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## HDAC Inhibition Upregulates PD-1 Ligands in Melanoma and Augments Immunotherapy with PD-1 Blockade

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### Abstract

Expression of PD-1 ligands by tumors and interaction with PD-1 expressing T cells in the tumor microenvironment can result in tolerance. Therapies targeting this co-inhibitory axis have proven clinically successful in the treatment of metastatic melanoma, non-small cell lung cancer and other malignancies. Therapeutic agents targeting the epigenetic regulatory family of histone deacetylases (HDACs) have shown clinical success in the treatment of some hematologic malignancies. Beyond direct tumor cell cytotoxicity, HDAC inhibitors have also been shown to alter the immunogenicity and enhance anti-tumor immune responses. Here we show that class I histone deacetylase inhibitors upregulated the expression of PD-L1 and, to a lesser degree, PD-L2 in melanomas. Evaluation of human and murine cell lines and patient tumors treated with a variety of HDAC inhibitors *in vitro* displayed upregulation of these ligands. This upregulation was robust and durable, with enhanced expression lasting past 96 hours. These results were validated *in vivo* in a B16F10 syngeneic murine model. Mechanistically, HDAC inhibitor treatment resulted in rapid upregulation of histone acetylation of the PD-L1 gene leading to enhanced and durable gene expression. The efficacy of combining HDAC inhibition with PD-1 blockade for treatment of melanoma was also explored in a murine B16F10 model. Mice receiving combination therapy had a slower tumor progression and increased survival compared to control and single agent treatments. These results highlight the ability of epigenetic modifiers to augment immunotherapies, providing a rationale for combining HDAC inhibitors with PD-1 blockade.

### Keywords

Melanoma; PD-L1; PD-L2; HDAC; histone deacetylase inhibitors; CD273; CD274; Panobinostat; Belinostat; Entinostat; Mocetinostat; LBH589; MS275; MGCD0103; PDX101; PD-1; CD279; B7-DC; B7-H1; Epigenetic

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## Introduction

Melanoma is associated with the highest mortality of any skin cancer, and is generally fatal once metastatic disease develops (1). Overall response rates to decarbazine, the standard chemotherapy for metastatic melanoma, are only 10%, with no demonstrated overall survival benefit (2, 3). In recent years the landscape of metastatic melanoma treatment has been altered by remarkable advances in immunotherapeutic approaches. Targeting the immune system by blocking checkpoint molecules has generated durable responses, with overall responses rates of 10–15% and 30–40% when blocking CTLA-4 or PD-1, respectively (4, 5). These results led to the FDA approval of anti-CTLA-4 antibody ipilimumab in 2011 and the anti-PD-1 antibodies pembrolizumab and nivolumab in 2014.

Binding by PD-1 to the costimulatory ligands programmed death 1 ligand (PD-L1), also known as CD274 or B7-H1, and programmed death 2 ligand (PD-L2), also known as CD273 or B7-DC, negatively regulate T-cell responses (6, 7). Mechanistically, recruitment of phosphatases by ligation of PD-L1 to the PD-1 receptor on T-cells reduces downstream phosphorylation and thereby shuts off the function of molecules involved in the pathways that mediate TcR engagement, such as ZAP-70, PKC- $\theta$  and Akt (8, 9). As a result, proliferation and cytokine production are inhibited, ultimately leading to dysfunction or apoptosis of T-cells (8–11). Under normal conditions PD-L1 acts to temper immune responses (12). Indeed, mice genetically deficient in PD-L1 signaling develop autoimmunity (13). However, in the context of cancer, expression of PD-L1 by tumors impairs tumor-reactive T-cells and allow for immune escape. In melanoma, PD-L1 is upregulated in tumor and stromal cells, creating an immunosuppressive microenvironment (14). Similar to PD-L1, PD-L2 is a critical negative regulator of T-cell responses (7, 15) and can be upregulated on tumor cells and, more commonly, on antigen presenting cells (7, 16).

