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Targeting Histone Deacetylases in Melanoma and T-cells

to Improve Cancer Immunotherapy

by

Andressa Sodre de Castro Laino

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Cell Biology, Microbiology, and Molecular Biology
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Date of Approval:
March 03, 2016

Keywords: HDACs, HDAC Inhibitors, Lymphocytes, Immune Checkpoint Blockade

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DEDICATION

I dedicate this work to graduate students, postdocs and researchers who are part of the scientific foundation. All their effort to maintain both ethics and progress of science should always be appreciated.

ACKNOWLEDGMENTS

I would like to thank my mentor, Dr. Eduardo Sotomayor, for the vast opportunities and training I received from him during the time I spent in his laboratory. His expertise and mentorship were and continue to be of great value to my journey during graduate school and my future career. I am also thankful for the support and expertise of my co-mentor, Dr. Sheng Wei, and my committee members, Dr. Shari Pilon-Thomas and Dr. P.K. Epiling-Burnette. Their assistance and insights were critical for the development of my graduate work. I would like to extend my appreciation to Dr. Lisa Butterfield, who kindly accepted the invitation to be part of my defense process.

I am also very grateful for having the privilege of collaborating and receive training from Dr. Jeffrey Weber. I have encountered exceptional opportunities and resources while conducting part of my studies in his lab. Much gratitude is expressed to the collaborators Dr. Amod Sarnaik, Bin Yu, Pingyan Cheng and Rupal Ramakrishnan.

Likewise, I appreciate the knowledge and inputs I encountered from joined lab meetings with Dr. Javier Pinilla's, Dr. Alejandro Villagra's, Dr. P.K. Epiling-Burnette, and Dr. Claudio Anasetti's labs. Especial appreciation is extended to Dr. Sotomayor's, Dr. Pinilla's and Dr. Villagra's lab current and former members: Jie Chen, Fengdong Cheng, Hongwei Wang, John Powers, Eva Sahakian, Susan Deng, Alison Dister, Kamira

Maharaj, Maritza Leinlaf and Patricio Perez-Villarroel. I would also like to thank Jodi Kroeger and the flow cytometry facility for providing valuable technical assistance.

I very much appreciate all the training and advice I received from all members of Cancer Biology PhD program. In particular, I would like to express my gratitude to Dr. Kenneth Wright and Cathy Gaffney, for the help I always received from them.

I am thankful for all the support I had from my father, Olavo, and his girlfriend, Juliana, from my mother, Andréa, and her husband, Eugenio, from my grandparents, Dulce and Olavo, and from all my family members. The reunions, jokes, laughs, FaceTimes and Whatsapps made everything better. I thank my supportive and loving aunt, Daniela, and her husband, Sergio. They helped me in so many ways when I came to United States and also through all my years in graduate school. I also would like to thank my Brazilian and American friends, Cecília, Débora, Eduardo, Paula Cristina and Adam Mailloux for all the good and funny moments. I would like to dedicate an especial thank you to João Paulo, my forever-best friend.

I am very grateful for having David Woods as my boyfriend, lab mate and also best friend. His help, support and love made a lot easier the hurdles I encountered during my graduate school.

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ABSTRACT

Histone deacetylases (HDACs) are key mediators of gene expression and, thus, major regulators of cell function. As such, HDACs play a role in orchestrating tumor biology, and the use of small inhibitors targeting theses proteins is attractive for the field of cancer therapy. Indeed, several HDAC inhibitors have received FDA-approval for the treatment of malignancies, while a myriad of these compounds continue to be evaluated in clinical trials. Besides their direct impact on tumor growth, HDAC inhibitors have been shown to increase immunogenicity of cancer cells, facilitating generation of a productive immune response against tumors. Immunotherapeutic approaches take advantage of the intrinsic ability of the immune system to manifest an anti-tumor response. Mechanisms of immune escape are often developed by cancer cells, neutralizing activity of the immune system. For example, upregulation of the PD1 ligands PDL1 and PDL2 by tumor cells negatively regulates the anti-tumor functions of PD1-expressing infiltrating T-cells. Importantly, strategies targeting this inhibitory axis have shown outstanding clinical benefit for the treatment of solid and hematological malignancies.

The mechanisms by which HDAC inhibitors modulate tumor and immune cells biology were explored herein. Initially, treatment of melanoma cells with pan- and class I-selective HDAC inhibitors resulted in upregulation of PDL1 and PDL2 molecules. These effects were observed in mouse and human cell lines, as well as in tumor cells

resected from metastatic melanoma patients. This upregulation was robust and sustained, lasting at least 96 hours *in vitro*, and validated *in vivo* using a B16F10 syngeneic mouse model. Enhanced expression of PDL1 mediated by HDAC inhibitors was found to result from enhanced histone acetylation at the PDL1 gene promoter region. Combination therapy of HDAC inhibition and PD1 blockade was explored in the tumor setting, leading to synergistic effects in terms of reducing melanoma progression and increasing survival of B16F10 melanoma-bearing mice. These data provide a clinical rationale for combination therapy of epigenetic modifiers (e.g. HDAC inhibitors) and PD1 blockade as means to augment cancer immunotherapy, improving patient outcomes.

As a second pillar of this research, the impacts of HDAC-selective inhibition were explored on immune cell biology, since the broad nature of pan-HDAC inhibitors was shown to be detrimental to T-cells *in vitro* and *in vivo*. Based on screening assay results, novel implications of treating melanoma patient T-cells *ex vivo* with the HDAC6-selective inhibitor ACY1215 were investigated. Treatment with this compound was unique among pan- and isotype-selective HDAC inhibitors in modulating T-cell cytokine production and showing minimal impact of T-cell viability. ACY1215 tempered Th2 cytokine production (i.e. IL-4, IL-6 and IL-10), and maintained Th1 effector cytokines (e.g. IFNγ and IL-2). Furthermore, ACY1215 increased expression of surface markers, including CD69 activation marker and ICOS co-stimulatory molecule. In addition, ACY1215 treatment enhanced accumulation of central memory T-cells during *ex vivo* expansion of tumor infiltrating T-cells harvested from resected tumors of metastatic

melanoma patients. Importantly, ACY1215-mediated inhibition improved tumor-killing capacity of T-cells.

These results highlight an unexplored ability of selective HDAC inhibitor ACY1215 to augment T-cell expansion during protocols of adoptive cell therapy. While the discoveries presented here warrant further investigation of cellular and molecular mechanisms associated with ACY1215-treated T-cells, the clinic implications are clear and rapidly translatable.

CHAPTER ONE:

INTRODUCTION

Introduction to Cancer

Cancer is characterized by an uncontrolled division of autologous cells. Instead of being defined as one homogeneous disease, cancer is a collection of diseases highly different and heterogeneous among and within patients. Reflective of that, new approaches to target malignancies are constantly being explored and developed. As a result, in a one-year interval between 2014 and 2015, the FDA approved 17 new drugs and products for treatment, prevention and imaging of cancer, including nine novel anti-cancer therapies and six new uses for previously approved anti-tumor treatments[1].

The occurrence of malignant cells and subsequent development and progression of cancer involve a variety of molecular and cellular modifications, initially described as hallmarks of cancer. Hanahan and Weinberg were pioneers in defining the multiple acquired capabilities of tumor cells required for cancers to establish and progress[2]. These hallmarks involve the capacity of cancer cells to sustain proliferation and indefinite proliferative capacity, resist apoptosis, induce angiogenesis in the tumor surroundings, being unresponsive to tumor growth suppressive molecules, modify cellular metabolism as means of self-preservation, evade an immune response, and ultimately, being able to invade and metastasize[3]. The identification and description of

these mechanisms contributed to the development of drugs targeting pathways and molecules involved in these hallmarks.

Even though cancer research has experienced progress through the years, as indicated by a reduction of the overall cancer death rates in the United States from the years of 2002 to 2011 and an increase of 5-year relative survival rate for all cancers combined from 49% in 1975 to 68% in 2010[1], cancer is an ongoing challenge, still accounting for one in four or one in seven deaths in United States[4] or worldwide[5], respectively[1].

Melanoma

Skin cancers have the highest incidence of all cancers and are usually not fatal, with the exception of melanoma. Melanoma is the most lethal type of skin cancers, having a high mortality associated with the occurrence of metastasis to the lungs, brain, liver, small bowel etc[6]. Even though melanoma has an incidence around 2% among all skin cancers, according to the American Cancer Society, there will be around 73,780 new diagnosed cases of melanoma in 2015, with an expected mortality of 13.4%.

The first resection of melanoma was reported in 1787[7], reflective of melanoma being a long-known type of solid cancer. Until the past decade, the standard treatment for advanced melanoma was limited to the administration of dacarbazine, an FDA-approved chemotherapy drug, not only restricted to the treatment of melanoma but also other types of cancer, such as sarcomas, neuroblastoma, Hodgkin's lymphoma, islet cell carcinoma etc[8]. Dacarbazine is a DNA alkylating agent and acts by adding alkyl groups to proteins forming the DNA double helix structure, thus resulting in

breakage of DNA strands, genetically instability and cell death. Until recently, the only FDA-approved treatment of unresectable melanoma consisted on the use of hydroxyurea as a cytotoxic drug, recombinant interleukin (IL)-2 as an immune regulator, or recombinant IFNα-2b as an adjuvant therapy in cases of high risk of cancer recurrence[9,10]. However, none of these lines of treatment resulted in overall survival benefit. Overall response rates are also low and not sustained, reaching 10% benefit with dacarbazine administration for stage IV melanoma[11,12].

One of the main hindrances to chemotherapy is the development of drug resistance, contributing to the failure of around 90% of patients treated for metastatic cancer[13]. Melanoma is similar in this regard, yet the mechanisms of resistance are not fully elucidated. After prolonged treatment, cancer cells can acquire resistance or cross-resistance to other drugs[14,15] by a variety of mechanisms, including disruption of apoptotic pathways by upregulation of survival molecules (e.g. BCL-e, BCL-X/L and survivin)[16], enhanced DNA repair ability[17], or reduced drug uptake capacity and increased expelling ability of the drug from the interior of the cell, through P-glycoprotein pumps commonly found on the tumor cell membrane[18]. Dacarbazine-treated melanoma cells can minimize the chemotherapy effects by increasing the levels of the DNA repair enzyme O^6-alkylguanine DNA alkyltransferase[16], or inhibiting drug transport into the cell, as previously mentioned[18]. Also, dacarbazine treatment may result in activation of RAF, MEK and ERK pathways and consequent secretion of IL-8 and vascular endothelial growth factor (VEGF)[19,20], selecting for tumor cells more tolerant or insensitive to the chemotherapy. Furthermore, conventional chemotherapy affects high

proliferative cells, an aspect characteristic of tumor cell biology, but also present in some healthy cells.

Under normal circumstances, extracellular signals provided by cytokines, growth factors, or hormones are known to activate the mitogen activated protein kinase (MAPK) pathway, regulating cell proliferation. However, mutations resulting in alteration and constitutive activation of the MAPK signaling pathway are found in 90% of melanoma tumors[21]. The three members of RAS family of GTPases comprise the oncogenes NRAS, KRAS and HRAS, which are usually mutated in different types of cancers and lead to activation of the MAPK pathway[22]. In melanoma, activating mutations in NRAS represent nearly 25% of tumors[21], while KRAS and HRAS mutations are found at lower rates of 2% and 1%, respectively[23]. Downstream of RAS signaling is BRAF protein, mutated in approximately 50% of cutaneous melanomas[24]. In the majority of melanomas, BRAF is altered due to a V600E mutation[25], resulting in activation of the MAPK signaling through a phosphorylation cascade activating MEK and ERK[26]. MAPK signaling can also be inappropriately activated as the result of loss of NF1 function, found in 15% of melanomas, activating mutations of KIT gene, and translocations or fusions of ALK or ROS with other genes[27].

Another pathway important for sustained proliferation is the PI3K/AKT/mTOR signaling cascade. Approximately 70% of melanomas have this pathway upregulated[21], as a result of mutated molecules involved in this signaling activation, such as c-KIT and PTEN[28,29]. Inactivation of PTEN, as well as upregulation of cyclin-D1, also results in melanoma evasion of growth suppression. Similarly, inactivation of the cyclin dependent kinase inhibitor 2A (CDKN2A) locus in melanoma, through hypermethylation-mediated

gene silencing, leads to disruption of the tumor suppressor p53 stabilization and of cell cycle regulation[30]. Mutations directly on p53 protein are also found in 5-25% of melanoma patients, and mediate apoptosis resistance on tumor cells[31].

Targeted Therapies in Melanoma

The rationale of targeting pathways involved in melanoma cell growth led to the development of inhibitors of the RAS signaling pathway. Vemurafenib acts by blocking mutated BRAF kinase and was approved by the FDA in 2011 for the treatment of unresectable, metastatic BRAF V600E-positive melanoma[32]. In a randomized phase III clinical trial for the treatment of metastatic melanoma, vemurafenib showed 48% response rate compared to 5% with dacarbazine administration, with median overall survival of 13.6 *versus* 9.7 months, respectively[33,34]. Dabrafenib is also a selective mutant-BRAF inhibitor and received FDA approval in 2013, as it reached 53% response rate *versus* 5% with dacarbazine treatment[35].

Alternatively, MEK inhibitors can be used in order to prevent tumors growth, as they also impair RAS signaling cascade downstream of BRAF protein. The MEK1/2 small molecule inhibitor trametinib was FDA-approved in 2013 for the treatment of metastatic, unresectable BRAF-mutated melanoma. Trametinib also showed clinical benefit over dacarbazine chemotherapy, as indicated by 22% *versus* 8% response rate, respectively, in a randomized phase III trial[36].

Combination therapies of BRAF and MEK inhibitors were also explored in phase III clinical trials during the past years. Combining dabrafenib with trametinib versus dabrafenib or vemurafenib alone resulted in a superior response rate of 64-67% versus

51% for the single-treatment groups[37,38]. Furthermore, treatment of vemurafenib plus cobimetinib – a MEK inhibitor – reached 64% response rate compared to 41% for administration of vemurafenib as a single agent. While targeted therapies for melanoma are very promising compared to standard dacarbazine treatment, they show limited clinical benefit in the vast majority of patients, as response lasts for six to eight months before tumors progress[39], resulting from tumor resistance[33,35].

In the past six years, melanoma therapy approaches targeting the tumor directly using small inhibitors in a personalized and rational manner have resulted in clinical benefit, especially relative to dacarbazine as the main line of treatment for over 30 years. Unfortunately, targeted therapies for melanoma have been limited to improving patient survival rather than generating durable responses, a reflection of resistance selection. Recently, the focus of melanoma therapy has expanded to new approaches, not only involving direct target of the tumor, but also boosting the immune. Indeed, recent advances in melanoma immunotherapy have been unprecedented.

Role of the Immune System in Melanoma

The immune system is responsible for neutralizing and eliminating infectious agents, which is accomplished by the recognition of antigens associated with pathogens. In a similar manner, both the innate and adaptive immune systems can recognize and combat tumor cells in a process termed immunosurveillance. The adaptive immune system can detect cancer cell antigens and mount an anti-tumor response, through a process involving antigen recognition and activation of dendritic cells (DCs), followed by antigen presentation in the lymph nodes to T and B

lymphocytes by major histocompatibility complex proteins (MHC), and, ultimately, T cell activation, clonal expansion and migration to the tumor in order to generate a productive response[40].

In melanoma, an association between the presence of a tumor infiltrate composed of reactive T-cells and an improved patient prognosis was first evidenced in 1989[41]. Initial studies have demonstrated that high levels of CD8+ tumor-infiltrating lymphocytes (TILs) promoted prolonged patient survival compared to patients lacking infiltrate[41,42]. Furthermore, evidence suggests that it is crucial that reactive T-cells infiltrate the tumor *milleu*, as the solely presence of circulating reactive T-cells does not suffice to improve survival[43]. Beyond melanoma, especially in the setting of immunogenic tumors, effector immune cells such as T-cells and NK can infiltrate solid tumors. For instance, tumor infiltration of CD8+ T-cells is associated with a favorable prognosis in epithelial ovarian carcinoma[44].

There are multiple ways in which immune cells can detect cancer antigens. Tumor associated antigens (TAAs) vary in their nature and can be recognized by T-cells. In melanoma, tumor infiltrating T-cells often recognize the non-mutated melanocyte differentiation proteins MART-1 and gp100 with low affinity binding, also expressed on normal cells derived from a common lineage (e.g. healthy melanocytes)[45,46]. However, studies have shown that those antigens are unlikely melanoma-specific targets of T-cell, as demonstrated by high toxicity against healthy tissues sharing the same antigens, after infusion of T-cells modified to express high affinity TCRs against MART-1 and gp100[47]. Furthermore, the ubiquitously expressed antigens survivin and hTERT are usually upregulated in cancer cells, while expressed at

low levels on healthy cells[48,49]. Also comprising TAAs are cancer/testis antigens, typically expressed on germline tissues, but frequently upregulated in tumor cells through epigenetic dysregulation. Examples include MAGE, BAGE GAGE and NY-ESO-1, having the latter being targeted in melanoma by adoptive transfer of genetic engineered T-cells with a NY-ESO-1 reactive TCR. In melanoma, results have demonstrated objective clinical response of 11 out of 20 patients undergoing T-cell therapy[50].

Antigens can also be tumor-specific (TSAs), and they may derive from viral genomes (e.g. EBV and HPV) or originate from non-synonymous mutations. Transcriptome analyses of tumor *versus* normal tissues of a variety of cancer patients have demonstrated that multiple tumor types express several neo-antigens generated by mutagenesis. Melanoma presents the highest frequency of non-synonymous mutations among all the cancers evaluated, achieving an average of 100 mutations per megabase[51]. Strategies involving whole-exome sequencing as means to screen for tumor neo-antigens and redirect the immune response against new targets open a new horizon for cancer therapy, especially in the setting of a highly mutated tumor such as melanoma. While identification of targetable antigens demands extensive screening and labor, these approaches are paving a new avenue for personalized medicine.

Research involving the intricate relationship between the immune system and cancer is constantly leading to new developments in cancer therapy redirecting immune components against tumor cells. Evidence shows that immunosuppressed mice and humans are more susceptible to the development of neoplasias[52-54]. Indeed, the concept of immunoediting explains the general process by which tumor and immune

system interact during cancer initiation, establishment and progression. In the setting of a productive immune response, immune cells can recognize and completely eradicate tumor cells, a process called elimination phase. Initial tumor development involves an inflammatory microenvironment, usually detected by the innate immune system, such as natural killer cells (NK), dendritic cells (DC) and macrophages. An orchestrated response involving the innate and adaptive immune systems arises, leading to death of tumor cells through effector cytokines (e.g. IFNγ, perforin) produced by NK and/or T-cells. Cytokines such as IFNγ and IL-12 are present in an inflammatory *milieu*, facilitating a type 1-like response and triggering direct tumor killing from CD8+ cytotoxic T-cells[55,56].

In the case of incomplete clearance of cancer cells, a temporary equilibrium phase occurs, in which the immune system exert a selective pressure to control tumor growth. Genetic instability and accumulation of DNA mutations may select for more resistant and immunosuppressive tumor cells. The immune system may succumb to this new microenvironment, where tumor is no longer visible and effector functions of immune cells are suppressed, and fail to mount a productive response. Tumor escape allows cancer to progress.

Immune Escape

Multiple factors contribute to tumor escape, mainly consisting of promoting a suppressive microenvironment in which the immune system is no longer able of properly respond against cancer cells. Many of these mechanisms involve reducing the recognition capacity and effector functions of cytotoxic T-cells. Commonly resulting from

selective pressure exerted by the immune system, melanoma and other cancer cells may lack appropriate T-cell activation signaling through downregulation of MHC molecule or loss of the MHC I invariant subunit b2-microglobulin[57,58]. Additionally, ineffective or weak T-cell signaling can result in T-cell anergy, impairing a productive response against the target[59].

A proper T-cell response is dependent on three signals, derived from initial engagement of the T-cell receptor (TCR) with the antigen-loaded MHC, subsequent expression and activation of costimulatory molecules, and stimuli by cytokines produced in the microenvironment to determine T-cell fate and function. Briefly, antigen stimulation of TCR leads to phosphorylation of tyrosine residues on the immunoreceptor tyrosine-based activation motifs (ITAMs), forming anchoring sites for activating molecules, such as ZAP-70, and leading to TCR signal transduction via the three main T-cell pathways of MAPK, protein kinase C (PKC) and calcineurin[60]. Costimulatory signaling is provided shortly after TCR engagement through two main groups of costimulatory receptors. One comprises the family of immunoglobulins, such as CD28 and ICOS, and the other is formed by the tumor necrosis factor family, including 4-1BB, OX40, CD27, CD30 and HVEM. Most costimulatory receptors are upregulated after T-cell activation via TCR and interact with ligands on the membrane of presenting cells. CD28 receptor is constitutively expressed on T-cell surface and is known to recognize CD80 and CD86 ligands[61]. In a type 1-like response, preferable in the setting of cancer, IL-2, IFNγ and TNF cytokines contribute to T-cell maintenance, proliferation and effector function.

As stated, an ideal T-cell response depend on a myriad of factors, including proper TCR stimulation and costimulatory signaling, as well as secretion of homeostatic cytokines. Tumors, on the contrary, display a suppressive microenvironment resultant from production of anti-inflammatory cytokines, attraction of suppressive immune cells, T-cell inhibitory signaling, induction of T-cell apoptosis, etc. Thus, an immunosuppressive environment frequently induces a state of unresponsiveness of tumor-specific T-cells and represents a hurdle for immunotherapy. In melanoma patients, T regulatory cells (Treg) can be found in primary[62,63] and metastatic lesions[64,65], as well as in affected lymph nodes[64]. The local chemokine setting found in the tumor *milieu* attracts CD4+CD25+Foxp3+ Tregs[66], which can mediate peripheral tolerance of effector T-cells. Moreover, tumor derived factors such as IL-10, TGFβ and indoleamine 2,3-dioxygenase (IDO) can induce Treg differentiation, tumor infiltration or proliferation[67,68], ultimately affecting melanoma patient survival[69]. Among their suppressive mechanisms, Tregs constitutively express cytotoxic T-lymphocyte-associated protein 4 receptor (CTLA4), which competes against CD28 receptor on the surface of T-cells for ligation to CD80/CD86 on the membrane of antigen presenting cells (e.g. DCs or tumor cells), increasing the threshold of activation of cytotoxic T-cells[70,71] and leading to degradation of CD80/CD86 ligands[72]. Moreover, CTLA4 engagement can recruit inhibitory proteins to the T-cell synapse and interfere with TCR and CD28 signaling[73], or even stimulates production of TGFβ inhibitory cytokine[74]. In the cancer setting, CTLA4 blockade has demonstrated efficacy *in vivo* as a single agent, as well as in combination with vaccines, antibody treatment, chemotherapy,

radiation, surgery etc[75]. Indeed, the use of CTLA4 blockade for melanoma treatment was recently approved by the FDA[76,77].

Furthermore, myeloid-derived suppressor cells (MDSC) or tumor-modulated immature DCs can accumulate in cancer lesions. They can downregulate T-cell activity and function through multiple mechanisms, including improper TCR signaling, arginase depletion as a result of arginase enzyme expression[78], production of reactive oxygen species (ROS)[79] and upregulation of inducible nitric oxide synthase (iNOS)[80]. Macrophages are also frequently present in the tumor infiltrate. As cancer develops, tumor associated macrophages (TAM) can polarize from a M1 to M2-like phenotype, in which they stop producing inflammatory factors and shift towards an anti-inflammatory and pro-tumorigenic setup through secretion on a variety of factors, including TGFβ, IL-10 and VEGF, ultimately inhibiting effector response and inducing angiogenesis[81-83].

Also among mechanisms of immune evasion, cancer cells can modulate the reactive immune infiltrate by production of inhibitory cytokines. IL-10 can be secreted by solid[84] and hematological[85] tumor cells, hindering production of pro-inflammatory cytokines, T-cell proliferation and cytotoxicity. It has been demonstrated that low doses of IL-10 can prevent T-cell apoptosis[86] and elicit CD8+ T-cell memory formation by insulating T-cells from signaling provided by inflammatory cytokines[87]. However, in the tumor setting, production of IL-10 and reduced levels of IL-12, coupled with the presence of DCs expressing low amounts of costimulatory molecules, can induce anergy of cytotoxic T-cells and prime a Th2 phenotype[88,89]. In melanoma, IL-10 production is predicative of prognosis, as higher levels of this cytokine are associated with reduced survival[90,91]. Also a major player in malignancies is IL-6, as it can inhibit

apoptosis of tumor cells and induce angiogenesis[92,93]. Even though IL-6 may have dual roles depending on the stage of tumor development, increased levels of IL-6 in the serum have been negatively associated with prognosis in multiple cancer types, including melanoma[90,94,95]. Besides these cytokines, melanoma frequently secretes transforming growth factor (TGFβ), a suppressive cytokine able to reduce T-cell effector function and modulate tumor motility and invasiveness[96]. TGFβ also presents growth inhibitory properties, although melanoma cells are not susceptible to this effect[97]. The presence of TGFβ in the tumor microenvironment can skew T-cell subsets into a Th2 phenotype, resulting in a less inflammatory response characterized by secretion of IL-4, IL-5, IL-6, IL-10, IL-12 and IL-13 cytokines[98]. These cytokines are associated with tumor promotion, as they temper the immune response. IL-4, for instance, facilitates the polarization of T-cells into a Th2 subset, and promotes the skewing of M1 to M2 macrophages. While M1 macrophages boost Th1 responses and enhance secretion of pro-inflammatory cytokines, M2 macrophages produce anti-inflammatory cytokines (e.g. IL-10, TGFβ), thus participating in immune suppression[99-101]. Generally, a Th2 phenotype is developed during response against extracellular pathogens, and is inappropriate in contexts necessitating a cellular response (e.g. viral infections, neoplasms). In the context of cancer, this type of response reduces tumor immunity, since it polarizes immune cells away from functions of the ultimately required Th1, effector phenotype.

The multifunctional role of cytokines in the tumor *milie*u is complex and comprises factors with described growth-promoting properties, including tumor necrosis factor (TNF), colony stimulating factor-1 (CSF1), IL-8 (also knows as CXCL8) and IL-

1[90,102,103]. In summary, mechanisms of tumor evasion cripple a responsive immune system through modulation of both cancer and immune cells, ultimately allowing progression of disease.

Immunotherapy in Melanoma

Immunotherapeutic approaches consist in manipulating components of the immune system to treat or control tumor cells or other diseases, such as infections. In the cancer setting, diverse strategies aiming to boost or redirect immune cells against the target have been developed, including the use of cytokines, monoclonal antibodies, vaccines, immune checkpoint blockade (e.g. PD1 and CTLA4) and adoptive transfer of immune cells.

Cytokine-directed therapy for melanoma, in which purified cytokines are systemically administered, is currently restricted to IFNα-2b[9] and IL-2[10]. Therapy using IFNα-2b was FDA-approved in 1995 as an adjuvant for completely resected melanoma stages II and III, when there is an intermediate or high risk of recurrence[104,105]. The use of IFNα-2b leads to an overall response rate of 22%, with an improved, but not consistent, overall survival. The toxicity derived from treatment is often high, and in some cases patients can experience serious side effects such as liver dysfunction and myelosuppression[106-108]. To reduce adverse effects, a long-acting pegylated (PEG) form of IFNα-2b[105] received FDA approval in 2011. Administration of IL-2 to metastatic melanoma patients was approved by the FDA in 1998, and it aims to stimulate and maintain activity of effector immune cells (e.g. T-cells and NKs). Also associated with

severe toxicity, IL-2 has shown a 16 to 23% overall response rate, coupled with durable responses in 5 to 10% of patients[109,110].

The understanding that cancer cells present tumor associated or specific antigens led to the development of another line of immunotherapy – patient vaccination. Cancer vaccines are mainly based on the strategy of eliciting CD8+ cytotoxic T-cell (CTL) response. As previously mentioned, melanoma presents tumor associated antigens that can be explored as targets of cancer vaccines. Several trials have studied vaccination based on the melanosomal protein gp100 in combination with other immunotherapy approaches (e.g. CTLA4 blockade and recombinant IL-2). In advanced melanoma patients, simultaneously administration of gp100 vaccine with IL-2 showed increased response rate and progression-free survival than treatment with IL-2 as a single agent[111]. However, when g100 vaccine administrated concomitantly with CTLA4 blockade – an immunotherapy approach potent as a single agent –, the benefits achieved were not significantly different to CTLA- blockade alone[112]. This may be a reflection of an already tolerant and exhausted subset of tumor-specific T-cells. As cancer progresses, mechanisms of immune evasion account for the generation of low quality T-cells lacking the capacity to respond against tumor. In this sense, approaches to recover CD4+ and CD8+ effector and memory T-cells are warranted. Recently, interest was placed in studies developing vaccines based on screened tumor neo-antigens. As part of personalized medicine, individualized vaccines would be produced targeting epitopes predicted by bioinformatics research, resulting from the mutatome of tumors from the patients themselves[113,114].

The use of oncolytic virus for the treatment of advanced melanoma has also been explored and has demonstrated clinical efficacy. This approach involves local administration of the virus on melanoma lesions. For instance, Talimogene laherparepvec, or T-VEC, is an attenuated form of herpes simplex virus 1 (HSV-1), engineered to lyse cancer cells and secrete GM-CSF in order to attract DCs to the tumor site. Although T-VEC infects both normal and cancer cells, it maintains its replication capabilities only on dividing cells, while sparing most of differentiated, non-dividing healthy tissues. A clinical trial has shown that T-VEC treatment of patients with stage III/IV unresectable melanoma promotes reduction of melanoma lesions on skin and lymph nodes, sustained at least for six months. In 2015, T-VEC received FDA approval for treatment of recurrent, unresectable melanoma[115].

Strategies seeking to induce a productive immune system are vastly explored in the tumor setting. The use of immunoregulatory antibodies targeting T-cell inhibitory molecules (e.g. PD1, PDL1 and CTLA4) has shown profound efficacy for the treatment of some cancers, especially melanoma. The immune checkpoint blockade antibodies consist of targeting either the CTLA4 receptor, reducing competition with CD28 costimulatory receptor on the surface of T-cells, or blocking PD1/PDL1 axis, thus minimizing negative regulation of activated T-cells. Clinical trials using these antibodies will be further discussed in detailed below.

As previously illustrated, the majority of immunotherapy approaches in the context of melanoma consist of modulating and enhancing activity of cytotoxic tumor-specific T-cells. A related strategy of melanoma therapy with profound impact in prolonged response and survival of some patients involves adoptive transfer of

autologous T-cells. These lymphocytes can be harvested from the tumor site (i.e. TILs) or genetically engineered to recognize the tumor. Both cases will be discussed in more details further in the chapter.

Immune Checkpoint Blockade

After approximately two decades since CTLA4 being described for the first time, a monoclonal antibody targeting this inhibitory receptor was assessed in a clinical setting. Ipilimumab, a human IgG1 monoclonal antibody against CTLA4, was approved by the FDA in 2011 for the treatment of metastatic melanoma. Initial phase I/II studies using scaled doses of ipilimumab for the treatment of advanced melanoma showed one partial, one complete responses, and seven patients with stable disease out of 88 individuals[76]. Furthermore, the efficacy of the drug was found to be dependent on dose with higher overall response rate of 11% followed administration of 10mg/kg of antibody, reaching one-year survival rate of 47%[116-118]. In a phase III clinical trial for the treatment of melanoma in stages III and IV, efficacy of ipilimumab plus dacarbazine was compared to dacarbazine alone. Results demonstrated prolonged overall survival for ipilimumab arm, at a dose of 10mg/kg[77]. Additionally, data from a meta-analysis of multiple clinical trials indicated durable response, with 3-year overall survival rate of 22% and a lower death rate during seven years after trial was ended[119,120]. The extent of ipilimumab applications continues to rise, as the FDA approved it in 2015 for the use as an adjuvant for treatment of cutaneous melanoma of patients with above one millimeter of pathology in the regional lymph nodes, or following complete resection. An ongoing phase III clinical trial for treatment of resected stage III melanoma patients has

reached a median recurrence-free survival of 26 *versus* 17 months with ipilimumab or placebo administration, respectively, reducing the risk of recurrence or decease by 25% (ClinicalTrials.gov Identifier: NCT00636168).

Tremelimumab is the second human monoclonal antibody developed targeting CTLA4, and not yet FDA-approved for melanoma treatment. Tremelimumab has a plasma half-life higher than ipilimumab, resulting in a scheduled dose significantly more sparse (once every three months at a dose of 15mg/kg)[121-123]. A phase II clinical trial for advanced melanoma reached 7% response rate and one- and two-year survival of 40% and 22%, respectively. When studies progressed to a phase III clinical trial, no benefit in overall survival was observed. Although promising, further research is warranted for melanoma treatment with tremelimumab.

Another strategy to target immune checkpoints consists of blocking PD1 receptor or the PD1 ligand, PDL1, the latter commonly found on the surface of tumor and stromal cells[124]. When the PD1 receptor interacts with its ligands, it triggers dephosphorylation of TCR signaling, thus downregulating T-cell activation. Pembrolizumab is a human IgG4 monoclonal antibody targeting PD1 and was FDA-approved for advanced or unresectable melanoma treatment in 2014. In a phase I clinical trial with a median follow-up time of eight months, treatment with pembrolizumab led to a overall response rate of 26%, regardless of the tested doses[125]. Nivolumab, also a human IgG4 monoclonal antibody against PD1, received approval by the FDA shortly after a phase I/II clinical trial for diverse types of solid cancers, such as melanoma, non-small-cell lung, renal-cell, prostate and colorectal carcinomas. In melanoma, 26 out of 94 patients presented clinical response at all doses evaluated[126].

In a follow-up, melanoma patients reached a 31% objective response rate, coupled with rates of 62% and 43% for one- and two-year survival, respectively[127]. This study attributed the likelihood of clinical benefit to the presence of PDL1 expression in immunohistochemical (IHC) slides derived from patient biopsies. However, this type of analysis is susceptible to limitations, as PDL1 evaluation is restricted to the tumor sites surgically removed and IHC technique provides low resolution of PDL1 staining. It is worth mentioning that this study did not account for expression of PDL2, the other known inhibitory ligand of the PD1 receptor. Research has demonstrated upregulation of PDL2 in the tumor *milieu*, frequently expressed on APCs, but also on tumor cells[128,129]. Moreover, a clinical trial defining the cutoff for PDL1 expression as the amounts of 5% or greater, or 1% or greater, demonstrated that objective responses were present in both groups with positive or negative PDL1 IHC staining[130]. Regardless, expression of PDL1, even though negative from the point of view of reducing T-cell function, may be a reflection of an active and productive immune response. This is demonstrated by a higher incidence of objective response and clinical benefit when PDL1 expression is present other than absent[131].

