated with hgp100 for the indicated number of hours and IFNγ, TNFα, and CD107a expression is depicted

in the CD8+ Thy1.1+ gated population. Each experiment described has been conducted at least three times with similar results and included at least four mice per group.

Figure 2.6: Priming of human PBMCs with IL-12 generates tumor specific cells phenotypically similar to their murine counterparts.

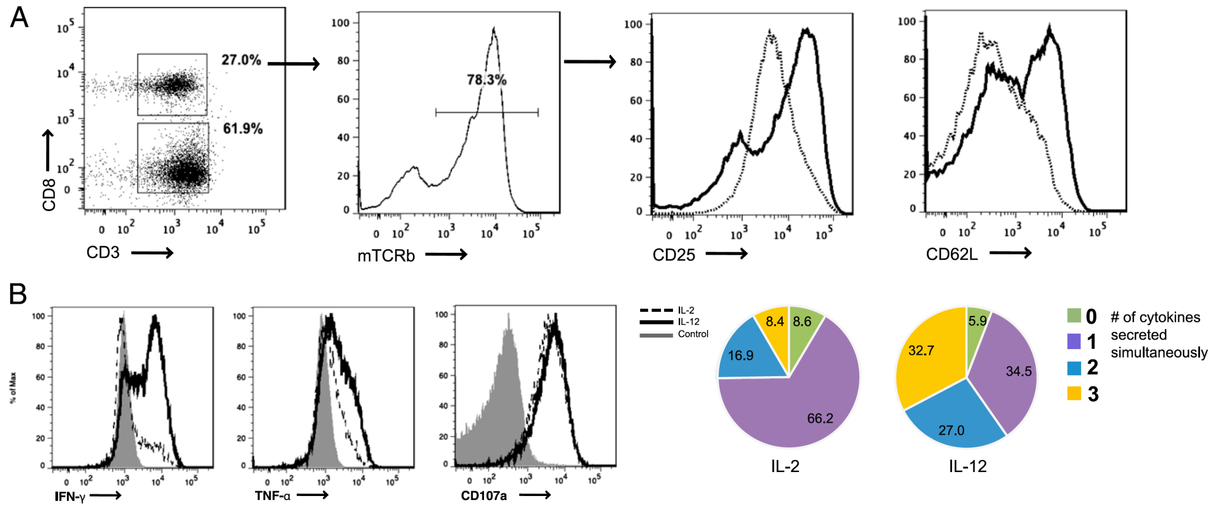

Figure 2.6: (A) Human PBMCs transduced with the chimeric gp100 TCR were analyzed on day 8. The phenotype of CD3+ CD8+ T-cells containing the mouse TCR-β chain (mTCRb) were analyzed for their expression of CD25 and CD62L. (B) Human PBMCs transduced with the gp100 chimeric TCR were pulsed with gp100 peptide loaded T2 cells for 4 hours. CD3+ CD8+ mTCRb+ cells were analyzed for IFNγ, TNFα, and CD107a. Pie charts for each priming condition indicate the number of cytokines being secreted simultaneously. These experiments were conducted three times with similar results.

Figure 2.7: Functional changes conferred as a result of priming with IL-12.

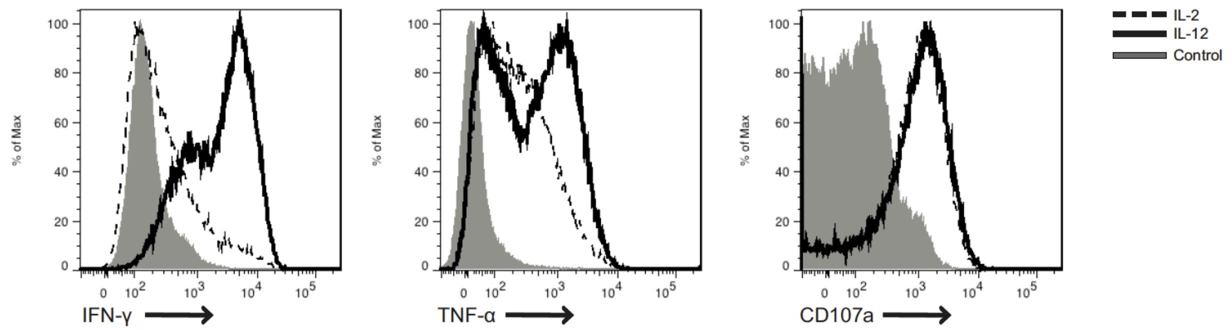

Figure 2.7: Representative FACS plots representing IFN-γ, TNF-α, and CD107a expression by IL-2 primed cells (dashed), IL-12 primed cells (black) and unstimulated (grey) cells. Information from these FACS plots allowed us to determine the number of cytokines each cell type secreted *simultaneously* as displayed by the pie charts in Figure 1C.

Figure 2.8: T-cells transduced with luciferase co-express GFP.

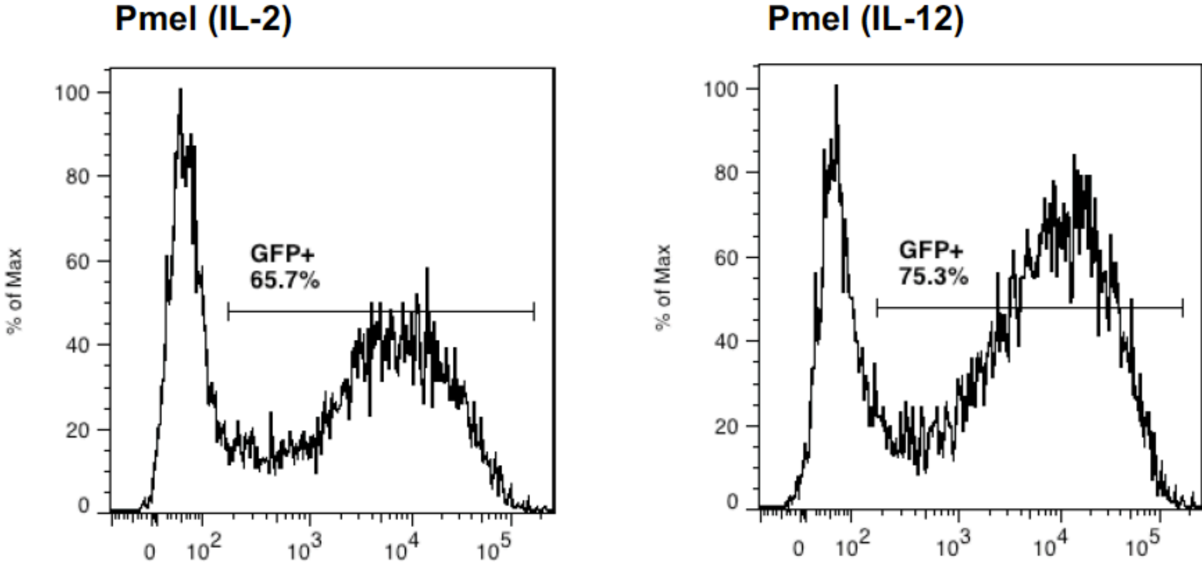

Figure 2.8: Prior to adoptive transfer and shortly following transduction with a vector expressing luciferase, Pmel T-cells were analyzed by FACS to assess GFP expression.

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Chapter 3:

Histone deacetylase inhibitor, LBH589, enhances the proliferative and polyfunctional capacity of adoptively transferred tumor specific T cells.

Introduction

The adoptive transfer of tumor specific T cells has become a viable treatment option for many solid tumors (1). However, despite the ability to specifically target tumors, adoptively transferred cells often fail to survive and persist in vivo (2). In addition, their ability to retain effector function diminishes quickly and often transitions into a suppressive state with an inability to mobilize IFN-γ (3–5). Many strategies have been employed to reverse this balance in favor of an effector T cell response. Strategies such as lymphodepletion prior to adoptive cell transfer and high doses of IL-2 have been shown to significantly increase retention and proliferation of adoptively transferred T cells (6, 7). In addition, combining these strategies with immunomodulation of the tumor itself has shown the potential to increase responsiveness to immunotherapy.