Inhibition of the PD-L1/PD-1 pathway through the use of the PD-1 blocking antibodies pembrolizumab or nivolumab has resulted in objective response rates in metastatic melanoma of 21–45% (5, 17). The two year survival of treatment refractory patients receiving nivolumab was 43% (18). In clinical trials targeting PD-L1, its blockade resulted in objective responses of 17.3% in metastatic melanoma patients, with a 5.7% complete response rate (19). While showing some efficacy, it is clear that PD-L1 blockade has not achieved the same levels of clinical benefit in melanoma as PD-1 blockade. Accumulating evidence suggests that PD-L1 expression on tumor, but not on the surrounding immune infiltrate, is significantly associated with objective response to PD-1 blocking antibodies (20). However, patient responses are observed in the absence of detectable PD-L1 on tumors (21).

Histone deacetylases (HDACs) are key mediators of epigenetic regulation that act by removing acetyl groups from the N-acetyl lysine amino acid on the tail of histones, and have proven to be attractive therapeutic targets for cancer. HDACs are phylogenetically classified as classes I, IIa, IIb, III and IV. Class I HDACs, with the exception of HDAC8, participate in chromatin remodeling as components of multi-protein complexes (22). HDAC inhibitors represent a chemically diverse group of drugs with several dozen HDAC inhibitors at various stages of development. To date, the HDAC inhibitors vorinostat and romipidesin

have been FDA approved for the treatment of cutaneous T-cell lymphoma, while belinostat was recently approved for the treatment of peripheral T-cell lymphoma and panobinostat for multiple myeloma. Additionally, several HDAC inhibitors are in mid-to late-phase clinical trials, including combination therapy for metastatic melanoma.

HDAC inhibitors induce cell cycle arrest and apoptosis in transformed cells, including melanoma (23). In addition to their selective cytotoxicity, HDAC inhibitors can induce a wide range of immunological changes in malignant cells. These changes include increased differentiation antigen expression, enhanced MHC class I and II surface expression as well as increases in other immunologically relevant costimulatory molecules such as CD80 and CD86 (23–25). These changes lead to augmented anti-tumor responses in models of murine melanoma adoptive cell therapy (26). HDAC inhibitors can also reduce ‘negative’ cell populations, such as myeloid-derived suppressor cells, and augment checkpoint blockade therapies, such as PD-1 blockade (27).

Herein we demonstrate a novel role for HDAC inhibitors in upregulating PD-L1 and PD-L2 in melanoma cells *in vitro* and in a B16F10 mouse model *in vivo*. HDAC inhibition induced prolonged PD-L1 expression in both human and mouse cell lines. *In vitro*, patient melanoma samples expressed high amounts of PD-L1 and PD-L2 in a dose-dependent manner in response to HDAC inhibitors. As a result of HDAC inhibition, a more relaxed chromatin state at the promoter regions of PD-L1 and PD-L2, and resultant increased gene expression, were observed. Combining the pan-HDAC inhibitor LBH589 with PD-1 blockade resulted in reduced tumor burden and improving survival in a murine B16F10 model of established tumor. Although PD-L1 and PD-L2 negatively orchestrate the immune response against tumor, the results herein demonstrate that combining PD-1 blockade with HDAC inhibition overcomes this hindrance to immunotherapy and provide a strong rationale for combinatorial therapy with these agents for the treatment of melanoma.

## Materials & Methods

### Cell Lines and Patient Samples

The human melanoma cell lines WM793, WM983A, WM35, WM1366, and the murine cell line B16F10 were purchased from ATCC (Manassass, VA). Mel-624 and Mel-888 were kindly provided by Dr. Shari Pilon-Thomas, and SkMel21 by Dr. Keiran Smalley at H. Lee Moffitt Cancer Center (Tampa, FL). Human cell lines were authenticated using STR profiling within six months prior to manuscript submission. Melanoma patient samples were obtained from surgical biopsies (clinical trial MCC15375; IRB approved protocol #106509; H. Lee Moffitt Cancer Center, Tampa, FL) and provided by Dr. Amod Sarnaik. All cells were cultured in RPMI 1640, supplemented with 10% fetal bovine serum, non-essential amino acids, penicillin, streptavidin, amphotericin B and Mycozap.