In a phase III clinical trial for the treatment of melanoma with nivolumab at a dose of 3mg/kg, durable responses were achieved, with an observed objective response rate of 32%[132]. In terms of adverse reactions, toxicity was manifested in all approaches targeting immune checkpoint blockade. Interestingly, a higher severity appears to be associated with enhanced objective response[133], potentially a reflection of an active and productive immune system. Moreover, the toxicity experienced by blockade of the PD1/PDL1 axis appears less severe adverse effects derived from

CTLA4 blockade. This may be a reflection of the outcome resultant from PD1/PDL1 blockade mainly being restricted to interfering with reactive T-cells immersed in the tumor *milieu*, while CTLA4 blockade is also occurring in lymphoid organs, a less focused location[134].

Clinical trials for the treatment of a variety of solid tumors were also designed for evaluation of developed compounds targeting PDL1 molecules. For instance, one of the compounds consists of a human IgG4 monoclonal antibody targeting PDL1 and was assessed in a phase I trial for the treatment of advanced melanoma. Nine out of fifty-two patients, or 17%, achieved an objective response, for which five lasted at least one year[135]. While these results represent a significant improve over standard chemotherapy, they were not as striking as PD1 blockade. This could be a reflection of blocking solely PDL1 ligand, still allowing interaction of PD1 receptor with PDL2, or it could be due to the avidity and affinity differences of the different antibodies, or other unknown mechanisms. Regardless, work is needed to continue improving efficacy of immune checkpoint blockade in melanoma and other types of cancer.

Research exploring the synergistic effects of combining PD1 and CTLA4 blockade is also under development. In a phase I clinical trial, a concomitant regimen of ipilimumab and nivolumab resulted in 11.5 months of median progression-free survival, compared to 6.9 and 2.9 months with nivolumab or ipilimumab treatment as single agents, respectively. Toxicity associated with a combinatory regimen was also higher, reaching 55% of patients in comparison to 16-27% of individuals undergoing monotherapy. These promising results led to the very recent FDA-approval of combination therapy of nivolumab plus ipilimumab for the treatment of metastatic or

unresectable melanoma[136]. Following a similar rationale, and aiming to reduce toxicity, ongoing research is exploring the effects of sequential administration of nivolumab and ipilimumab. Surprisingly, nivolumab being administrated prior to ipilimumab led to an objective response in 40 to 50% of patients, while the contrary (i.e. ipilimumab followed by nivolumab) resulted in less than half of this rate[137]. So far, the mechanisms responsible for this impaired response in the arm of ipilimumab-first treated patients are not fully elucidated.

Finally, immunotherapy blocking immune checkpoint brings a promising, exciting, perspective to melanoma treatment. As such, studies understanding the molecular and cellular biology behind treatment, and novel approaches exploring these agents in combination with other anti-melanoma strategies, are warranted.

Adoptive T-cell Therapy

The ability to grow T-cells *ex vivo* was first evidenced in 1976 with the discovery of IL-2 as a cytokine capable of expanding T-cells *in vitro*, while maintaining effector function[138]. Initial studies in melanoma demonstrated that CD4+ and CD8+ TILs could be harvested from tumor biopsies and specifically recognize autologous melanoma *in vitro*[139]. In 1988, adoptive T-cell transfer (ACT) using harvested TILs was able to trigger tumor regression in metastatic melanoma patients[140]. A growing body of data has taken place since then, with constant improvements in ACT regimens. Even though TIL therapy is not approved by the FDA for the treatment of melanoma, it has a profound impact in cancer immunotherapy, in some cases reaching object responses in up to 72% of metastatic melanoma patients[141].

There are a few approaches in the field of ACT, including harvesting and expanding pre-existent autologous tumor-reactive T-cell populations for subsequent infusion (i.e. TIL therapy), but also genetically engineering T-cells to recognize tumor cells (e.g. TCR or CAR transfer). Both strategies count on the ability of T-cells to successfully exert an anti-tumor, cytolytic function. In order to redirect the immune system against tumors, artificial T-cell receptors such chimeric antigen receptors (CARs) can be introduced in the membrane of T lymphocytes to recognize a specific antigen present on the tumor surface[142,143]. Currently, most CARs consist of an extracellular binding moiety, a transmembrane region and a signaling endodomain to trigger activation. These receptors recognize the target antigen on the tumor surface with high affinity and in a MHC-independent manner, leading to activation of T-cells through the same pathways triggered by TCR and costimulatory molecules. Typically CAR signaling endodomains are composed of a CD3-zeta tail to initiate signal 1 of T-cell activation coupled to a costimulatory molecule to trigger signal 2. CAR-modified T-cells are able to overcome mechanisms of tumor evasion, such as downregulation of MHC I by the tumor cells, in addition to not being susceptible to mechanisms of central tolerance. The clinical use of CARs has thus far been applied to leukemias[144-146] and lymphomas[147,148], renal carcinoma[149], neuroblastoma[150] and colon carcinoma[151]. The results from these clinical trials have been encouraging, with rapid tumor eradication and complete remission in patients with poor diagnosis of chronic lymphocytic leukemia (CLL) or acute lymphoblastic leukemia (ALL) through the use of CARs against CD19. However, a major concern in respect to CAR therapies is that generation of a potent anti-tumor response often leads to off-target function of the modified T-cells. In a study

of CAR therapy for the treatment of CLL, clinical responses were accompanied by a long-term depletion of normal B-cells that shared the CD19 antigen[147]. Adverse effects significantly more severe against normal lung tissues were observed in a clinical trial using a CAR against ERBB2 for the treatment of colon carcinoma[151]. Despite the promising results reported so far, some aspects of treatments using CARs should still be improved to make the therapy as safe and effective as possible. Surface molecules shared by tumors and healthy tissues are not suitable targets to CARs with current used design, since they could promote off-target immune reactions.

Alternatively, genes encoding alpha and beta chains of TCR can be transferred to T-cells in order to redirect response against tumor[152]. Research has shown that TCR genes can be isolated from patients with a successful clinical profile resultant from ACT therapy, or from immunized mice[47,153]. Research has shown that T-cells genetically engineered with MART-1-specific TCR produce IFNγ in the presence of HLA-matched melanoma cell lines *in vitro*[154]. Moreover, preclinical studies demonstrated higher T-cell proliferation, increased cytolytic capacity and prolonged persistence *in vivo* of TCR-transgenic T-cell in an artificial model using ovalbumine (OVA) peptide[155]. A clinical trial for the treatment of metastatic melanoma using TCR-transgenic T-cells demonstrated that infusion of less differentiated T-cells (e.g. expressing naïve markers such as CD45RA+CD45RO-) led to an objective response in 30% of patients, with prolonged T-cell survival *in vivo* and acquisition of memory phenotype (e.g. CD45RA-CD45RO+)[47]. Furthermore, approaches involving genetically engineering of T-cells with a CAR or TCR specific for the melanoma antigen NY-ESO-1 were explored as well in preclinical models and clinical trials. Both approaches involve

laborious work, since they add an extra step of artificially modifying those cells with TCR or CAR transgenes[156,157].

TIL Therapy in Melanoma

Adoptive transfer of autologous TILs in the setting of metastatic melanoma has proven exceptional success. Especially in the case of melanoma, a highly immunogenic type of cancer, reactive T-cells can sometimes be yielded from tumor biopsies. Initial clinical trials using TILs in combination with high dose of IL-2 for the treatment of metastatic melanoma were considered a failure due to lack of *in vivo* persistence of the transferred T-cells. Short-term responses were characteristic of treatment, a result of the inability to maintain viability of infused TILs[140]. Almost ten years later, a phase I clinical trial demonstrated that a non-myeloablative, temporary lymphopenia was essential for efficacy of TIL therapy[158]. This conditioned regimen reduces T-cell competition for homeostatic cytokines, minimizes Treg suppression and augments the presentation and stimulation capacity of APCs[159]. Clinical trials involving TIL therapy for metastatic melanoma patients have adopted a chemotherapy-based lymphodepletion regimen, followed by increasing doses of total-body irradiation, prior autologous TIL infusion combined with systemic administration of a high dose of IL-2. According to the Response Evaluation Criteria in Solid Tumors (RECIST) criteria, objective responses were achieved in 49-72% patients, varying among conditioned-lymphodepleting regimens, yet not statistically different. Among 93 patients enrolled in these trials, 22% reached complete regression and 20% presented sustained responses, lasting for five to ten years, and possibly cured from the disease[160].

Furthermore, TIL therapy conducted across four independent research institutes also demonstrated substantial efficacy, reaching objective responses in 55, 48, 40 and 38% of patients, depending on the study[161-163].

The protocol of harvesting, expanding and infusing TILs has been optimized over the years since its first description in 1988[140]. Currently, surgically removed melanoma samples are cultured in high dose of IL-2 (i.e. 6000IU/mL) either as multiple small fragments or as a single-cell suspension through digestion of extracellular proteins (e.g. collagenase, hyaluronidase). Within two to three weeks, TIL clones are usually the only cells that yield from the tumor cultures. Harvested TILs are then assessed for reactivity against HLA-matched melanoma cell lines or patient-derived tumor, usually with IFNγ production used as readout. This step is called pre-rapid expansion (pre-REP) and, even though it promotes TIL expansion, the resultant number of cells are insufficient for ACT therapy. TIL cultures are then rapidly expanded, a process aided by irradiated feeder cells and activation via CD3 complex of the TCR by antibody use (i.e. OKT3 antibody clone), in the presence of high dose IL-2. This step also takes two to three weeks and generates up to 10^{11} lymphocytes. Following this protocol of activation and expansion, TILs are referred as post-REP and can be infused back into patients. Prior to ACT, patients undergo a lymphodepletion regimen through administration of two days of cyclophosphamide and five days of fludarabine treatment. TILs are then adoptively transferred to patients, concomitantly with high dose IL-2[164].

TIL therapy has proven to be highly effective and even curative in some cases. However, a major hurdle of immunotherapy involving adoptive T-cell transfer is lack of persistence *in vivo* following infusion. Indeed populations of T-cells able to maintain a

high proliferative capacity are sought to mediate a robust response against tumor[165,166]. In this regard, exploring mechanisms to facilitate a less differentiated, memory-like, reactive phenotype is attractive in the field of immunotherapy.

Introduction to Epigenetics

Epigenetic modulation refers to reversible, heritable changes regulating gene expression by mechanisms other than directly altering the sequence of nucleotides. Dysregulated expression of genes is an intrinsic characteristic of tumors cells. Such dysregulation can derive from point mutations, translocations, amplification and deletions, as a reflection of genetic instability of transformed cells. Alternatively, gene expression is also often modulated by epigenetic changes interfering with chromatin structure, including acetylation of histones and methylation of CpG islands proceeding promoter regions. Not defined as epigenetics *per se*, post-translation modifications such as acetylation of non-histone proteins and gene silencing through microRNA also influence gene regulation, and may utilize of component originally described as part of epigenetic machinery (e.g. histone deacetylases). In eukaryotic cells, nuclear DNA is wrapped around histones, which are grouped into five classes (i.e. H1, H2A, H2B, H3 and H4). The structural and functional unit of chromatin is formed by two sets of the four histones H2A, H2B, H3 and H4, the nucleosome, while H1 histone facilitates DNA packaging outside of the nucleosome core. Epigenetic modifications occurring on the DNA or histone tails trigger conformational changes in the chromatin, allowing for a more relaxed or condensed DNA, and lead to gene activation or repression, respectively[167-169]. The acetylation status of histones is a major contributor of chromatin

conformation and can be oppositely regulated through histone acetyltransferases (HAT) and histone deacetylases (HDAC). While HATs act by transferring an acetyl group from acetyl-CoA molecule to the ε-amino group at the N-terminal at histones tail, HDACs remove N-acetyl lysine amino acid on the tail on histones. Deacetylated histones result in electrostatic attraction of positively charged lysine residue to negatively charged, nearby DNA. Usually, histone acetylation leads to a structurally open and transcriptionally active chromatin (i.e. euchromatin), in opposition to HDAC-mediated histone deacetylation, associated with a repressed chromatin and gene transcription (i.e. heterochromatin)[170-172]. Furthermore, post-translational regulation of histone proteins is not restricted to acetylation and methylation, in the sense they are susceptible to modifications involving ubiquitination, phosphorylation, sumoylation, citrullitation, among others[173,174].

Histone Deacetylases in Cancer and Immune System

Histones modification as a result of acetylation and deacetylation processes has been demonstrated as part of cancer development and other abnormalities, including immune disorders, diabetes and neurodegenerative diseases[175]. In this regard, studies involving therapies to reverse epigenetic modifications (e.g. acetylation) are attractive in the field of cancer research. A total of eighteen human HDACs have been described so far, phylogenetically grouped in four classes (i.e. classes I, IIa, IIb, III and IV) according to their homology to yeast orthologs, and varying in structure, cellular localization, tissue distribution, specificity and enzymatic mechanism. Classes I, IIa, IIb and IV comprise the classically described HDACs, presenting a conserved catalytic

domain of approximately 390 amino acids and zinc-dependency for deacetylases activity. Class III is composed by seven members of the sirtuin family (i.e. SIRT1 through SIRT7), also displaying a conserved catalytic domain of around 275 amino acids, unrelated to the classical HDACs, and being instead dependent of NAD+ enzyme[176,177]. HDACs 1, 2, 3 and 8 belong to class I and are ubiquitously expressed, mainly exerting their function in the nucleus. With exception of HDAC8, class I HDACs participate in chromatin remodeling as components of multiprotein complexes, as genuine epigenetic mediators. HDACs 4, 5, 6, 7, 9 and 10 are members of class IIa and IIb of HDACs and have a broad array of substrates, not only limited to histone proteins. In fact, HDAC function lies outside of chromatin remodeling, as they can promote deacetylation of non-histone proteins[178]. Several of these substrates participate in diverse biological events influencing both tumor and normal cells, and include molecules such as α-tubulin, β-catenin, chaperon HSP90, p53 tumor suppressor, the transcription factors c-Myc, NFkB, E2F, etc[179]. Finally, the most recently identified HDAC11 is the sole member of class IV of HDACs and it is shown to be involved in regulation of tumor and immune cell biology[180,181]. Classification and characteristics of the eleven HDACs are illustrated in figure 1.

In the cancer setting, research involving a comprehensive panel of histone H4 post-translational modifications in both normal and cancer cells has identified loss of monoacetylated lysine 16 in malignant cell lines and primary tumors as a hallmark of human cancer cells[182]. Accordingly, multiple types of cancer display aberrant HDAC expression. For instance, upregulation of class I HDACs is observed in several solid tumors, as indicated by overexpression of HDAC1 in breast, colon, prostate and gastric

carcinomas, HDAC2 in colorectal, cervical and also gastric cancers, and HDAC3 in colon carcinoma as well[183-188]. Moreover, HDAC6 is present at high levels in breast cancer and HDAC11 expression is elevated in colon, prostate, breast and ovarian tumors[180,189]. Conversely, downregulation or lack of HDACs 1 and 2 were reported in cancer cells[190,191]. In melanoma, expression of class I HDACs is associated with increased survival of patients with advanced disease. A descriptive study demonstrated upregulation of HDAC8 in BRAF-mutated melanoma samples, as well as correlation with HDAC1 expression and p65 phosphorylation, a subunit of NFkB complex that can be associated with drug resistance to MAPK inhibition[192,193]. Indeed, protein acetylation is reported as an important mediator of resistance to targeted therapies, as HDAC inhibitors directly alter cell growth pathways (e.g. MAPK) involved in drug resistance. For instance, HDAC-mediated inhibition promotes hyperacetylation of the chaperone HSP90, triggering degradation of downstream proteins and upstream tyrosine kinase receptors involved on RAF and AKT pathways[194,195].

The role of several HDACs has been demonstrated in immune cells, but implications on a tumor context are still being explored. It is documented that both HDACs 1 and 2 have overlapping functions during T-cell development, and are capable of promoting compensatory mechanisms to avoid dysregulation of this process. Indeed, T-cell development is arrested in HDACs 1 and 2 double knockout (KO) mice. This is likely resulted from genomic instability generated by loss of HDACs and 2, as well as disruption of TCR signaling[196]. Furthermore, HDAC1 is suggested to inhibit cytokine production from activated effector T-cells, since abrogation of HDAC1 on T-cells in a mouse model of asthma triggers an enhanced Th2-type response, as indicated by

increased production of IL-4, IL-5 and IL-10[197]. A recent study exploring the role of HDAC1 on T-cell activation demonstrated that HDAC1 and mSIN3A function in a protein complex to repress IL-2 production. During T-cell activation, mSIN3A is phosphorylated by CDK5, disrupting this complex and triggering IL-2 expression[198].

The role of HDAC6 has been studied in a specific T-cells subset, defined as CD4+CD25+FoxP3+ Tregs, through *in vitro* treatment of Tregs with the HDAC6-specific inhibitor tubacin or by harvesting Tregs from HDAC6 KO mice. As a consequence of downregulation or lack of HDAC6, expression of the transcription factor Foxp3, CTLA4 inhibitory molecule, and IL-10 production were increased, resulting in enhanced Treg suppressive capacity and prevention of colitis development *in vivo* in a dextran sulfate induced mouse models. Moreover, inhibition of HDAC6 or its downstream target (i.e. HSP90) was able to minimize autoimmunity and transplant rejection, as a reflection of enhanced Treg suppression[199]. HDAC6 has also been implicated in deacetylation of molecules involved in the immunological synapse between T-cells and APCs. Induced overexpression of HDAC6 results in disruption of CD3 and LFA-1 in the contact site, and impairs IL-2 production[200].

HDAC7 is expressed in high levels on CD4+CD8+ double-positive thymocytes and participate on T-cell development, in a process involving its recruitment to Nur77 promoter by interaction with the transcription factor MEF2D. As a regulator of Nur77, HDAC7 inhibition results in increased apoptosis during TCR activation of developing thymocytes[201]. The role of HDAC9 was evaluated on Tregs and, similarly to the aforementioned HDAC6 studies, HDAC9KO mouse Tregs displayed enhanced suppressive capacity in a colitis mouse model. Mechanistically, expression of the heat-

shock protein HSP70 in Tregs lacking HDAC9 was enhanced, and HSP70 was found to interact with Foxp3, leading to a more suppressive T-cell phenotype[202]. Research has also been conducted to assess functions of HDAC11 on immune cells. Studies using KO mouse models or HDAC *in vitro* inhibition have demonstrated that HDAC11 abrogation triggers upregulation of IL-10 production on APCs and macrophages, impairing T-cell effector response in an antigen-specific context and inducing immune tolerance[181,203]. Furthermore, inhibition of HDAC11 expression *in vitro* through the use of small interfering RNAs (siRNA) induced apoptosis of hodgkin's lymphoma tumor cells and upregulation of OX40L, a costimulatory T-cell ligand. Also reported was inhibition of IL-10-producing Tregs generation, reflective of an enhanced OX40L expression[204].

Figure 1. Scheme of HDAC Classification and Catalytic Domains. The eleven HDACs are phylogenetically classified in four classes. Class I comprises HDACs 1, 2, 3 and 8, class IIa members are HDACs 4, 5, 7 and 9, class IIb is composed by HDACs 6 and 10, and the sole member of class IV is HDACs.

HDAC Inhibitors

The development of HDAC inhibitors (HDACi) became attractive with the understanding of epigenetics effects in tumor biology, especially considering the aberrant pattern of HDAC expression in cancer cells. HDACi directly target tumor growth by inducing cell cycle arrest during transition from G1 to S phase. Mechanistically, alteration of the chromatin structure results in G1/S arrest, as well as changes in molecules involved in cell cycle, including CDKN1A-mediated retinoblastoma protein dephosphorylation, and cyclin A and D repression. Furthermore, HDACi can directly trigger apoptosis of tumor cells through distinct pathways, including ROS-induced mitochondria dysfunction, downregulation of Bcl-2, upregulation of Bim, and enhanced expression of Fas and FasL[205-207]. Intriguingly, tumor cells are more vulnerable to apoptosis and senescence mediated by HDACi than normal tissues. A postulated explanation is that maintenance of gene expression on tumor cells heavily relies on epigenetic regulators, lacking compensatory pathways to overcome any major epigenetic disruption[208]. Not solely restricted to effects on tumor cells, HDACi also impact on the tumor microenvironment. A variety of HDACi interfere with molecules involved in the process of angiopoieses, such as VEGF, angiopoetin and HIF-1a, therefore negatively regulating angiogenesis[209-211].

The broad effects of HDACi also extend to immunoregulatory modulation. HDAC inhibition triggers upregulation of MHC I, MHC II, CD80, CD86 and CD40 molecules, increasing immunogenicity of tumor cells[212-214]. Conversely, HDACi has shown to reduce inflammatory response, by decreasing the circulating levels of IFNγ, TNF, IL-1β and IL-6 in autoimmunity context[215]. Moreover, HDACi can impair IL-12p70

secretion and, thus, DC maturation[216], as well as reduce IL-2 production in T-cells[217]. Interestingly, signal transducer and activator of transcription (STAT) proteins are shown to interact with HDACs. STAT signaling is downstream of cytokine receptors (e.g. IFN, IL-2 and IL-4 receptors) and lead to transcription of a myriad of genes, often involved in T-cell function and fate. A study has demonstrated that association of HDAC1 with STATs 1 and 2 enhance IFNα-mediated gene expression, and that this is impaired after HDAC inhibition[218]. HDACi also interferes with STA3-dependent gene transcription, and research has demonstrated involvement of HDACs 1, 2 and 3 in IFNγ/STAT1 signaling[219]. Furthermore, STAT5 transcription recruits HDAC1, resulting in acetylation of histones and other proteins forming the transcription complex[220]. Moreover, HDAC3 inhibition reduces phosphorylation and promotes hyperacetylation of STAT3, an effect not reversible by exogenous IL-6, which is a cytokine upstream of STAT3 signaling[221]. These studies demonstrate the importance of class I HDACs in contributing to an inflammatory response, at least in a STAT-dependent fashion. With a few exceptions, most of the aforementioned anti-tumor and immunoregulatory HDACi effects are due to pan- rather than specific-inhibition. However, the impacts of HDAC pan-inhibition in directly impairing tumor growth could also extend to the immune cells, thus being detrimental.

HDACi are generally classified in five different groups, depending on chemical structure and specificity. As such, the classes include hydroxyamic acids, benzamides, cyclic tetrapeptides, ketones and aliphatic acids[222]. Several HDACi have been developed and described as pan-, class-, or isotype-specific. Examples of HDACi with a broad specificity include panobinostat (LBH589)[223], belinostat (PDX101)[224], trichostatin

A (TSA)[225], vorinostat (SAHA)[226] and quisinostat (JNJ26481585)[223]. Some of the HDACi reported as class I selective are sodium butyrate[227], valproic acid (VPA)[228], mocetinostat (MGCD0103)[223], etinostat (MS275)[229] and romidepsin (FK228)[230]. A few HDACi have been described as class IIa selective, such as TMP195 and TMP269[225]. Due to the similarity across HDACs, there are fewer isotype-selective HDACi available. As such, PCI34051 has been described as an HDAC8 potent inhibitor over other HDAC isoforms[231], while activity of RGFP966 has been reported to be selective for HDAC3[232]. Particularly HDAC6 is unique in containing two catalytic domains, allowing for the development of a greater variety of isotype-specific inhibitors. For instance, tubacin[233], tubastatin A[233], nexturastat A[234] and rocilinostat (ACY1215)[235] have been described as HDAC6 specific- or selective-inhibitors. The HDACi ACY1215, however, also displays some activity against class I HDACs. A semi-comprehensive description of HDAC inhibitors specificity is illustrated in table I.

Currently, there are four HDACi approved by the FDA. A clinical trial using vorinostat for the treatment of cutaneous T-cell lymphoma (CTCL) resulted in 30% rate of objective response, leading to its FDA approval in 2006[236]. Approximately three years later, romidepsin was approved by the FDA also for the treatment of CTCL, reaching 34% of overall response rate as a single agent[237,238]. Another HDACi, belinostat, received FDA-approval in 2014 for the treatment of relapsed or refractory peripheral T-cell lymphoma (PTCL), demonstrating 26% overall response rate[239]. Finally, panobinostat was approved by the FDA for the treatment of patients with multiple myeloma, in combination with bortezomib, a proteasome inhibitor prescribed for multiple myeloma and mantle cell lymphoma therapy. In a phase II clinical trial, simultaneous

treatment with panobinostat and bortezomib promoted 59% of tumor shrinkage rate and a median progression-free survival of 10.6 months. Both results were higher than the other arm of the study, in which patients received bortezomib and dexamethasone, without panobinostat administration (i.e. 41% tumor shrinkage rate and 5.8 months median progression-free survival)[240].

Unfortunately, approaches addressing the hindrances of HDACi treatment to inflammatory and desirable immunoregulatory effects are still needed. Herein, mechanisms to overcome such limitations and improve immune response in the cancer setting are explored.

Table 1. Specificity of HDAC Inhibitors. Table describes the reported selectivity of commercially available HDAC inhibitors, and their current FDA approval status.

HDAC Inhibitor	Reported Selectivity	FDA Status
Panobinostat (LBH589)	Pan-selectivity	Approved
Belinostat (PDX101)	Pan-selectivity	Approved
Quisinostat (JNJ26481585)	Pan-selectivity, with minimal effect on HDAC6, HDAC7 and HDAC9	-
Tricostatin A (TSA)	Class I, class Iib, and class IV selective	-
Vorinostat (SAHA)	Class I selective, with minimal effect of HDAC3	Approved
Valproic acid (VPA)	Class I selective	-
Sodium butyrate	Class I selective	-
Mocetinostat (MGCD0103)	Class I selective, with low effect on HDAC3, HDAC4, HDAC5 and HDAC11	-
Etinostat (MS275)	Class I selective, with minimal effect on HDAC8	-
Romidepsin (FK228)	Class I selective	Approved
TMP195	Class IIa selective	-
TMP269	Class IIa selective	-
RGFP966	HDAC3 selective	-
Ricolinostat (ACY1215)	HDAC6 selective, with low effect on class I HDAC	-
Tubacin	HDAC6 selective	-
Tubastatin A	HDAC6 selective	-
Nexturastat A	HDAC6 selective	-
PCI34051	HDAC8 selective	-

CHAPTER TWO:

THE USE OF PAN-HDAC INHIBITOR LBH589 (PANOBINOSTAT) AS AN ADJUVANT TO MELANOMA IMMUNOTHERAPY AUGMENTS PD1 BLOCKADE

Background and Rationale

The antitumor activity of LBH589 has been vastly studied, comprising research on both hematological malignancies and solid tumors. For instance, a phase I clinical trial using escalating doses of LBH589 was conducted for the treatment of Japanese patients with advanced solid cancers, including colon, stomach, gall bladder, lung, oesophagus, ovary, and others. As a single agent, LBH589 treatment failed in promoting partial or complete responses. However, six out of fourteen patients presented stable disease for at least four months[241]. In a phase II clinical trial, LBH589 demonstrated modest tumor reduction and maintained disease stable in patients with small-cell lung cancer[242]. Another phase II clinical trial based on LBH589 treatment for patients with relapse or refractory Hodgkin's lymphoma promoted tumor shrinkage in 74% of patients and 78% rate of 1-year survival[243].

Experiments evaluating LBH589 treatment *in vitro* of melanoma cells, in combination BRAF inhibition, demonstrated synergistic effects of double-treatment resulting in tumor cell death through necrosis[244]. Moreover, preclinical studies assessed anti-tumor and immunoregulatory functions of LBH589 *in vitro* and in a mouse model of

B16F10 melanoma. As expected, melanoma cell growth was delayed, as a result of cell cycle arrest and apoptosis mediated by LBH589 treatment. Corroborating previously published data, LBH589-treated human and mouse melanoma cell lines upregulated expression of immunologically relevant surface markers, such as MHC I, MHC II, CD40, CD80 and CD86, as well as melanoma antigens, including gp100 and MART-1. Possibly as a result of both direct and indirect effects on the cancer cells, melanoma growth *in vivo* was delayed and B16F10 tumor-bearing mice displayed prolonged survival after systemic LBH589 administration[214].

There is an increasing body of evidence demonstrating the activity of HDACi in upregulating immunogenic and antigenic molecules. However, whether these immunomodulatory effects are extended to regulation of inhibitory molecules remains unclear. The importance of such studies lays on the fact that attenuation of immune response through expression of inhibitory molecules, such as PDL1/PDL2, hinders response against tumor. In order to maintain homeostasis, expression of PDL1, also known as CD274, negatively modulates T-cell activation and tempers immune response. Mechanistically, phosphatases are recruited upon ligation of PDL1 to its receptor PD1 on the surface of T-cells, reducing downstream phosphorylation of molecules involved in the TCR-mediated signaling (e.g. ZAP-70, Akt and PKCtheta) and, thus, attenuating immune response[245,246]. While disruption of PDL1 signaling often triggers autoimmunity, its upregulation in the setting of cancer represents a well-established mechanism of immune evasion, frequently generating tolerance and non-reactive T-cells[124,247,248]. Moreover, PDL2, also known as CD274, is another ligand for

PD1 receptor and it has also been described as a negative regulator of T-cell response[128].

Herein, an unexplored role of LBH589 in modulating expression of PDL1 and PDL2 on melanoma cells is described, as well as inhibitory immune effects of several HDACi[249]. Class I HDAC inhibition through MS275 and MGCD0103 were shown to upregulate PDL1 and PDL2. MS275 is selective for HDACs 1 and 3[229], while MGCD0103 is most potent against HDACs 1, 2, 3 and 11[223]. PDL1 and PDL2 expression on melanoma cells was upregulated *in vitro* and *in vivo* in response to HDACi, in a dose-dependent fashion. This sustained upregulation was a result of increased gene expression triggered by HDACi-mediated acetylation of the promoter regions of PDL1 and PDL2 genes. Despite the benefits promoted by HDACi in a cancer context, upregulation of the inhibitory molecules PDL1 and PDL2 may represent a limitation to T-cell mediated anti-tumor response. Thus, combination therapy of LBH589 and PD1 blockade using a melanoma mouse model was reported here as able to overcome this hindrance, creating a rationale for their simultaneous use in the clinical setting.

Materials and Methods

Cell Lines and Patient Samples

The mouse melanoma cell line B16F10, and the human cell lines WM983A, WM793, WM1366 and WM35 were acquired from ATCC (Manassass, VA). The cell line SkMel21 was provided by Dr. Keiran Smalley, and Mel-624 and Mel-888 by Dr. Shari

Pilon-Thomas, both researches at H. Lee Moffitt Cancer Center (Tampa, FL). Primary melanoma samples were obtained from resected biopsies from patients undergoing the clinical trial MCC15375 at H. Lee Moffitt Cancer Center, and provided by Dr. Amod Sarnaik (IRB approval protocol number 106509). All cells were cultured in RPMI 1640, in the presence of 10% fetal bovine serum, non-essential amino acids, streptomycin, penicillin, and amphotericin B.

Mouse Models

All animal research was performed according to the IACUC protocols approved at University of South Florida (protocols 4380R and 4100M). C57BL/6 mice were acquired from NCI Laboratories and Charles River Laboratories (Wilmington, MA), and maintained at H. Lee Moffitt Cancer Center animal facility. Subcutaneous inoculation of $1x10^5$ B16F10 melanoma cells was performed for *in vivo* experiments assessing tumor growth and mouse survival outcomes. After seven days, intraperitoneal treatment of 15mg/kg of LBH589 thrice weekly as a single agent or combined with 3mg/kg of PD1 blocking antibody from BioXCell (West Lebanon, NH) twice weekly, for a total of three weeks. The control group received injections with drug vehicle (i.e. dextrose 5%). For tumor volume analysis, caliper measurements were calculated using the formula ($width^2$ x length)/2. Expression of PDL1 and PDL2 was also evaluated *in vivo*. Mice were subcutaneously injected with $1x10^5$ B16F10 cells and, following ten days, treated with 15mg/kg of LBH589 or dextrose 5% for three consecutive days. Within two hours after the last treatment was performed, tumors were harvested and assessed by flow cytometry.

HDAC Inhibitors

Novartis (Basel, Switzerland) provided LBH589 for *in vitro* and *in vivo* experiments. MS275, MGCD0103, ACY1215, PCI34051, and PXD101 were obtained from Selleck Chemicals (Houston, TX). All HDACi were reconstituted in DMSO for *in vitro* use, and stored in aliquots at -80°C. Stock dilution was performed immediately prior to use, as indicated. For *in vivo* experiments, 5% dextrose was used as vehicle for LBH589, and drug dissolution was aided by sonication.

Flow Cytometry Analyses

For *in vitro* experiments evaluating expression of surface molecules, melanoma cells were treated with HDACi, at indicated concentrations and time-points. Accutase was used to harvest cells and surface flow cytometry staining was performed in the presence of FACS buffer (PBS, 2mM EDTA, 2% FBS). For analysis of surface molecules, cells were stained with antibodies against PDL1 and PDL2 for 30 minutes at 4°C. Antibodies were purchased from eBioscienece (San Diego, CA) and were conjugated with phycoerythryn, fluorescein isothiocyanate (FITC) or allophycocyanin (APC). Viability was assessed through the use of 50ng/mL DAPI. Intracellular staining was performed for validation of melanoma cells derived from patients, using the transcription factor staining buffer set from eBioscience (San Diego, CA) and instructions provided by the manufacture. Antibodies against S100 and Mart-1, conjugated to FITC and alexa fluor 405, were purchased from Abcam (Cambridge, MA) and Novusbio (Littleton, CO), respectively. Acquisition of cells was performed in a LSR

II flow cytometer instrument from BD Biosciences (San Jose, CA). FlowJo software was used for data analyses.

Western Blot

Protein was extracted through the use of lysis buffer (1% SDS, 4M Urea, 100nM dithiothrietol in 100nM Tris) and sonication, in the presence of proteinase inhibitors. Gel loading buffer (0.2% (weight/volume) bromophenol blue (200mM DTT, 20% glycerol) was then diluted in a ratio of 5:1 with the lysates, and boiled for 15 minutes. Electrophoresis was performed in a SDS-PAGA gel, protein was transferred to a nitrocellulose membrane, followed by primary antibody incubation overnight at 4°C. Antibodies were reactive against β-actin, total histone 3, acetylated histone 3 and acetylated α-tubulin, and obtained from Cell Signaling (Danvers, MA). At the next day, incubation with IRDYE secondary antibody was performed for two hours at room temperature. Immunoblots were then developed using a LI-COR instrument.

Chromatin Immunoprecipitation

The protocol for chromatin immunoprecipitation (ChIP) was previously described in Desai, S. et al.[250]. The protocol was corrected for cell numbers and used a concentration of 0.5mM EGTA for buffers containing this reagent. Briefly, $5x10^6$ melanoma cells were treated with 12.5nM LBH589 or DMSO control for two hours. The primary antibodies for acetylated histone 3 and rabbit control IgG were purchased from Active Motif (Carlsbad, CA) and from Fisher Scientific (Waltham, MA), respectively, and each immunoprecipitation was incubated with 5ug overnight at 4^oC. Samples were then

incubated with 50uL of A/G plus beads obtained from Santa Cruz Biotechnology (Santa Cruz, CA), for two hours at 4°C. For DNA purification, MiniElute PCR Purification Kit purchased from Qiagen (Valencia, CA) was used according to the manufacturer's instructions. SYBERGreen-based quantitative real-time PCR from BioRad Laboratories (Hercules, CA) was used for analysis of the chromatin immunoprecipitates, using a BioRad CFX96 PCR instrument and software. ChIP primers covered a region of 1800bp upstream the start codon of either PDL1 or PDL2 human genes, generating amplicons between 60 to 150bp. NCBI-Blast database was used for primer design, and sequences are as follow.