Histone deacetylase inhibitors (HDACis), vorinostat (SAHA) and romidepsin, have been approved for the treatment of cutaneous T-cell lymphomas through mechanisms including cell cycle arrest and activation of the intrinsic death pathway (8, 9). Acetylation and deacetylation of lysine residues on histone tails and non-histone substrates controls a number of cellular processes including the regulation of transcription, transcription factor stability and cell survival. Histone deacetylases are a class of enzymes responsible for deacetylation of histone proteins and other non-histone protein substrates (10). Inhibition of histone deacetylases with HDACis, such as vorinostat, can preferentially induce cell cycle arrest, apoptosis, and differentiation in leukemic malignancies and solid tumors (11). More importantly, HDACis have been shown to enhance tumor immunity by upregulating the expression of major histocompatibility class (MHC) molecules, costimulatory molecules and components involved in tumor necrosis factor (TNF) superfamily signaling (12–15). However, HDACis have also been shown to increase the function of T regulatory cells and increase IDO immunosuppression by dendritic cells (16–18). These

potentially dichotomous functions of HDACis in the context of tumor immunity have complicated their inclusion in immunotherapy protocols.

In order to test whether an HDACi could synergize with immunotherapy in an *in vivo* melanoma model, we utilized panobinostat (LBH589) in combination with T cell transfer therapy. LBH589 is a cinnamic hydroxamic acid derivative with broad inhibitory activity of class I, II, and IV HDACs in the low nanomolar range (19). It has shown clinical efficacy for the treatment of multiple myeloma and Hodgkin's lymphoma and has been generally well tolerated (20). However, whether LBH589 could similarly enhance adoptive T cell transfer without generating a potentially immunosuppressive milieu had yet to be addressed. We utilized gp100 tumor associated antigen specific Pmel T cell immunotherapy in an *in vivo* melanoma model in order to address these concerns. Adjuvant administration of LBH589 potently synergized with adoptive cell transfer, but to our surprise, created a highly pro-inflammatory environment that could be measured by significant modulation of serum cytokine levels. This was accompanied by a significant expansion and enhancement of effector function which occurred in the presence and absence of tumor. Notably, peptide specific release of TNF following restimulation of Pmel T cells and serum cytokine levels of TNF were significantly increased and sustained over time. Taken together with an increase in the T cell specific expression of the TNF superfamily receptor, OX-40, inclusion of LBH589 highlights the potential new role of HDAC inhibitors in modulating and sustaining *in vivo* T cell function.

Results

LBH589 synergizes with an adoptive cell transfer therapy to reduce tumor burden

Significant controversy exists about whether HDACi tolerize or enhance endogenous immunity. In addition, the mechanisms by which HDACi alter immune responsiveness are not well understood. We previously reported that another HDACi similar to LBH589 (LAQ824) could enhance ACT in a mouse model (21). We wanted to understand the immunological mechanisms by which LBH589 acts in vivo, and to ascertain whether HDACi could promote ACT in other models. Mice with 10 day established B16 tumors were lymphodepleted with 500 cGy irradiation the day prior to adoptive transfer. Treated groups then received 5 x 10^6 Pmel T cells, supported by a $gp100_{25\text{-}33}$ peptide pulsed dendritic cell vaccination, and high dose IL-2 with or without a subtherapeutic dose of LBH589 (5 mg/kg). One week later, this was followed by a second vaccination with peptide pulsed dendritic cells and high dose IL-2 (Fig. 3.1A). As a single agent, LBH589 did not decrease tumor burden in comparison to groups that did not receive treatment ($p=0.45$). However, LBH589 in combination with Pmel adoptive transfer therapy, showed significantly greater control of tumor growth over the course of 40 days ($p=0.019$) (Fig. 3.1B). Thus, while unable to show antitumor activity by itself, LBH589 was able to enhance a T cell based therapy.

LBH589 alters and extends in vivo peripheral cytokine production

Widely varied immunomodulatory effects of HDACi have been reported in a number of different models. The majority of these studies have highlighted the anti-inflammatory properties of HDACis (22). In particular, the role of HDACis in the functional enhancement and generation of T regulatory cells and the production of IDO by dendritic cells suggests their ability to dampen the anti-tumor immune response. However, given our results in an in vivo tumor model, an initial lymphopenic environment may prove to be different. In order to assess whether global changes in the inflammatory environment were occurring, we quantified peripheral blood serum cytokine levels at 3 distinct time points (Fig. 3.2A). The first sample was obtained one hour prior to

vaccination with a peptide pulsed vaccine. The second and third serum samples were then obtained 4 hours and 36 hours following revaccination respectively. A dramatic shift towards pro-inflammatory cytokine production was observed 4 hours the second dendritic cell vaccination (Fig. 3.2B). This shift was highlighted by a significant release of TNF and IL-2, and a significant reduction in IL-5 and IL-10 in groups treated with LBH589 and adoptive transfer compared with groups only receiving Pmel adoptive transfer. Furthermore, these significant shifts in pro-inflammatory cytokine production were still noticeable 36 hours following vaccination. Notably, serum levels of IFN-γ and TNF remained significantly elevated in mice treated with LBH589 and adoptive transfer. These results are impressive considering the serum half-life of TNF is approximately 20 minutes. These surprising results point towards a prolonged and sustained global shift towards a pro-inflammatory environment in vivo.

Expansion of adoptively transferred cells and reduction of T regulatory cells with LBH589 treatment

The reported anti-inflammatory properties of HDAC inhibitors have provided little enthusiasm for their concurrent use in immunotherapeutic strategies for tumors. However, with such extensive changes observed in the cytokine environment, we investigated whether the co-administration of LBH589 influenced the expansion of Pmel T cells and other endogenous lymphocyte populations. We quantified the percentages and absolute numbers of Pmel T cells and T regulatory cell populations in mice with established B16-F10 tumors (Fig. 3.3A and 3.7A) We were surprised to find that there was a significant increase in the percentage of Pmel T cells (CD8+ Thy1.1+) recovered from the spleen 3 days following re-vaccination, 45% for groups receiving LBH589 and 14% for groups not receiving LBH589 ($p=0.0007$) (Fig. 3.3A). In contrast to what has been reported, this expansion of adoptively transferred T cells was accompanied by a decrease in both the percentage and absolute number of endogenous T regulatory cells

(CD4+ FoxP3+) recovered in the spleen from 2.1% to 1.1% in mice treated with LBH589 (p=0.039) (Fig. 3.3A and 3.7A). This significantly altered the tumor specific T cell to T regulatory cell ratio in the periphery from 121.9 to 8.1 in mice treated with and without LBH589 respectively (p=0.0064). Furthermore, this was accompanied by a greater percentage of Pmel T cells within the tumor when groups were treated with LBH589 than treated without, 39% to 11.7% respectively (p=0.0021), and an increased, though not significant, number of absolute Pmel T cells per mg of tumor (Fig. 3.3B and 3.7B). Although the T regulatory cell population was negligible in the tumor, the overwhelming tumor infiltrating lymphocyte population increased the Pmel to T reg cell ratio from 235.5 to 49.7 for mice treated with and without LBH589, respectively (p=0.06). This data suggests that the result of HDACi coadministration may be context dependent, and can provide a pro-inflammatory environment during an antitumor response in lymphodepleted hosts.