### Mouse Models

C57BL/6 mice were purchased from NCI Laboratories and Charles River Laboratories (Wilmington, MA), and housed at H. Lee Moffitt Cancer Center animal facility. For *in vivo* studies, mice were subcutaneously inoculated with  $1 \times 10^5$  B16F10 melanoma cells.

Assessment of tumor growth and survival was performed after intraperitoneal administration of 15mg/kg of LBH589 three times a week (Monday, Wednesday, Friday) alone or in combination with 3mg/kg of PD-1 blocking antibody from BioXCell (West Lebanon, NH) twice weekly (Tuesday, Thursday), for three weeks. Treatments started seven days after B16F10 inoculation. Dextrose 5% was used in the treatment control group. Tumor volume was assessed by caliper measurement and calculated by the formula  $(\text{width}^2 \times \text{length})/2$ . For analysis of PD-L1 and PD-L2 expression *in vivo*, mice were treated three times with 15mg/kg of LBH589 or dextrose 5% ten days after B16F10 inoculation. Mice were euthanized and tumors were harvested within two hours after the last treatment for flow cytometry analysis. All animal studies were in agreement with protocols approved by the IACUC at the University of South Florida.

### HDAC Inhibitors

LBH589 was kindly provided by Novartis (Basel, Switzerland). MGCD0103, MS275, PXD101, PCI34051, and ACY1215 were purchased from Selleck Chemicals (Houston, TX). DMSO-reconstituted HDACi aliquots were stored at  $-80^{\circ}\text{C}$ . For *in vitro* studies, stocks were diluted to final concentration immediately prior to use. For *in vivo* use, LBH589 was dissolved and sonicated in 5% dextrose.

### Flow Cytometry Analyses

For cell surface analyses, melanoma cells were treated with HDAC inhibitors for 24, 48 or 72 hours, as indicated. Cells were harvested with Accutase, washed and resuspended in FACS buffer (PBS, 2mM EDTA, 2% FBS). Cells were stained with phycoerythryn, fluorescein isothiocyanate or allophycocyanin conjugated antibodies from eBioscience (San Diego, CA) against PD-L1 and PD-L2, for 30 minutes at  $4^{\circ}\text{C}$ . Cells were then washed, resuspended in FACS buffer containing DAPI (50ng/mL) and immediately acquired using an LSR II flow cytometer from BD Biosciences (San Jose, CA).

Patient-derived melanoma cells were also verified by flow staining with fluorescein isothiocyanate and alexa fluor 405 conjugated antibodies against S100 and Mart-1, from Abcam (Cambridge, MA) and Novusbio (Littleton, CO), respectively. Intracellular staining was performed using the transcription factor staining buffer set from eBioscience (San Diego, CA), according to the manufacture's instructions. Analyses were performed using FlowJo software.

### Western Blot

Cells were lysed with lysis buffer (1% SDS, 4M Urea, 100nM dithiothriitol in 100nM Tris) and sonicated on ice for 16 minutes of alternated on/off 30 seconds pulses. Lysates were mixed 5:1 with gel loading buffer (0.2% (weight/volume) bromophenol blue, 200mM DTT, 20% glycerol) and boiled for 15 minutes. Samples were electrophoresed in a SDS-PAGE gel and transferred to a nitrocellulose membrane. Incubation with primary antibody was performed overnight at  $4^{\circ}\text{C}$ . Antibodies against acetylated histone 3, total histone 3, acetylated  $\alpha$ -tubulin and  $\beta$ -actin were purchased from Cell Signaling (Danvers, MA). Immunoblots were incubated with appropriate IRDYE secondary antibody for 2 hours and developed using a LI-COR instrument.