PDL-1 promoter region: Fw 5'- GGCAAATTCCGTTTGCCTCA-3' Rv 5'-TCCTCCTAGATGGCCTGGAT-3', Fw 5'- GCTGGGCCCAAACCCTATT-3' Rv 5'-TTTGGCAGGAGCATGGAGTT-3', Fw 5'- CTAGAAGTTCAGCGCGGGAT-3' Rv 5'-GGCCCAAGATGACAGACGAT-3', Fw 5'- ATGGGTCTGCTGCTGACTTT-3' Rv 5'-GGCGTCCCCCTTTCTGATAA-3', Fw 5'- GGGGGACGCCTTTCTGATAA-3' Rv 5'-AAGCCAACATCTGAACGCAC-3', Fw 5'- ACTGAAAGCTTCCGCCGATT-3' Rv 5'-CCCAAGGCAGCAAATCCAGT-3', Fw 5'- AGGACGGAGGGTCTCTACAC-3' Rv 5'-ATTGGCTCTACTGCCCCCTA-3', Fw 5'- GTAGGGAGCGTTGTTCCTCC-3' Rv 5'-GTGTAGAGACCCTCCGTCCT-3', Fw 5'- TAGGGGGCAGTAGAGCCAAT-3' Rv 5'-CAAAACTGAATCGCGCCTGG-3';

PDL2 promoter region: Fw 5'-CCTGGCACAGCACTAAGACA-3', Rv 5'-CTTCCCCATTGTCCCTGGAG-3', Fw 5'- GGCAGCAGGAGAAGGATTGA-3', Rv 5'-GCCCCACTATACCTTCAGGC-3', Fw 5'- TGGCTGTTCATTTTGGTGGC-3', Rv 5'-

ATGAGGACTTGCCACAGCTC-3’, Fw 5’- AAGGGTGGCCTACCTTCTCT-3’, Rv 5’-TCTGGGGCAGGAGGACATTA-3’.

Quantitative Real Time PCR

Cells were lysed using TRIzol, following manufacture’s instructions. Isolation of RNA was performed using a standard phenol-chloroform separation protocol, and cDNA was generated by an iScript kit from Bio-Rad, according to the provided instructions. SYBERGreen-based quantitative real-time PCR was used for expression analyses, on a Bio-Rad CFX96 platform and software. Relative mRNA expression was calculated using the formula 2^[-(delta delta Ct)]. The reference gene of choice was 18S ribosomal RNA. NCBI-Blast database was also used for the design of primers, which are as follow.

PDL1: Fw 5’-TCCTGAGGAAAACCATACAGC-3’ Rv 5’-GATGGCTCCCAGAATTACCA-3’. 18S: Fw 5’-GTAACCCGTTGAACCCCATT-3’ Rv 5’-CCATCCAATCGGTAGTAGCG-3’.

Melanoma Cytokine Production

Melanoma cells were plated and cultured in the presence of DMSO control or the HDACi LBH589, MGDC0103 or MS275, at the indicated concentrations. Supernatant was harvested 72 hours after treatment started, and cytokine production was evaluated by cytokine bead assay (CBA), according to the manufacture’s instructions. Sample acquisition was performed in a LSR II flow cytometer instrument and analyzed using FCAP software from BD Biosciences.

Statistical Analysis

Statistical analysis based on unpaired, two-tailed, student's t-test determined significance of PDL1 and PDL2 expression. Differences in tumor growth were assessed by one-way analysis of variance, at indicated time-points. Kaplan-Meier survival analysis log rank test evaluated mouse survival. Analyses of correlation significance, Pearson's R-square values and linear regression were performed for data correlation of gene expression, gene acetylation and PDL1 surface expression. GraphPad Prism 6.0 software was used to all statistical analyses, and p-values lower than 0.05 were considered significant.

Results

Expression of PDL1 is Increased by HDAC Inhibitors on Melanoma Cell Lines

To initially evaluate specificity of HDAC inhibitors on melanoma cells, the B16F10 cell line was treated *in vitro* with LBH589, MGCD0103, MS275, ACY1215 and PCI34051 for 2 or 24 hours, at the indicated concentrations. A few protein targets for HDACs have been previously described in the literature[251,252]. As a result of class I HDAC inhibition, with exception of HDAC8, the levels of histone acetylation became increased. Moreover, acetylation of α-tubulin is reported as consequence of HDAC6 activity. As demonstrated in figure 2A, immunoblot analyses revealed that as early as two hours of treatment with LBH589 (pan-HDACi) or MGCD0103 (class I selective), histone 3 (H3) acetylation was enhanced. After 24 hours, MS275 (class I selective) was

able to increase the levels of acetylated H3, and LBH589 still maintained its activity. As expected, no effects were seen following treatment with ACY1215 (HDAC6 selective) and PCI34051 (HDAC8 selective). Furthermore, ACY1215 treatment led to an increase of α-tubulin acetylation at 24 hours after HDAC inhibition. Staining for total H3 and β-actin were used as loading control of the experiment, and no major differences in protein levels were observed.

The immunoregulatory effects of pan- and class I selective HDACi have been previously described in melanoma[214,253]. To expand on these findings, the human cell lines WM983A, WM793, WM35, WM1366, Mel-624, Mel-888, SkMel-21, and the mouse lines B16F10 and B78H1 were treated with HDACi and evaluated for PDL1 expression. The human cell lines used in this study comprise diverse mutational status[254-258], listed in table II. Briefly, melanoma cells were treated with 10nM LBH589, 500nM MGCD0103 and 500nM MS275 for 72 hours, and then harvested using Accutase in order to avoid loss of membrane protein. Expression of PDL1 was performed by flow cytometry. Histograms in figure 2B demonstrate that treatment with the aforementioned HDACi upregulated PDL1 expression in all cell lines at various degrees, compared to DMSO control treatment. Interestingly, basal expression of PDL1 in the presence of DMSO control varied among all the cell lines tested, in comparison with autofluorescence, which was determined by fluorescence minus one (FMO). The mean fluorescence intensity (MFI) values for all samples are listed in table III. Additionally, a dose dependency of PDL1 upregulation was observed when WM793 cells were treated with twice as much LBH589, MGCD0103 and MS275 inhibitors. By doubling the

concentration of these HDACi, PDL1 expression increased even further, as demonstrated in figure 2C.

To determine kinetics of PDL1 expression, the cell lines WM983A, WM793 and B16F10 were treated with 10nM LBH589, 500nM MGCD0103 and 500nM MS275 for 24, 48, 72 or 96 hours. Cells were once more collected using Accutase and PDL1 expression was evaluated by flow cytometry. Graphs in figure 2D represent MFI of PDL1 expression subtracted from autofluorescence for each time-point. The zero-hour value was determined by MFI results obtained following treatment with DMSO control. PDL1 upregulation was seen as early as 24 hours after HDAC inhibition, and continue to increase at least until 96 hours, when the later time-point was calculated. Depending on the cell line or HDACi used, peaks of expression were observed at 48 or 96 hours, and once more, the degree of expression varied as well. In B16F10, MGCD0103 induced the most robust upregulation of PDL1 in all time-points assessed, while MS275 showed the least impressive effect. In WM983A, PDL1 upregulation was very consistent among all HDACi, with a peak of expression at 72 hours in the presence of MS275. Finally, LBH589 was able to upregulate PDL1 to higher levels than the other two inhibitors in WM793, which was least affected by MS275 treatment.

Table 2. Mutational Status of Melanoma Cell Lines. Table describes the reported mutations present on established human melanoma cell lines.

Human Melanoma Cell Line	Mutational Status
WM983A	BRAF and p53
WM793	BRAF, PTEN and CDK4
WM35	BRAF and PTEN
WM1366	NRAS
Mel-624	BRAF
Mel-888	BRAF
SkMel-21	NRAS

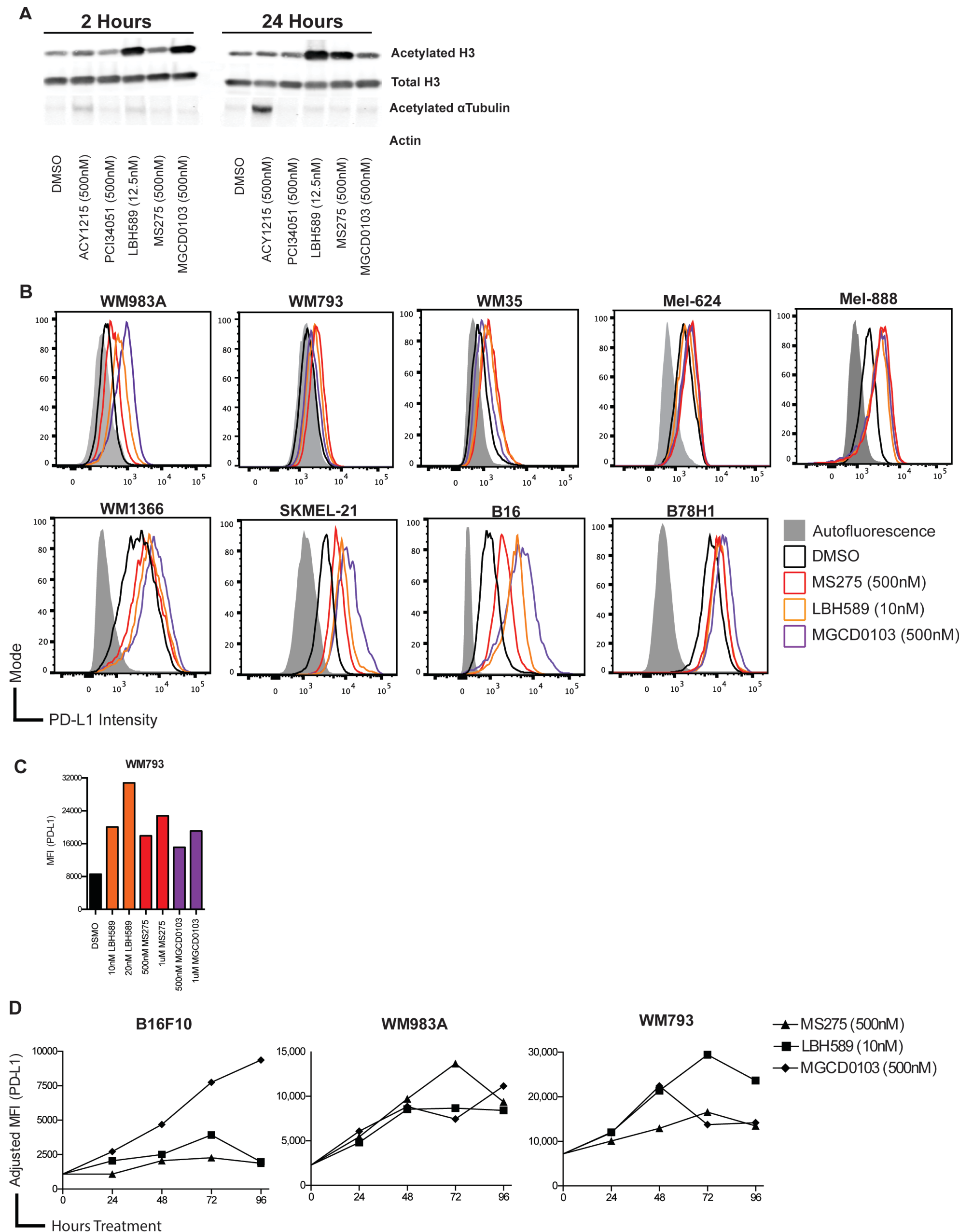


Figure 2. HDAC Inhibitors Upregulate PDL1 in Melanoma. (A) B16F10 melanoma cells were cultured for 2 and 24 hours in the presence of indicated HDAC inhibitors. Cells were washed, lysed and analyzed by immunoblotting for acetylated histone 3, total histone 3, acetylated α-tubulin and β-actin. **(B)** Indicated melanoma cell lines were treated with 500nM MS275 (red), 10nM LBH589 (orange), 500nM MGCD0103 (purple) or DMSO control (black) for 72 hours *in vitro* and PDL1 expression was evaluated. **(C)** WM793 cells were treated with DMSO or the indicated HDAC inhibitors and concentrations for 72 hours. Cells were then washed and evaluated for expression of PDL1 by flow cytometry. Values are graphed as mean fluorescent intensity (MFI). **(D)** Indicated melanoma cell lines were plated and treated with 500nM MS275 (triangles), 10nM LBH589 (squares), or 500nM MGCD0103 (diamonds) at 96, 72, 48, or 24 hours prior to evaluation of PDL1. Expression of DMSO- treated cells was graphed as zero hour treatment. All values are graphed as mean fluorescence intensity (MFI) with autofluorescence values subtracted. Results shown are representative of 2-3 independent experiments.

Table 3. PDL1 Expression in Melanoma Cell Lines as a Result of HDAC Inhibition. Mean fluorescent intensity (MFI) and percent (%) change over DMSO control are illustrated for various melanoma cell lines treated for 72 hours with LBH589, MS275 and MGCD0103 HDAC inhibitors, at indicated concentrations.

Cell Line	Treatment	PDL1 MFI	% Change over DMSO
WM983A	Autofluorescence	328	NA
	DMSO	386	NA
	LBH589 (10nM)	835	216%
	MS275 (500nM)	546	141%
	MGCD0103 (500nM)	1151	298%
WM793	Autofluorescence	1568	NA
	DMSO	1744	NA
	LBH589 (10nM)	2361	135%
	MS275 (500nM)	2730	157%
	MGCD0103 (500nM)	2108	121%
B78H1	Autofluorescence	651	NA
	DMSO	9044	NA
	LBH589 (10nM)	13839	153%
	MS275 (500nM)	13051	144%
	MGCD0103 (500nM)	18364	203%
SkMel21	Autofluorescence	1404	NA
	DMSO	3686	NA
	LBH589 (10nM)	10616	288%
	MS275 (500nM)	16508	448%
	MGCD0103 (500nM)	7171	195%
WM35	Autofluorescence	698	NA
	DMSO	1095	NA
	LBH589 (10nM)	1654	151%
	MS275 (500nM)	1693	155%
	MGCD0103 (500nM)	1261	115%
WM1366	Autofluorescence	648	NA
	DMSO	4788	NA
	LBH589 (10nM)	8184	171%
	MS275 (500nM)	6999	146%
	MGCD0103 (500nM)	10169	212%
B16	Autofluorescence	206	NA
	DMSO	1297	NA
	LBH589 (10nM)	4139	319%
	MS275 (500nM)	2480	191%
	MGCD0103 (500nM)	7350	567%
624	Autofluorescence	1337	NA
	DMSO	4241	NA
	LBH589 (10nM)	6211	146%
	MS275 (500nM)	6446	152%
	MGCD0103 (500nM)	7142	168%
888	Autofluorescence	1027	NA
	DMSO	2097	NA
	LBH589 (10nM)	2999	143%
	MS275 (500nM)	3608	172%
	MGCD0103 (500nM)	3281	156%

Expression of PDL1 and PDL2 is Enhanced on Melanoma Patient Samples Treated with Inhibitors with Specificity for Class I HDACs

To build upon these results and address whether the effects of HDACi on PDL1 expression were also extended to patient samples, several primary human melanomas were treated with a more comprehensive panel of HDACi. Briefly, melanoma cells from surgically removed biopsies were culture *in vitro*. To verify whether expanded cells were indeed melanoma instead of tumor fibroblasts or other adherent cells, expression of the melanoma markers S100 and Mart1[259] was assessed by flow cytometry. Histograms for patient samples tested are shown in figure 3. Melanoma cells were then treated for 24 hours with DMSO control or HDACi at the indicated doses, and then washed twice for drug removal. Fresh media was added and cells were cultured for additional 48 hours prior flow cytometry analysis of PDL1 and PDL2 expression. Evaluation of PDL2 expression on patient samples was performed due to its emerging importance as a negative regulator of T-cell response[128,129,260]. Results are graphed in figure 4 and represent acquired MFI values subtracted for autofluorescence. As expected, all doses of LBH589, MGCD0103 and MS275 triggered PDL1 upregulation on melanoma patient samples. In addition, the pan-HDACi PDX101 similarly enhanced PDL1 expression. Moreover, a dose-dependent effect was observed for all pan- and class I selective HDACi, as illustrated by increasing concentrations resulting in higher levels of PDL1 expression. Interestingly, LBH589 generated the most impressive effects, reaching the highest peak of PDL1 expression in a much lower dose than the other HDACi. Similar to the effects described above, treatment of patient melanomas with pan- and class I selective HDACi also increased PDL2 expression in a dose-

dependent manner, yet at a lesser degree. Conversely, neither ACY1215, Nexturastat A nor PCI34051 seemed to have any substantial effect on PDL1 and PDL2 (figure 4A and B). ACY1215 and Nexturastat A have reported HDAC6-selectivity, while PCI34051 is described as selective for HDAC8. These findings suggest that PDL1 upregulation is mainly due to inhibition of the class I HDACs 1, 2 and/or 3.

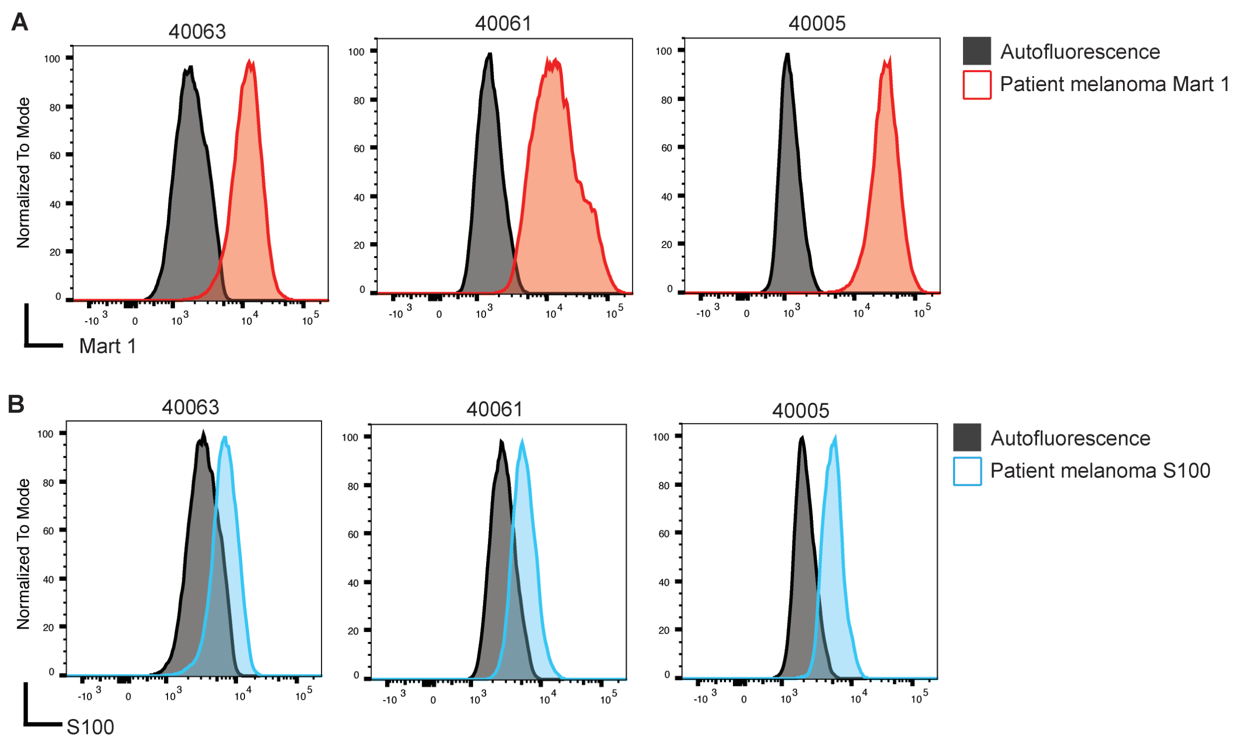


Figure 3. Verification of Patient Melanomas. Patient derived tumor cells were stained for intracellular **(A)** MART-1 and **(B)** S100, and expression was evaluated by flow cytometry.

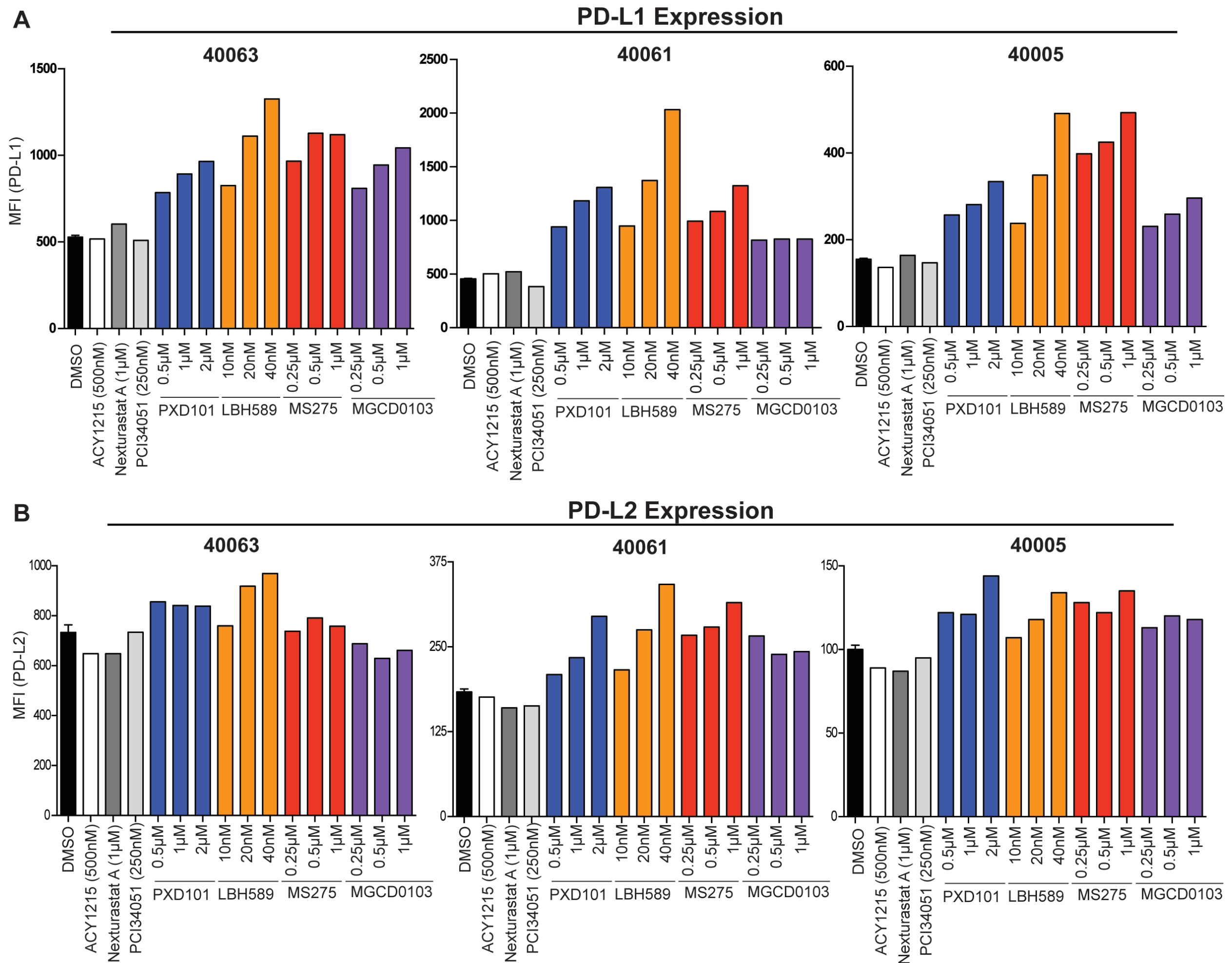


Figure 4. Inhibition of Class I HDACs Increases PDL1 and PDL2 Expression in Patient Melanomas in a Dose Dependent Manner. Patient melanomas obtained from biopsies and expanded in culture were plated and treated with indicated HDAC inhibitors and concentrations for 24 hours. Cells were then washed and cultured for a further 48 hours. At 72 hours past initial treatment, melanomas were evaluated for expression of **(A)** PDL1 and **(B)** PDL2. DMSO controls were run in triplicate. MFI values are graphed with autofluorescence values subtracted.

Systemic HDAC Inhibition Upregulates PDL1 and PDL2 on Tumor Cells *in vivo*

Considering the high potency of LBH589 in the described *in vitro* experiments and its clinical relevance on ongoing clinical trials, this HDACi was chosen for further investigation. The effects of LBH589 on PDL1 and PDL2 expression on tumor cells *in*

vivo were evaluated using a B16F10 mouse model. In order to determine the ideal dose of LBH589 for *in vivo* treatment, several concentrations ranging from to 1 to 25mg/kg were addressed. Loss of body mass was assessed as a surrogate of toxicity, and is reported in figure 5. Since no differences in mouse weight were observed for any of the doses, in comparison with vehicle control (5% dextrose), the previously described concentration of 15mg/kg for a mouse model[214] was chosen for *in vivo* studies. Briefly, a total of 10^5 B16F10 melanoma cells were subcutaneously inoculated in C57BL/6 mice and tumors were allowed to grow for 10 days, when they become visible or palpable. LBH589 (15mg/kg) or vehicle were intraperitoneally administered for three consecutive days. Tumors were then resected and physically dissociated by passing the mass repeatedly through a 70µm sterile filter. Expression of PDL1 and PDL2 was performed by flow cytometry and assessed on viable CD45- cells. Graphs on figures 6A and B illustrate the acquired average of MFI ± the standard error of the mean (SEM). In summary, PDL1 was upregulated following *in vivo* treatment with LBH589, reaching an average MFI of twice as the value in the dextrose control group. Additionally, PDL2 expression was also enhanced on tumor cells when mice were treated with LBH589, in comparison with dextrose administration. However, similar to results obtained *in vitro*, the magnitude of PDL2 upregulation was inferior than the effects observed on PDL1 expression.

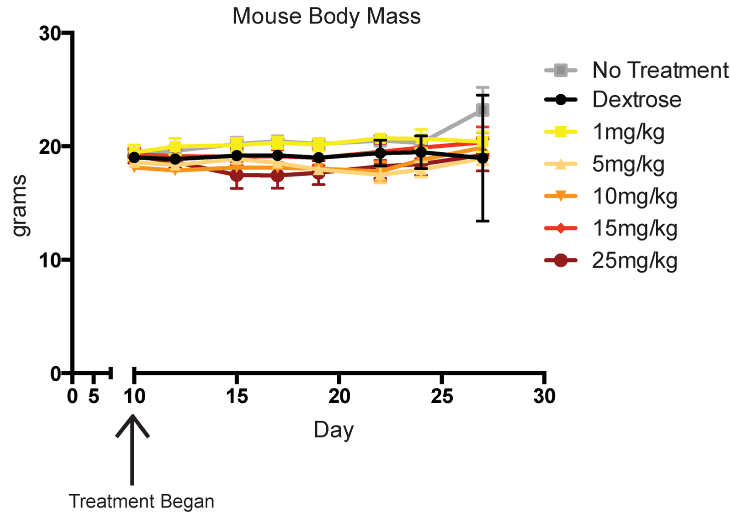


Figure 5. *In vivo* LBH589 Toxicity Assessment. B16F10 bearing mice (5 per group) were treated with indicated doses of LBH589 or dextrose by intraperitoneal injection three times weekly (Monday, Wednesday, Friday) beginning on day 10. Mouse body mass was monitored.

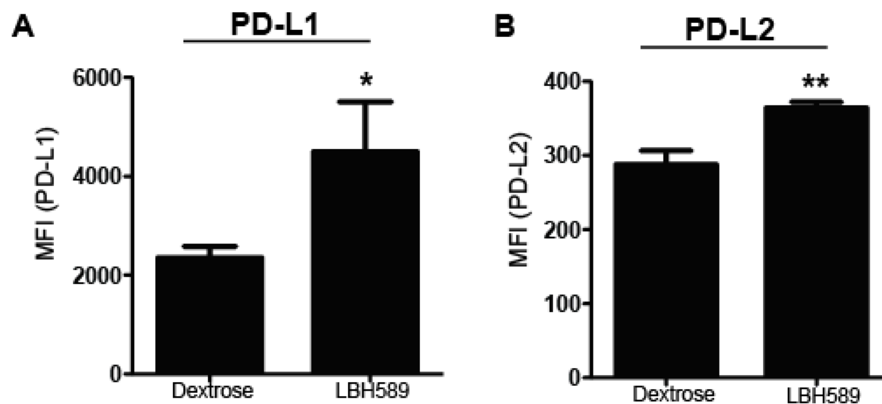


Figure 6. HDAC Inhibitors Upregulate PDL1 and PDL2 Expression *in vivo*. C57BL/6 mice were inoculated subcutaneously with B16F10 melanoma. When tumors were visible, 10 days post inoculation, mice received treatment with 15mg/kg LBH589 or dextrose control (five mice per group) for three consecutive days. On the third day of treatment, tumors were harvested. **(A)** PDL1 and **(B)** PDL2 expression were evaluated by flow cytometry. *P<0.05, **P<0.01.

LBH589 Treatment Augments Histone Acetylation at the PDL1 and PDL2 Gene Promoters

HDACs play an important role in modulating the acetylation status of histones, and thus, chromatin structure. As such, changes in histone acetylation as a result of LBH589 treatment were evaluated at the promoter regions of PDL1 and PDL2 genes. The melanoma cell line WM983A was treated with 12.5nM LBH589 or DMSO control for two hours, prior to cell fixation for chromatin immunoprecipitation (ChIP) analysis. Fixed cells were then pulled-down for pan-acetylated histone 3, as described in the methods section. Primers covering the promoter regions of PDL1 and PDL2 were designed in order to evaluate histone 3 acetylation in these areas. As demonstrated in figure 7A, LBH589 *in vitro* treatment resulted in increased histone acetylation in the PDL1 promoter region in comparison to DMSO control, reaching a peak around 455bp upstream the first exon of human PDL1 gene. Such acetylation was also observed into the gene region and up to approximately 1700bp upstream the first exon. Furthermore, histone 3 acetylation was marginally enhanced on PDL2 promoter region after *in vitro* treatment with LBH589, and may be a reflection of the low basal acetylation observed upon DMSO control treatment (figure 7B). To build upon these findings, histone 3 acetylation at the PDL1 and PDL2 promoters was assessed in several melanoma cell lines, including WM793, Mel-624, Mel-888 and SkMel-21. As demonstrated in figures 7C and D, higher levels of acetylated histone 3 were observed at the gene promoters after *in vitro* treatment with LBH589, in comparison with DMSO control. In accordance with the previous results, increase in acetylation of histone 3 at the PDL2 gene was modest for the majority of the cell lines.

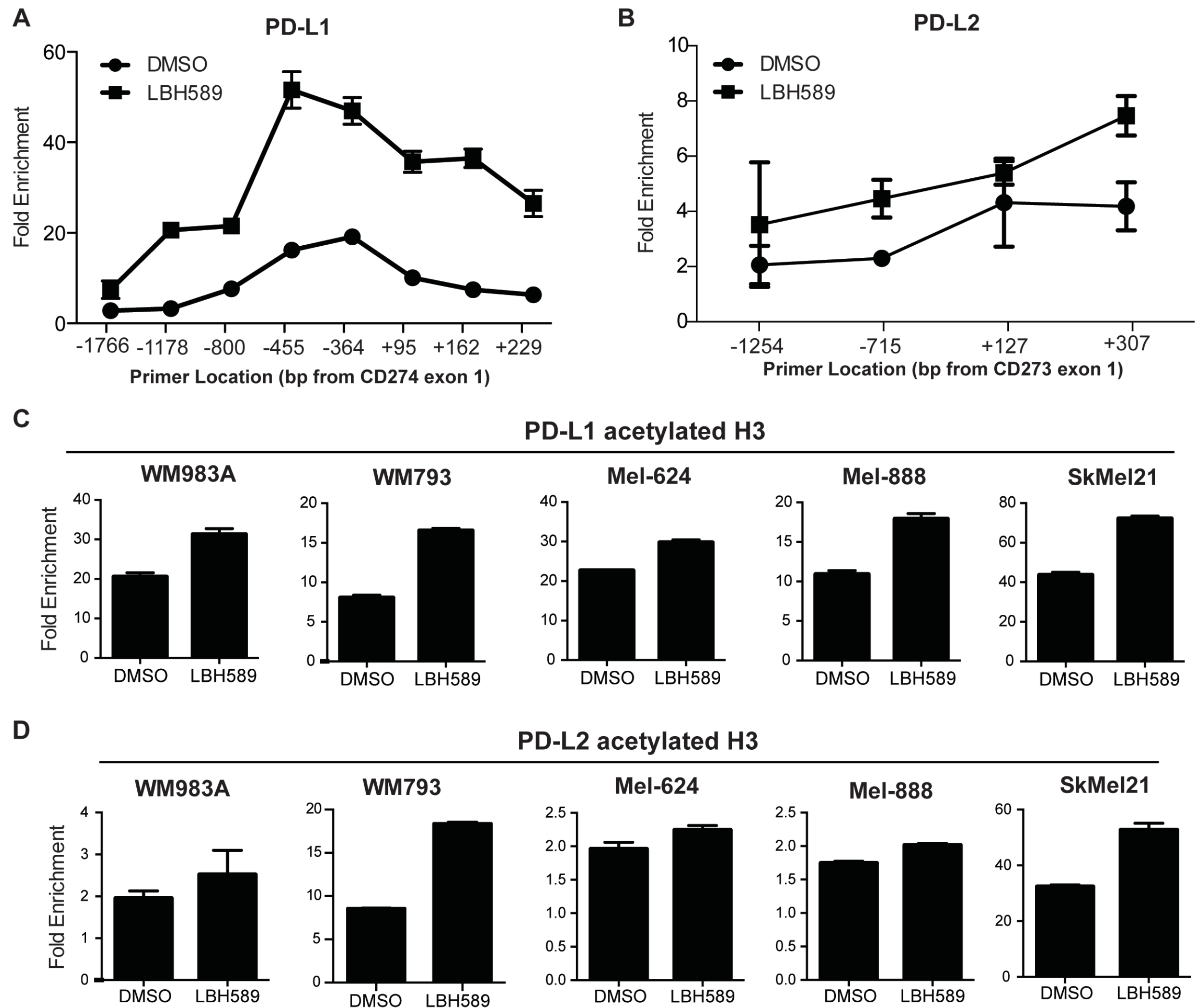


Figure 7. HDAC Inhibition Increases Histone Acetylation at the PDL1 and PDL2 Promoters. Indicated melanoma cell lines were treated *in vitro* for two hours with 12.5nM LBH589 (squares) or DMSO control (circles). Cells were then fixed and chromatin immunoprecipitated for acetylated histone 3 or IgG control. DNA pull-down was quantified by qRT-PCR. Fold enrichment over corresponding IgG pull-down at the **(A)** PDL1 and **(B)** PDL2 gene regions for WM983A are graphed. Results shown are representative of two independent experiments. Five other cell lines were assessed once for acetylation at the **(C)** -455 gene region of PDL1 and **(D)** +307 gene region of PDL2. For all graphs, error bars are representative of technical replicates.