Enhanced T cell expansion with LBH589 is not dependent on the presence of tumor

The ability of particular HDACi to immunosensitize tumors has been documented and may explain the observed synergism between ACT immunotherapy and LBH589. However, whether LBH589 directly influenced T cell proliferation in the absence of tumor was still unknown. In order to test whether LBH589 mediated its effects regardless of tumor, we adoptively transferred 5×10^6 Pmel T cells into lymphodepleted, non-tumor bearing hosts. These cells were again supported by a peptide pulsed vaccination and IL-2 with or without LBH589. Ten days following adoptive transfer, and 3 days following vaccination, in groups receiving LBH589, Pmel T cells represented 81% of the spleen ($2.5\text{x}10^7$ cells), in comparison to only 19% ($1.5\text{x}10^6$ cells) without LBH589 ($p<0.001$) (Fig. 3.4A). Furthermore, this expansion was not limited to the spleen, but was also observed in the percentage of Pmel T cells in the overall CD8 population from peripheral blood (Fig. 3.4B). This expansion was the most dramatic following vaccination.

In addition to enhanced expansion, retention of Pmel cells also persisted 21 days following adoptive transfer (11 days post vaccination). Thus the, combination of an HDACi with a T cell based immunotherapy was not immunosuppressive. Instead, the coadministration of LBH589 resulted in increased proliferation and retention of adoptively transferred T cells.

Increased ex vivo polyfunctionality of tumor specific T cells with LBH589

Realizing that LBH589 mediated such a profound effect on the retention of adoptively transferred Pmel T cells, we also addressed whether the function of these cells was likewise enhanced. Optimal expansion of Pmel T cells occurred in the days following revaccination, providing an ideal timeframe for us to assess their ability to degranulate (CD107a+/LAMP-1+) and/or secrete the cytokines IFN-γ and TNF. Lymphodepleted, non-tumor bearing mice were adoptively transferred with $5x10^6$ Pmel T cells and supported with a dendritic cell vaccine and IL-2 with or without LBH589. Ten days following adoptive transfer, splenocytes from mice were obtained and restimulated with $gp100_{25-33}$ peptide for five hours. During this time, anti-CD107a was utilized in order to quantify the percentage of T cells undergoing degranulation. In groups treated with LBH589, we observed significant increases in Pmel T cell cytokine secretion and degranulation. The percentage and MFI of Pmel T cells secreting IFN-γ was significantly increased in mice treated with LBH589 compared with those treated without (88.9% vs. 74.1% respectively, $p<0.005$) (Fig. 3.5A). Furthermore, the percentage of Pmel T cells exhibiting mobilization of the degranulation marker, CD107a, was also increased in mice treated with LBH589 compared with those treated without (92.5% vs 86.8% respectively, $p<0.05$) (Fig. 3.5C). The most dramatic difference we observed for LBH589 treated groups was more than a 2 fold increase in the percentage of cells secreting TNF in comparison to those not treated (82.2% vs. 36.9% respectively, $p<0.001$) (Fig 3.5B). When considering the number of cells able to secrete both cytokines and demonstrate the ability to degranulate, more than 70.9% of Pmel cells

simultaneously showed polyfunctionality when treated with LBH589 in comparison to 30.2% in groups not treated with LBH589 ($p<0.0001$) (Fig. 3.5D). Furthermore, this is in stark contrast to three days following adoptive transfer when only 10% of untreated and 20% of LBH589 treated Pmel T cells were able to secrete TNF (Fig. 3.8). This demonstrated a significant enhancement in the ability to respond to a peptide specific vaccination with elevated TNF secretion. For the first time, we have shown the ability of an HDAC inhibitor to increase the *in vivo* polyfunctional capacity of activated CD8 T cells *in vivo*.

LBH589 enhances markers of activation on tumor specific T cells

The TNF superfamily of ligands and receptors, as well as TNF itself, are known to be modulated by HDACis in numerous cell lines (12, 23–25). Our results already demonstrated superior enhancement of TNF secretion by restimulated Pmel T cells, so we turned our attention towards the TNF superfamily of ligands and receptors as well as other markers of activation. We showed previously that high affinity IL-2 receptor α chain (CD25) was important for proliferative and *in vivo* antitumor activity of CD8 T cells (26). In order to assess whether this was also the case in mice treated with LBH589, we recovered splenocytes from non-tumor bearing mice 10 days following adoptive cell transfer (Fig. 3.2A). Pmel T cells recovered from groups treated with LBH589 showed a significantly increased percentage and MFI of CD25 expression (Fig 3.6A). We examined TNF family members normally expressed on activated CD8 T cells. Significant changes in expression could not be detected for CD40L (Fig. 3.6C), 4-1BB, or CD137 (data not shown). However, OX-40 was significantly upregulated on Pmel T cells following vaccination in groups treated with LBH589. CD25 and OX-40 are known to play important roles in the survival, proliferation and memory formation of T cells (27). OX-40 agonistic antibodies have already been investigated in their ability to potentiate CD8 T cell memory generation and antitumor

activity (28–30). Therefore, inclusion of LBH589 in current adoptive cell transfer protocols may enhance the anti-tumor activity of T cells in vivo.

Discussion

Utilization of a pan-HDAC inhibitor, LBH589, enhanced the antitumor activity of an adoptive T cell transfer therapy in a murine B16-F10 subcutaneous melanoma model. The exact targets and mechanisms of HDAC activities are still relatively unknown in immune cells. Multiple studies have suggested that HDAC inhibitors possess potent immunomodulatory properties, both stimulatory and suppressive in nature. Our study addressed whether an HDAC inhibitor could synergize with an adoptive cell transfer therapy, without suppressing or compromising a tumor specific effector T cell response. We demonstrated that inclusion of LBH589 induced a significant regression of established B16 melanoma tumors while generating a systemic proinflammatory cytokine milieu defined by the sustained release of IFN-γ and TNF. Enhanced antitumor activity was even further exemplified by an increased recovery of adoptively transferred Pmel cells both systemically and intratumorally, with a drastic reduction in the T regulatory cell population. It has been shown that high levels of peripheral T regulatory cell populations are indicative of clinical responsiveness to adoptive cell transfer in patients (31). This ratio has also been shown to have a positive predictive factor for control of tumor growth (32). With LBH589, pharmacological modulation of both T effector and T regulatory cell populations has the potential to significantly increase anti-tumor activity.

Our results indicate an enhanced inflammatory cytokine environment and state of T cell responsiveness, which may be attributed to an LBH589 mediated immunomodulation of tumor cells. As stated previously, histone deacetylase inhibitors have been shown to increase the expression of MHC, co-stimulatory molecules, and tumor associated antigens on tumor cells

themselves. In this study, we instead tested the ability of LBH589 to modulate T cell function in non-tumor bearing hosts. We determined that the majority of immunological effects seen in our treatment regimen were due to an overwhelming enhancement of T cell proliferation and function by LBH589, even in the absence of tumor. Furthermore, this enhancement of function was dominated by a more than 2 fold change in the peptide specific secretion of TNF, closely mirroring the data observed in serum cytokine levels. An increase in HDACi-mediated IFN-γ secretion has already been observed in an immunotherapy model utilizing pan-HDACi LAQ824 (21). However, a pharmacological enhancement of polyfunctional T cell status has not been previously documented for an HDAC inhibitor. This warrants further investigation into the molecular mechanisms for HDACi mediated regulation of TNF expression. Further insights into effector T cell regulation of TNF expression has the potential to significantly alter the design of genetically engineered lymphocytes for tumor immunotherapies.