## Chromatin Immunoprecipitation

Chromatin preparation was performed as described by Desai, S. et al. (28), adjusted for the number of cells for each immunoprecipitation and substituted with a concentration of 0.5mM EGTA for buffers containing this reagent. Briefly,  $5 \times 10^6$  cells were treated for two hours with LBH589 12.5nM or DMSO control. A total of 5ug of primary antibodies for acetylated histone 3 from Active Motif (Carlsbad, CA) and rabbit control IgG from Fisher Scientific (Waltham, MA) were used for each immunoprecipitation. After overnight antibody incubation, reactions were incubated for two hours at 4°C with 50uL of protein A/G plus beads from Santa Cruz Biotechnology (Santa Cruz, CA). DNA purification was done by using the MiniElute PCR Purification Kit from Qiagen (Valencia, CA), following the manufacturer's instructions. Evaluation of the ChIP was performed by SYBERGreen-based quantitative real-time PCR from BioRad Laboratories (Hercules, CA) using a BioRad CFX96 PCR instrument. ChIP primers were designed using NCBI-Blast and covered 1800bp upstream the start codon of PDL-1 and PDL-2 human genes. Amplicons were between 60 and 150 base pairs. Primers were 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'- GCGTCCCCCTTCTGATAA-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'- GTAGGGAGCGTTGTTCTCC-3' Rv 5'-  
GTGTAGAGACCCTCCGTCCT-3',

Fw 5'- TAGGGGCAGTAGAGCCAAT-3' Rv 5'-  
CAAACTGAATCGCGCCTGG-3';

PD-L2 promoter region:

Fw 5'-CCTGGCACAGCACTAAGACA-3', Rv 5'-  
CTTCCCCATTGTCCCTGGAG-3',

Fw 5'- GGCAGCAGGAGAAGGATTGA-3', Rv 5'-  
GCCCCACTATACCTTCAGGC-3',

Fw 5'- TGGCTGTTCAATTTGGTGGC-3', Rv 5'-  
ATGAGGACTTGCCACAGCTC-3',

Fw 5'- AAGGGTGGCCTACCTTCTCT-3', Rv 5'-  
TCTGGGGCAGGAGGACATTA-3'.

### Quantitative Real Time PCR

Cells were lysed by TRIzol from Invitrogen (Grand Island, NY). RNA was isolated from samples by a standard phenol-chloroform separation protocol, and cDNA generated by an iScript kit from Bio-Rad. Expression was assessed by quantitative real time PCR using a SYBRGreen platform on a Bio-Rad CFX96 system. Resultant data was assessed by Bio-Rad CFX software and calculated by the formula  $2^{[-(\Delta\Delta Ct)]}$  for relative mRNA expression. Correlation of mRNA levels with PD-L1 surface expression and gene acetylation was calculated by the formula  $2^{(-\Delta Ct)}$ , adjusted by  $\times 10^5$ . 18S ribosomal RNA was used as the reference gene in all experiments. Primers were designed using NCBI-Blast and are as follows:

PD-L1: Fw 5'-TCCTGAGGAAAACCATACAGC-3' Rv 5'-  
GATGGCTCCCAGAATTACCA-3';

18S: Fw 5'-GTAACCCGTTGAACCCATT-3' Rv 5'-  
CCATCCAATCGGTAGTAGCG-3'.

### Statistical Analysis

Significance of PD-L1 and PD-L2 expression was determined by unpaired, two-tailed, student's t-test. Difference in tumor growth was evaluated by one-way analysis of variance, at the indicated time-points. Mice survival was assessed by Kaplan-Meier survival analysis log rank test. For correlation of PD-L1 surface expression, gene acetylation and gene expression data, analyses of correlation significance, Pearson's R-square values and linear regression were performed. All statistical analyses were performed using GraphPad Prism 6.0 software. Significance was considered for values of p less than 0.05.

## Results

### HDAC inhibitors upregulate expression of PD-L1 in melanoma cell lines

To determine the differential effects of HDAC inhibitors on melanoma, B16F10 murine melanoma cells were treated *in vitro* with pan and selective HDAC inhibitors for 2 or 24 hours at the indicated doses. Increases in acetylated histone 3 is a surrogate for class I HDAC inhibition, whereas increased acetylated alpha-tubulin is a surrogate for inhibition of HDAC6 (29). At two hours of treatment, both LBH589 (panobinostat) and MGCD0103 (mocetinostat) increased acetylated histone 3, whereas MS275 (etinostat) resulted in no observable increase (Fig. 1A). However, at 24 hours of treatment, MS275 treated cells displayed higher acetylated histone 3, LBH589-treated melanoma continued to display increased acetylation of histone 3, and MGCD0103 regressed to near basal levels. Neither the selective HDAC6 inhibitor ACY1215 (rocilinostat), nor the selective HDAC8 inhibitor PCI34051 had any observable effect on acetylated histone 3 at 2 or 24 hours of treatment. ACY1215-treated cells did display higher levels of acetylated  $\alpha$ -tubulin. At two hours of treatment, LBH589 also resulted in a less profound, but observable increase in acetylated  $\alpha$ -tubulin. Total histone 3 and  $\beta$ -actin were utilized as loading controls.