Acetylated Chromatin Status Resulted from LBH589 Treatment Leads to Enhanced PDL1 Gene Expression

Increased levels of histone acetylation usually promote relaxation of the chromatin structure and, thus, gene expression. Since LBH589 *in vitro* treatment of melanoma cells resulted in substantial differences in the levels of acetylated histone 3 at the PDL1 promoter region, impacts on gene expression were explored. First, kinetics of mRNA levels of PDL1 were assessed by qRT-PCR in LBH589 or DMSO treated WM983A cells for 6, 14, 24 or 48 hours. As demonstrated in figure 8A, PDL1 mRNA expression was upregulated as early as 6 hours and continued to increase at least until 48 hours after inhibition through 12.5nM LBH589 treatment, in comparison to DMSO control. The earliest time-point assessed was chosen for further evaluation of PDL1 expression in additional melanoma cell lines, represented on figure 8B. Indeed, LBH589-treated cells displayed higher levels of PDL1 mRNA at 6 hours, largely variable among the cell lines.

Gene transcriptional activity is often modulated through the acetylation status of histones. Considering that all the melanoma cell lines tested displayed varied levels of acetylation of histone 3 at the promoter region of PDL1, protein and mRNA expression, the association of these three observed values was explored at a basal level for the cell lines WM983A, WM793, Mel-624, Mel-888, SkMel-21 and WM1366. Correlations across the autofluorescence-adjusted MFI values of PDL1 surface expression shown in figure 2C, the relative fold units of PDL1 mRNA from figure 8B, and the PDL1 gene associated acetylated histone 3 fold enrichment values graphed on figure 7C were assessed. As shown in figures 8C-E, correlations were identified

between PDL1 gene acetylation and mRNA expression (R^2=0.861), PDL1 surface expression and mRNA expression (R^2=0.5649), and PDL1 surface expression and acetylation (R^2=0.5958).

In order to address whether PDL1 transcription was mainly due to a direct epigenetic effect on the chromatin structure or other indirect mechanism were involved, IFNγ secretion was evaluated on melanoma cells treated with pan- or class I HDACi. IFNγ is an inflammatory cytokine capable of stimulating PDL1 expression on tumor cells[261,262]. Since HDAC inhibitors can alter the chromatin structure and thus regulate gene expression, the levels of IFNγ were assessed after HDACi treatment, even though melanoma cells are not known to secrete this cytokine. For analysis of cytokine secretion, melanoma cells were treated *in vitro* with 12.5nM LBH589, 250nM MGCD0103 or 250nM MS275 for 72 hours, and assessed for IFNγ present on supernatant. The pan- and class I selective inhibitors failed to induce IFNγ production melanoma cells from a patient, and on the cell lines WM983A, Mel-624 and Mel-888. Since the amount of secreted IFNγ was undetectable for DMSO and HDACi treated cells, this datum was not graphed. To verify the reliability of the technique and whether the melanoma cells were capable of cytokine production, the levels of other cytokines were also assessed, including TNF, IL-2, IL-4, IL-6 and IL-10. While no measurable amounts of TNF, IL-2 and IL-4 were detected, IL-10 and IL-6 production were observed at basal levels (DMSO control treatment) and following HDAC inhibition for 72 hours. The cell line Mel-624 and melanoma cells derived from two patient samples were evaluated. The levels of IL-10 and IL-6 secretion ranged from 50 to 2500 and 20 to 800 pg/mL, respectively, and variations were dependent on the sample and treatment.

Interestingly, there was no distinguished pattern in the regulation of these cytokines across LBH589, MGCD0103 and MS275 treatments. The acquired values for cytokine secretion were consistent among sample triplicates for each treatment, but largely varied between cell lines and treatment. The results herein described indicate that HDACi-mediated PDL1 upregulation was likely due to a direct mechanism facilitating gene expression.

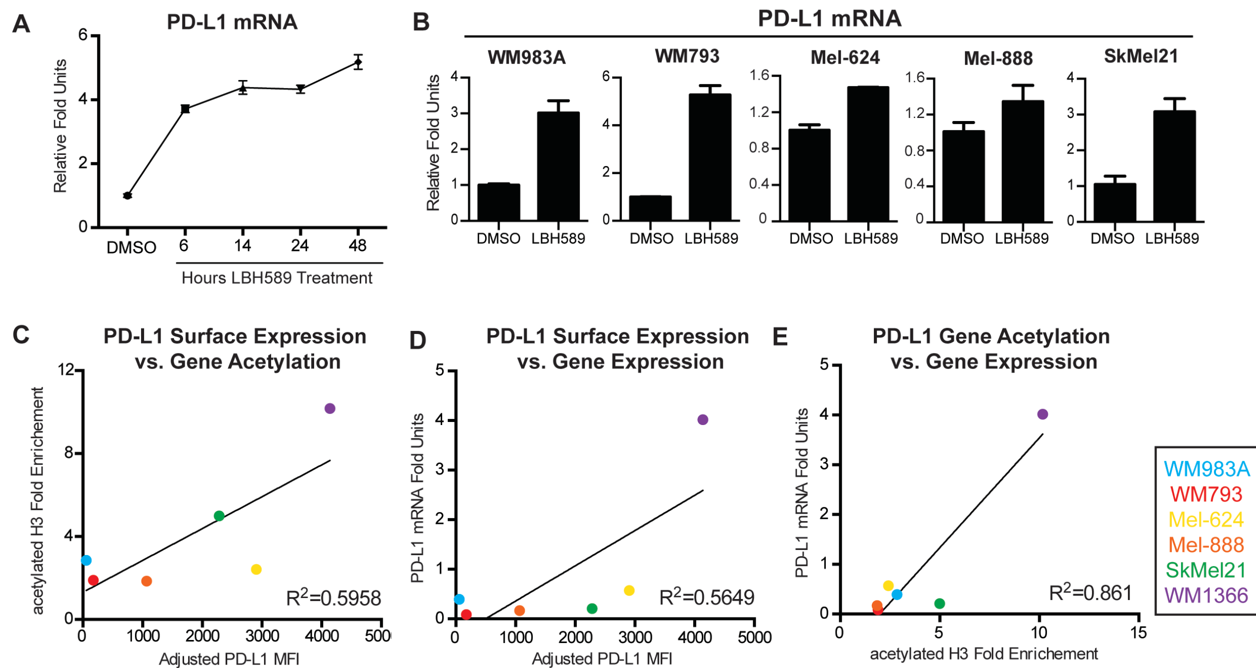


Figure 8. PDL1 mRNA Expression Increased Following HDAC Inhibition, Correlating with Protein Expression and Gene Acetylation. (A) WM983A cells were treated with DMSO or 12.5nM LBH589 for indicated time points. Cells were assessed by qRT-PCR for PDL1 expression. **(B)** Indicated cell lines were treated with DMSO or 12.5nM LBH589 for six hours and subsequently assessed by qRT-PCR for PDL1 expression. For all graphs, error bars are representative of technical replicates. Correlations of **(C)** PDL1 surface expression *versus* gene acetylation, **(D)** PDL1 surface expression *versus* gene expression and **(E)** PDL1 gene acetylation *versus* gene expression were assessed for various melanoma cell lines at basal state (DMSO control). Acetylated H3 was graphed as fold enrichment over corresponding IgG pull-down at the -455 region of PDL1 gene. Gene expression was determined by qRT-PCR and calculated as fold units relative to 18S endogenous ribosomal RNA. Flow cytometry analysis of PDL1 surface expression was indicated as mean fluorescence intensity (MFI).

Systemic Treatment of LBH589 in Combination with PD1 Blockade Delays Tumor Growth and Increases Mouse Survival

As an attempt to disrupt the upregulation of PD1 ligands mediated by HDAC inhibition, while maintaining the anti-tumor effects promoted by HDAC inhibitors, combination therapy of PD1 blockade and LBH589 treatment was explored *in vivo*. Initially, B16F10 melanoma cells were subcutaneously inoculated in C57BL/6 mice. After tumors established, ten mice per group were treated with intraperitoneal injections of 15mg/kg LBH589, 3mg/kg PD1 blocking antibody, a combination of both compounds, or vehicle control (5% dextrose). As reported in figure 9A, combinatory therapy of LBH589 and PD1 blockade significantly reduced tumor progression in comparison to the control group ($p<0.05$ at days 21, 24 and 27). As a single agent, neither LBH589 nor PD1 blockade reached significance over control, although a trend towards minimizing tumor burden was observed in LBH589-treated mice. No discernable differences in melanoma growth over the control group were seen during treatment solely with PD1 blocking antibody. As demonstrated on figure 9B, mouse survival was also improved as a result of combination therapy of LBH589 and PD1 blockade *versus* vehicle control ($p<0.05$). The median survival times were greater than 37 days for combination therapy, 34.5 days for LBH589, 30.5 days for PD1 blocking antibody, and 29 days for dextrose.

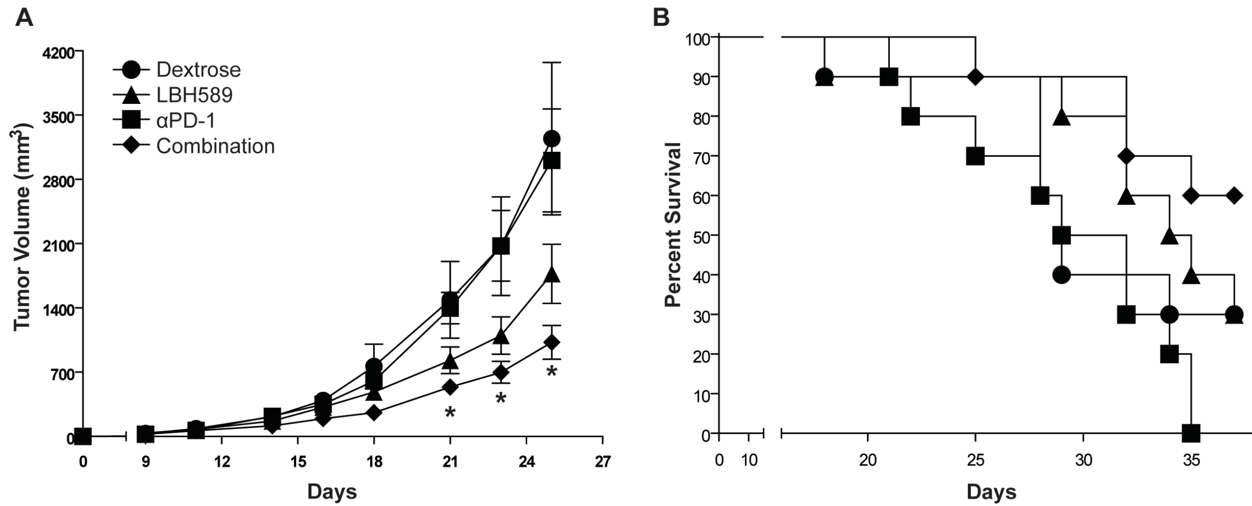


Figure 9. Combining HDAC Inhibition with PD1 Blockade *in vivo* Results in Delayed Tumor Growth and Enhanced Survival. C57BL/6 mice were inoculated subcutaneously with B16F10 melanoma. Seven days after inoculation mice began treatment with LBH589 (15mg/kg, triangles) (Monday, Wednesday and Friday), PD1 blocking antibody (3mg/kg, squares) (Tuesday and Thursday), a combination of these agents (diamonds) or dextrose control (circles) for three weeks. **(A)** Tumor growth was measured and **(B)** survival monitored. Log rank test of survival curve differences was $p<0.05$. Ten mice were assessed per group and results shown are representative of two independent experiments. *$p<0.05$.

Discussion

The ability of pan- and class I-HDAC inhibitors to upregulate the PD1 ligands PDL1 and PDL2 on melanoma cells was demonstrated here in experiments both *in vitro* and *in vivo*. PDL2 expression was enhanced at a lesser degree than PDL1, with the latter being robust and sustained. Moreover, all murine and human melanoma cells lines evaluated, as well as tumor cells obtained from melanoma patients, displayed HDAC inhibition mediated upregulation of PDL1 and PDL2 regardless of the mutational status (table II; figures 2 and 4). Mechanistically, enhanced PDL1 expression was associated with higher levels of histone acetylation at its gene promoter region, as a reflection of HDAC inhibition. In line with acetylated histone relaxing chromatin structure, the amounts of PDL1 mRNA were also elevated, suggesting a link between

increased expression of this protein and the HDAC inhibitor-mediated histone acetylation. Supportive of this hypothesis, correlations at basal levels between acetylated histone 3 at the gene promoter region, PDL1 message, and protein expression, were found to be significant (figure 8). Interestingly, PDL2 gene displayed lower histone acetylation than PDL1 promoter region at basal levels, and this may be the reason for the observed reduced degree of PDL2 expression. Moreover, LBH589-mediated inhibition resulted in only a mild increase of histone acetylation at the gene promoter region of PDL2 (figure 7). Collectively, these results suggest a direct mechanism of HDAC inhibition in upregulating PDL1, through relaxation of chromatin at the gene promoter region mediated by increased acetylation of histone 3.

In the cell lines and patient samples evaluated, expression of PDL1 was detectable at a basal level, and proven to be upregulated following class I HDAC inhibition. For instance, WM793 cell line displayed low amounts of PDL1 protein at a basal state, and HDAC inhibition induced the lowest levels of acetylated histone 3 at the gene promoter among all the cell lines tested. Intriguingly, the ability of HDAC inhibition to enhance PDL1 surface expression was still present, although the basal levels of this protein were minimally above background (fluorescence minus one). In contrast, PDL1 upregulation induced by HDAC inhibition on the mouse cell line B78H1 did not seem to be related to the initial expression of the protein. This may be a result of dysfunctional gene transcripts often present in B78H1 cell line[263]. Therefore, further studies need to be conducted in order to elucidate whether the reported upregulation is dependent on initial expression of PDL1. While the effects mediated by HDAC inhibitors granted enhanced accessibility of the transcriptional machinery to PDL1 promoter, upregulation

of this molecule might still rely on functional transcription factors, such as STATs proteins. While beyond the focus of this work, research exploring the differences in the machinery regulating PDL1 expression among the diverse melanoma cell lines is relevant for the basic understanding of this molecule. Answering these questions will provide a strong rationale regarding strategies to target the immune-tumor interactions in the clinical setting.

The results herein presented demonstrated the ability of HDAC inhibitors to modulate PD1 expression. However, further investigation of the specific HDACs orchestrating this effect is necessary to increase specificity and minimize undesirable events. Here, the pan-HDAC inhibitor LBH589 was able to increase expression of PDL1, an effect also extended to treatment with the class I HDAC inhibitors MGCD0103 and MS275. These inhibitors display most potency against HDACs 1, 2, 3, 11, and HDACs 1, 3, respectively. As the class I HDAC8 is not reported targeted by these inhibitors, it is postulated that the impacts on PDL1 expression induced by HDAC inhibition were likely due to the activity of HDACs 1, 2 and/or 3. Indeed, the HDAC8-selective inhibitor PCI34051, and also HDAC6-selective inhibitors, failed to modulate PDL1 or PDL2 expression, at least at the evaluated concentrations. In support to this hypothesis, HDACs 1, 2 and 3 are mainly localized in the cell nucleus, in contrast to the generally cytoplasm localization of the remaining classical HDACs. It is likely that alterations on the acetylation levels of histones induced by the use of pan- and class I-HDAC inhibitors partially control their transcriptional activity. However, further identification of the particular HDACs regulating expression of PD1 ligands and

contribution of other epigenetic mechanisms (e.g. DNA methylation) to modulation of these molecules need to be fully addressed.

In the clinical setting, the presence of PDL1 on tumor cells has been associated with improved objective response of patients undergoing PD1 blockade therapy[131,264]. This is likely a reflection of an active and productive immune system, resulting in upregulation of PDL1 as means to temper reactive immune cells. Lack of PDL1 may reflect the absence of a pro-inflammatory immune response, in which no stimuli for PDL1 and PDL2 expression (e.g. IFNγ and TNF secretion) are provided[265]. Hence, the benefits of PD1 blockade may be irrelevant in a *milieu* where T-cells are unable to properly respond against tumor. The herein model demonstrates that upregulation of PDL1 and PDL2 is a direct effect of HDAC inhibition, other than associated with an active immune system. In this case, PDL1 expression is an undesirable effect mediated by HDAC inhibitors, and can be circumvent by the use to PD1 blockade therapy. Indeed, blocking this pathway concomitantly with HDAC inhibition for the treatment of B16F10 melanoma in a mouse model resulted in synergistic response, providing rationale for combining these agents as an immunotherapeutic strategy. Indeed, concomitant *in vivo* administration of the pan-HDAC inhibitor LBH589 and PD1 blocking antibody improved response against tumor in comparison with either drug alone or vehicle control, leading to reduced tumor burden and enhanced overall survival of melanoma-bearing mice (figure 9).

The relevance of an intact immune system for the treatment of melanoma using LBH589 monotherapy has been previously shown in mouse models. Under these models, overall survival of melanoma-bearing mice was improved following LBH589 *in*

vivo, but the HDAC inhibitor failed to generate an anti-tumor response in immunodeficient mice[214]. Accordingly, treatment of B16F10-bearing mice with LBH589 as a single agent resulted in a mild reduction of tumor burden and increased survival in the herein studies, while PD1 blockade alone did not improve anti-tumor response in comparison to the control group (figure 9). In support of these results, PD1 blockade has been shown to be unable to provide benefits as a single agent in mouse B16F10 melanoma models, reaching anti-tumor response only in combination with vaccine therapy[266]. Interestingly, combining PD1 blocking antibody with LBH589 *in vivo* resulted in improved outcome, even in the absence of adjuvant vaccination (figure 9). It is postulated that HDAC inhibitors activities confer superior T-cell activation, as they are known to augment tumor antigens and MHC expression in melanoma cells[214], thus acting as an adjuvant for PD1 blockade therapy.

Finally, these data corroborate previous evidence demonstrating the ability of HDAC inhibitors to influence the immune landscape in the context of cancer, through changes including cytokine secretion from tumor cells, as wells as expression of differentiation antigens, MHC and costimulatory molecules. Furthermore, the intimate relationship between epigenetic regulation and immune outcome is highlighted herein, supporting the assessment of HDAC inhibition and PD1 blockade in the clinical setting.

CHAPTER THREE:

SELECTIVE HDAC INHIBITION IMPROVES T-CELL FUNCTION IN THE SETTING OF MELANOMA IMMUNOTHERAPY

Background and Rationale

Epigenetic modifications are key players in regulating gene expression. In this regard, modulation of histone deacetylases (HDACs) has raised attention for its implications in tumor biology. While HDAC pan-inhibition directly affects tumor growth, the immune system may succumb to its broad nature. Recently developed HDAC-selective inhibitors can minimize undesirable effects, being attractive for cancer immunotherapies. Adoptive transfer of T-cells (ACT) has been used in several clinical trials and has demonstrated potent responses against tumor[141,144-147]. ACT therapy is especially effective treatment for metastatic melanoma, achieving objective clinical responses as high as 72% of patients undergoing tumor infiltration lymphocyte (TIL) therapy[141]. However, lack of persistence of reactive T-cells is a major reason why patients fail to sustain long term responses to treatment. Acquisition of T-cell memory characteristics contributes to the effectiveness of ACT, as illustrated in preclinical and clinical studies[160,166,267-270]. Indeed, a meta-analysis study involving 16 publications and 4248 cancer patients with diverse types of tumors correlated the presence of memory TILs with prediction of disease prognosis. An association between accumulation of

memory-defined TILs in the tumor sites and favorable clinical outcomes of overall survival and disease-free survival was observed[271].

Research has shown the importance of the level of differentiation of CD8+ and CD4+ T-cells for an effective response against tumor. There is no clear cut in terms of a defined T-cell population capable of the most potent anti-tumor activity, however, a growing body of evidence suggests that the presence of early differentiation markers may confer improved responses. In a preclinical study of ACT therapy for melanoma-bearing mouse model, transfer of naïve T-cells promoted superior anti-tumor activity than transfer of T-cells comprising more advanced stages of differentiation. This may be due to reduced homing to lymphoid organs, lower production of IL-2 and increased susceptibility to apoptosis[272]. Subsequent preclinical studies showed that both naïve and central memory T-cells were effective for ACT protocols. Interestingly, low expression of the terminal differentiation and senescence marker KLRG1 was associated with higher proliferative capacity and cytokine production following ACT[273]. Moreover, several other parameters may confer increased T-cell proliferation, such as low levels of CD57 and longer telomeres[274]. In murine and primate animal models, infusion of central memory T-cells presented superior persistence *in vivo* when compared to transfer of effector memory T-cells. Furthermore, central memory T-cells maintained their phenotypic and functional characteristics, including expression of lymphoid homing molecules[275,276]. While phenotypic markers of differentiation are constantly being explored and there is no strict classification of T-cell subsets, differences in expression of CD45RA and CD45RO lineage markers, and CD62L and

CCR7 molecules can aid in distinguishing between naïve, central memory, effector memory and effector cells[277].

The results described herein highlight benefits in modulation of T-cells through the HDAC6 selective inhibitor ACY1215, including upregulation of a central memory phenotype, reduced production of type-2 T-cell response, enhanced expression of activation and costimulatory proteins, as well as effector molecules, ultimately leading to improved cytotoxicity against tumor cells.

ACY1215 has been used as a single agent and in combination with pomalidomide, lenalidomide or bortezomib in several ongoing clinical trials for the treatment of hematological malignancies (ClinicalTrials.gov Identifiers: NCT01323751, NCT01997840, NCT02091063, NCT01583283, NCT02189343). Preliminary results of a phase I clinical trial using ACY1215 combined with either lenalidomide or dexamethasone for the treatment of multiple myeloma demonstrated safety and biological activity of ACY1215 administration[278]. Also for the treatment of multiple myeloma, ACY1215 was given in combination with bortezomib or dexamethasone. Once more, all doses assessed were well tolerated[279]. Furthermore, preclinical studies using ACY1215 in combination with bortezomib demonstrated synergistic effects on the treatment of human multiple myeloma on immunodeficient mice, as indicated by reduced tumor burden and increased overall survival[235]. Although administration of ACY1215 has demonstrated direct impact on tumor viability and growth, its effects on immune cells remain to be investigated in the cancer setting.

Materials and Methods

Human Samples

Peripheral blood mononuclear cells (PBMC) were obtained from apheresis of healthy donors and isolated from buffy coats, or collected in heparin tubes from blood samples of melanoma patients. Samples derived from healthy donors were provided from OneBlood (Tampa, FL) and samples derived from melanoma patients were obtained from clinical trials performed at H. Lee Moffitt Cancer Center. All studies involving the use of primary human cells were in agreement with protocols approved by the IRB at H. Lee Moffitt Cancer Center and University of South Florida (IRB approval protocol number 106509). Briefly, PBMC samples were separated via centrifugation in a density gradient using 1.077g/mL Ficoll Histopaque. CD3+ T-cells were then harvested through negative-isolation using magnetic columns. For experiments involving tumor infiltrating lymphocytes (TILs), melanoma patient samples were obtained from surgical biopsies. Tumor fragments were cultured in media containing 6000IU/mL recombinant human IL-2. Yielded reactive TILs were either frozen at this step or rapidly expanded (REP) through activation with 30ng/mL OKT3 antibody and irradiated (5000 rads) feeder cells in a ratio of 200:1 of feeders:TILs. All human T-cells were cultured in RPMI media, supplemented with 10% human serum, 55μM beta-mercaptoethanol, non-essential amino acids, HEPES, penicillin, streptomycin and gentamicin.

HDAC Inhibitors

HDACi were obtained and utilized as described previously on chapter 2. Additionally, the inhibitors SAHA, quisinostat, LMK235, TMP269, nexturastat A, tubastatin A, RGFP966 and BG45 were also purchased from Selleck Chemicals (Houston, TX).

T-cell Cytokine Production

T-cells from healthy donors and melanoma patients were plated, activated via CD3/CD28 dynabeads (ThermoFisher Scientific, Waltham, MA), and cultured for 72 hours in the presence of DMSO control or HDACi, at the indicated concentrations. Supernatant was then collected for evaluation of cytokine production by luminex multiplex assay, performed as indicated by the manufacture. A Luminex 100 instrument was used for sample acquisition.

Flow Cytometry Analyses

For surface analyses, cells were stained in the presence of FACS buffer (PBS, 2nM EDTA, 2% FBS), as described previously in chapter 2. Antibodies against CD3, CD8, CD4, CD69, CD278 (ICOS), CD45RA, CD45RO, CD62L, and/or CCR7 that were conjugated to a variety of fluorochromes and purchased from BD Biosciences (San Jose, CA) or eBioscience (San Diego, CA). For evaluation of acetylated histone 3 and acetylated α-tubulin, intracellular staining was performed using the transcription factor staining buffer set from eBioscience (San Diego, CA), as indicated by the manufacture. Briefly, a two-step protocol was performed for intracellular staining with

the primary antibodies alexa fluor 647-conjugated acetylated histone 3 (Novusbio, Littleton, CO) and unconjugated acetylated α-tubulin, followed by secondary staining with PE-conjugated anti-Fab2 (Cell Signaling, Danvers, MA). To assess T-cell production of effector molecules, intracellular staining was also performed as described above, using fluorochrome-conjugated antibodies against IFNγ and CD107a purchased from BD Biosciences. All human T-cells used for *in vitro* studies were cultured in the presence of 6000IU/mL IL-2, and either not activated or stimulated via aCD3/CD28 or phorbol myristate acetage (PMA)/ionomycin (Cell Stimulation Cocktail; eBioscience; San Diego, CA), as indicated.

T-cell Viability Assays *in vitro*

Viability analyses were performed by flow cytometry of DAPI (50ng/mL) labeled cells or by MTS colorimetric assay obtained from Promega (Fitchburg, WI), according to the manufacture's instructions. Briefly, mouse or human T-cells were cultured for 72 hours in the presence of HDACi or DMSO control. Cells were then incubated with MTS reagent for three hours, and reduction of MTS tetrazolium compound by the live cells was measured by the Synergy HTX spectrophotometer (BioTek, Winooski, VT). Absorbance was set at 490nM and background subtraction, at 670nM. Acquired values were represented as relative percentage of DMSO control treated cells.

T-cell Cytotoxicity Against Melanoma

To assess T-cell cytotoxicity capabilities, pre-REP TILs were thawed and cultured in the presence of 500nM ACY1215 and 6000IU/mL IL-2 for seven days. Cells were washed with PBS and cultured for five more days with fresh media containing 6000IU/mL IL-2, 1ug/mL OKT3 and 500nM ACY1215. HLA-matched melanoma cell line (Mel-624) was labeled with Cell Trace Far Red (ThermoFisher Scientific, Waltham, MA), plated and co-cultured with TILs for 48 hours, at indicated ratios. Melanoma cell death was then evaluated by flow cytometry analysis of annexin V and propidium iodide (PI).

Microarray Analysis

For microarray analysis, TILs were cultured for seven days in the presence of 6000IU/mL IL-2 and 500nM ACY1215 or DMSO control and then lysed for RNA was extraction. Briefly, RNA was converted into cDNA, amplified and biotin-labeled, using the Ambion Message Amp Premier RNA Amplification Kit (Life Technologies, Grand Island, NY). The protocol was adapted from Van Gelder et al[280]. For Affymetrix-based array, biotin-labeled RNA hybridization, staining and chip scanning was performed, as previously described in Warrington et al[281]. The Human Genome U133 Plus 2.0 oligonucleotide probe arrays were used, containing probes for transcripts designed based on GenBank, dbEST and RefSeq sequences. For microarray data analysis, hybridization artifacts were inspected on the output files and subsequently analyzed through the Affymetrix GeneChip Operating Software (GCOS), using the MAS 5.0 algorithm for background correction. Statistical analysis based on paired, student's t-test determined significant probes, and considered p-value and q-value, 50% false discovery

rate cut-off, and p-value<0.001. The selected significant probes averaged fold change>1.5x and the probe sets were mapped to known single gene. For pathway analysis, gene set enrichment analysis (GSEA) computational methods was used.

Mouse Studies *in vitro* and *in vivo*

To evaluate tumor immune infiltrate in a melanoma-bearing mouse model, C57BL/6 mice were subcutaneously inoculated with 10^5 B16F10 cells. After seven days from injections, intraperitoneal administration of 15mg/kg LBH589 or vehicle control (5% dextrose) was performed, thrice weekly for one week. At the last day of treatment, tumors were harvested and physically dissociated through repeated passages in a 70um filter. Flow cytometry analysis of live cells stained for CD45, CD8, CD4, PD1, PDL1 and PDL2 was performed using a LSR II instrument (BD Biosciences) and FlowJo software. Antibodies were obtained from BD Bioscience (San Jose, CA) or eBioscience (San Diego, CA). Viability was determined by the use of 50ng/mL DAPI dye. For evaluation of circulating lymphocytes, mice were treated with LBH589 or 5% dextrose, as described above, and cell numbers were determined by complete blood count (CBC).

For *in vitro* experiments, lymph nodes and spleens were harvested from C57BL/6, and CD3+ T-cells were negatively isolated through magnetic columns. T-cells were plated and cultured for 72 hours in the presence of increasing doses of LBH589 (0.3 to 20nM) or DMSO control. Analysis of live cells was performed by flow cytometry of DAPI-stained cells, as described above.

Statistical Analysis

Statistical analysis based on paired, two-tailed, student's t-test determined significance of DMSO control *versus* ACY1215-treated primary human T-cells. Differences in DMSO control *versus* ACY1215-treated mouse T-cells during *in vitro* and *in vivo* experiments were assessed by unpaired, two-tailed, student's t-test. GraphPad Prism 6.0 software was used for all statistical analyses, and p-values lower than 0.05 were considered significant.

Results

Pan-HDAC Inhibition through LBH589 Upregulates PDL1 and PDL2 on Immune Cells, with no Impact on PD1 Expression

To build upon the previous results of LBH589 effects on PDL1 and PDL2 expression by melanoma cells, the immune infiltrate of tumor-bearing mice was assessed for expression of these inhibitory proteins. To this end, 10^5 B16F10 melanoma cells were subcutaneously injected on mice flanks. After tumors were established, treatment with 15mg/kg LBH589 or 5% dextrose began, thrice weekly for one week. Resected melanoma was then evaluated for expression of PDL1 and PDL2 on non-tumor cells, and PD1 on T-cells. As demonstrated in figure 10A, systemic treatment with LBH589 resulted in upregulation of PDL1 and PDL2 on CD45+ non-T-cells (CD8-CD4-) immune cells ($p<0.01$ and $p<0.05$, respectively). Interestingly, no detectable changes on PD1 receptor expression occurred (figure 10B). All MFI values for PDL1, PDL2 and PD1 expression were above background (fluorescence minus

one), and are illustrated in table IV. Intriguingly, the percent of CD45+ cells out of live cells was reduced in LBH589-treated group, in comparison to control ($p<0.05$), as illustrated in figure 10C. This could be a result from skewing of a specific immune subset or of stromal cells. Another possible explanation is that HDACi cytotoxic effects may be detrimental to immune cells viability.

Pan-HDAC Inhibitors Impact T-cell Viability *in vitro* and *in vivo*

Although HDAC inhibitors have established anti-tumor properties[282], impairment of the class I HDACs 1 and 2 is known to promote apoptosis on activated and proliferating T-cells[283]. To address the effects of pan-HDAC inhibition through LBH589, mouse CD3+ T-cells were cultured *in vitro* for 72 hours in the presence of maintenance dose of IL-2 (100IU/mL) and increasing concentrations of LBH589, ranging from 0.3nM to 20nM. The 0 (zero) point on the *x axis* represents treatment with DMSO control. As shown in figure 11A, LBH589 doses ranging from 2.5nM to 20nM resulted in approximately 20 to 50% of cell viability. The graph is representative of three to four experiments, and cell death is usually variable and/or higher than 50% from concentrations raging from 2.5nM to 20nM. To evaluate the impact of LBH589 on T-cells following *in vivo* treatment, melanoma-bearing mice were injected with 15mg/kg LBH589, as described in the methods section. After LBH589 treatment, the number of circulating total lymphocytes was reduced to about 50% in comparison to the control group ($p<0.01$), as demonstrated in figure 11B.

To further these results, human T-cells obtained from PBMC of melanoma patients were treated *in vitro* with an expanded panel of HDACi. Isolated CD3+ T-cells were treated

with several HDACi or DMSO control for 72 hours. As illustrated in figure 11C, all pan-HDAC inhibitors tested, including LBH589, SAHA, belinostat and quisinostat, enhanced T-cell death. At the doses evaluated, the class I HDAC inhibitors MS275 and MGCD0103 promoted minimal effects on T-cell viability. Similarly, the class IIa HDAC selective inhibitors LMK235 and TMP269, the HDAC6 selective inhibitors ACY1215, nexturastat A and tubastatin A, the HDAC3 specific inhibitors RGFP966 and BG45, and the HDAC8 selective inhibitor PCI34051 displayed low impact on T-cell viability, at least at the assessed concentrations. The concentration of DMSO used as control for the *in vitro* studies was calculated based on the highest amount used for the HDACi treatments. This amount had no effect on T-cell viability, being similar to media only condition for all parameters evaluated (data not shown).

Table 4. Expression of PD1, PDL1 and PDL2 on Mouse Tumor Infiltrating Immune Cells. Mean fluorescent intensity (MFI) values obtained from 9-10 mice treated with dextrose or LBH589 are illustrated as mean ± SEM.