TNF has been shown to be crucial for the priming and effector function of CD8 T cells during an antitumor immune response (33, 34). This is especially significant considering that proper costimulation and cytokine support for effective T cell responses is severely lacking during tumor surveillance. HDAC inhibitors have been shown to preferentially upregulate the expression of TNF superfamily members, including TNF itself, TNF receptors, and TNF associated ligands. Depsipeptide has been shown to increase the expression of TNF-related apoptosis-inducing ligand (TRAIL) on chronic lymphocytic leukemia (CLL) and acute lymphoblastic leukemia cell lines sensitizing them to death receptor induced apoptosis (15). Additionally, an increase in OX-40 ligand in Hodgkin lymphoma has been shown to inhibit the generation of IL-10 producing T regulatory cells *in vitro* (25). In these studies, we demonstrated that in a lymphodepleted environment with a high dose of IL-2, LBH589 is also able to specifically increase the expression of two TNF family members – TNF and OX-40. OX-40 is a critical co-stimulatory molecule that is necessary for effector function, survival, and memory

generation (33). Combined with an increase in the expression of CD25, we believe that LBH589 may specifically modulate the proliferation, retention and responsiveness of CD8 T cells.

We have demonstrated that LBH589 does in fact potentiate the function of CD8 T cells in an adoptive cell transfer therapy. In the case of a graft versus host disease (GVHD) model, LBH589 was also able to potently activate T cell function, cytokine secretion, and accelerate disease pathology (35). However, utilizing the hydroxamic based HDAC inhibitor, SAHA, was immunosuppressive and protected against GVHD and increased survival. Such results suggest that different HDAC inhibitors may have varying effects upon activated T cells. Together, our studies have begun to define the mechanisms of immunomodulation in different cell types, differentiation states, and cytokine environments.

Acetylation of the N-terminal region of histone proteins regulates the kinetics of transcription. A dynamic interchange between histone acetyltransferases (HATs) and HDACs, such as LBH589, regulates this process of acetylation and deacetylation. It has been hypothesized that histone acetylation is involved in T cell tolerization and inhibition of T cell function (36). We demonstrated that a HDAC inhibitor has the potential to sensitize tumor specific cells to peptide specific vaccination and acquisition of full effector function. Further investigations need to be conducted in order to determine whether differential histone acetylation is involved in T cell effector function and T cell exhaustion and whether TNF superfamily members are more sensitive to this type of regulation. We also demonstrated that the utilization of an HDAC inhibitor is a safe and effective mechanism to regulate the proliferation and function of adoptively transferred tumor specific T cells, and could potentially warrant its inclusion in future ACT human clinical trials.

Materials and Methods

Animals and cell lines

All mice were bred and kept under defined-flora pathogen-free conditions at the Association for Assessment and Accreditation of Laboratory Animal Care-approved animal facility of the Division of Experimental Radiation Oncology at the University of California Los Angeles. Mice were handled in accordance with the University of California Los Angeles animal care policy and approved animal protocols. The B16-F10 murine melanoma cell line and the GL26 glioma cell line were obtained from American Type Culture Collection (Rockville, MD).

Tumor implantation and lymphodepletion

For studies analyzing tumor growth over time in an *in vivo* melanoma model, C57BL/6 mice (6-12 weeks of age) were implanted subcutaneously in the lower left flank with $2x10^5$ B16-F10 melanoma cells and allowed to establish for 10 days. For studies analyzing tumor infiltrating lymphocytes, $2.5x10^5$ B16-F10 melanoma cells were implanted in the same location and allowed to establish for 10 days.

One day prior to adoptive T-cell transfer, lymphopenia was induced by 500 cGy total body irradiation.

Adoptive T cell transfer and dendritic cell vaccination

Pmel-1 T cells and bone marrow derived dendritic cells were generated as previously described. In brief, naïve Pmel-1 splenocytes were activated with human $gp100_{25-33}$ peptide (NH_2-KVPRNQDWL-OH, 1 *u*g/ml; Biosynthesis, Lewisville, TX) and 100 IU/ml human IL-2 (National

Cancer Institute Preclinical Repository, Developmental Therapeutics Program) for 72 hours (26). These cells were then re-cultured for an additional 48 hours in 100 IU/ml IL-2 without hgp100. Pmel-1 T-cells ($5x10^6$) were injected i.v. in 0.1 ml PBS.

Bone marrow derived dendritic cells were generated as previously described (26). In brief, bone marrow cells were initially cultured overnight in a petri dish in RPMI 1640 supplemented with 10% FBS. Once harvested, dendritic cells were pulsed with human $gp100_{25–33}$ peptide at a concentration of 10 uM for 90 min at room temperature. Approximately $5x10^5$ were injected subcutaneously at four sites on the back (37). IL-2 ($5x10^5$ IU) was administered in 500 *u*l and given as an intraperitoneal injection. LBH589 (a kind gift from Novartis) was given at a dose of 5 mg/kg and administered at the same time as IL-2.

Pmel-1 T cell ex vivo stimulation and intracellular FACS staining

After the indicated time periods following adoptive T cell transfer, splenocytes were enumerated and restimulated with or without $hgp100_{25-33}$ peptide. GolgiPlug protein transport inhibitor (BD Biosciences) and allophycocyanin-conjugated anti-CD107a Ab (2 ug, clone 1D4B; BD Biosciences) were added to each well containing T cells. Cells were stimulated at 37°C for 0, 1, 2, or 5 hours. After each time period, cells were placed on ice in the dark until all cells could be stained at the same time. Cells were washed with PBS containing 2% FBS and stained with CD8 Ab, Thy1.1 Ab, and a fixable dead cells stain (Live/Dead, Invitrogen) on ice. Cells were fixed and permeabilized with commercial buffers (eBioscience). Intracellular staining was thencompleted by staining with IFN-γ, TNF-α and IL-2 Abs on ice in the dark.

Flow cytometry and mAbs

Spleens and tumors were harvested from mice after adoptive transfer. Spleens were passed through 70 *u*m cell strainers and lymphocytes were obtained after hypotonic lysis. Approximately $1x10^6$ were used for each staining. To determine the number of tumor-infiltrating lymphocytes (TILs), tumors were weighed and minced with a scalpel. The tumor was then digested in collagenase with DNase for 2 hours on a rotator. Small mononuclear cells within the tumor were enumerated by trypan blue exclusion, with approximately $1x10^6$ lymphocytes used for staining. TILs were calculated by determining the absolute number of CD8+ Thy1.1+ cells per milligram of tumor.

Fluorochrome conjugated Abs to CD4 (clone RM4-5), CD8 (clones 5H10 and 53-6.7), CD107a (clone 1D4B), IFN-γ (clone XMG1.2), TNF-α (clone MP6-XT22) and IL-2 (clone JES6-5H4) were obtained from BD Biosciences or Biolegend. Fluorochrome conjugated Abs to Thy1.1 (clone HIS51) and FoxP3 (clone FJK-16s) were obtained from eBioscience.

All FACS analysis was performed with the use of an LSRII (BD Biosciences). Gates were set based on samples stained with all fluorophores minus one. Only viable cells, as determined by negative staining with a dead cell stain (Live/Dead Fixable Near-IR Dead Cell Stain Kit, Invitrogen), were included in subsequent analyses. Data were analyzed using FlowJo software (Treestar).

Assessment of cytokine serum levels

Serial blood measurements were obtained by retro-orbital sinus collection at the time points indicated. Collected blood was allowed to clot for 30 minutes before centrifugation for 10 minutes at 1000 x g. Serum was removed immediately and stored at -20°C until utilized.

Cytokine serum levels were assayed with a Milliplex MAP Mouse Cytokine Magnetic Bead Panel (Millipore) and analyzed at the University of California Los Angeles Immuno/BioSpot Core.

Figure 3.1: LBH589 in combination with an adoptive cell transfer therapy reduces tumor burden in a subcutaneous B16-F10 melanoma model.