Using doses of HDAC inhibitors chosen for optimal specificity, a panel of murine and human melanoma cell lines was evaluated for PD-L1 expression after treatment. The growth phase of the tumor from which they are derived and the mutational status of the human cell lines tested are as follows: WM983A – vertical growth phase – mutations in BRAF and p53; WM793 – vertical growth phase – mutations in BRAF, PTEN, and CDK4; WM35 – radial growth phase – mutations in BRAF and PTEN; Mel-624 – metastatic – mutation in BRAF; Mel-888 – combination of three subcutaneous lesions – mutation in BRAF; WM1366 – vertical growth phase – mutations in NRAS ; SkMel-21 – metastatic – mutation in NRAS (30–34). The murine melanoma cell lines B16F10 and B78H1 were also evaluated. Melanoma cell lines were cultured in the presence of 10nM LBH589, 500nM MS275, 500nM MGCD0103 or DMSO as control. After 72 hours, melanomas were disassociated from plates by Accutase to preserve surface markers. Cells were stained and assessed by flow cytometry for expression of PD-L1 in live cells. Autofluorescence was determined by the use of fluorescence minus one (FMO) controls. In all cell lines tested, the three HDAC inhibitors upregulated surface expression of PD-L1, though to varying degrees (Fig. 1B and Table 1). While expression was elevated above autofluorescence in all evaluated cell lines, varying degrees of basal PD-L1 expression were seen. Additionally, doubling the dose of inhibitor used for LBH589, MS275 and MGCD0103 further elevated the expression of PD-L1 (Supplemental Fig. 1).

In addition to evaluating the effects of HDAC inhibitors, the impact of DNA methylation inhibitors on melanoma PD-L1 expression were evaluated. At 48 hours of treatment with 5 $\mu$ M azacitidine or 500nM decitabine, both Mel-624 and Mel-888 experienced significant upregulation of PD-L1 and PD-L2 surface expression (Supplemental Fig. 2). However, 1 $\mu$ M RG108 did not produce a change in either marker.

### Upregulation of PD-L1 by HDAC inhibition is durable

To determine the kinetics of the observed PD-L1 upregulation, the melanoma cell lines WM983A, WM793 and B16F10 were plated and treated for the indicated durations with LBH589, MS275 or MGCD0103 at the indicated doses and harvested for analysis as described above. The MFI of PD-L1 expression was determined (Fig. 1C). Subtracting cell line-specific autofluorescence provided a zero value indicating no expression of PD-L1. Initial, zero-hour expression was determined by DMSO treated controls' MFI. Detectable upregulation of PD-L1 was observed as early as 24 hours in all three cell lines. Peak expression was seen as early as 48 hours or as late as 96 hours, dependent on inhibitor and cell line. Additionally, differences existed in the degree of upregulation induced by the different HDAC inhibitors. In B16F10, MS275 induced the least amount of upregulation, with LBH589 intermediate and MGCD0103 the most robust. Indeed, MGCD0103 treatment was still increasing at the 96-hour time point. In contrast to B16F10, WM983A PD-L1 expression had the highest degree of upregulation in response to MS275. WM793 was most affected by LBH589, with peak expression at 72 hours.