	CD8+	CD4+	Non T-cells CD45+	
Treatment	**PD1 MFI**	**PD1 MFI**	**PDL1 MFI**	**PDL2 MFI**
Autofluorescence	114	109	265	268
Dextrose Control	559.3 (± 25.1)	359.2 (± 17.5)	952.9 (± 11.7)	715.4 (± 14.1)
LBH589 (15mg/kg)	532.4 (± 48.5)	349.5 (± 15.1)	1131.7 (± 17.0)	879.7 (± 17.4)

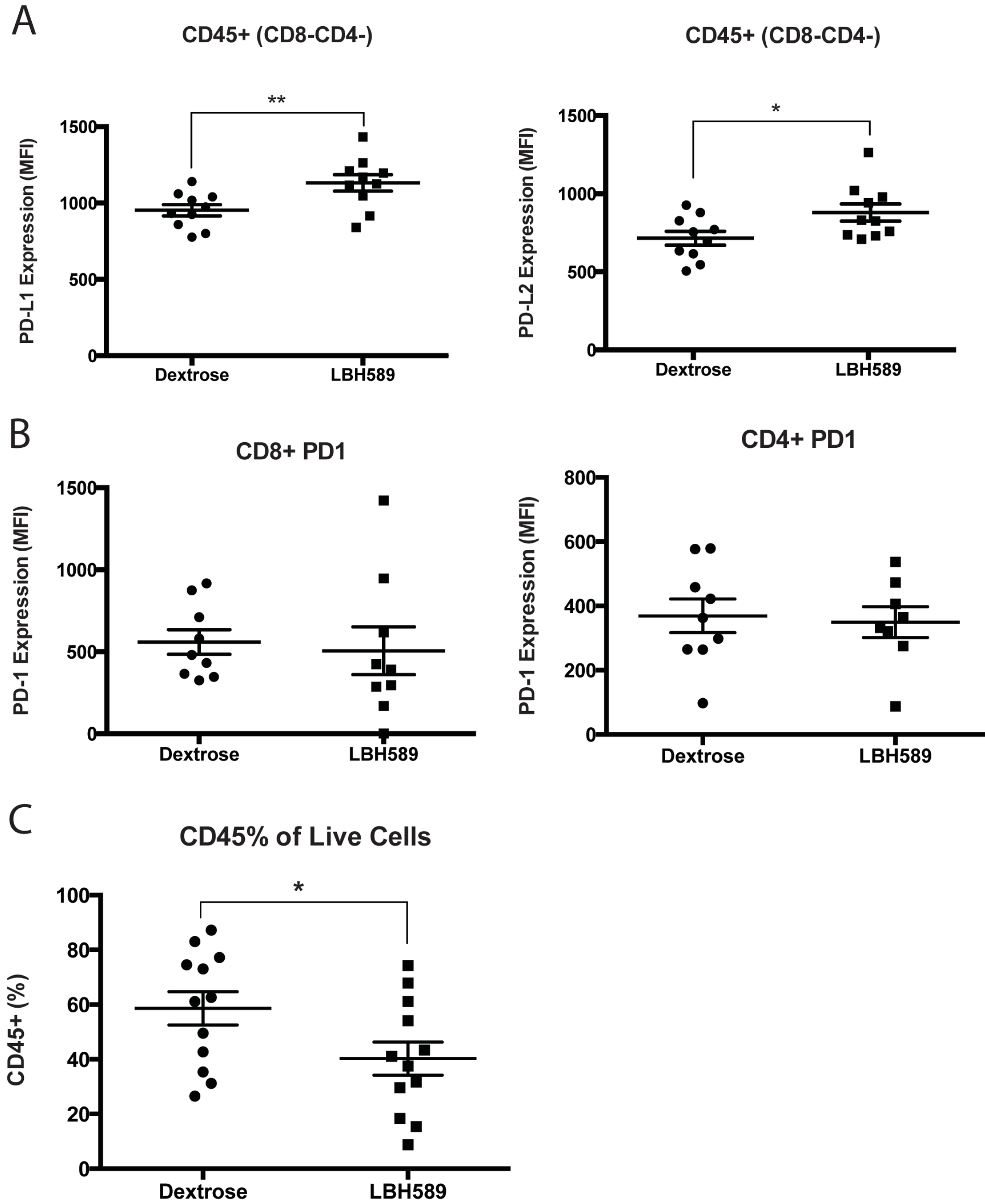


Figure 10. Pan-HDAC Inhibition *in vivo* through LBH589 Promotes Diverse Effects on Tumor Infiltrating Immune Cells. B16F10-bearing mice were treated *in vivo* with 15mg/kg LBH589 or dextrose control and evaluated by flow cytometry for expression of **(A)** PDL1 and PDL2 on CD45+CD8-CD4- cells, **(B)** PD1 on CD45+CD8+ or CD45+CD4+ T-cells, and **(C)** percent of CD45+ cells. *$p<0.05$, **$p<0.01$, two-tailed student's t test.

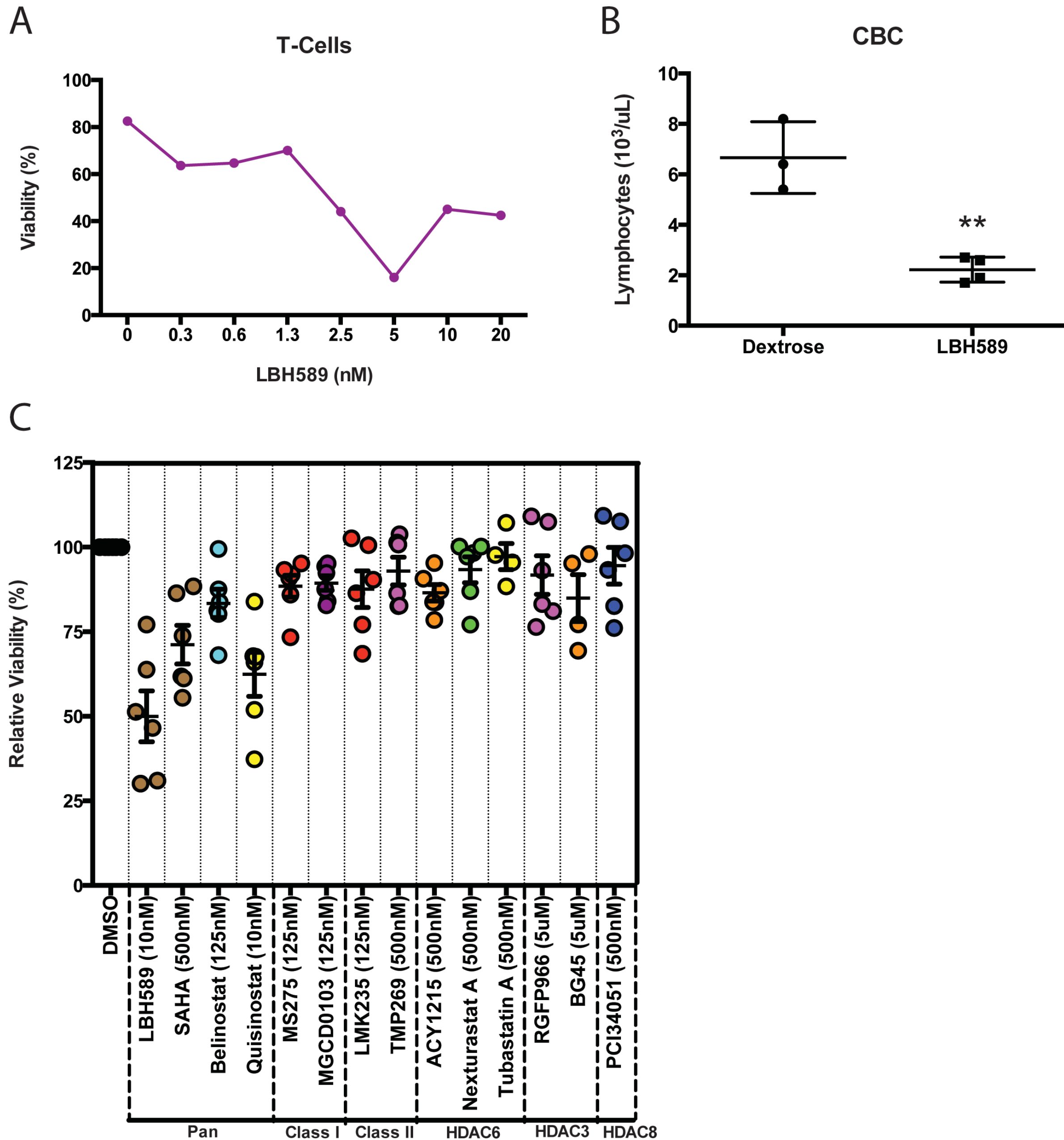


Figure 11. Pan-HDAC Inhibition Reduces T-cell Viability. (A) CD3+ T-cells harvested from lymph nodes and spleens of C57BL/6 were treated with LBH589 for 72 hours at the indicated doses. The percent of viable T-cells was assessed through DAPI staining by flow cytometry. **(B)** B16F10-bearing mice were treated in vivo with 15mg/kg LBH589 and the number circulating lymphocytes was evaluated by complete blood count (CBC). **$p<0.01$, two-tailed student's t test. **(C)** CD3+ T-cells were negatively isolated from PBMC of melanoma patients and treated with pan- or isotype-selective HDAC inhibitors for 72 hours, at the indicated doses. Viability was determined by MTS colorimetric assay.

HDAC Inhibitors Have Disparate Effects on T-cell Cytokine Production

The next step was to evaluate the effects of these HDACi on global T-cell cytokine production in the setting of cancer. Human T-cells obtained from PBMC of melanoma patients were activated via CD3/CD28 stimulation and treated *in vitro* with several HDACi with diverse selectivity. Samples from seven to twelve patients were assessed for cytokines commonly produced by T-cells, including IFNγ, IL-2, IL-4, IL-6, IL-10 and IL-17A. These cytokines determine T-cell function and are important during response against tumors. High levels of Th2 cytokines (e.g. IL-4, IL-10) are usually present in cancers at advanced stages and associated with worse prognosis[284]. Conversely, a Th1 response is characterized by the production of effector cytokines, such as IFNγ and IL-2, and is preferable for a productive response against the tumor. Indeed, these cytokines have been used over the past 30 years to augment cancer immunotherapy[285]. In addition to the classic Th1 and Th2 phenotypes, T-cells can polarize to a Th17 phenotype, characterized by secretion of IL-17. The roles of IL-17 are complex and note fully understood. Indeed IL-17 has been shown to be context-dependent, and capable of suppressing or promoting tumor progression[286,287]. Although the mechanisms underlying these effects are not elucidated, the importance of IL-17-expressing T-cells in the cancer context is becoming evident.

Representative data are illustrated in figure 12, and the standard error of the mean (SEM) was calculated based on independent treatments of the same patient samples. With exception of IL-2, treatment with pan-HDAC inhibitors resulted in decreased levels of most cytokines. In fact, IL-2 secretion was induced regardless of HDACi treatment, reaching amounts of 2000pg/mL. Inhibition of class I HDACs, class

IIa HDACs, HDAC3 and HDAC8 led to an unclear pattern of cytokine production, with variable effects especially in IL-17A, IL-4, IL-6 and IL-10. Induction of IL-17A was indeed inconsistent or minimal following HDAC inhibition through all the tested compounds. Surprisingly, the HDAC6 selective inhibitors nexturastat A and tubastatin A displayed similar behavior on production of IL-4, IL-6 and IL-10, while the HDAC6 selective inhibitor ACY1215 resulted in distinct effects. That is, IL-4 secretion was downregulated in the presence of nexturastat A, tubastatin A and ACY1215, but the levels of IL-10 were higher following inhibition with the first two and lower in ACY1215-treated samples.

It is important to note that these studies did not assess the percentages of CD4+ and CD8+ T-cells expressing the aforementioned cytokines. Although interesting to couple cytokine production data with T-cell subset analyses, the percentages of CD4+ and CD8+ T-cells were similar between ACY1215 and DMSO treatment in previous experiments. For this reason, it is not anticipated that the observed differences are attributed to skewing of the numbers of CD4+ and CD8+ T-cells after ACY1215 treatment. Future work utilizing intracellular cytokine staining may be warranted to address these questions, but remains out of the scope of the current study.

ACY1215 Reduces T-cell Type-2 Cytokine Production, in Contrast to Other HDAC6 Selective Inhibitors

To determine the significance of HDAC6 selective inhibition on cytokine production, T-cells from PBMC of at least seven melanoma patients were evaluated for IL-4, IL-6, IL-10, IL-17A, IL-2 and IFNγ secretion after HDACi or DMSO control

treatment (figure 13). Each dot plotted on the graph represents one melanoma patient. Following ACY1215-mediated inhibition, the levels of IL-4, IL-6 and IL-10 were reduced, reaching statistical difference for IL-4 and IL-6 amounts ($p<0.001$ and $p<0.05$, respectively). No major changes occurred in IFNγ, IL-2 and IL-17A production. While other HDAC6 selective inhibitors, such as nexturastat A and tubastatin A also led to a significant decrease on IL-4 levels ($p<0.01$ and $p<0.001$, respectively), mixed effects were observed on IL-6, and IL-10 was secreted in higher amounts ($p<0.001$ and $p<0.01$, respectively) comparing to DMSO control. Considering that lower levels of Th2-cytokines are preferable in a tumor setting, and that ACY1215 caused minimal impacts on secreted IFNγ and IL-2, as well as on cell viability, this inhibitor was pursued for further study.

In order to determine ACY1215 specificity, T-cells obtained from PBMC of healthy donors were treated with class I HDAC and HDAC6 selective inhibitors, including ACY1215, MGCD0103, nexturastat A and tubastatin A. As a surrogate of class I HDAC and HDAC6 inhibition, respectively, acetylated histone 3 and acetylated α-tubulin were evaluated by flow cytometry after HDACi or DMSO *in vitro* treatment for 2 and 24 hours. As demonstrated in figure 14A, inhibition mediated by three HDAC6 selective drugs resulted in enhanced acetylation of α-tubulin as early as 2 hours and as late as 24 hours. This effect was dependent on the dose, and the HDAC inhibitors appeared to have similar potency in regards to the HDAC6 target. The graphs indicate MFI values for all treatments, and the standard error of the mean (SEM) was calculated based on independent treatments of the same donor samples. As expected, the levels of acetylated α-tubulin following MGCD0103 treatment were comparable to DMSO

control. Interestingly, the impact of ACY1215 was also extended to acetylation of histone 3, as shown in figure 14B. The class I HDAC inhibitor MGCD0103 led to time-dependent histone 3 acetylation, and treatment with ACY1215 displayed similar MFI levels, indicating that the drug specificity is not solely restricted to HDAC6 at this concentration on T-cells. Nexturastat A also generated a mild upregulation of acetylated histone 3. Finally, the impacts of tubastatin A on acetylation of histone were minimal, as the MFI levels were similar to DMSO control in both 2 and 24 hours after *in vitro* treatment.

T-cells from Melanoma Patients Display Sustained Upregulation of Activation and Costimulatory Markers Following ACY1215 *in vitro* Treatment

To address ACY1215 effects on phenotype, T-cells isolated from PBMC of melanoma patients were treated with this compound or DMSO, activated via CD3/CD28, and assessed for kinetics of expression of CD69 and ICOS. The former is a molecule upregulated in early stages of activation and the latter is a costimulatory receptor expressed following T-cell activation. Each graph in figure 15 represents the results obtained from each of the four evaluated patients. Displayed are the MFI values, acquired in 4, 24 and 72 hours. The standard error of the mean (SEM) was calculated based on independent treatments of the same patient samples, and p-values are *<0.05, ***<0.001, ****<0.0001. As illustrated in figure 15A, CD69 expression is increased in later time-points (24 and 72 hours) after ACY1215 treatment in comparison with DMSO. Intriguingly, most of the patients assessed also expressed higher levels of ICOS at 72 hours after activation and HDAC inhibition through ACY1215, as indicated in

figure 15B. Other co-stimulatory molecules were also evaluated, including 4-1BB and OX40, but no notable changes in expression resulting from ACY1215 treatment were observed (data not shown).

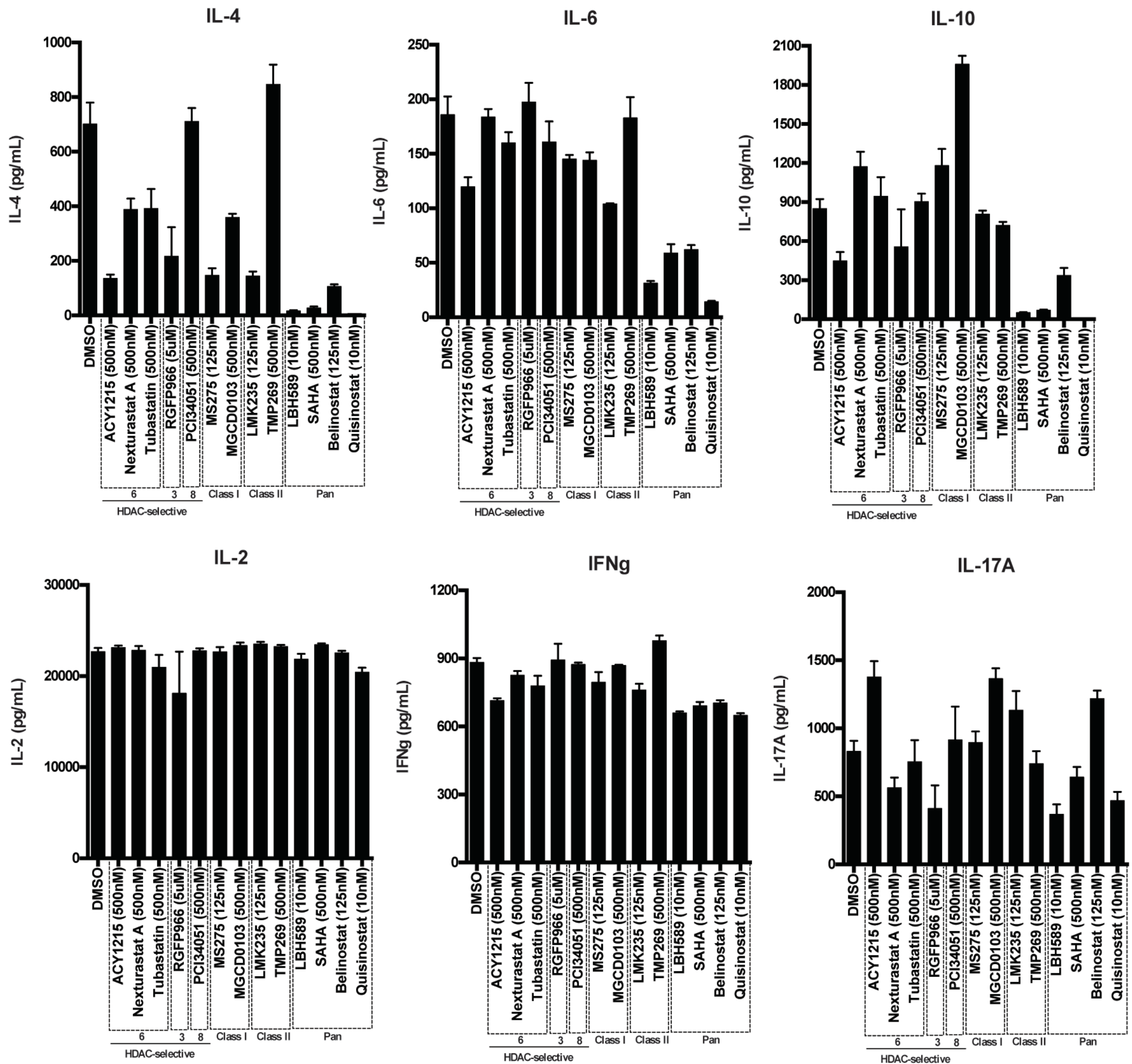


Figure 12. HDAC Inhibitors Have Disparate Effects on Cytokine Production. CD3+ T-cells were negatively isolated form PBMC of melanoma patients, treated with pan- and isotype-selective HDAC inhibitors at the indicated doses, and activated with aCD3/28 dynabeads for 72 hours. Supernatant was then harvested and the secreted cytokines IL4, IL-6, IL-10, IL-17A, IL-2, IFNγ were evaluated by luminex. The graphs represent Representative data of cytokine production following treatment with diverse HDAC inhibitors, as described above.

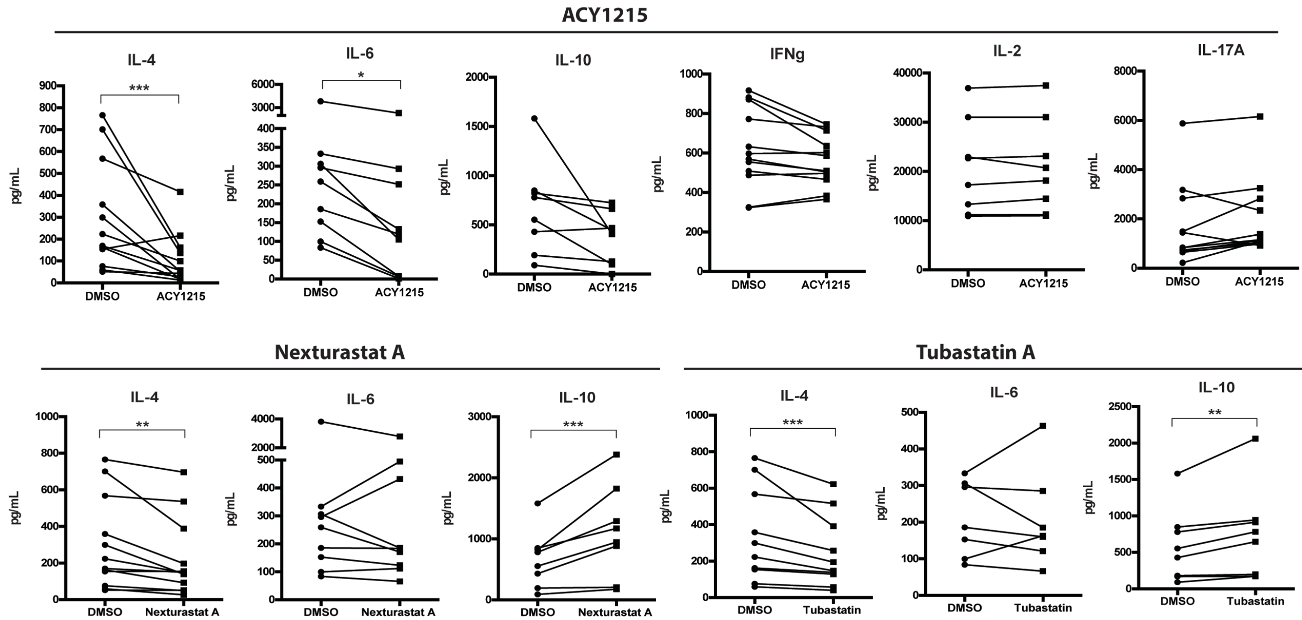


Figure 13. The HDAC Inhibitor ACY1215 is Unique in Reducing Th2 Cytokine Production, with Minimal Impact on Other Cytokines. CD3+ T-cells were negatively isolated form PBMC of melanoma patients and treated HDAC inhibitors, as described in figure 12. Graphs represent patient samples treated with the HDAC6-selective inhibitors ACY1215, nexturastast A and tubastatin A at 500nM concentration. All experiments were performed in triplicates. Nine to eleven patients were assessed. *p<0.05, ***p<0.001; two-tailed student's t-test.

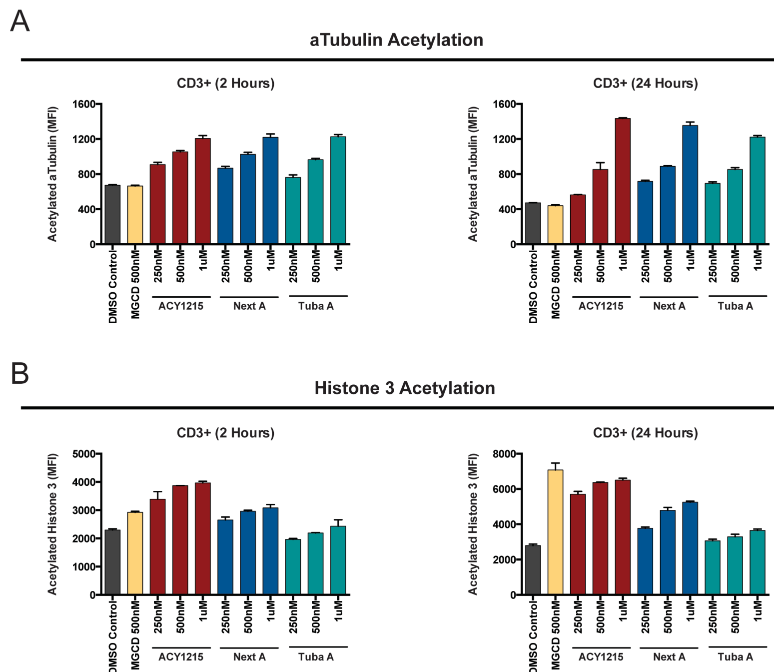


Figure 14. Specificity of HDAC Inhibitors. PBMC was collected from healthy donors and treated with MGCD0103, ACY1215, nexturastat A, tubastatin A or DMSO control, at the indicated concentrations, for 2 and 24 hours. Analysis of acetylated α-tubulin and acetylated histone 3 on CD3+ T-cells was performed by flow cytometry.

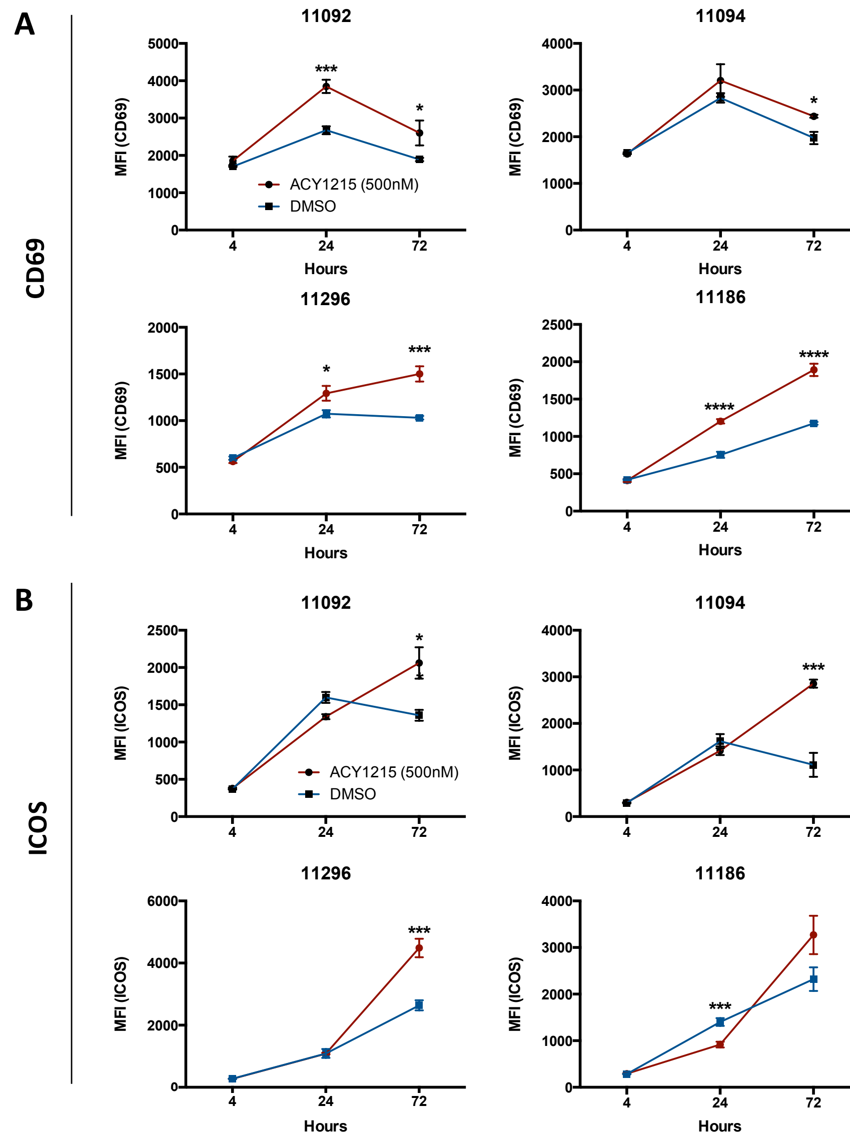


Figure 15. ACY1215 Enhances Expression of Surface Molecules on Activated T-cells. CD3+ T-cells were negatively isolated from PBMC of melanoma patients, treated with ACY1215 and activated with aCD3/28 dynabeads. Expression of **(A)** CD69 activation marker and **(B)** ICOS co-stimulatory molecule were evaluated by flow cytometry after 4, 24 and 72 hours, as indicated. *p<0.05, ***p<0.001, ****p<0.0001; two-tailed student's t-test.

ACY1215-mediated Inhibition Increases Central Memory Accumulation of T-cells

Since ACY1215 treatment altered surface T-cell molecules, the next step was to investigate whether other phenotypic changes occurred following inhibition using this drug. T-cells harvested from PBMC of melanoma patients were treated with DMSO control or ACY1215 and expanded in high dose of IL-2. After one week of treatment, expression of CD45RO, CD45RA, CD62L and CCR7 was evaluated by flow cytometry to determine any changes on T-cell subsets. Differences in the levels of these markers are characteristic of distinct T-cell populations. The phenotype CD45RA+CD45RO-CCR7+CD62L+ is present on naïve T-cells, CD45RA-CD45RO+CCR7+CD62L+ on central memory, CD45RA-CD45RO+CCR7-CD62L- on effector memory, and CD45RA+CD45RO+CCR7-CD62L- on effector T-cells[277]. Paired analyses in figure 16 illustrate the percent of central memory CD45RA-CD45RO+CCR7+CD62L+ T-cells for each patient (black dots) after treatment. As demonstrated in figure 16A, an enhanced percentage of central memory CD8+ and CD4+ T-cells from melanoma patients was observed following inhibition by ACY1215, in comparison with DMSO treatment ($p<0.05$ for CD8+ and $p<0.01$ for CD4+). This increase was also reported on T-cells derived from healthy donors (figure 16B; $p<0.05$ for CD8+ and $p<0.01$ for CD4+), suggesting that ACY1215 effects occur on T-cells in general.

Due to the importance of central memory phenotypes in protocols of ACT, the impact of ACY1215 *in vitro* treatment was further evaluated on TILs harvested from tumor biopsies of melanoma patients. As shown in figure 16C, TIL treatment with ACY1215 resulted in an enhance in central memory percent of CD4+ and CD8+

lymphocytes ($p<0.05$ and $p<0.01$, respectively), regardless of the initial percent of this population. In order to illustrate changes also occurring in the other evaluated T-cell subsets (i.e. naïve, effector memory and effector), the percent of these populations after TIL treatment with ACY1215 versus DMSO were graphed in figure 17. While there is a consistent increase on central memory T-cells percent, there is no clear pattern on alterations occurring in the other T-cell populations. For instance, the percent of effector memory T-cells was found unaltered or increased (TIL 1 CD4+ and CD8+, respectively), or even decreased (TILs 2 and 3) after ACY1215 *in vitro* treatment. Several TIL samples displayed lower percent of effector T-cells, but a mild increase on this subset was also observed (CD8+ TIL 1). Finally, the naïve T-cell percentage remained largely unchanged in the majority of samples, being reduced on CD8+ TIL 1 and increased on CD8+ TIL 2.

Accumulation of Central Memory and Phenotypic Alterations on TILs Mediated by ACY1215 Treatment are Maintained after Rapid Expansion *ex vivo*

To further evaluate whether phenotypic changes as a result of ACY1215 treatment were maintained after rapid expansion phase (REP), TILs were treated with ACY1215 or DMSO control and activated via CD3/CD28 using Dynabeads, in order to mimic REP protocols. After one week, central memory percent was higher in both CD8+ and CD4+ TILs treated with ACY1215 inhibitor (15% vs 20% for CD8+, $p<0.05$, and 22% vs 32% for CD4+, $p<0.05$; figure 18B), suggesting these alterations are sustained even following T-cell activation. To address whether other phenotypic changes previously observed were also maintained after activation and expansion, ICOS

expression was evaluated. Surprisingly, ICOS levels were higher on post-REP CD4+ TILs ($p<0.0001$), but no impact was observed on CD8+ lymphocytes (figure 18B). In all graphs, the standard error of the mean (SEM) was calculated based on independent treatments of the same patient sample.

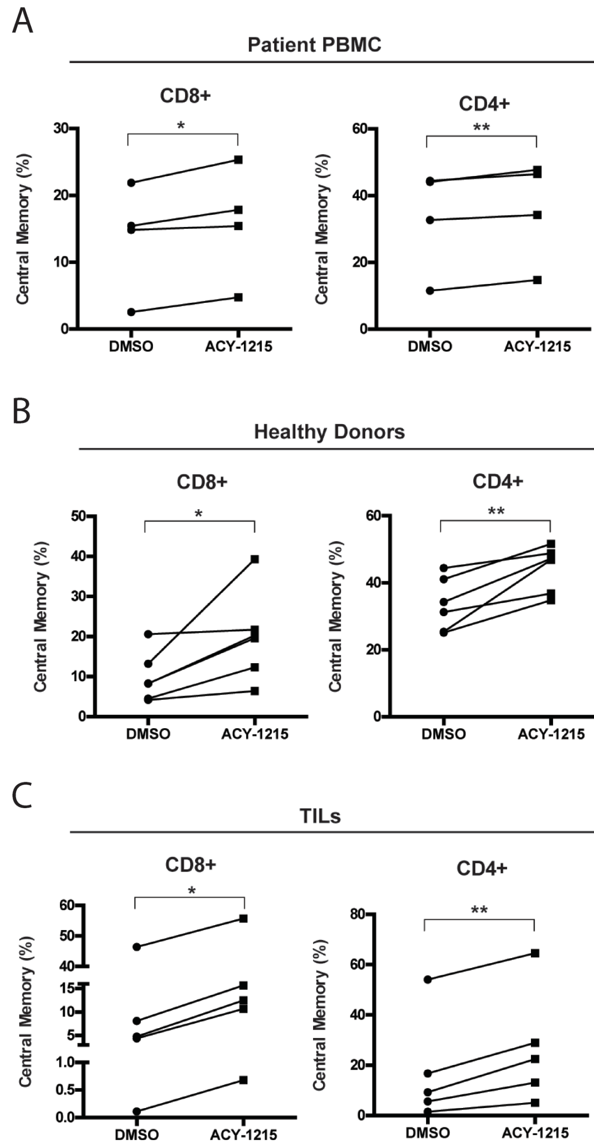


Figure 16. ACY1215 Increases Accumulation of Central Memory T-cells. CD3+ T-cells were negatively isolated from **(A)** PBMC of melanoma patients or **(B)** healthy donors, or **(C)** harvested from tumor biopsies. T-cells were treated with 500nM ACY1215 and expanded with 6000IU/mL IL-2 for one week. Expression of the memory markers CD62L, CD45RO, CD45RA and/or CCR7 was assessed by flow cytometry. Graphed dots indicate individual patient samples. *$p<0.05$, **$p<0.01$, ****$p<0.0001$; two-tailed, paired student's t-test.