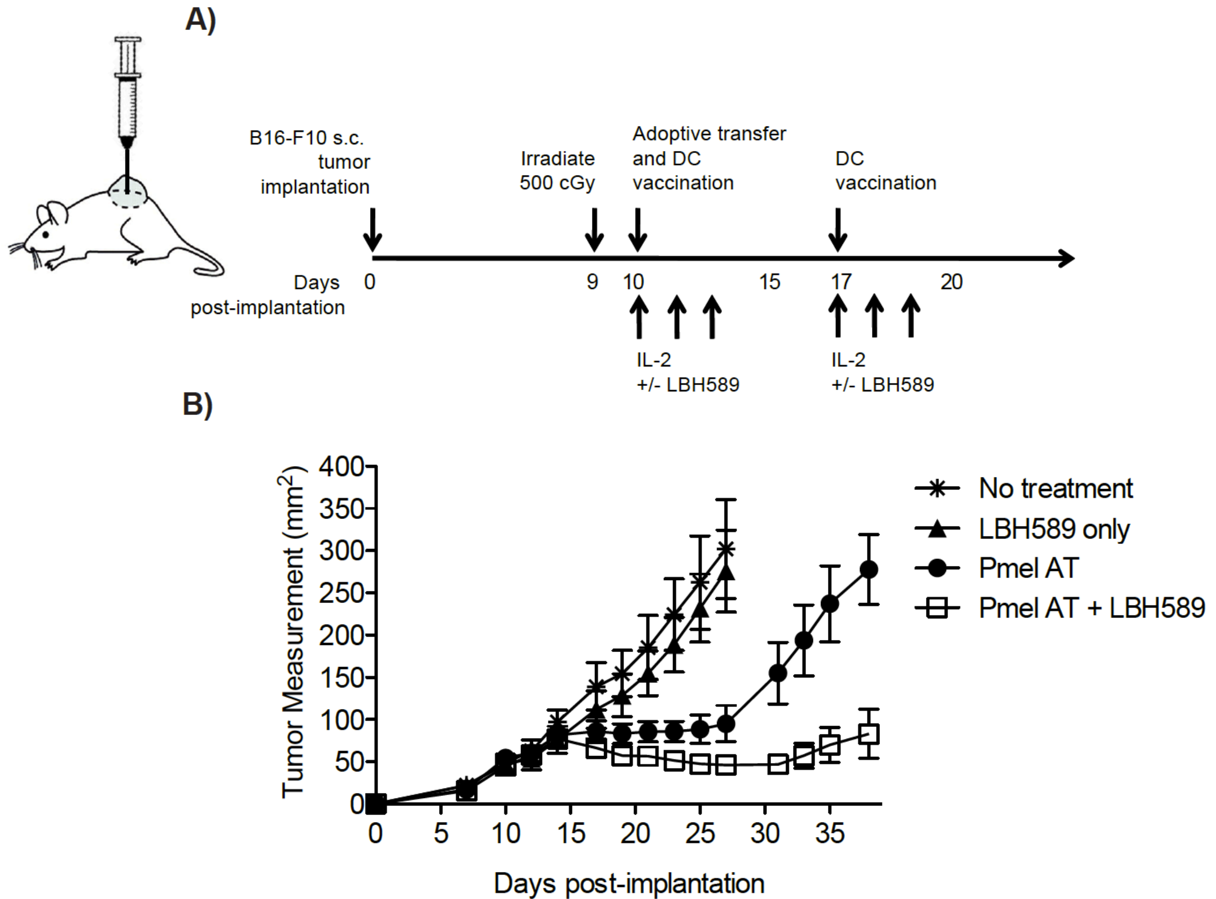

Figure 3.1: A. Treatment schedule. B. Tumor growth measurements of the mean area of tumors in control mice treated with irradiation only, LBH589 following irradiation, Pmel T cell adoptive transfer, or Pmel T cell adoptive transfer therapy with LBH589. Results are representative of at least three similar experiments.

Figure 3.2: Treatment with LBH589 alters the peripheral cytokine milieu of non-tumor bearing mice treated with LBH589.

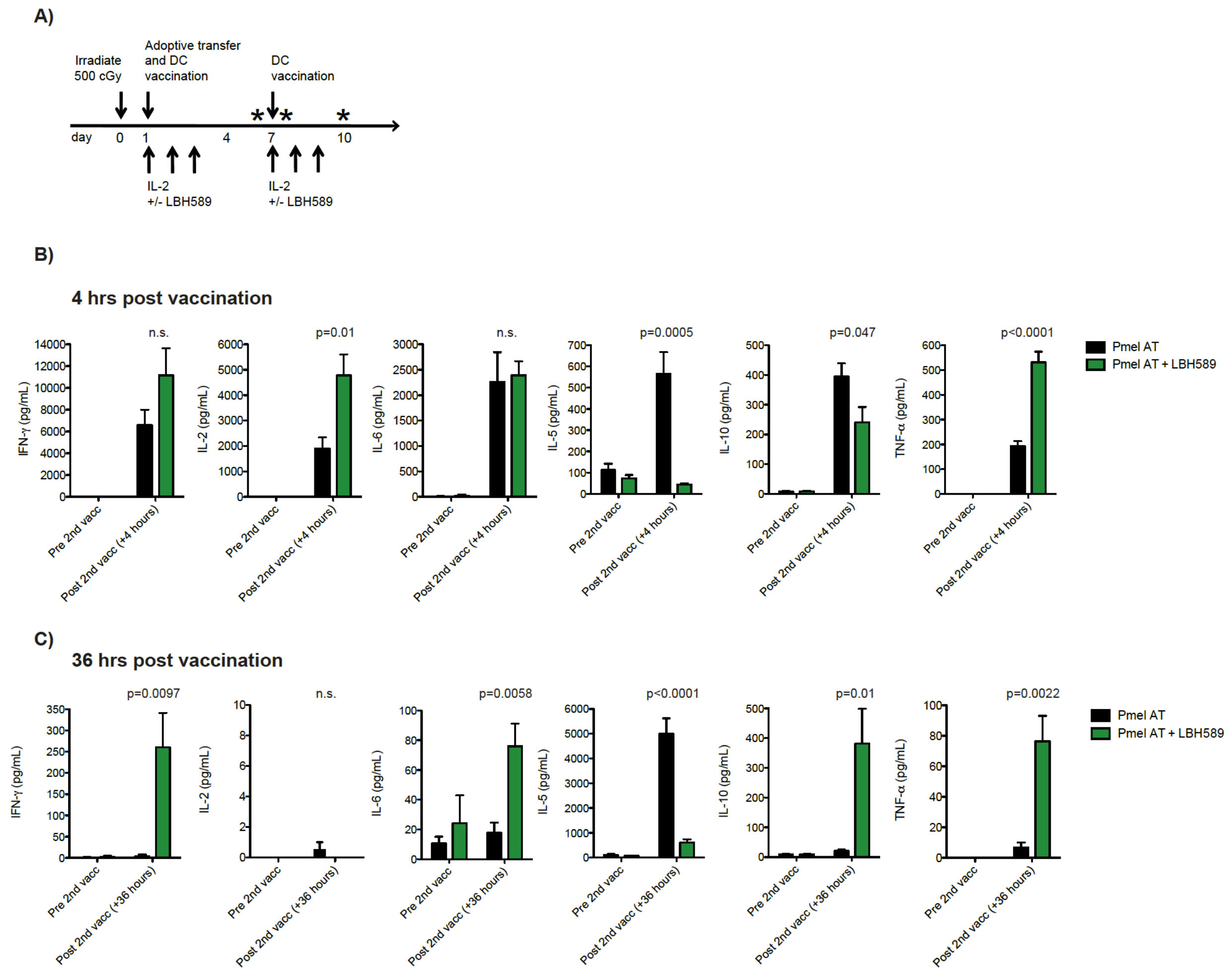

Figure 3.2: A. Treatment scheme. Asterisks indicate timepoints in which serum was collected. B. Serum levels of cytokines in groups treated with and without LBH589. Serum was taken one day prior to dendritic cell vaccination, and then four hours post vaccination. C. Serum cytokine levels of the same treatment groups show prolonged levels of circulating IFN-γ and TNF 3 days post dendritic cell vaccination. Each group contained 6 mice with similar data obtained in an

independent experiment. Student’s t-tests were performed to determine statistical significance between groups.