## HDAC inhibitors with specificity for class I HDACs increase PD-L1 and PD-L2 expression on patient melanomas in a dose-dependent manner

To expand on these findings, several primary human melanomas were treated with an expanded panel of HDAC inhibitors. Patient melanomas were expanded *in vitro* and verified for expression of the melanoma markers Mart1 and S100 (35) (Supplemental Fig. 3). Cultured cells were treated for 24 hours with the indicated HDAC inhibitors or DMSO controls. After 24 hours, media and inhibitors were removed, cells were washed twice, and fresh media added to cultures. After an additional 48 hours (72 hours after the initial treatment) cells were harvested as described above, stained for PD-L1 and PD-L2, and data was acquired by flow cytometry. For all cell lines DMSO controls were run in triplicate. Autofluorescence MFI values were subtracted and adjusted MFI values graphed (Figure 2). As before, PD-L1 expression was enhanced with LBH589, MS275 and MGCD0103 treatment (three representative patient samples in Fig. 2A). PDX101 (belinostat), a pan-HDAC inhibitor, similarly upregulated PD-L1 expression. Furthermore, these four inhibitors had a clear dose-dependent effect, with higher concentrations resulting in even greater expression of PD-L1. Of the inhibitors evaluated, LBH589 had the greatest ability to enhance PD-L1 expression, with the highest levels achieved at 20 times less than the concentration of the other evaluated inhibitors. Unlike these four agents, ACY1215, Nexturastat A and PCI34051 produced no changes in PD-L1 expression at the concentrations evaluated. ACY1215 and Nexturastat A are isotype-selective for HDAC6, while PCI34051 is selective for HDAC8, and the concentrations evaluated were chosen to remain within their described specificities (36, 37). These results suggest that the observed upregulation of PD-L1 is confined to inhibition of the class I HDAC1, HDAC2 and/or HDAC3. Similar to PD-L1, PD-L2 expression was also enhanced by LBH589, MS275, MGCD0103 and PDX101, but not ACY1215, Nexturastat A or PCI34051 (Fig. 2B). PD-L2 displayed less consistent, but still largely dose-dependent upregulation after HDAC inhibition.

### PD-L1 and PD-L2 upregulation occurs *in vivo*

The ability of HDAC inhibitors to upregulate PD-1 ligands *in vivo* was investigated using a B16F10 murine model. LBH589 was chosen for continued evaluation due to its high potency. Five C57BL/6 mice per group were subcutaneously injected with  $10^5$  B16F10 melanoma cells. Tumors were allowed to grow, and on day 10, once tumors became visible, treatment with LBH589 (15mg/kg) or 5% dextrose control was given for three consecutive days. On the third day of treatment, tumors were excised, physically disassociated by repeated passage of tumors through a 70 $\mu$ m sterile filter and stained. Expression of PD-L1 (Fig. 3A) and PD-L2 (Fig. 3B) on viable, CD45<sup>-</sup> cells was assessed by flow cytometry and reported as the average MFI  $\pm$  the standard error of the mean (SEM). LBH589 treatment resulted in a significant increase of PD-L1 expression compared to control treated tumors. Indeed, the average approximately doubled the PD-L1 expression of the dextrose control. Likewise, a significant increase in PD-L2 expression in the LBH589 treated group compared to the control was seen ( $p < 0.01$ ). PD-L2 expression, though enhanced, was upregulated to a lesser magnitude than PD-L1. Basal PD-L2 expression was also low, relative to PD-L1 expression.



### HDAC inhibitors relax the chromatin state at the PD-L1 and PD-L2 gene promoters

To determine potential changes in the chromatin state by HDAC inhibition in the PD-L1 and PD-L2 genes, the histone acetylation changes resulting from treatment were analyzed. WM983A melanoma cells were treated with DMSO control or 12.5nM LBH589 for two hours, at which time cells were fixed. Chromatin immunoprecipitation (ChIP) for pan-acetylated histone 3 was performed (Fig. 4A). LBH589-treated cells displayed more acetylation in the promoter region of the PD-L1 gene. Peak acetylation in the LBH589 treated sample was observed at approximately 455 base pairs upstream of the first exon of the PD-L1 gene, with acetylation changes tapering off at ~1700 base pairs towards the 5' end and into the gene region. The PD-L2 gene had low basal acetylation, which was marginally increased after LBH589 treatment (Fig. 4B). To further upon these findings, additional melanoma cell lines were also evaluated by ChIP in a similar fashion as above (Fig. 4C). All melanomas tested displayed higher histone acetylation at the PD-L1 promoter after LBH589 treatment. Likewise, higher histone acetylation at the PD-L2 gene, resulting from LBH589 treatment, was also seen (Fig. 4D).