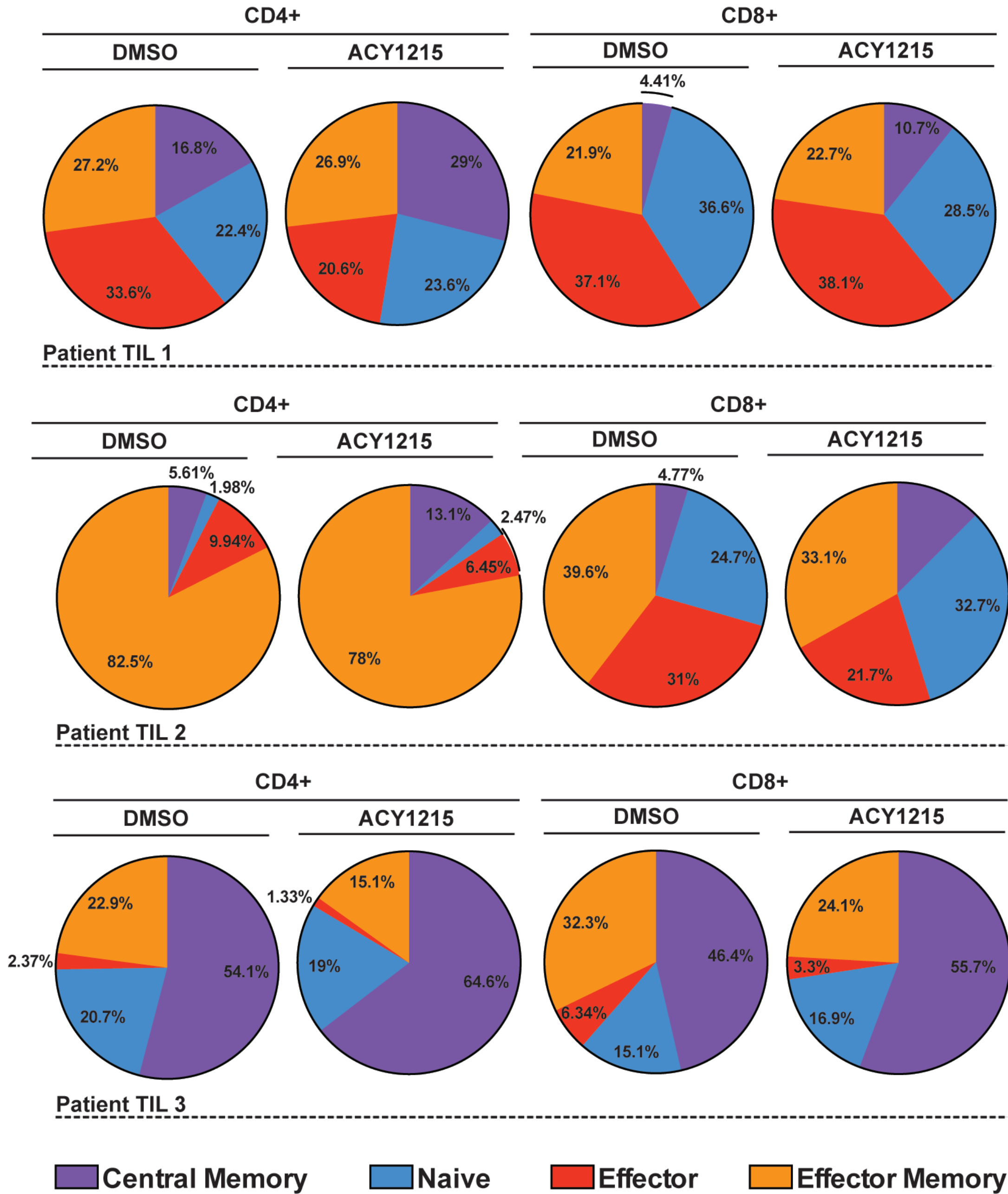


Figure 17. Effects of ACY1215-mediated Inhibition on TIL Populations. TILs were obtained from resected melanoma tumors, treated and evaluated as described in figure 16. Pie graphs are representative of TILs from three patients.

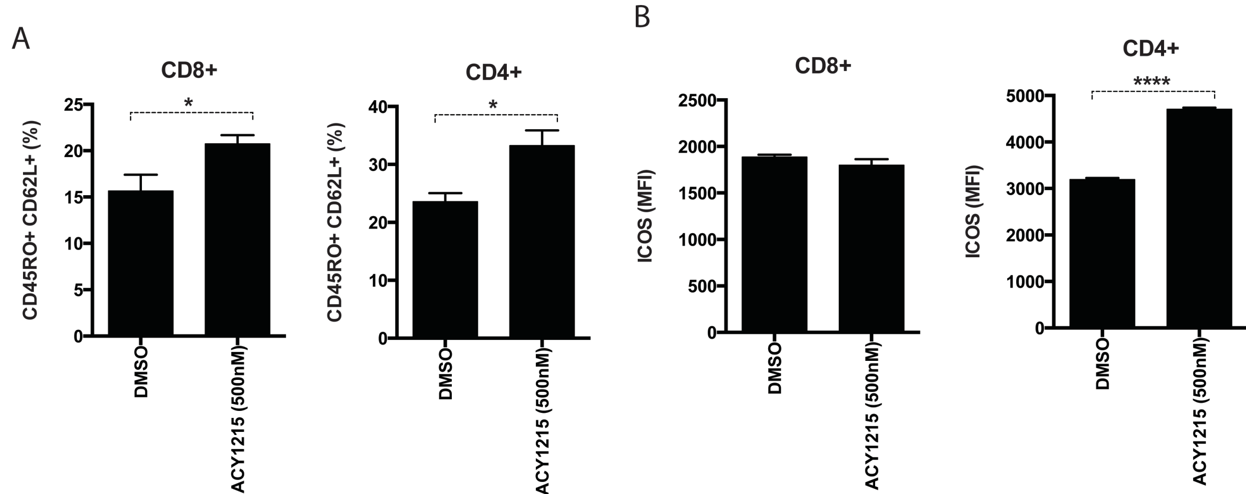


Figure 18. Post-REP TILs Maintain Phenotypic Changes Induced by ACY1215 Treatment *in vitro*. TILs isolated from melanoma biopsies were treated with ACY1215, activated via CD3/CD28 and expanded for one week with 6000IU/mL IL-2. Analyses of **(A)** expression of CD45RO+CD62L+ double-positive T-cells and **(B)** levels of ICOS were performed by flow cytometry. *p<0.05, ****p<0.0001; two-tailed, paired student's t-test.

ACY1215-treated Post-REP TILs Produce Higher Levels of Effector Molecules and Confer Superior Cytotoxicity Against Melanoma

To build upon these results and assess T-cell function after ACY1215-mediated inhibition, TILs harvested from melanoma patients were treated with ACY1215 or DMSO and underwent an adapted TIL protocol, using CD3/CD28 Dynabeads for activation. Intracellular production of IFNγ and expression of the degranulation marker CD107a were evaluated, as readout of effector function. Figure 19A illustrates that CD8+ post-REP TILs produced higher percent of double-positive IFNγ and CD107a after treatment with ACY1215 in comparison to DMSO control (p<0.05). Furthermore, the percent of IFNγ-expressing CD8+ and CD4+ post-REP TILs were also increased after ACY1215-mediated inhibition (p<0.05 and p<0.01, respectively). Calculation of

standard error of the mean (SEM) was based on independent treatments of the same patient sample. To address whether this result was generally extended to T-cells, CD3+ lymphocytes isolated from PBMC of healthy donors were evaluated for expression of IFNγ and CD107a. As expected, the percent of CD8+ T-cells expressing these molecules were enhanced after treatment with ACY1215 and high dose of IL-2 for one week, and subsequent pharmacological activation using PMA and ionomycin, as seen in figure 19B ($p<0.05$). Each dot on the graph represents paired analysis of one patient.

The aforementioned results suggest that ACY1215-mediated inhibition may improve cytolytic capacity of T-cells. Thus, post-REP TILs treated with ACY1215 or DMSO during *in vitro* expansion were co-cultured with the HLA-matched melanoma cell line Mel-624 for 48 hours, at the ratios of 0.2:1 and 0.8:1 (TIL:Melanoma). Melanoma cells were previously labeled with a cell tracker and the calculated percent of cell death was relative to viability of melanoma cells in the absence of TILs. As demonstrated in figure 19C, TILs harvested from a melanoma patient were capable of improving tumor cell killing from approximately 50 to 70% or 70 to 80% depending on the cell ratio, after treatment with DMSO or ACY1215, respectively. This enhanced ACY1215-mediated response against melanoma seemed to be dose-dependent, as TIL treatment with 250nM ACY1215 resulted in reduced melanoma death in comparison to 500nM ACY1215, while still presenting superior cytotoxicity than DMSO control.

Microarray Analysis Reveals Enhanced Expression of Genes Associated with Inflammatory Response and T-cell Memory Following TIL Treatment with ACY1215

Finally, to mechanistically investigate whether the ACY1215-medited effects were impacting gene expression, microarray analyses were performed in TILs harvested from four melanoma patients following treatment with ACY1215 or DMSO for one week, in the presence of high dose of IL-2. After normalization for gene expression, paired TILs samples (DMSO *versus* ACY1215) of each patient were graphed in a scatter plot, as shown in figure 20A. A linear behavior was observed for all patients, with the presence of a few probesets differently expressed between the matched pairs. Accordingly, the principal component analysis (PCA) seen in figure 20B demonstrates that the separation reached for the first three evaluated components (t[1], t[2] and t[3]) is based on the patient samples, while the forth component (t[4]) separates samples regarding the treatment received (DMSO *versus* ACY1215). This indicates that patient samples substantially vary among each other, with modulation of gene expression mediated by ACY1215 being a less differentiating factor. In order to illustrate gene expression, the results obtained from the four TIL samples are displayed in a heat map (figure 20C). The microarray analysis revealed 163 significant probesets ($p<0.001$), in which 153 were unique genes. TIL treatment with ACY1215 resulted in downregulation of 55 genes, while 108 genes were found upregulated. Furthermore, gene set enrichment analysis (GSEA) was performed to evaluate the impacts on pathways. Interestingly, genes involved in inflammatory response were altered following ACY1215 treatment, and are described in table V.

In order to explore whether changes in gene expression could be involved on the cellular alterations described previously, a specific set of genes governing memory T-cells phenotypes was assessed. Evaluation of gene expression of *sell*, *lef1* and *cd300a* demonstrated that the first two were upregulated (+1.57 and +1.32 fold difference, respectively, in comparison with DMSO control), while the latter was downregulated (-1.06 fold difference compared to DMSO treatment). According to published literature, upregulation of *sell* and *lef1* genes, and downregulation of *cd300a* gene, is found in a central memory phenotype rather than effector memory[268]. These results are in agreement with the ACY1215-mediated phenotypic changes on T-cells leading to accumulation of a central memory phenotype.

Table 5. Differential Expression of Genes Involved in Inflammatory Signaling. GSEA pathway analysis identified nine overlapping genes defining inflammatory response. p-value 1.18E-08, false discovery rate (FDR) q-value 5.88-07.

Gene Symbol	Description	Fold Difference	p(paired)
SELL	selectin L	1.57	0.0036
TNFRSF9	tumor necrosis factor receptor superfamily, member 9	0.85	0.0025
SRI	sorcin	0.80	0.0071
GPR183	G-protein-coupled receptor 183	0.66	0.0034
CCL20	chemokine (C-C motif) ligand 20	0.63	0.0055
GNA15	guanine nucleotide binding protein (G-protein), alpha 15 (Gq class)	-0.73	0.0015
IRF7	interferon regulatory factor 7	-0.77	0.0083
P2RX4	purinergic receptor P2X, ligand-gated ion channel, 4	-0.81	0.0036
CXCL8	chemokine (C-X-C motif) ligand 8	-1.36	0.0068

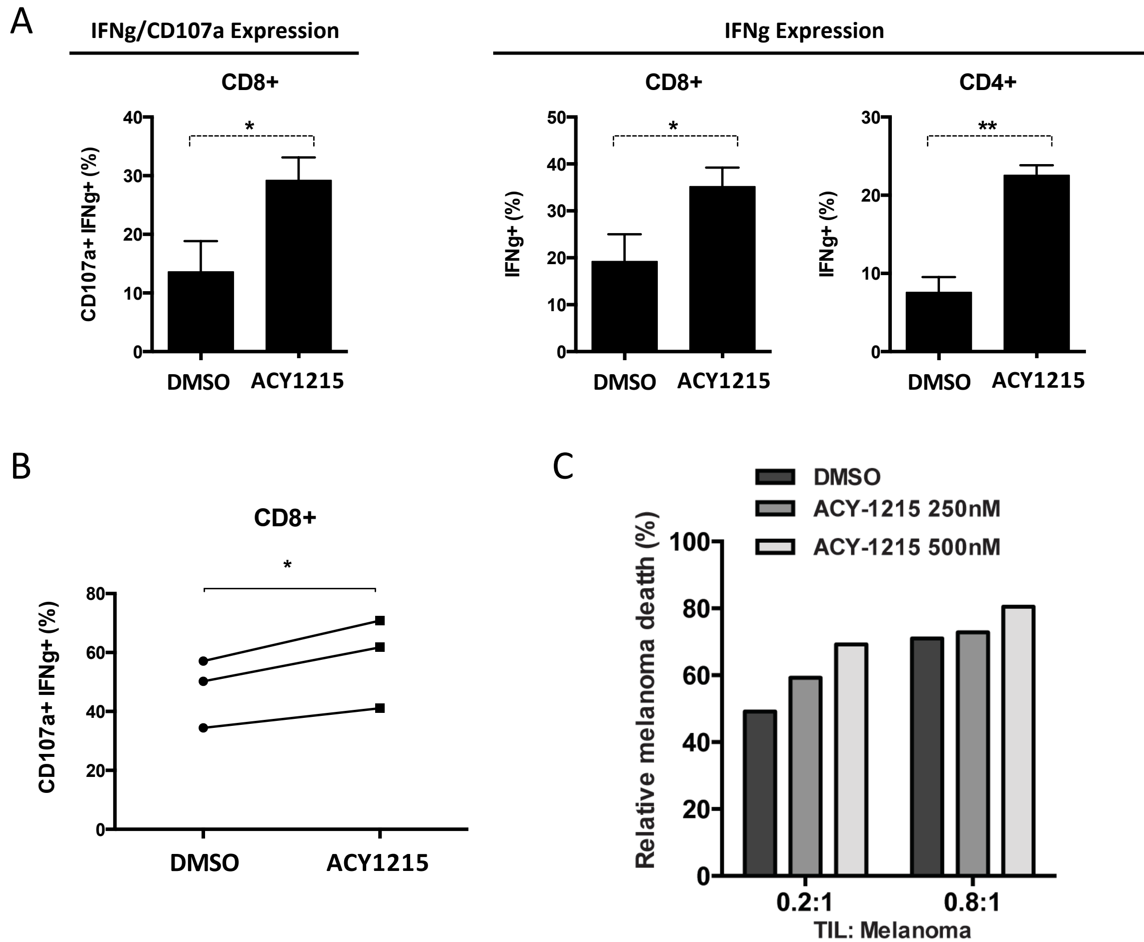


Figure 19. Post-REP TILs Treated with ACY1215 Produce Increased Levels of Effector Molecules and Mediate Enhanced Tumor Cytotoxicity. (A) TILs were harvested from melanoma biopsies, treated with 500nM ACY1215 or DMSO control, activated via CD3/CD28 and expanded *in vitro*. IFNγ-producing T-cells and double expression of IFNγ and CD107a were assessed by flow cytometry. **(B)** CD3+ T-cells were negatively isolated from PBMC of healthy donors, treated with 500nM ACY1215 or DMSO and expanded for one weeks. T-cells were then activated with PMA/ionomycin and monensin-treated for two hours, prior analysis of CD107a and IFNγ expression by flow cytometry. **(C)** Pre-REP TILs were expanded, treated with 500nM ACY1215 or DMSO, and activated. TILs were then co-cultured with HLA-matched melanoma for 48 hours. Relative melanoma death was assessed by flow cytometry, determined by expression of annexin V and viability marker incorporation. *$p<0.05$, **$p<0.01$; two-tailed student's t-test.

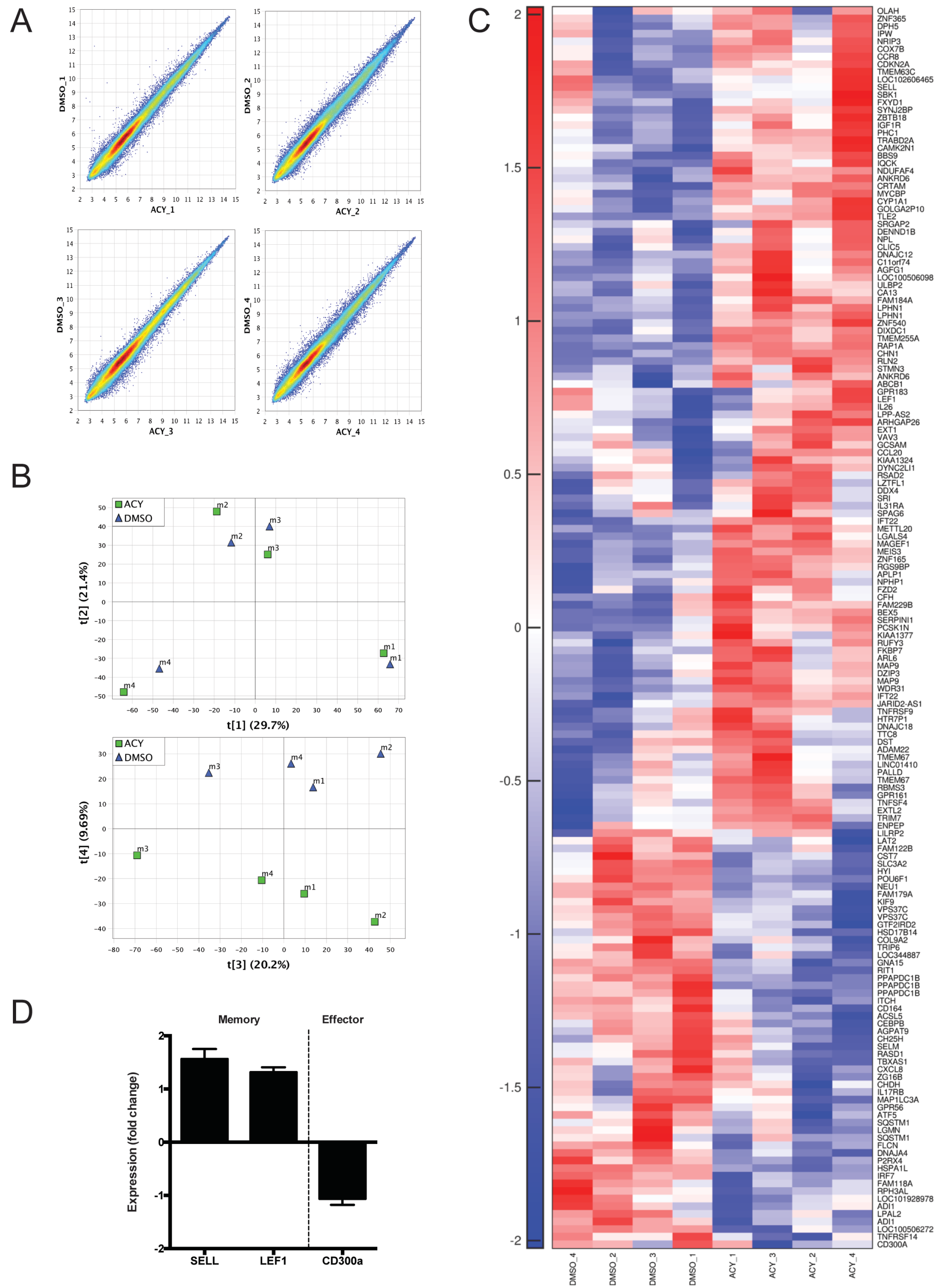


Figure 20. Culturing TILs in the Presence of ACY1215 Enhances Expression of Genes Associated with Inflammatory Response and T-cell Memory. TILs were harvested from tumor fragments from melanoma patients, cultured for one week with 500nM ACY1215 and 6000IU/mL IL-2, and evaluated for gene expression by Affimetrix-based microarray. **(A)** Scatter plots of gene probesets from paired analysis (DMSO *vs* ACY1215) of each patient TILs are shown. **(B)** Principal component analyses separating samples based on patient variability (t[1], t[2], t[3]) or DMSO *vs* ACY1215 treatment (t[4]) were performed. **(C)** Heat map is representative of relative gene expression obtained from DMSO or ACY1215 treated TILs. **(D)** Graphs demonstrate expression of genes associated with memory and effector T-cell phenotypes, relative to DMSO control (*sell*, p=0.0036; *lef1*, p=0.0008; *cd300a*, p=0.0026).

Discussion

The research presented herein sought to explore the role of HDAC inhibitors in regulating the immune response. In the first section of these studies a mechanism by which LBH589 inhibitor upregulates PDL1 and PDL2 expression on melanoma cells was elucidated. Tumor infiltrating immune cells were also evaluated for expression of these inhibitory molecules. Considering that class I and pan-HDAC compounds were found to lead to acetylation of histone 3 at the promoter regions of PDL1 and PDL2 genes and thus lead to gene expression, it was not surprising that immune cells were also susceptible to their effects. Indeed, LBH589 treatment *in vivo* using a B16F10 melanoma mouse model led to enhanced expression of PDL1 and PDL2 on CD45+ non T-cells (figure 10). While the mechanism for this upregulation remains to be validated, it is likely similar to the gene regulation described for melanoma cells in chapter 2.

Because combinatory therapy *in vivo* using LBH589 and PD1 blockade for the treatment of melanoma resulted in synergistic effects (chapter 2), expression of PD1 was assessed on tumor infiltrating T-cells on a B16F10 mouse model. Although LBH589 did not alter PD1 expression, this receptor was detected on T-cells (MFI values were higher than fluorescence minus one technical control; table IV). This highlights the importance of blocking PD1 and PDL1/PDL2 axis as means to improve therapy using HDAC inhibitors. However, as a result of LBH589 treatment, the percentage of CD45+ immune infiltrating cells was reduced (figure 10). This could be due to higher susceptibility of immune cells to the cytotoxic effects of HDAC inhibitors, lower migration of immune cells in general or of a specific subset to the tumor sites, or even decreased percent of immunosuppressive cells. HDAC inhibition has been shown to decrease the

number of CD4+Foxp3+CD25+ Tregs *in vivo* in a mouse model[288]. Nonetheless interesting, this study focused on the impacts of pan-HDAC inhibitors on immune cells viability, specifically T-cells, and investigated whether negative effects could be circumvented by the use of selective HDAC inhibitors. Indeed, LBH589, SAHA, belinostat and quisinostat (pan-HDAC inhibitors) treatments reduced T-cell viability to approximately 50% at low concentrations (figure 11). Conversely, all the class- or isotype-selective HDAC inhibitors promoted minimal impacts on T-cell viability. Although inhibition of class I HDACs[196] or HDAC8 through the use of PCI34051[231] are shown to promote T-cell death, the doses tested were sufficiently low to not impact viability and still exert an effect.

As exposure to cytokines can determine T-cells function, investigative research evaluating the effects of several HDAC inhibitors was performed (figures 12 and 13). Likely due to the negative effects of pan-HDAC inhibitors on T-cell viability, treatment with these inhibitors decreased production of IL-4, IL-6, IL-10, IFNγ and IL-17A to some degree. Interestingly, the compounds targeting class I HDACs, class IIa HDACs, HDAC3 and HDAC8 had disparate effects of T-cells cytokine production. This may be due to the fact that HDAC inhibitors display different potency. Furthermore, HDAC selectivity assays are usually performed in cell-free assays, which may fail to represent the variability according to cell types. Nevertheless, HDACs are shown to be involved with cytokine production, such as IL-4[197], IL-6[289], IL-10[181], IL-17A[290], IL-2[291] and IFNγ[292], and the results presented here corroborate their role in orchestrating production of these cytokines. Interestingly, the HDAC6 selective nexturastat A and tubastatin A displayed a clear role in decreasing IL-4 levels, while increasing IL-10 production. The

other HDAC6 selective inhibitor, ACY1215, was unique in reducing the levels of IL-4, IL-6 and IL-10. Tempering type-2 T-cell response is advantageous in the tumor setting, since it can reduce a suppressive environment. IL-4 stimuli promote Th2 differentiation[293] and a balance of more IFNγ and less IL-4 is preferred in the context of cancer. Although the role of IL-6 is dependent on the stage of tumor, decreased levels of this cytokine can aid anti-tumor response[92]. Moreover, IL-10 is an anti-inflammatory cytokine, capable of inhibiting antigen presentation and Th1 cytokine production[293]. These unique effects of ACY1215 contrasting with other HDAC6 selective inhibitors are possibly due to the dual ability of this compound to inhibit HDAC6 and promote acetylation of histone 3 on T-cells, at the assessed concentrations (figure 14).

The presented results described an unexplored role of ACY1215-mediated inhibition in improving T-cell function, while sparing them from cytotoxic effects promoted by pan- and class I HDAC inhibitors[244,294]. T-cell treatment with ACY1215 at concentrations of 250nM or 500nM displayed minimal effects on viability, however, these doses were sufficient to alter the phenotype and functions of T-cells. Besides dampening Th2 cytokine production, ACY1215 treatment of circulating T-cells or TILs of melanoma patients led to an accumulation of a central memory subset (figures 16 and 18). Here, central memory subset was defined based on expression of CD45RO, CD45RA, CD62L and CCR7. While no striking differences were observed in CD127, a marker of long-living T-cells[295] (data not shown), an expanded panel of molecules defining less differentiated, stem-memory cells (e.g. CD45RA+CD95+CD122+CCR7+CD62L+)[296] can still be explored in the context of ACY1215 treatment. The phenotype skewing to this population may be a result of a

decrease distributed along other subsets (i.e. naïve, effector memory and/or effector) rather than a shift occurring solely in a specific population (figure 17). Possibly, a reduction on effector memory, effector or even naïve T-cells may be allowing for central memory T-cells to preferentially expand. Regardless, accumulation of central memory TILs can optimize adoptive T-cell therapy, since the presence of memory T-lymphocytes is associated with prorogued *in vivo* persistence and, thus, improved patient outcome and survival[271]. In this regard, strategies to improve *ex vivo* expansion of TILs are warranted and can be explored with the use of ACY1215.

Furthermore, treatment of circulating T-cells and TILs with ACY1215 resulted in higher levels of the costimulatory molecule ICOS (figures 15 and 18). Expression of ICOS has been shown to be associated with improved anti-tumor response in melanoma mouse models. Engagement of ICOS receptor with its ligand (ICOSL) is necessary to induce a type-1 response during CTLA4 blockade in B16F10-bearing mice[297,298]. Indeed, ICOS expression on CD4+ T-cells is required for orchestration of Th1-response against tumor in mice undergoing anti-CTLA4 immunotherapy[299]. In humans, expression of ICOS and the proliferation marker Ki67 is associated with ipilimumab treatment of melanoma patients[300]. Based on these preclinical and clinical studies, ACY1215-mediated ICOS upregulation represents an attractive route to enhance TIL therapy, especially when it concerns to combination therapies using ipilimumab in the setting of melanoma. The levels of the activation marker CD69 were also upregulated as a result of ACY1215-mediated inhibition (figure 15), and may be indicative of prolonged activated status of these T-cells. However, evaluation of other

activation molecules (e.g. CD44, CD25), or costimulatory receptors remains to be investigated.

Functionally, the percent of TILs producing IFNγ and CD107a was increased following treatment with ACY1215 in comparison to control. In line with this result, an enhanced cytotoxicity *in vitro* activity against HLA-matched melanoma cell line was observed (figure 19). While evaluation of these effects on TILs from multiple patients, as well as TIL response against other reactive and non-reactive cell lines or even melanoma cells derived from resected tumors is warranted, these data indicate the ability of ACY1215 to generate optimal characteristics for T-cell response in the cancer setting. Most importantly, the aforementioned impacts of ACY1215 were maintained following *ex vivo* expansion of TILs (post-REP). Although the protocols used in this study were an adapted version of REP protocols utilized for TIL therapy of melanoma patients, the results suggest that ACY1215-mediated inhibition can promote benefits even after massive expansion of TILs for infusion into patients. Furthermore, these effects seem to be unrestricted to diseased patients, as they were also observed in T-cells derived from healthy donors (figures 16 and 19), suggesting that ACY1215 treatment approach is not limited to the context of melanoma.

In line with the observed phenotypic changes, ACY1215 treatment of TILs altered gene expression (figure 20). Only a small set of genes were differentially modulated by ACY1215 compared to control, which is in agreement with the subtle alterations seen in expression of surface molecules and cytokine production. Corroborating the reported accumulation of central memory T-cells, ACY1215 treatment upregulated *sell* and *lef1* genes and downregulated *cd300a* expression. A similar gene expression pattern has

been shown to be associated with a central memory phenotype[268]. Also in accordance with the observed tempering of the anti-inflammatory cytokines IL-4, IL-6 and IL-10, and enhanced percent of IFNγ-producing T-cells, GSEA pathway analysis revealed a role of ACY1215 in modulating inflammatory response. While exact pathway(s)/mechanism(s) by which ACY1215 exerts these T-cell anti-tumor enhancing characteristics remain to be fully elucidated, these results create a rationale of using this compound as mean to augment T-cell response in immunotherapy approaches, especially concerning TIL therapy for melanoma.

CHAPTER FOUR:

CONCLUSIONS & FUTURE PERSPECTIVES

The data generated from these studies elucidated a mechanism by which HDAC inhibitors modulate tumor biology, thus impacting on T-cell response. Upregulation of the inhibitory molecules PDL1 and PDL2 as a result of HDAC inhibition is detrimental to generation of a productive T-cell response against tumor. Understanding these negative effects in the context of cancer grants rationale to combinatory therapies able to circumvent such limitations. Furthermore, both LBH589 and PD1-blockade are approved by the FDA (as separate agents with different indications), facilitating the translation from the presented preclinical investigation to clinical investigation. Upregulation of PDL1 was found to be a result of class I inhibition. In fact, HDAC1, HDAC2 and HDAC3, with the exception of HDAC8, appear to be the major modulators of the observed results, as inhibitors with specificity for these HDACs were able to induce PDL1 expression. Studies addressing the role of individual HDACs are in progress. Elucidating whether PDL1 and PDL2 upregulation results from HDAC1, HDAC2 or HDAC3, or a combination of these, are relevant to modulate these molecules with more specific inhibitors in order to avoid undesired effects. Indeed, being able to promote the anti-tumor effects mediated by HDAC inhibitors, while circumventing upregulation of inhibitory molecules is attractive for the setting of cancer. In contrast,

targeting the specific HDACs involved in upregulation of PDL1 is attractive for treatment of autoimmunity.

These studies also highlight the advantages of using ACY1215 HDAC-selective inhibitor to improve T-cell function and response against melanoma. Previous research has demonstrated the ability of pan- and class I HDAC inhibitors to promote tumor cell death[214,301]. However, if extended to immune cells, these effects can be disadvantageous during a productive immune response against tumor. The unique impacts of ACY1215 on T-cell biology are advantageous to adoptive T-cell therapy approaches, and should be leveraged to translate these results to a clinical setting. The data herein described provide rationale to incorporate the use of ACY1215 during protocols of *ex vivo* TIL expansion. Hence, this extra step would require little additional effort and possibly significant benefits to patient outcomes. The fact of ACY1215 being already used in several clinical trials also facilitates its transition from translational to clinical research. Since the herein reported improvements on T-cell response were solely based on *in vitro* treatment, systemic administration may not be necessary, thus avoiding undesirable side effects often accompanying chemotherapy. Regardless, future work is warranted to elucidate the mechanisms by which ACY1215 is modulating T-cell molecular and cellular biology. Ultimately, these studies revealed novel functions and mechanisms of pan-, class I- and isotype-selective HDAC inhibitors in both tumor and immune cells.

REFERENCES

1 Baselga, J. *et al.* AACR Cancer Progress Report 2015. *Clin Cancer Res* **21**, S1-S128, doi:10.1158/1078-0432.CCR-15-1846 (2015).
2 Hanahan, D. & Weinberg, R. A. The hallmarks of cancer. *Cell* **100**, 57-70 (2000).
3 Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144**, 646-674, doi:10.1016/j.cell.2011.02.013 (2011).
4 in *American Cancer Society. Cancer Facts & Figures.* (American Cancer Society, Atlanta (GA), 2015).
5 in *World Health Organization. Global status report on noncommunicable diseases.* (World Health Organization, Geneva (Switzerland), 2014).
6 Howlader N, N. A., Krapcho M, Neyman N, Aminou R, Waldron W, Altekruse SF, Kosary CL, Ruhl J, Tatalovich Z, Cho H, Mariotto A, Eisner MP, Lewis DR, Chen HS, Feuer EJ, Cronin KA, Edwards BK *SEER Cancer Statistics Review*, 2011).
7 Home, E. Observations on cancer, case VIII. (1805).
8 Stewart, L. A. Chemotherapy in adult high-grade glioma: a systematic review and meta-analysis of individual patient data from 12 randomised trials. *Lancet* **359**, 1011-1018 (2002).
9 Kirkwood, J. M. *et al.* Comparison of intramuscular and intravenous recombinant alpha-2 interferon in melanoma and other cancers. *Ann Intern Med* **103**, 32-36 (1985).
10 Rosenberg, S. A. *et al.* Observations on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer. *The New England journal of medicine* **313**, 1485-1492, doi:10.1056/NEJM198512053132327 (1985).
11 Costanza, M. E. *et al.* Results of a randomized study comparing DTIC with TIC mustard in malignant melanoma. *Cancer* **37**, 1654-1659 (1976).
12 Patel, P. M. *et al.* Extended schedule, escalated dose temozolomide versus dacarbazine in stage IV melanoma: final results of a randomised phase III study (EORTC 18032). *European journal of cancer* **47**, 1476-1483, doi:10.1016/j.ejca.2011.04.030 (2011).
13 Andersen, M. H. *et al.* Cancer treatment: the combination of vaccination with other therapies. *Cancer immunology, immunotherapy : CII* **57**, 1735-1743, doi:10.1007/s00262-008-0480-y (2008).
14 Bredel, M. Anticancer drug resistance in primary human brain tumors. *Brain Res Brain Res Rev* **35**, 161-204 (2001).
15 Helmbach, H., Sinha, P. & Schadendorf, D. Human melanoma: drug resistance. *Recent Results Cancer Res* **161**, 93-110 (2003).