Figure 3.3: Dramatic expansion and recovery of tumor specific T cells *in vivo* as a result of LBH589 treatment.

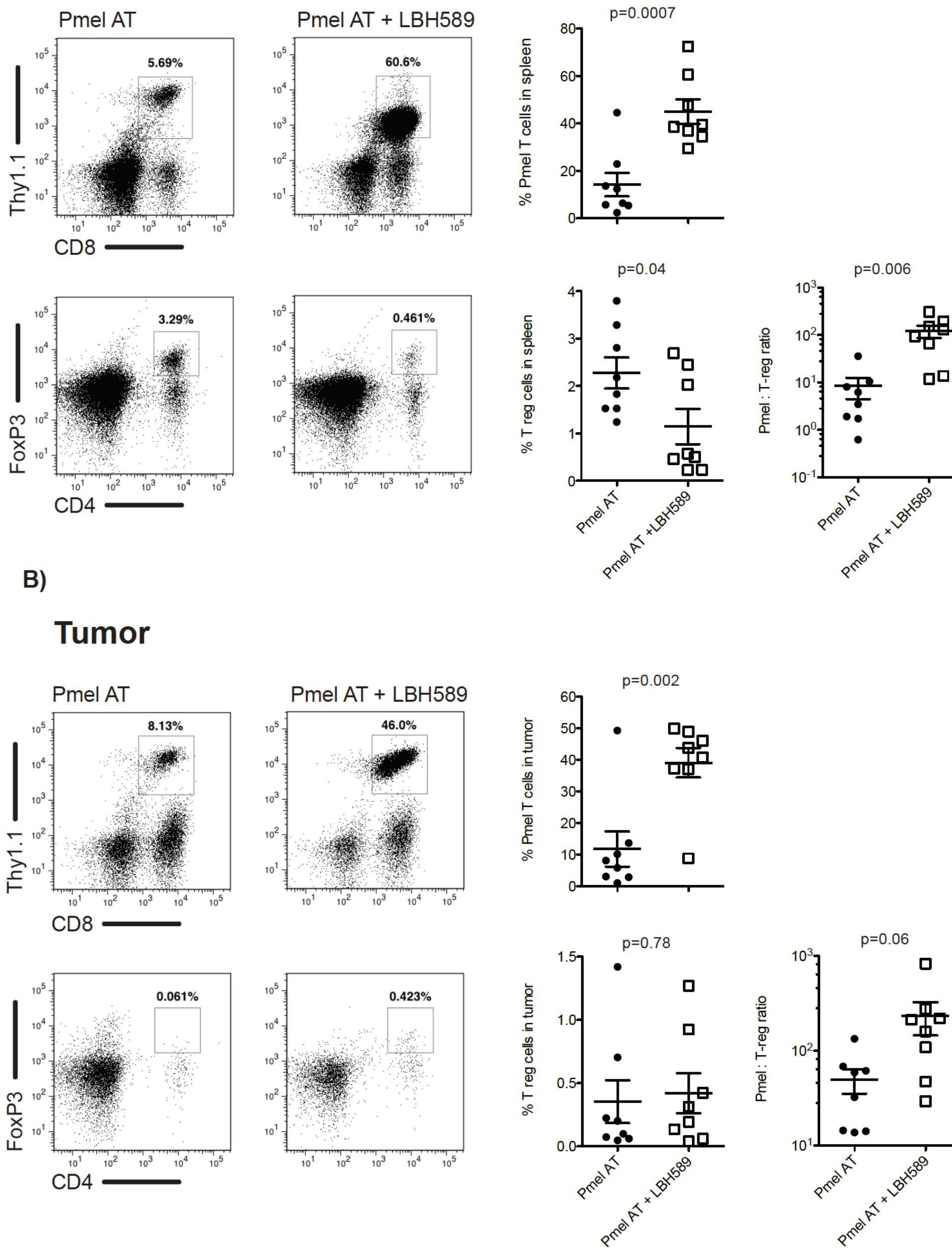

Figure 3.3: A. Quantification of Pmel T cells recovered from splenocytes collected 10 days after adoptive cell transfer according to the treatment scheme on Fig. 1A. Representative flow

cytometry plots shown are derived from a lymphocyte gate followed by a live cell gate. Pmel : T reg ratios are determined by utilizing absolute numbers of Pmel T cells (CD8+ Thy1.1+) to the absolute number of T reg cells (CD4+ CD25hi FoxP3+). B. Quantification of lymphocytes within the tumor. Representative flow cytometry plots of lymphocytes isolated from subcutaneous B16 melanomas digested with collagenase 10 days following adoptive cell transfer. Dot plots shown are derived from lymphocyte and live cell gates. The Pmel : T reg ratio was calculated using absolute number of Pmel T cells and T reg cells per mg of tumor. A and B *dark circles* indicate mice treated with adoptive cell transfer and *open squares* indicate mice treated with adoptive cell transfer with LBH589 and each symbol represents one mouse. Each experiment described has been conducted two times with similar results and included at least four mice per group. Student's t-tests were performed to determine statistical significance between groups.

Figure 3.4: Recovery and systemic expansion of Pmel T cells with LBH589 also occurs in the absence of tumor.

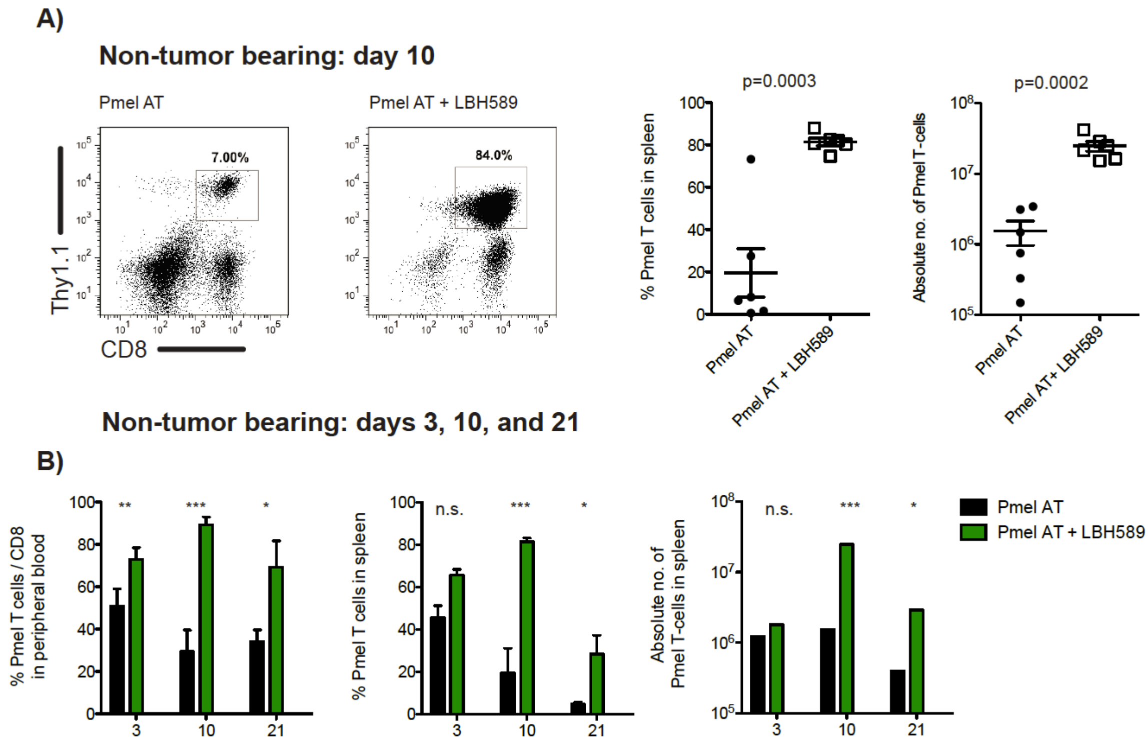

Figure 3.4: A. Expansion of Pmel T cells treated by the same scheme as shown in Fig. 1A, but in non-tumor bearing irradiated hosts. Representative dot plots indicate the percentage and absolute number of splenocytes that are Pmel T cells on day 10 following adoptive cell transfer. B. Analysis of Pmel T cell expansion in both peripheral blood and spleen over time. Pmel T cell expansion in peripheral blood is expressed as the percentage of Pmel T cells in the overall CD8 T cell population days 3, 10, and 21 days following adoptive cell transfer. Results described are representative of at least three experiments with similar results. Student's t-tests were performed to determine statistical significance between groups.