16 Grossman, D. & Altieri, D. C. Drug resistance in melanoma: mechanisms, apoptosis, and new potential therapeutic targets. *Cancer metastasis reviews* **20**, 3-11 (2001).
17 Curtin, J. A. *et al.* Distinct sets of genetic alterations in melanoma. *The New England journal of medicine* **353**, 2135-2147, doi:10.1056/NEJMoa050092 (2005).
18 Rockmann, H. & Schadendorf, D. Drug resistance in human melanoma: mechanisms and therapeutic opportunities. *Onkologie* **26**, 581-587, doi:74156 (2003).
19 Lev, D. C. *et al.* Dacarbazine causes transcriptional up-regulation of interleukin 8 and vascular endothelial growth factor in melanoma cells: a possible escape mechanism from chemotherapy. *Molecular cancer therapeutics* **2**, 753-763 (2003).
20 Lev, D. C. *et al.* Exposure of melanoma cells to dacarbazine results in enhanced tumor growth and metastasis in vivo. *J Clin Oncol* **22**, 2092-2100, doi:10.1200/JCO.2004.11.070 (2004).
21 Hocker, T. L., Singh, M. K. & Tsao, H. Melanoma genetics and therapeutic approaches in the 21st century: moving from the benchside to the bedside. *J Invest Dermatol* **128**, 2575-2595, doi:10.1038/jid.2008.226 (2008).
22 Cox, A. D. & Der, C. J. Ras family signaling: therapeutic targeting. *Cancer biology & therapy* **1**, 599-606 (2002).
23 Milagre, C. *et al.* A mouse model of melanoma driven by oncogenic KRAS. *Cancer Res* **70**, 5549-5557, doi:10.1158/0008-5472.CAN-09-4254 (2010).
24 Davies, H. *et al.* Mutations of the BRAF gene in human cancer. *Nature* **417**, 949-954, doi:10.1038/nature00766 (2002).
25 Schrama, D. *et al.* BRAFV600E mutations in malignant melanoma are associated with increased expressions of BAALC. *Journal of carcinogenesis* **7**, 1, doi:10.1186/1477-3163-7-1 (2008).
26 Dhomen, N. & Marais, R. BRAF signaling and targeted therapies in melanoma. *Hematol Oncol Clin North Am* **23**, 529-545, ix, doi:10.1016/j.hoc.2009.04.001 (2009).
27 Bastian, B. C. The molecular pathology of melanoma: an integrated taxonomy of melanocytic neoplasia. *Annu Rev Pathol* **9**, 239-271, doi:10.1146/annurev-pathol-012513-104658 (2014).
28 Wu, H., Goel, V. & Haluska, F. G. PTEN signaling pathways in melanoma. *Oncogene* **22**, 3113-3122, doi:10.1038/sj.onc.1206451 (2003).
29 Omholt, K., Krockel, D., Ringborg, U. & Hansson, J. Mutations of PIK3CA are rare in cutaneous melanoma. *Melanoma research* **16**, 197-200, doi:10.1097/01.cmr.0000200488.77970.e3 (2006).
30 Kyrgidis, A., Tzellos, T. G. & Triaridis, S. Melanoma: Stem cells, sun exposure and hallmarks for carcinogenesis, molecular concepts and future clinical implications. *Journal of carcinogenesis* **9**, 3, doi:10.4103/1477-3163.62141 (2010).
31 Meyle, K. D. & Guldberg, P. Genetic risk factors for melanoma. *Human genetics* **126**, 499-510, doi:10.1007/s00439-009-0715-9 (2009).

32 Sullivan, R. J., Lorusso, P. M. & Flaherty, K. T. The intersection of immune-directed and molecularly targeted therapy in advanced melanoma: where we have been, are, and will be. *Clin Cancer Res* **19**, 5283-5291, doi:10.1158/1078-0432.CCR-13-2151 (2013).

33 Chapman, P. B. *et al.* Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *The New England journal of medicine* **364**, 2507-2516, doi:10.1056/NEJMoa1103782 (2011).

34 McArthur, G. A. *et al.* Safety and efficacy of vemurafenib in BRAF(V600E) and BRAF(V600K) mutation-positive melanoma (BRIM-3): extended follow-up of a phase 3, randomised, open-label study. *The lancet oncology* **15**, 323-332, doi:10.1016/S1470-2045(14)70012-9 (2014).

35 Hauschild, A. *et al.* Dabrafenib in BRAF-mutated metastatic melanoma: a multicentre, open-label, phase 3 randomised controlled trial. *Lancet* **380**, 358-365, doi:10.1016/S0140-6736(12)60868-X (2012).

36 Flaherty, K. T. *et al.* Improved survival with MEK inhibition in BRAF-mutated melanoma. *The New England journal of medicine* **367**, 107-114, doi:10.1056/NEJMoa1203421 (2012).

37 Long, G. V. *et al.* Combined BRAF and MEK inhibition versus BRAF inhibition alone in melanoma. *The New England journal of medicine* **371**, 1877-1888, doi:10.1056/NEJMoa1406037 (2014).

38 Robert, C. *et al.* Improved overall survival in melanoma with combined dabrafenib and trametinib. *The New England journal of medicine* **372**, 30-39, doi:10.1056/NEJMoa1412690 (2015).

39 Sullivan, R. J. & Flaherty, K. T. Resistance to BRAF-targeted therapy in melanoma. *European journal of cancer*, doi:10.1016/j.ejca.2012.11.019 (2013).

40 Neagu, M. The immune system--a hidden treasure for biomarker discovery in cutaneous melanoma. *Adv Clin Chem* **58**, 89-140 (2012).

41 Clark, W. H., Jr. *et al.* Model predicting survival in stage I melanoma based on tumor progression. *Journal of the National Cancer Institute* **81**, 1893-1904 (1989).

42 Clemente, C. G. *et al.* Prognostic value of tumor infiltrating lymphocytes in the vertical growth phase of primary cutaneous melanoma. *Cancer* **77**, 1303-1310, doi:10.1002/(SICI)1097-0142(19960401)77:7<1303::AID-CNCR12>3.0.CO;2-5 (1996).

43 Haanen, J. B. *et al.* Melanoma-specific tumor-infiltrating lymphocytes but not circulating melanoma-specific T cells may predict survival in resected advanced-stage melanoma patients. *Cancer immunology, immunotherapy : CII* **55**, 451-458, doi:10.1007/s00262-005-0018-5 (2006).

44 Zhang, L. *et al.* Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *The New England journal of medicine* **348**, 203-213, doi:10.1056/NEJMoa020177 (2003).

45 Kawakami, Y. *et al.* Identification of a human melanoma antigen recognized by tumor-infiltrating lymphocytes associated with in vivo tumor rejection. *Proc Natl Acad Sci U S A* **91**, 6458-6462 (1994).

46 Kawakami, Y. *et al.* Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor. *Proc Natl Acad Sci U S A* **91**, 3515-3519 (1994).

47 Johnson, L. A. *et al.* Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. *Blood* **114**, 535-546, doi:10.1182/blood-2009-03-211714 (2009).

48 Vonderheide, R. H. Telomerase as a universal tumor-associated antigen for cancer immunotherapy. *Oncogene* **21**, 674-679, doi:10.1038/sj.onc.1205074 (2002).

49 Andersen, M. H. *et al.* Spontaneous cytotoxic T-cell responses against survivin-derived MHC class I-restricted T-cell epitopes in situ as well as ex vivo in cancer patients. *Cancer Res* **61**, 5964-5968 (2001).

50 Robbins, P. F. *et al.* A pilot trial using lymphocytes genetically engineered with an NY-ESO-1-reactive T-cell receptor: long-term follow-up and correlates with response. *Clin Cancer Res* **21**, 1019-1027, doi:10.1158/1078-0432.CCR-14-2708 (2015).

51 Lawrence, M. S. *et al.* Mutational heterogeneity in cancer and the search for new cancer-associated genes. *Nature* **499**, 214-218, doi:10.1038/nature12213 (2013).

52 Goedert, J. J. The epidemiology of acquired immunodeficiency syndrome malignancies. *Seminars in oncology* **27**, 390-401 (2000).

53 Shankaran, V. *et al.* IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature* **410**, 1107-1111, doi:10.1038/35074122 (2001).

54 Girardi, M. *et al.* Regulation of cutaneous malignancy by gammadelta T cells. *Science* **294**, 605-609, doi:10.1126/science.1063916 (2001).

55 Street, S. E., Trapani, J. A., MacGregor, D. & Smyth, M. J. Suppression of lymphoma and epithelial malignancies effected by interferon gamma. *J Exp Med* **196**, 129-134 (2002).

56 Airoldi, I. *et al.* Lack of Il12rb2 signaling predisposes to spontaneous autoimmunity and malignancy. *Blood* **106**, 3846-3853, doi:10.1182/blood-2005-05-2034 (2005).

57 Algarra, I., Cabrera, T. & Garrido, F. The HLA crossroad in tumor immunology. *Human immunology* **61**, 65-73 (2000).

58 Porgador, A., Mandelboim, O., Restifo, N. P. & Strominger, J. L. Natural killer cell lines kill autologous beta2-microglobulin-deficient melanoma cells: implications for cancer immunotherapy. *Proc Natl Acad Sci U S A* **94**, 13140-13145 (1997).

59 Schwartz, R. H. T cell anergy. *Annual review of immunology* **21**, 305-334, doi:10.1146/annurev.immunol.21.120601.141110 (2003).

60 Halloran, P. F. Immunosuppressive drugs for kidney transplantation. *The New England journal of medicine* **351**, 2715-2729, doi:10.1056/NEJMra033540 (2004).

61 Acuto, O. & Michel, F. CD28-mediated co-stimulation: a quantitative support for TCR signalling. *Nature reviews. Immunology* **3**, 939-951, doi:10.1038/nri1248 (2003).

62 Ladanyi, A. *et al.* FOXP3+ cell density in primary tumor has no prognostic impact in patients with cutaneous malignant melanoma. *Pathol Oncol Res* **16**, 303-309, doi:10.1007/s12253-010-9254-x (2010).
63 Mougiakakos, D. *et al.* Intratumoral forkhead box P3-positive regulatory T cells predict poor survival in cyclooxygenase-2-positive uveal melanoma. *Cancer* **116**, 2224-2233, doi:10.1002/cncr.24999 (2010).
64 Jandus, C., Bioley, G., Speiser, D. E. & Romero, P. Selective accumulation of differentiated FOXP3(+) CD4 (+) T cells in metastatic tumor lesions from melanoma patients compared to peripheral blood. *Cancer immunology, immunotherapy : CII* **57**, 1795-1805, doi:10.1007/s00262-008-0507-4 (2008).
65 Ahmadzadeh, M. *et al.* FOXP3 expression accurately defines the population of intratumoral regulatory T cells that selectively accumulate in metastatic melanoma lesions. *Blood* **112**, 4953-4960, doi:10.1182/blood-2008-06-163048 (2008).
66 Curiel, T. J. *et al.* Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* **10**, 942-949, doi:10.1038/nm1093 (2004).
67 Nizar, S., Meyer, B., Galustian, C., Kumar, D. & Dalgleish, A. T regulatory cells, the evolution of targeted immunotherapy. *Biochimica et biophysica acta* **1806**, 7-17, doi:10.1016/j.bbcan.2010.02.001 (2010).
68 Munn, D. H. & Mellor, A. L. Indoleamine 2,3-dioxygenase and tumor-induced tolerance. *The Journal of clinical investigation* **117**, 1147-1154, doi:10.1172/JCI31178 (2007).
69 Brody, J. R. *et al.* Expression of indoleamine 2,3-dioxygenase in metastatic malignant melanoma recruits regulatory T cells to avoid immune detection and affects survival. *Cell Cycle* **8**, 1930-1934 (2009).
70 Walunas, T. L. *et al.* CTLA-4 can function as a negative regulator of T cell activation. *Immunity* **1**, 405-413 (1994).
71 Oaks, M. K. & Hallett, K. M. Cutting edge: a soluble form of CTLA-4 in patients with autoimmune thyroid disease. *J Immunol* **164**, 5015-5018 (2000).
72 Qureshi, O. S. *et al.* Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. *Science* **332**, 600-603, doi:10.1126/science.1202947 (2011).
73 Chuang, E. *et al.* The CD28 and CTLA-4 receptors associate with the serine/threonine phosphatase PP2A. *Immunity* **13**, 313-322 (2000).
74 Chen, W., Jin, W. & Wahl, S. M. Engagement of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) induces transforming growth factor beta (TGF-beta) production by murine CD4(+) T cells. *J Exp Med* **188**, 1849-1857 (1998).
75 Grosso, J. F. & Jure-Kunkel, M. N. CTLA-4 blockade in tumor models: an overview of preclinical and translational research. *Cancer Immun* **13**, 5 (2013).
76 Weber, J. S. *et al.* Phase I/II study of ipilimumab for patients with metastatic melanoma. *J Clin Oncol* **26**, 5950-5956, doi:10.1200/JCO.2008.16.1927 (2008).
77 Robert, C. *et al.* Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *The New England journal of medicine* **364**, 2517-2526, doi:10.1056/NEJMoa1104621 (2011).

78 Bronte, V., Serafini, P., Mazzoni, A., Segal, D. M. & Zanovello, P. L-arginine metabolism in myeloid cells controls T-lymphocyte functions. *Trends in immunology* **24**, 302-306 (2003).
79 Rodriguez, P. C. & Ochoa, A. C. T cell dysfunction in cancer: role of myeloid cells and tumor cells regulating amino acid availability and oxidative stress. *Seminars in cancer biology* **16**, 66-72, doi:10.1016/j.semcancer.2005.10.001 (2006).
80 Mazzoni, A. *et al.* Myeloid suppressor lines inhibit T cell responses by an NO-dependent mechanism. *J Immunol* **168**, 689-695 (2002).
81 Rigo, A. *et al.* Macrophages may promote cancer growth via a GM-CSF/HB-EGF paracrine loop that is enhanced by CXCL12. *Molecular cancer* **9**, 273, doi:10.1186/1476-4598-9-273 (2010).
82 Joimel, U. *et al.* Stimulation of angiogenesis resulting from cooperation between macrophages and MDA-MB-231 breast cancer cells: proposed molecular mechanism and effect of tetrathiomolybdate. *BMC cancer* **10**, 375, doi:10.1186/1471-2407-10-375 (2010).
83 Sica, A. *et al.* Macrophage polarization in tumour progression. *Seminars in cancer biology* **18**, 349-355, doi:10.1016/j.semcancer.2008.03.004 (2008).
84 Kim, J. *et al.* IL-10 production in cutaneous basal and squamous cell carcinomas. A mechanism for evading the local T cell immune response. *J Immunol* **155**, 2240-2247 (1995).
85 Visco, C. *et al.* Elevated serum levels of IL-10 are associated with inferior progression-free survival in patients with Hodgkin's disease treated with radiotherapy. *Leuk Lymphoma* **45**, 2085-2092, doi:10.1080/10428190410001712234 (2004).
86 Taga, K. *et al.* Interleukin-10 inhibits apoptotic cell death in infectious mononucleosis T cells. *The Journal of clinical investigation* **94**, 251-260, doi:10.1172/JCI117315 (1994).
87 Laidlaw, B. J. *et al.* Production of IL-10 by CD4(+) regulatory T cells during the resolution of infection promotes the maturation of memory CD8(+) T cells. *Nat Immunol* **16**, 871-879, doi:10.1038/ni.3224 (2015).
88 Steinbrink, K., Graulich, E., Kubsch, S., Knop, J. & Enk, A. H. CD4(+) and CD8(+) anergic T cells induced by interleukin-10-treated human dendritic cells display antigen-specific suppressor activity. *Blood* **99**, 2468-2476 (2002).
89 Pawelec, G., Hambrecht, A., Rehbein, A. & Adibzadeh, M. Interleukin 10 protects activated human T lymphocytes against growth factor withdrawal-induced cell death but only anti-fas antibody can prevent activation-induced cell death. *Cytokine* **8**, 877-881, doi:10.1006/cyto.1996.0117 (1996).
90 Kucera, R. *et al.* Evaluation of IL-2, IL-6, IL-8 and IL-10 in Malignant Melanoma Diagnostics. *Anticancer Res* **35**, 3537-3541 (2015).
91 Torisu-Itakura, H. *et al.* Monocyte-derived IL-10 expression predicts prognosis of stage IV melanoma patients. *J Immunother* **30**, 831-838, doi:10.1097/CJI.0b013e318158795b (2007).
92 Naugler, W. E. & Karin, M. The wolf in sheep's clothing: the role of interleukin-6 in immunity, inflammation and cancer. *Trends Mol Med* **14**, 109-119, doi:10.1016/j.molmed.2007.12.007 (2008).

93 Aggarwal, B. B. *et al.* Targeting signal-transducer-and-activator-of-transcription-3 for prevention and therapy of cancer: modern target but ancient solution. *Annals of the New York Academy of Sciences* **1091**, 151-169, doi:10.1196/annals.1378.063 (2006).

94 Blay, J. Y. *et al.* Serum level of interleukin 6 as a prognosis factor in metastatic renal cell carcinoma. *Cancer Res* **52**, 3317-3322 (1992).

95 Salgado, R. *et al.* Circulating interleukin-6 predicts survival in patients with metastatic breast cancer. *International journal of cancer. Journal international du cancer* **103**, 642-646, doi:10.1002/ijc.10833 (2003).

96 Busse, A. & Keilholz, U. Role of TGF-beta in melanoma. *Curr Pharm Biotechnol* **12**, 2165-2175 (2011).

97 Berking, C. *et al.* Transforming growth factor-beta1 increases survival of human melanoma through stroma remodeling. *Cancer Res* **61**, 8306-8316 (2001).

98 Abbas, A. K., Murphy, K. M. & Sher, A. Functional diversity of helper T lymphocytes. *Nature* **383**, 787-793, doi:10.1038/383787a0 (1996).

99 Gordon, S. Alternative activation of macrophages. *Nature reviews. Immunology* **3**, 23-35, doi:10.1038/nri978 (2003).

100 Mantovani, A., Sica, A. & Locati, M. New vistas on macrophage differentiation and activation. *Eur J Immunol* **37**, 14-16, doi:10.1002/eji.200636910 (2007).

101 Goerdt, S. *et al.* Alternative versus classical activation of macrophages. *Pathobiology* **67**, 222-226, doi:28096 (1999).

102 Lind, M. H. *et al.* Tumor necrosis factor receptor 1-mediated signaling is required for skin cancer development induced by NF-kappaB inhibition. *Proc Natl Acad Sci U S A* **101**, 4972-4977, doi:10.1073/pnas.0307106101 (2004).

103 Lin, E. Y., Gouon-Evans, V., Nguyen, A. V. & Pollard, J. W. The macrophage growth factor CSF-1 in mammary gland development and tumor progression. *J Mammary Gland Biol Neoplasia* **7**, 147-162 (2002).

104 Kirkwood, J. M. *et al.* Interferon alfa-2b adjuvant therapy of high-risk resected cutaneous melanoma: the Eastern Cooperative Oncology Group Trial EST 1684. *J Clin Oncol* **14**, 7-17 (1996).

105 Eggermont, A. M. *et al.* Adjuvant therapy with pegylated interferon alfa-2b versus observation alone in resected stage III melanoma: final results of EORTC 18991, a randomised phase III trial. *Lancet* **372**, 117-126, doi:10.1016/S0140-6736(08)61033-8 (2008).

106 Kirkwood, J. M. *et al.* High- and low-dose interferon alfa-2b in high-risk melanoma: first analysis of intergroup trial E1690/S9111/C9190. *J Clin Oncol* **18**, 2444-2458 (2000).

107 Eggermont, A. M. *et al.* Long-term results of the randomized phase III trial EORTC 18991 of adjuvant therapy with pegylated interferon alfa-2b versus observation in resected stage III melanoma. *J Clin Oncol* **30**, 3810-3818, doi:10.1200/JCO.2011.41.3799 (2012).

108 Creagan, E. T. *et al.* Phase II trials of recombinant leukocyte A interferon in disseminated malignant melanoma: results in 96 patients. *Cancer treatment reports* **70**, 619-624 (1986).

109 Atkins, M. B., Kunkel, L., Sznol, M. & Rosenberg, S. A. High-dose recombinant interleukin-2 therapy in patients with metastatic melanoma: long-term survival update. *The cancer journal from Scientific American* **6 Suppl 1**, S11-14 (2000).

110 Joseph, R. W. *et al.* Correlation of NRAS mutations with clinical response to high-dose IL-2 in patients with advanced melanoma. *J Immunother* **35**, 66-72, doi:10.1097/CJI.0b013e3182372636 (2012).

111 Schwartzentruber, D. J. *et al.* gp100 peptide vaccine and interleukin-2 in patients with advanced melanoma. *The New England journal of medicine* **364**, 2119-2127, doi:10.1056/NEJMoa1012863 (2011).

112 Hodi, F. S. *et al.* Improved survival with ipilimumab in patients with metastatic melanoma. *The New England journal of medicine* **363**, 711-723, doi:10.1056/NEJMoa1003466 (2010).

113 Schumacher, T. N. & Schreiber, R. D. Neoantigens in cancer immunotherapy. *Science* **348**, 69-74, doi:10.1126/science.aaa4971 (2015).

114 Kreiter, S. *et al.* Mutant MHC class II epitopes drive therapeutic immune responses to cancer. *Nature* **520**, 692-696, doi:10.1038/nature14426 (2015).

115 Andtbacka, R. H. *et al.* Talimogene Laherparepvec Improves Durable Response Rate in Patients With Advanced Melanoma. *J Clin Oncol* **33**, 2780-2788, doi:10.1200/JCO.2014.58.3377 (2015).

116 Wolchok, J. D. *et al.* Guidelines for the evaluation of immune therapy activity in solid tumors: immune-related response criteria. *Clin Cancer Res* **15**, 7412-7420, doi:10.1158/1078-0432.CCR-09-1624 (2009).

117 Wolchok, J. D. *et al.* Ipilimumab monotherapy in patients with pretreated advanced melanoma: a randomised, double-blind, multicentre, phase 2, dose-ranging study. *The lancet oncology* **11**, 155-164, doi:10.1016/S1470-2045(09)70334-1 (2010).

118 O'Day, S. J. *et al.* Efficacy and safety of ipilimumab monotherapy in patients with pretreated advanced melanoma: a multicenter single-arm phase II study. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO* **21**, 1712-1717, doi:10.1093/annonc/mdq013 (2010).

119 Schadendorf, D. *et al.* Pooled Analysis of Long-Term Survival Data From Phase II and Phase III Trials of Ipilimumab in Unresectable or Metastatic Melanoma. *J Clin Oncol* **33**, 1889-1894, doi:10.1200/JCO.2014.56.2736 (2015).

120 McDermott, D. *et al.* Durable benefit and the potential for long-term survival with immunotherapy in advanced melanoma. *Cancer Treat Rev* **40**, 1056-1064, doi:10.1016/j.ctrv.2014.06.012 (2014).

121 Ribas, A. *et al.* Antitumor activity in melanoma and anti-self responses in a phase I trial with the anti-cytotoxic T lymphocyte-associated antigen 4 monoclonal antibody CP-675,206. *J Clin Oncol* **23**, 8968-8977, doi:10.1200/JCO.2005.01.109 (2005).

122 Ribas, A. *et al.* Phase III randomized clinical trial comparing tremelimumab with standard-of-care chemotherapy in patients with advanced melanoma. *J Clin Oncol* **31**, 616-622, doi:10.1200/JCO.2012.44.6112 (2013).

123 Camacho, L. H. *et al.* Phase I/II trial of tremelimumab in patients with metastatic melanoma. *J Clin Oncol* **27**, 1075-1081, doi:10.1200/JCO.2008.19.2435 (2009).

124 Taube, J. M. *et al.* Colocalization of inflammatory response with B7-h1 expression in human melanocytic lesions supports an adaptive resistance mechanism of immune escape. *Science translational medicine* **4**, 127ra137, doi:10.1126/scitranslmed.3003689 (2012).
125 Robert, C. *et al.* Anti-programmed-death-receptor-1 treatment with pembrolizumab in ipilimumab-refractory advanced melanoma: a randomised dose-comparison cohort of a phase 1 trial. *Lancet* **384**, 1109-1117, doi:10.1016/S0140-6736(14)60958-2 (2014).
126 Topalian, S. L. *et al.* Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *The New England journal of medicine* **366**, 2443-2454, doi:10.1056/NEJMoa1200690 (2012).
127 Topalian, S. L. *et al.* Survival, durable tumor remission, and long-term safety in patients with advanced melanoma receiving nivolumab. *J Clin Oncol* **32**, 1020-1030, doi:10.1200/JCO.2013.53.0105 (2014).
128 Latchman, Y. *et al.* PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat Immunol* **2**, 261-268, doi:10.1038/85330 (2001).
129 Yamazaki, T. *et al.* Expression of programmed death 1 ligands by murine T cells and APC. *J Immunol* **169**, 5538-5545 (2002).
130 Weber, J. S. *et al.* Safety, efficacy, and biomarkers of nivolumab with vaccine in ipilimumab-refractory or -naive melanoma. *J Clin Oncol* **31**, 4311-4318, doi:10.1200/JCO.2013.51.4802 (2013).
131 Taube, J. M. *et al.* Association of PD-1, PD-1 ligands, and other features of the tumor immune microenvironment with response to anti-PD-1 therapy. *Clin Cancer Res* **20**, 5064-5074, doi:10.1158/1078-0432.CCR-13-3271 (2014).
132 Weber, J. S. *et al.* A Phase 3 Randomized, Open-Label Study of Nivolumab (Anti-Pd-1; Bms-936558; Ono-4538) Versus Investigator's Choice Chemotherapy (Icc) in Patients with Advanced Melanoma after Prior Anti-Ctla-4 Therapy. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO* **25** (2014).
133 Weber, J. *et al.* A randomized, double-blind, placebo-controlled, phase II study comparing the tolerability and efficacy of ipilimumab administered with or without prophylactic budesonide in patients with unresectable stage III or IV melanoma. *Clin Cancer Res* **15**, 5591-5598, doi:10.1158/1078-0432.CCR-09-1024 (2009).
134 Weber, J. S. Current perspectives on immunotherapy. *Seminars in oncology* **41 Suppl 5**, S14-29, doi:10.1053/j.seminoncol.2014.09.003 (2014).
135 Brahmer, J. R. *et al.* Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *The New England journal of medicine* **366**, 2455-2465, doi:10.1056/NEJMoa1200694 (2012).
136 Larkin, J. *et al.* Combined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma. *The New England journal of medicine* **373**, 23-34, doi:10.1056/NEJMoa1504030 (2015).
137 Hodi, F. S. *et al.* An open-label, randomized, phase 2 study of nivolumab (NIVO) given sequentially with ipilimumab (IPI) in patients with advanced melanoma (CheckMate 064). *The European Cancer Congress* **Late Breaking Abstract** (2015).

138 Morgan, D. A., Ruscetti, F. W. & Gallo, R. Selective in vitro growth of T lymphocytes from normal human bone marrows. *Science* **193**, 1007-1008 (1976).
139 Muul, L. M., Spiess, P. J., Director, E. P. & Rosenberg, S. A. Identification of specific cytolytic immune responses against autologous tumor in humans bearing malignant melanoma. *J Immunol* **138**, 989-995 (1987).
140 Rosenberg, S. A. *et al.* Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. A preliminary report. *The New England journal of medicine* **319**, 1676-1680, doi:10.1056/NEJM198812223192527 (1988).
141 Rosenberg, S. A. & Dudley, M. E. Adoptive cell therapy for the treatment of patients with metastatic melanoma. *Current opinion in immunology* **21**, 233-240, doi:10.1016/j.coi.2009.03.002 (2009).
142 Gross, G., Gorochov, G., Waks, T. & Eshhar, Z. Generation of effector T cells expressing chimeric T cell receptor with antibody type-specificity. *Transplant Proc* **21**, 127-130 (1989).
143 Eshhar, Z., Waks, T., Gross, G. & Schindler, D. G. Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the gamma or zeta subunits of the immunoglobulin and T-cell receptors. *Proc Natl Acad Sci U S A* **90**, 720-724 (1993).
144 Brentjens, R., Yeh, R., Bernal, Y., Riviere, I. & Sadelain, M. Treatment of chronic lymphocytic leukemia with genetically targeted autologous T cells: case report of an unforeseen adverse event in a phase I clinical trial. *Molecular therapy : the journal of the American Society of Gene Therapy* **18**, 666-668, doi:10.1038/mt.2010.31 (2010).
145 Brentjens, R. J. *et al.* CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia. *Science translational medicine* **5**, 177ra138, doi:10.1126/scitranslmed.3005930 (2013).
146 Grupp, S. A. *et al.* Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. *The New England journal of medicine* **368**, 1509-1518, doi:10.1056/NEJMoa1215134 (2013).
147 Kochenderfer, J. N. *et al.* Eradication of B-lineage cells and regression of lymphoma in a patient treated with autologous T cells genetically engineered to recognize CD19. *Blood* **116**, 4099-4102, doi:10.1182/blood-2010-04-281931 (2010).
148 Till, B. G. *et al.* Adoptive immunotherapy for indolent non-Hodgkin lymphoma and mantle cell lymphoma using genetically modified autologous CD20-specific T cells. *Blood* **112**, 2261-2271, doi:10.1182/blood-2007-12-128843 (2008).
149 Lamers, C. H. *et al.* Treatment of metastatic renal cell carcinoma with autologous T-lymphocytes genetically retargeted against carbonic anhydrase IX: first clinical experience. *J Clin Oncol* **24**, e20-22, doi:10.1200/JCO.2006.05.9964 (2006).
150 Pule, M. A. *et al.* Virus-specific T cells engineered to coexpress tumor-specific receptors: persistence and antitumor activity in individuals with neuroblastoma. *Nat Med* **14**, 1264-1270, doi:10.1038/nm.1882 (2008).

151 Morgan, R. A. *et al.* Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. *Molecular therapy : the journal of the American Society of Gene Therapy* **18**, 843-851, doi:10.1038/mt.2010.24 (2010).
152 Dembic, Z. *et al.* Transfer of specificity by murine alpha and beta T-cell receptor genes. *Nature* **320**, 232-238, doi:10.1038/320232a0 (1986).
153 Parkhurst, M. R. *et al.* T cells targeting carcinoembryonic antigen can mediate regression of metastatic colorectal cancer but induce severe transient colitis. *Molecular therapy : the journal of the American Society of Gene Therapy* **19**, 620-626, doi:10.1038/mt.2010.272 (2011).
154 Clay, T. M. *et al.* Efficient transfer of a tumor antigen-reactive TCR to human peripheral blood lymphocytes confers anti-tumor reactivity. *J Immunol* **163**, 507-513 (1999).
155 Coccoris, M. *et al.* Long-term functionality of TCR-transduced T cells in vivo. *J Immunol* **180**, 6536-6543 (2008).
156 Robbins, P. F. *et al.* Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. *J Clin Oncol* **29**, 917-924, doi:10.1200/JCO.2010.32.2537 (2011).
157 Stewart-Jones, G. *et al.* Rational development of high-affinity T-cell receptor-like antibodies. *Proc Natl Acad Sci U S A* **106**, 5784-5788, doi:10.1073/pnas.0901425106 (2009).
158 Dudley, M. E. *et al.* A phase I study of nonmyeloablative chemotherapy and adoptive transfer of autologous tumor antigen-specific T lymphocytes in patients with metastatic melanoma. *J Immunother* **25**, 243-251 (2002).
159 Gattinoni, L., Powell, D. J., Jr., Rosenberg, S. A. & Restifo, N. P. Adoptive immunotherapy for cancer: building on success. *Nature reviews. Immunology* **6**, 383-393, doi:10.1038/nri1842 (2006).
160 Rosenberg, S. A. *et al.* Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. *Clin Cancer Res* **17**, 4550-4557, doi:10.1158/1078-0432.CCR-11-0116 (2011).
161 Pilon-Thomas, S. *et al.* Efficacy of adoptive cell transfer of tumor-infiltrating lymphocytes after lymphopenia induction for metastatic melanoma. *J Immunother* **35**, 615-620, doi:10.1097/CJI.0b013e31826e8f5f (2012).
162 Radvanyi, L. G. *et al.* Specific lymphocyte subsets predict response to adoptive cell therapy using expanded autologous tumor-infiltrating lymphocytes in metastatic melanoma patients. *Clin Cancer Res* **18**, 6758-6770, doi:10.1158/1078-0432.CCR-12-1177 (2012).
163 Besser, M. J. *et al.* Adoptive transfer of tumor-infiltrating lymphocytes in patients with metastatic melanoma: intent-to-treat analysis and efficacy after failure to prior immunotherapies. *Clin Cancer Res* **19**, 4792-4800, doi:10.1158/1078-0432.CCR-13-0380 (2013).
164 Rosenberg, S. A. & Restifo, N. P. Adoptive cell transfer as personalized immunotherapy for human cancer. *Science* **348**, 62-68, doi:10.1126/science.aaa4967 (2015).

165 Chapuis, A. G. *et al.* Transferred melanoma-specific CD8+ T cells persist, mediate tumor regression, and acquire central memory phenotype. *Proc Natl Acad Sci U S A* **109**, 4592-4597, doi:10.1073/pnas.1113748109 (2012).
166 Klebanoff, C. A. *et al.* Central memory self/tumor-reactive CD8+ T cells confer superior antitumor immunity compared with effector memory T cells. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 9571-9576, doi:10.1073/pnas.0503726102 (2005).
167 Richmond, T. J. & Davey, C. A. The structure of DNA in the nucleosome core. *Nature* **423**, 145-150, doi:10.1038/nature01595 (2003).
168 Khorasanizadeh, S. The nucleosome: from genomic organization to genomic regulation. *Cell* **116**, 259-272 (2004).
169 Kouzarides, T. Chromatin modifications and their function. *Cell* **128**, 693-705, doi:10.1016/j.cell.2007.02.005 (2007).
170 Struhl, K. Histone acetylation and transcriptional regulatory mechanisms. *Genes & development* **12**, 599-606 (1998).
171 Hebbes, T. R., Thorne, A. W. & Crane-Robinson, C. A direct link between core histone acetylation and transcriptionally active chromatin. *The EMBO journal* **7**, 1395-1402 (1988).
172 Alland, L. *et al.* Role for N-CoR and histone deacetylase in Sin3-mediated transcriptional repression. *Nature* **387**, 49-55, doi:10.1038/387049a0 (1997).
173 Ray-Gallet, D. & Almouzni, G. Nucleosome dynamics and histone variants. *Essays Biochem* **48**, 75-87, doi:10.1042/bse0480075 (2010).
174 Sanchez, R. & Zhou, M. M. The role of human bromodomains in chromatin biology and gene transcription. *Curr Opin Drug Discov Devel* **12**, 659-665 (2009).
175 Khan, S. N. & Khan, A. U. Role of histone acetylation in cell physiology and diseases: An update. *Clin Chim Acta* **411**, 1401-1411, doi:10.1016/j.cca.2010.06.020 (2010).
176 Gregoretti, I. V., Lee, Y. M. & Goodson, H. V. Molecular evolution of the histone deacetylase family: functional implications of phylogenetic analysis. *J Mol Biol* **338**, 17-31, doi:10.1016/j.jmb.2004.02.006 (2004).
177 Frye, R. A. Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. *Biochemical and biophysical research communications* **273**, 793-798, doi:10.1006/bbrc.2000.3000 (2000).
178 Yao, Y. L. & Yang, W. M. Beyond histone and deacetylase: an overview of cytoplasmic histone deacetylases and their nonhistone substrates. *J Biomed Biotechnol* **2011**, 146493, doi:10.1155/2011/146493 (2011).
179 Singh, B. N. *et al.* Nonhistone protein acetylation as cancer therapy targets. *Expert Rev Anticancer Ther* **10**, 935-954, doi:10.1586/era.10.62 (2010).
180 Deubzer, H. E. *et al.* HDAC11 is a novel drug target in carcinomas. *International journal of cancer. Journal international du cancer*, doi:10.1002/ijc.27876 (2012).
181 Villagra, A. *et al.* The histone deacetylase HDAC11 regulates the expression of interleukin 10 and immune tolerance. *Nat Immunol* **10**, 92-100, doi:10.1038/ni.1673 (2009).
182 Fraga, M. F. *et al.* Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. *Nature genetics* **37**, 391-400, doi:10.1038/ng1531 (2005).