Figure 3.5: Functional enhancement of Pmel T cell cytokine production.

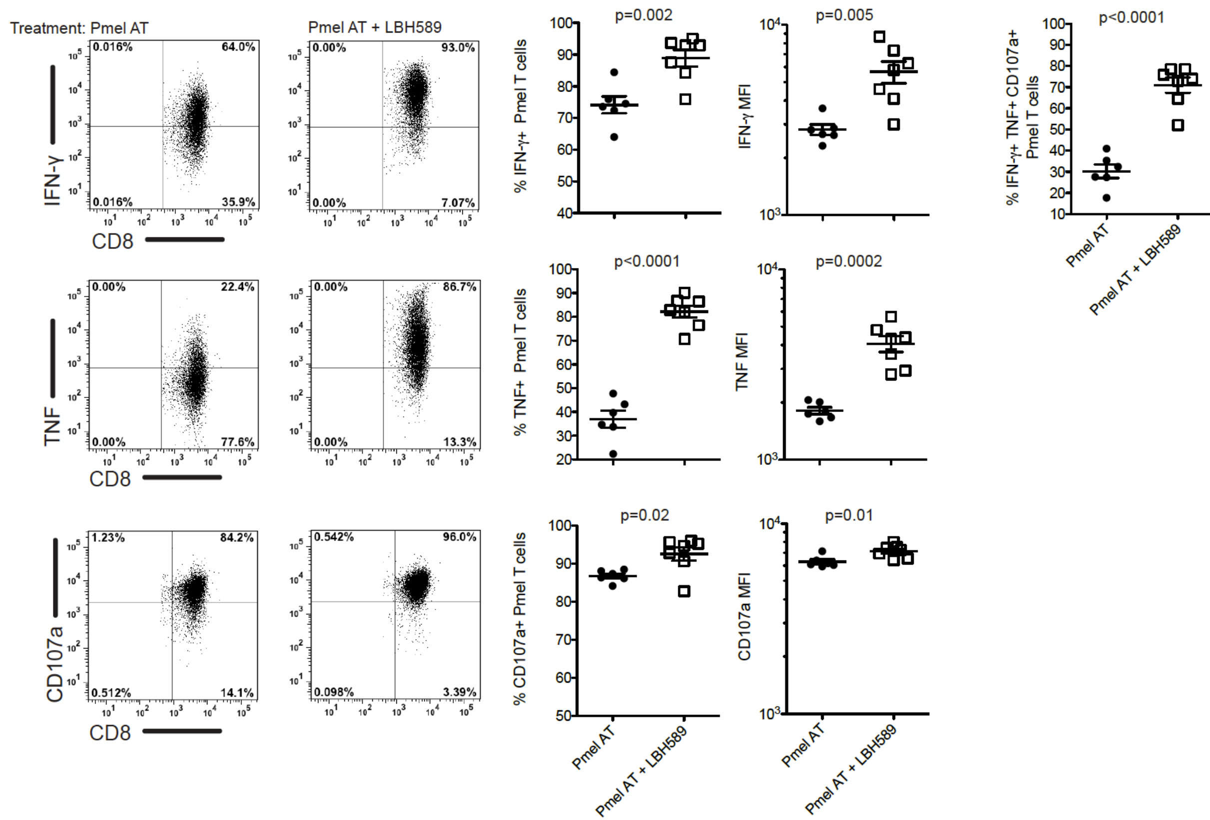

Figure 3.5: Restimulation of Pmel T cell splenocytes with cognate $gp100_{25\text{-}33}$ peptide *ex vivo* for five hours. Representative plots indicate splenocytes restimulated 10 days following adoptive cell transfer and are gated from CD8+ Thy1.1+ Pmel T cells. Gates were set based on Pmel cells that lacked peptide stimulation for the 5 hour duration of the restimulation. CD107a staining, as a marker for degranulation, was achieved by addition of CD107a antibody prior to the restimulation period. Serum from at least 6 mice was measured per cytokine. Student's t-tests were performed to determine statistical significance between groups.

Figure 3.6: LBH589 increases expression of markers of T-cell activation and costimulation.