183 Wilson, A. J. *et al.* Histone deacetylase 3 (HDAC3) and other class I HDACs regulate colon cell maturation and p21 expression and are deregulated in human colon cancer. *J Biol Chem* **281**, 13548-13558, doi:10.1074/jbc.M510023200 (2006).

184 Choi, J. H. *et al.* Expression profile of histone deacetylase 1 in gastric cancer tissues. *Japanese journal of cancer research : Gann* **92**, 1300-1304 (2001).

185 Halkidou, K. *et al.* Upregulation and nuclear recruitment of HDAC1 in hormone refractory prostate cancer. *The Prostate* **59**, 177-189, doi:10.1002/pros.20022 (2004).

186 Huang, B. H. *et al.* Inhibition of histone deacetylase 2 increases apoptosis and p21Cip1/WAF1 expression, independent of histone deacetylase 1. *Cell death and differentiation* **12**, 395-404, doi:10.1038/sj.cdd.4401567 (2005).

187 Zhu, P. *et al.* Induction of HDAC2 expression upon loss of APC in colorectal tumorigenesis. *Cancer cell* **5**, 455-463 (2004).

188 Song, J. *et al.* Increased expression of histone deacetylase 2 is found in human gastric cancer. *APMIS : acta pathologica, microbiologica, et immunologica Scandinavica* **113**, 264-268, doi:10.1111/j.1600-0463.2005.apm_04.x (2005).

189 Zhang, Z. *et al.* HDAC6 expression is correlated with better survival in breast cancer. *Clin Cancer Res* **10**, 6962-6968, doi:10.1158/1078-0432.CCR-04-0455 (2004).

190 Ropero, S. *et al.* A truncating mutation of HDAC2 in human cancers confers resistance to histone deacetylase inhibition. *Nature genetics* **38**, 566-569, doi:10.1038/ng1773 (2006).

191 Ozdag, H. *et al.* Differential expression of selected histone modifier genes in human solid cancers. *BMC genomics* **7**, 90, doi:10.1186/1471-2164-7-90 (2006).

192 Wilmott, J. S. *et al.* Expression of the class 1 histone deacetylases HDAC8 and 3 are associated with improved survival of patients with metastatic melanoma. *Mod Pathol* **28**, 884-894, doi:10.1038/modpathol.2015.34 (2015).

193 Konieczkowski, D. J. *et al.* A melanoma cell state distinction influences sensitivity to MAPK pathway inhibitors. *Cancer discovery* **4**, 816-827, doi:10.1158/2159-8290.CD-13-0424 (2014).

194 Gallagher, S. J., Tiffen, J. C. & Hersey, P. Histone Modifications, Modifiers and Readers in Melanoma Resistance to Targeted and Immune Therapy. *Cancers (Basel)* **7**, 1959-1982, doi:10.3390/cancers7040870 (2015).

195 Yu, X. *et al.* Modulation of p53, ErbB1, ErbB2, and Raf-1 expression in lung cancer cells by depsipeptide FR901228. *Journal of the National Cancer Institute* **94**, 504-513 (2002).

196 Dovey, O. M. *et al.* Histone deacetylase 1 and 2 are essential for normal T-cell development and genomic stability in mice. *Blood* **121**, 1335-1344, doi:10.1182/blood-2012-07-441949 (2013).

197 Grausenburger, R. *et al.* Conditional deletion of histone deacetylase 1 in T cells leads to enhanced airway inflammation and increased Th2 cytokine production. *J Immunol* **185**, 3489-3497, doi:10.4049/jimmunol.0903610 (2010).

198 Lam, E., Pareek, T. K. & Letterio, J. J. Cdk5 controls IL-2 gene expression via repression of the mSin3a-HDAC complex. *Cell Cycle* **14**, 1327-1336, doi:10.4161/15384101.2014.987621 (2015).

199 de Zoeten, E. F. *et al.* Histone deacetylase 6 and heat shock protein 90 control the functions of Foxp3(+) T-regulatory cells. *Mol Cell Biol* **31**, 2066-2078, doi:10.1128/MCB.05155-11 (2011).
200 Serrador, J. M. *et al.* HDAC6 deacetylase activity links the tubulin cytoskeleton with immune synapse organization. *Immunity* **20**, 417-428 (2004).
201 Dequiedt, F. *et al.* HDAC7, a thymus-specific class II histone deacetylase, regulates Nur77 transcription and TCR-mediated apoptosis. *Immunity* **18**, 687-698 (2003).
202 de Zoeten, E. F., Wang, L., Sai, H., Dillmann, W. H. & Hancock, W. W. Inhibition of HDAC9 increases T regulatory cell function and prevents colitis in mice. *Gastroenterology* **138**, 583-594, doi:10.1053/j.gastro.2009.10.037 (2010).
203 Lian, Z. R., Xu, Y. F., Wang, X. B., Gong, J. P. & Liu, Z. J. Suppression of histone deacetylase 11 promotes expression of IL-10 in Kupffer cells and induces tolerance following orthotopic liver transplantation in rats. *J Surg Res* **174**, 359-368, doi:10.1016/j.jss.2010.12.035 (2012).
204 Buglio, D. *et al.* HDAC11 plays an essential role in regulating OX40 ligand expression in Hodgkin lymphoma. *Blood* **117**, 2910-2917, doi:10.1182/blood-2010-08-303701 (2011).
205 Rosato, R. R., Almenara, J. A., Dai, Y. & Grant, S. Simultaneous activation of the intrinsic and extrinsic pathways by histone deacetylase (HDAC) inhibitors and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) synergistically induces mitochondrial damage and apoptosis in human leukemia cells. *Molecular cancer therapeutics* **2**, 1273-1284 (2003).
206 Kim, H. R. *et al.* Trichostatin A induces apoptosis in lung cancer cells via simultaneous activation of the death receptor-mediated and mitochondrial pathway? *Exp Mol Med* **38**, 616-624, doi:10.1038/emm.2006.73 (2006).
207 Insinga, A. *et al.* Inhibitors of histone deacetylases induce tumor-selective apoptosis through activation of the death receptor pathway. *Nat Med* **11**, 71-76, doi:10.1038/nm1160 (2005).
208 Weinstein, I. B. Cancer. Addiction to oncogenes--the Achilles heal of cancer. *Science* **297**, 63-64, doi:10.1126/science.1073096 (2002).
209 Bolden, J. E., Peart, M. J. & Johnstone, R. W. Anticancer activities of histone deacetylase inhibitors. *Nature reviews. Drug discovery* **5**, 769-784, doi:10.1038/nrd2133 (2006).
210 Deroanne, C. F. *et al.* Histone deacetylases inhibitors as anti-angiogenic agents altering vascular endothelial growth factor signaling. *Oncogene* **21**, 427-436, doi:10.1038/sj.onc.1205108 (2002).
211 Qian, D. Z. *et al.* Class II histone deacetylases are associated with VHL-independent regulation of hypoxia-inducible factor 1 alpha. *Cancer Res* **66**, 8814-8821, doi:10.1158/0008-5472.CAN-05-4598 (2006).
212 Maeda, T., Towatari, M., Kosugi, H. & Saito, H. Up-regulation of costimulatory/adhesion molecules by histone deacetylase inhibitors in acute myeloid leukemia cells. *Blood* **96**, 3847-3856 (2000).
213 Magner, W. J. *et al.* Activation of MHC class I, II, and CD40 gene expression by histone deacetylase inhibitors. *J Immunol* **165**, 7017-7024 (2000).

214 Woods, D. M. *et al.* The antimelanoma activity of the histone deacetylase inhibitor panobinostat (LBH589) is mediated by direct tumor cytotoxicity and increased tumor immunogenicity. *Melanoma research*, doi:10.1097/CMR.0b013e328364c0ed (2013).
215 Leoni, F. *et al.* The antitumor histone deacetylase inhibitor suberoylanilide hydroxamic acid exhibits antiinflammatory properties via suppression of cytokines. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 2995-3000, doi:10.1073/pnas.052702999 (2002).
216 Saemann, M. D. *et al.* Bacterial metabolite interference with maturation of human monocyte-derived dendritic cells. *Journal of leukocyte biology* **71**, 238-246 (2002).
217 Diakos, C. *et al.* Novel mode of interference with nuclear factor of activated T-cells regulation in T-cells by the bacterial metabolite n-butyrate. *J Biol Chem* **277**, 24243-24251, doi:10.1074/jbc.M200191200 (2002).
218 Nusinzon, I. & Horvath, C. M. Interferon-stimulated transcription and innate antiviral immunity require deacetylase activity and histone deacetylase 1. *Proc Natl Acad Sci U S A* **100**, 14742-14747, doi:10.1073/pnas.2433987100 (2003).
219 Klampfer, L., Huang, J., Swaby, L. A. & Augenlicht, L. Requirement of histone deacetylase activity for signaling by STAT1. *J Biol Chem* **279**, 30358-30368, doi:10.1074/jbc.M401359200 (2004).
220 Xu, M., Nie, L., Kim, S. H. & Sun, X. H. STAT5-induced Id-1 transcription involves recruitment of HDAC1 and deacetylation of C/EBPbeta. *The EMBO journal* **22**, 893-904, doi:10.1093/emboj/cdg094 (2003).
221 Minami, J. *et al.* Histone deacetylase 3 as a novel therapeutic target in multiple myeloma. *Leukemia* **28**, 680-689, doi:10.1038/leu.2013.231 (2014).
222 Drummond, D. C. *et al.* Clinical development of histone deacetylase inhibitors as anticancer agents. *Annual review of pharmacology and toxicology* **45**, 495-528, doi:10.1146/annurev.pharmtox.45.120403.095825 (2005).
223 Arts, J. *et al.* JNJ-26481585, a novel "second-generation" oral histone deacetylase inhibitor, shows broad-spectrum preclinical antitumoral activity. *Clin Cancer Res* **15**, 6841-6851, doi:10.1158/1078-0432.CCR-09-0547 (2009).
224 Plumb, J. A. *et al.* Pharmacodynamic response and inhibition of growth of human tumor xenografts by the novel histone deacetylase inhibitor PXD101. *Molecular cancer therapeutics* **2**, 721-728 (2003).
225 Lobera, M. *et al.* Selective class IIa histone deacetylase inhibition via a nonchelating zinc-binding group. *Nature chemical biology* **9**, 319-325, doi:10.1038/nchembio.1223 (2013).
226 Huber, K. *et al.* Inhibitors of histone deacetylases: correlation between isoform specificity and reactivation of HIV type 1 (HIV-1) from latently infected cells. *J Biol Chem* **286**, 22211-22218, doi:10.1074/jbc.M110.180224 (2011).
227 Davie, J. R. Inhibition of histone deacetylase activity by butyrate. *J Nutr* **133**, 2485S-2493S (2003).
228 Gurvich, N., Tsygankova, O. M., Meinkoth, J. L. & Klein, P. S. Histone deacetylase is a target of valproic acid-mediated cellular differentiation. *Cancer Res* **64**, 1079-1086 (2004).

229 Beckers, T. *et al.* Distinct pharmacological properties of second generation HDAC inhibitors with the benzamide or hydroxamate head group. *International journal of cancer. Journal international du cancer* **121**, 1138-1148, doi:10.1002/ijc.22751 (2007).
230 Furumai, R. *et al.* FK228 (depsipeptide) as a natural prodrug that inhibits class I histone deacetylases. *Cancer Res* **62**, 4916-4921 (2002).
231 Balasubramanian, S. *et al.* A novel histone deacetylase 8 (HDAC8)-specific inhibitor PCI-34051 induces apoptosis in T-cell lymphomas. *Leukemia* **22**, 1026-1034, doi:10.1038/leu.2008.9 (2008).
232 Malvaez, M. *et al.* HDAC3-selective inhibitor enhances extinction of cocaine-seeking behavior in a persistent manner. *Proc Natl Acad Sci U S A* **110**, 2647-2652, doi:10.1073/pnas.1213364110 (2013).
233 Butler, K. V. *et al.* Rational design and simple chemistry yield a superior, neuroprotective HDAC6 inhibitor, tubastatin A. *J Am Chem Soc* **132**, 10842-10846, doi:10.1021/ja102758v (2010).
234 Bergman, J. A. *et al.* Selective histone deacetylase 6 inhibitors bearing substituted urea linkers inhibit melanoma cell growth. *Journal of medicinal chemistry* **55**, 9891-9899, doi:10.1021/jm301098e (2012).
235 Santo, L. *et al.* Preclinical activity, pharmacodynamic, and pharmacokinetic properties of a selective HDAC6 inhibitor, ACY-1215, in combination with bortezomib in multiple myeloma. *Blood* **119**, 2579-2589, doi:10.1182/blood-2011-10-387365 (2012).
236 Olsen, E. A. *et al.* Phase IIb multicenter trial of vorinostat in patients with persistent, progressive, or treatment refractory cutaneous T-cell lymphoma. *J Clin Oncol* **25**, 3109-3115, doi:10.1200/JCO.2006.10.2434 (2007).
237 Piekarz, R. L. *et al.* Phase II multi-institutional trial of the histone deacetylase inhibitor romidepsin as monotherapy for patients with cutaneous T-cell lymphoma. *J Clin Oncol* **27**, 5410-5417, doi:10.1200/JCO.2008.21.6150 (2009).
238 Whittaker, S. J. *et al.* Final results from a multicenter, international, pivotal study of romidepsin in refractory cutaneous T-cell lymphoma. *J Clin Oncol* **28**, 4485-4491, doi:10.1200/JCO.2010.28.9066 (2010).
239 Lee, H. Z. *et al.* FDA Approval: Belinostat for the Treatment of Patients with Relapsed or Refractory Peripheral T-cell Lymphoma. *Clin Cancer Res* **21**, 2666-2670, doi:10.1158/1078-0432.CCR-14-3119 (2015).
240 San-Miguel, J. F. *et al.* Panobinostat plus bortezomib and dexamethasone versus placebo plus bortezomib and dexamethasone in patients with relapsed or relapsed and refractory multiple myeloma: a multicentre, randomised, double-blind phase 3 trial. *The lancet oncology* **15**, 1195-1206, doi:10.1016/S1470-2045(14)70440-1 (2014).
241 Morita, S. *et al.* Phase I dose-escalating study of panobinostat (LBH589) administered intravenously to Japanese patients with advanced solid tumors. *Invest New Drugs* **30**, 1950-1957, doi:10.1007/s10637-011-9751-0 (2012).
242 de Marinis, F. *et al.* A phase II study of the histone deacetylase inhibitor panobinostat (LBH589) in pretreated patients with small-cell lung cancer. *J Thorac Oncol* **8**, 1091-1094, doi:10.1097/JTO.0b013e318293d88c (2013).

243 Younes, A. *et al.* Panobinostat in patients with relapsed/refractory Hodgkin's lymphoma after autologous stem-cell transplantation: results of a phase II study. *J Clin Oncol* **30**, 2197-2203, doi:10.1200/JCO.2011.38.1350 (2012).

244 Lai, F. *et al.* Cotargeting histone deacetylases and oncogenic BRAF synergistically kills human melanoma cells by necrosis independently of RIPK1 and RIPK3. *Cell Death Dis* **4**, e655, doi:10.1038/cddis.2013.192 (2013).

245 Sheppard, K. A. *et al.* PD-1 inhibits T-cell receptor induced phosphorylation of the ZAP70/CD3zeta signalosome and downstream signaling to PKCtheta. *FEBS letters* **574**, 37-41, doi:10.1016/j.febslet.2004.07.083 (2004).

246 Parry, R. V. *et al.* CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. *Mol Cell Biol* **25**, 9543-9553, doi:10.1128/MCB.25.21.9543-9553.2005 (2005).

247 Latchman, Y. E. *et al.* PD-L1-deficient mice show that PD-L1 on T cells, antigen-presenting cells, and host tissues negatively regulates T cells. *Proc Natl Acad Sci U S A* **101**, 10691-10696, doi:10.1073/pnas.0307252101 (2004).

248 Keir, M. E. *et al.* Tissue expression of PD-L1 mediates peripheral T cell tolerance. *J Exp Med* **203**, 883-895, doi:10.1084/jem.20051776 (2006).

249 Woods, D. M. *et al.* HDAC Inhibition Upregulates PD-1 Ligands in Melanoma and Augments Immunotherapy with PD-1 Blockade. *Cancer immunology research* **3**, 1375-1385, doi:10.1158/2326-6066.CIR-15-0077-T (2015).

250 Desai, S., Bolick, S. C., Maurin, M. & Wright, K. L. PU.1 regulates positive regulatory domain I-binding factor 1/Blimp-1 transcription in lymphoma cells. *J Immunol* **183**, 5778-5787, doi:10.4049/jimmunol.0901120 (2009).

251 Hubbert, C. *et al.* HDAC6 is a microtubule-associated deacetylase. *Nature* **417**, 455-458, doi:10.1038/417455a (2002).

252 Deardorff, M. A. *et al.* HDAC8 mutations in Cornelia de Lange syndrome affect the cohesin acetylation cycle. *Nature* **489**, 313-317, doi:10.1038/nature11316 (2012).

253 Shen, L. *et al.* Class I histone deacetylase inhibitor entinostat suppresses regulatory T cells and enhances immunotherapies in renal and prostate cancer models. *PloS one* **7**, e30815, doi:10.1371/journal.pone.0030815 (2012).

254 Tsao, H., Goel, V., Wu, H., Yang, G. & Haluska, F. G. Genetic interaction between NRAS and BRAF mutations and PTEN/MMAC1 inactivation in melanoma. *J Invest Dermatol* **122**, 337-341, doi:10.1046/j.0022-202X.2004.22243.x (2004).

255 Tanami, H. *et al.* Involvement of overexpressed wild-type BRAF in the growth of malignant melanoma cell lines. *Oncogene* **23**, 8796-8804, doi:10.1038/sj.onc.1208152 (2004).

256 Herlyn, M. Melanoma cell lines grouped for BRAF/-N-ras/PTEN/CDk4 mutations. *The Wistar Institute* (2015).

257 Herlyn, M. *et al.* Characteristics of cultured human melanocytes isolated from different stages of tumor progression. *Cancer Res* **45**, 5670-5676 (1985).

258 Rivoltini, L. *et al.* Quantitative correlation between HLA class I allele expression and recognition of melanoma cells by antigen-specific cytotoxic T lymphocytes. *Cancer Res* **55**, 3149-3157 (1995).

259 Shidham, V. B. *et al.* Evaluation of micrometastases in sentinel lymph nodes of cutaneous melanoma: higher diagnostic accuracy with Melan-A and MART-1 compared with S-100 protein and HMB-45. *The American journal of surgical pathology* **25**, 1039-1046 (2001).

260 Saunders, P. A., Hendrycks, V. R., Lidinsky, W. A. & Woods, M. L. PD-L2:PD-1 involvement in T cell proliferation, cytokine production, and integrin-mediated adhesion. *Eur J Immunol* **35**, 3561-3569, doi:10.1002/eji.200526347 (2005).

261 Cheng, X. *et al.* The PD-1/PD-L pathway is up-regulated during IL-12-induced suppression of EAE mediated by IFN-gamma. *Journal of neuroimmunology* **185**, 75-86, doi:10.1016/j.jneuroim.2007.01.012 (2007).

262 Muhlbauer, M. *et al.* PD-L1 is induced in hepatocytes by viral infection and by interferon-alpha and -gamma and mediates T cell apoptosis. *Journal of hepatology* **45**, 520-528, doi:10.1016/j.jhep.2006.05.007 (2006).

263 Chiang, E. Y., Henson, M. & Stroynowski, I. Correction of defects responsible for impaired Qa-2 class Ib MHC expression on melanoma cells protects mice from tumor growth. *J Immunol* **170**, 4515-4523 (2003).

264 Suzanne L. Topalian, M. D., F. Stephen Hodi, M.D., Julie R. Brahmer, M.D., Scott N. Gettinger, M.D.,, David C. Smith, M. D., David F. McDermott, M.D., John D. Powderly, M.D., Richard D. Carvajal, M.D., Jeffrey A. Sosman, M.D., Michael B. Atkins, M.D., Philip D. Leming, M.D., David R. Spigel, M.D.,, Scott J. Antonia, M. D., Ph.D., Leora Horn, M.D., Charles G. Drake, M.D., Ph.D., Drew M. Pardoll, M.D., Ph.D., Lieping Chen, M.D., Ph.D., William H. Sharfman, M.D., Robert A. Anders, M.D., Ph.D., Janis M. Taube, M.D., Tracee L. McMiller, M.S., Haiying Xu, B.A., Alan J. Korman, Ph.D., Maria Jure-Kunkel, Ph.D., Shruti Agrawal, Ph.D., Daniel McDonald, M.B.A., Georgia D. Kollia, Ph.D., Ashok Gupta, M.D., Ph.D., Jon M. Wigginton, M.D., & and Mario Sznol, M. D. Safety, Activity, and Immune Correlates of Anti–PD-1 Antibody in Cancer. *The New England journal of medicine* **366**, 2443-2245 (2012).

265 Rodig, N. *et al.* Endothelial expression of PD-L1 and PD-L2 down-regulates CD8+ T cell activation and cytolysis. *Eur J Immunol* **33**, 3117-3126, doi:10.1002/eji.200324270 (2003).

266 Curran, M. A., Montalvo, W., Yagita, H. & Allison, J. P. PD-1 and CTLA-4 combination blockade expands infiltrating T cells and reduces regulatory T and myeloid cells within B16 melanoma tumors. *Proc Natl Acad Sci U S A* **107**, 4275-4280, doi:10.1073/pnas.0915174107 (2010).

267 Chapuis, A. G. *et al.* Transferred melanoma-specific CD8+ T cells persist, mediate tumor regression, and acquire central memory phenotype. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 4592-4597, doi:10.1073/pnas.1113748109 (2012).

268 Crompton, J. G. *et al.* Akt inhibition enhances expansion of potent tumor-specific lymphocytes with memory cell characteristics. *Cancer Res* **75**, 296-305, doi:10.1158/0008-5472.CAN-14-2277 (2015).

269 Chodon, T. *et al.* Adoptive transfer of MART-1 T cell receptor transgenic lymphocytes and dendritic cell vaccination in patients with metastatic melanoma. *Clin Cancer Res*, doi:10.1158/1078-0432.CCR-13-3017 (2014).

270 Klebanoff, C. A. *et al.* Determinants of successful CD8+ T-cell adoptive immunotherapy for large established tumors in mice. *Clin Cancer Res* **17**, 5343-5352, doi:10.1158/1078-0432.CCR-11-0503 (2011).
271 Jia, Q., Yang, Y. & Wan, Y. Tumor-infiltrating memory T-lymphocytes for prognostic prediction in cancer patients: a meta-analysis. *Int J Clin Exp Med* **8**, 1803-1813 (2015).
272 Gattinoni, L. *et al.* Acquisition of full effector function in vitro paradoxically impairs the in vivo antitumor efficacy of adoptively transferred CD8+ T cells. *The Journal of clinical investigation* **115**, 1616-1626, doi:10.1172/JCI24480 (2005).
273 Hinrichs, C. S. *et al.* Adoptively transferred effector cells derived from naive rather than central memory CD8+ T cells mediate superior antitumor immunity. *Proc Natl Acad Sci U S A* **106**, 17469-17474, doi:10.1073/pnas.0907448106 (2009).
274 Hinrichs, C. S. *et al.* Human effector CD8+ T cells derived from naive rather than memory subsets possess superior traits for adoptive immunotherapy. *Blood* **117**, 808-814, doi:10.1182/blood-2010-05-286286 (2011).
275 Klebanoff, C. A. *et al.* Central memory self/tumor-reactive CD8+ T cells confer superior antitumor immunity compared with effector memory T cells. *Proc Natl Acad Sci U S A* **102**, 9571-9576, doi:10.1073/pnas.0503726102 (2005).
276 Berger, C. *et al.* Adoptive transfer of effector CD8+ T cells derived from central memory cells establishes persistent T cell memory in primates. *The Journal of clinical investigation* **118**, 294-305, doi:10.1172/JCI32103 (2008).
277 Appay, V., van Lier, R. A., Sallusto, F. & Roederer, M. Phenotype and function of human T lymphocyte subsets: consensus and issues. *Cytometry. Part A : the journal of the International Society for Analytical Cytology* **73**, 975-983, doi:10.1002/cyto.a.20643 (2008).
278 Yee, A. *et al.* ACY-1215, a Selective Histone Deacetylase (HDAC) 6 Inhibitor, In Combination With Lenalidomide and Dexamethasone (dex), Is Well Tolerated Without Dose Limiting Toxicity (DLT) In Patients (Pts) With Multiple Myeloma (MM) At Doses Demonstrating Biologic Activity: Interim Results Of a Phase 1b Trial. *Blood* **122** (2013).
279 Vogl, D. T. *et al.* Phase 1B Results of Ricolinostat (ACY-1215) Combination Therapy with Bortezomib and Dexamethasone in Patients with Relapsed or Relapsed and Refractory Multiple Myeloma (MM). *Blood* **124** (2014).
280 Van Gelder, R. N. *et al.* Amplified RNA synthesized from limited quantities of heterogeneous cDNA. *Proc Natl Acad Sci U S A* **87**, 1663-1667 (1990).
281 Warrington, J. A., Nair, A., Mahadevappa, M. & Tsyganskaya, M. Comparison of human adult and fetal expression and identification of 535 housekeeping/maintenance genes. *Physiol Genomics* **2**, 143-147 (2000).
282 Olzscha, H., Sheikh, S. & La Thangue, N. B. Deacetylation of chromatin and gene expression regulation: a new target for epigenetic therapy. *Critical reviews in oncogenesis* **20**, 1-17 (2015).
283 Boucheron, N. *et al.* CD4(+) T cell lineage integrity is controlled by the histone deacetylases HDAC1 and HDAC2. *Nat Immunol* **15**, 439-448, doi:10.1038/ni.2864 (2014).

284 Shurin, M. R., Lu, L., Kalinski, P., Stewart-Akers, A. M. & Lotze, M. T. Th1/Th2 balance in cancer, transplantation and pregnancy. *Springer Semin Immunopathol* **21**, 339-359 (1999).
285 Rosenberg, S. A., Spiess, P. & Lafreniere, R. A new approach to the adoptive immunotherapy of cancer with tumor-infiltrating lymphocytes. *Science* **233**, 1318-1321 (1986).
286 Gnerlich, J. L. *et al.* Induction of Th17 cells in the tumor microenvironment improves survival in a murine model of pancreatic cancer. *J Immunol* **185**, 4063-4071, doi:10.4049/jimmunol.0902609 (2010).
287 DeNardo, D. G., Andreu, P. & Coussens, L. M. Interactions between lymphocytes and myeloid cells regulate pro- versus anti-tumor immunity. *Cancer metastasis reviews* **29**, 309-316, doi:10.1007/s10555-010-9223-6 (2010).
288 Liu, Z., Zhang, C. & Sun, J. Deacetylase inhibitor trichostatin A down-regulates Foxp3 expression and reduces CD4+CD25+ regulatory T cells. *Biochemical and biophysical research communications* **400**, 409-412, doi:10.1016/j.bbrc.2010.08.090 (2010).
289 Glauben, R., Sonnenberg, E., Wetzel, M., Mascagni, P. & Siegmund, B. Histone deacetylase inhibitors modulate interleukin 6-dependent CD4+ T cell polarization in vitro and in vivo. *J Biol Chem* **289**, 6142-6151, doi:10.1074/jbc.M113.517599 (2014).
290 Hou, X. *et al.* Histone deacetylase inhibitor regulates the balance of Th17/Treg in allergic asthma. *Clin Respir J*, doi:10.1111/crj.12227 (2014).
291 Lam, E., Pareek, T. K. & Letterio, J. J. Cdk5 controls IL-2 Gene Expression via Repression of the mSin3a-HDAC Complex. *Cell Cycle*, 0, doi:10.4161/15384101.2014.987621 (2015).
292 Dahllof, M. S. *et al.* HDAC inhibitor-mediated beta-cell protection against cytokine-induced toxicity is STAT1 Tyr701 phosphorylation independent. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research* **35**, 63-70, doi:10.1089/jir.2014.0022 (2015).
293 Lee, S. & Margolin, K. Cytokines in cancer immunotherapy. *Cancers (Basel)* **3**, 3856-3893, doi:10.3390/cancers3043856 (2011).
294 Boucheron, N. *et al.* CD4(+) T cell lineage integrity is controlled by the histone deacetylases HDAC1 and HDAC2. *Nat Immunol* **15**, 439-448, doi:10.1038/ni.2864 (2014).
295 Huster, K. M. *et al.* Selective expression of IL-7 receptor on memory T cells identifies early CD40L-dependent generation of distinct CD8+ memory T cell subsets. *Proc Natl Acad Sci U S A* **101**, 5610-5615, doi:10.1073/pnas.0308054101 (2004).
296 Restifo, N. P., Dudley, M. E. & Rosenberg, S. A. Adoptive immunotherapy for cancer: harnessing the T cell response. *Nature reviews. Immunology* **12**, 269-281, doi:10.1038/nri3191 (2012).
297 Fu, T., He, Q. & Sharma, P. The ICOS/ICOSL pathway is required for optimal antitumor responses mediated by anti-CTLA-4 therapy. *Cancer Res* **71**, 5445-5454, doi:10.1158/0008-5472.CAN-11-1138 (2011).

298 Fan, X., Quezada, S. A., Sepulveda, M. A., Sharma, P. & Allison, J. P. Engagement of the ICOS pathway markedly enhances efficacy of CTLA-4 blockade in cancer immunotherapy. *J Exp Med* **211**, 715-725, doi:10.1084/jem.20130590 (2014).

299 Chen, H. *et al.* CD4 T cells require ICOS-mediated PI3K signaling to increase T-Bet expression in the setting of anti-CTLA-4 therapy. *Cancer immunology research* **2**, 167-176, doi:10.1158/2326-6066.CIR-13-0155 (2014).

300 Wang, W. *et al.* Biomarkers on melanoma patient T cells associated with ipilimumab treatment. *Journal of translational medicine* **10**, 146, doi:10.1186/1479-5876-10-146 (2012).

301 Frys, S. *et al.* Entinostat, a novel histone deacetylase inhibitor is active in B-cell lymphoma and enhances the anti-tumour activity of rituximab and chemotherapy agents. *British journal of haematology* **169**, 506-519, doi:10.1111/bjh.13318 (2015).

APPENDIX A: INSTITUTIONAL REVIEW BOARD APPROVAL

UNIVERSITY OF
SOUTH FLORIDA

RESEARCH INTEGRITY AND COMPLIANCE
Institutional Review Boards, FWA No. 00001669
12901 Bruce B. Downs Blvd., MDC035 • Tampa, FL 33612-4799
(813) 974-5638 • FAX(813)974-7091

11/30/2015

Amod Sarnaik, M.D.
H Lee Moffitt Cancer Center
12902 Magnolia Drive
Tampa, FL 33612

RE: **Expedited Approval for Continuing Review**
IRB#: CR4_106509
Title: Use of Excess Melanoma Tumor Specimens Not Required for Diagnostic Purposes for Validation of Tumor Infiltrating Lymphocyte (TIL) Growth Procedures (MCC #15375)

Study Approval Period: 12/14/2015 to 12/14/2016

Dear Dr. Sarnaik:

On 11/30/2015, the Institutional Review Board (IRB) reviewed and **APPROVED** the above application and all documents contained within including those outlined below.

Approved Item(s):
Protocol Document(s):
15375.pr.v08.2015-03-17.clean.pdf

Consent/Assent Document(s)*:
15375.icf.v07.2015-04-30.clean.doc.pdf

*Please use only the official IRB stamped informed consent/assent document(s) found under the "Attachments" tab on the main study's workspace. Please note, these consent/assent document(s) are only valid during the approval period indicated at the top of the form(s) and replace the previously approved versions.

The IRB determined that your study qualified for expedited review based on federal expedited category number(s):

(3) Prospective collection of biological specimens for research purposes by noninvasive means.

As the principal investigator of this study, it is your responsibility to conduct this study in accordance with USF HRPP policies and procedures and as approved by the USF IRB. Any changes to the approved research must be submitted to the IRB for review and approval by an amendment. Additionally, all unanticipated problems must be reported to the USF IRB within five (5) calendar days.

We appreciate your dedication to the ethical conduct of human subject research at the University of South Florida and your continued commitment to human research protections. If you have any questions regarding this matter, please call 813-974-5638.

Sincerely,

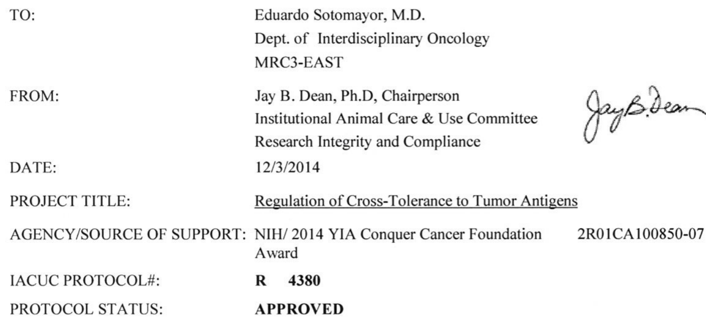
VJorgensen MD

E. Verena Jorgensen, M.D., Chairperson
USF Institutional Review Board

APPENDIX B: INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL

RESEARCH INTEGRITY AND COMPLIANCE
INSTITUTIONAL ANIMAL CARE USE COMMITTEE

MEMORANDUM



TO:	Eduardo Sotomayor, M.D. Dept. of Interdisciplinary Oncology MRC3-EAST
FROM:	Jay B. Dean, Ph.D, Chairperson Institutional Animal Care & Use Committee Research Integrity and Compliance
DATE:	12/3/2014
PROJECT TITLE:	Regulation of Cross-Tolerance to Tumor Antigens
AGENCY/SOURCE OF SUPPORT:	NIH/ 2014 YIA Conquer Cancer Foundation Award 2R01CA100850-07
IACUC PROTOCOL#:	**R 4380**
PROTOCOL STATUS:	**APPROVED**

Your request for continuation of this study was received and will be reported to the Institutional Animal Care and Use Committee (IACUC) at its 2/2015 meeting. The IACUC acknowledges that this study is currently on going as previously approved. Please be advised that **continuation of this study is in effect for a one-year period beginning 2/26/2015 .**

addition, please take note of the following:

- **IACUC approval is granted for a one-year period at the end of which, an annual renewal form must be submitted for years two (2) and three (3) of the protocol.** After three years all continuing studies must be completely re-described in a new application and submitted to IACUC for review.

- **All changes to the IACUC-Approved Protocol must be pre-approved by the IACUC [IACUC policyIII.11].** Minor changes can be submitted to the IACUC for review and approval as an amendment or procedural change, whereas major changes to the protocol require submission of a new IACUC application. Minor changes are changes considered to be within the scope of the original research hypothesis or involve the original species and are submitted to the IACUC as an Amendment or Procedural change. Any change in the IACUC–approved protocol that does not meet the latter definition is considered a major protocol change and requires the submission of a new application. More information on what constitutes a minor versus major protocol change and procedural steps necessary for IACUC review and approval are available on the Comparative Medicine web site at http://www3.research.usf.edu/cm/changes-protocol.asp

cc: Comparative Medicine

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