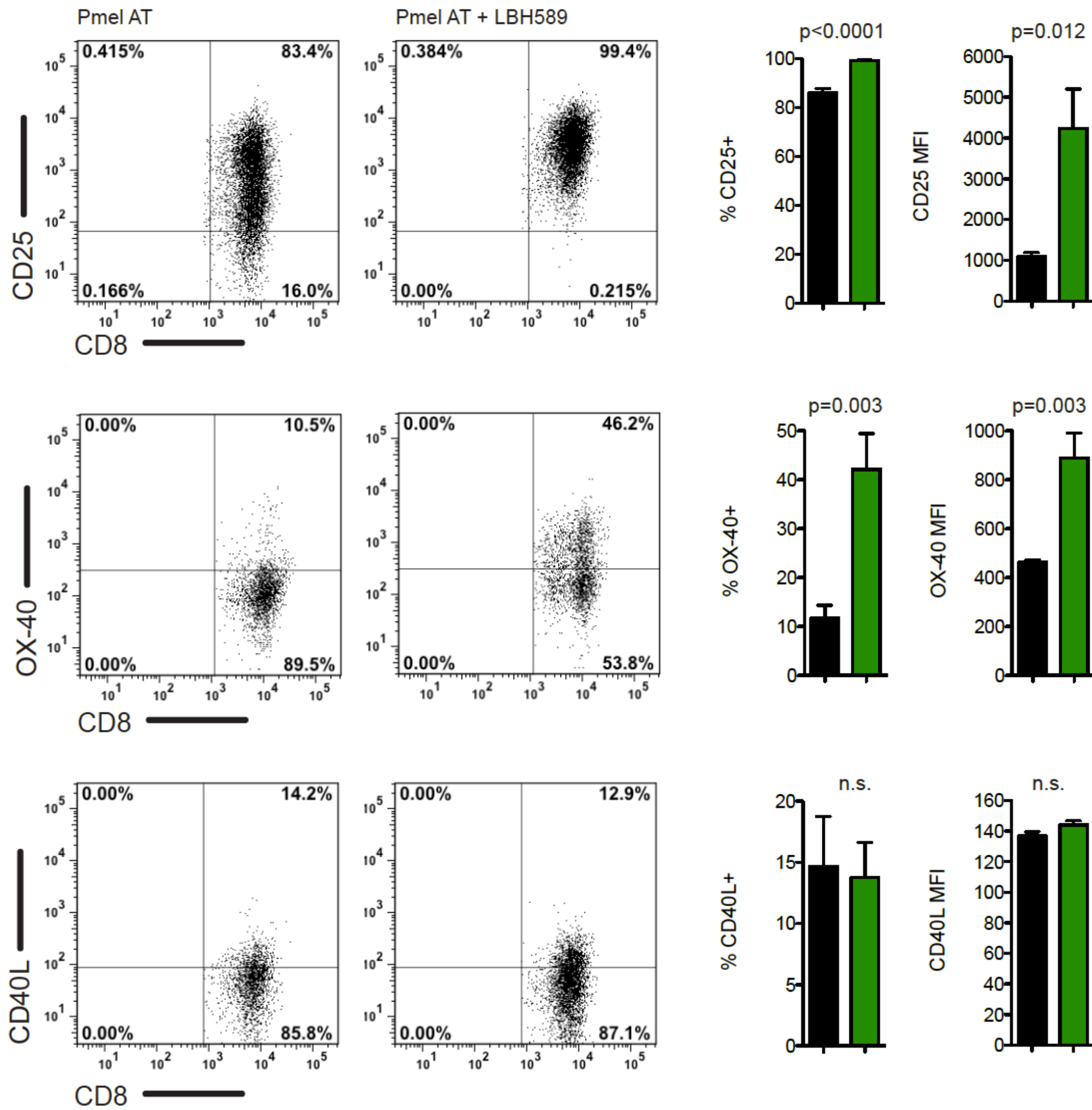

Figure 3.6: Splenocytes from mice treated with and without LBH589 were assayed ten days after adoptive cell transfer. Representative plots are gated on CD8+ Thy1.1+ Pmel T cells. Each group contained 6 mice with similar data obtained in an independent experiment. Student's t-tests were performed to determine statistical significance between groups.

Figure 3.7: Dramatic expansion and recovery of tumor specific T cells *in vivo* as a result of LBH589 treatment.

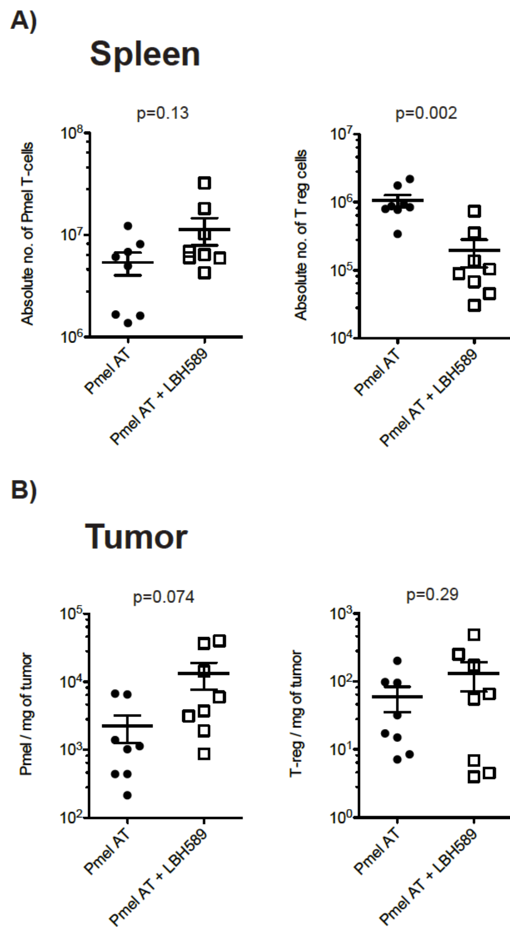

Figure 3.7: A. Quantification of the absolute numbers of Pmel T cells and T regulatory cells recovered from splenocytes collected 10 days after adoptive cell transfer according to the treatment scheme on Fig. 3.1A. B. Quantification of the absolute numbers of Pmel T cells and T regulatory cells per milligram of tumor.

Figure 3.8: Functional enhancement of Pmel T cell cytokine production.

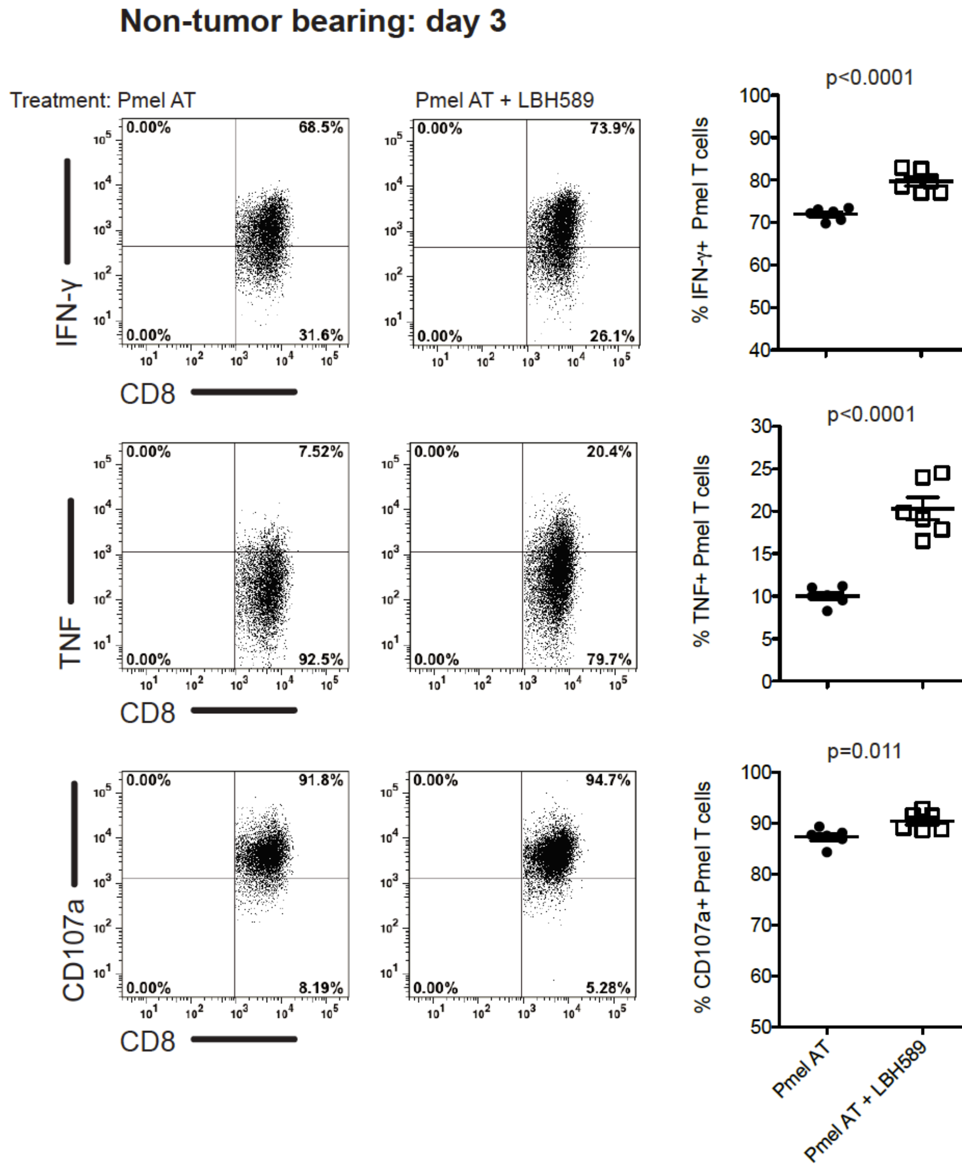

Figure 3.8: A. Restimulation of Pmel T cell splenocytes with cognate $gp100_{25-33}$ peptide *ex vivo* for five hours. Representative plots indicate splenocytes restimulated 3 days following adoptive

cell transfer and are gated from CD8+ Thy1.1+ Pmel T cells. Gates were set based on Pmel cells that lacked peptide stimulation for the 5 hour duration of the restimulation.

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