### HDAC inhibitor-mediated chromatin acetylation leads to increases in PD-L1 gene expression

As increased histone acetylation is indicative of a relaxed chromatin structure and enhanced gene expression, the impact of the observed increased acetylation of histone 3 on PD-L1 gene expression was evaluated. To determine the kinetics of PD-L1 expression after inhibiting HDAC, WM983A cells were treated with 12.5nM LBH589 for 6, 14, 24 or 48 hours. Expression of PD-L1 was elevated as early as six hours after treatment, and expression continued to increase up to or past 48 hours (Fig. 5A). To further validate these findings, additional melanoma cell lines were also investigated for PD-L1 expression after six hours of LBH589 treatment (Fig. 5B). Expression of PD-L1 was upregulated after LBH589 treatment, to varying degrees, in all cell lines.

As histone acetylation is a known mechanism governing gene transcriptional activity, and thus protein expression, the association of these three observed values was explored. To this end, the DMSO treatment MFI values (minus autofluorescence) of PD-L1 surface expression from Fig. 1B, PD-L1 promoter associated acetylated histone 3 fold enrichment values from Fig. 4C, and PD-L1 mRNA expression from Fig. 5B were evaluated for correlation. Correlations were found between PD-L1 surface expression and acetylation ( $R^2=0.5958$ ), PD-L1 surface expression and mRNA expression ( $R^2=0.5649$ ), and PD-L1 gene acetylation and mRNA expression ( $R^2=0.861$ ) (Fig. 5 C–E).

### Combining HDAC inhibition with PD-1 blockade delays tumor progression and enhances survival

Given the previous demonstration of the anti-melanoma effects of HDAC inhibitors and the observed upregulation of PD-1 ligands, the therapeutic efficacy of disrupting the PD-1 inhibitory axis in combination with HDAC inhibition was explored. To this end, C57BL/6 mice were injected subcutaneously with B16F10 melanoma cells. Seven days later, 10 mice per group were treated with LBH589 (15mg/kg), PD-1 blocking antibody (3mg/kg), a combination of both agents or a dextrose control. This dose of LBH589 has been previously

described (23), and did not affect mouse weight, a surrogate of toxicity (Supplemental Fig. 4). Mice receiving treatment with a combination of PD-1 blockade and LBH589 had significantly ( $p < 0.05$ ) less tumor progression than control treated mice (Fig. 6A). Although neither single agent group achieved significance, LBH589-treated mice trended towards lower tumor volumes, in agreement with previous publication (23). PD-1 blockade as a single agent achieved no discernable difference in tumor progression versus control. Combination therapy also significantly improved survival ( $p < 0.05$ ) over control treated mice (Fig. 6B). Median survival times were as follows: dextrose 29 days, LBH589 34.5 days, PD-1 blockade 30.5 days, and combination therapy  $>37$  days.

## Discussion

The results presented herein demonstrate the ability of HDAC inhibitors of class I HDACs to increase the expression of the PD-1 ligands, PD-L1 and PD-L2, *in vitro* and *in vivo* on melanoma cells. This enhanced expression is sustained and robust in the case of PD-L1. Furthermore, this upregulation was seen in all melanoma cell lines and patient samples tested, regardless of mutational status. An increased histone acetylation at the promoter regions of the PD-L1 and PD-L2 genes was associated with increased PD-1 ligand expression. Accompanying the increased histone acetylation, an increase in PD-L1 mRNA was seen, linking the enhanced surface expression with histone acetylation changes. Supporting this model, correlations between the basal PD-L1 surface expression, mRNA expression, and promoter acetylation of evaluated cell lines were observed. Compared to the PD-L1 gene, the PD-L2 gene had lower basal histone acetylation. This may explain low basal PD-L2 expression. While LBH589 treatment increased acetylation levels in the PD-L2 gene, the increase was mild. Collectively, these data support a model where HDAC inhibition increases chromatin relaxation, enhancing gene expression and resultantly PD-1 ligand surface expression on melanoma cells.

Intriguingly, both of the DNA methyltransferase inhibitors azacitidine and decitabine were also able to augment PD-L1 and PD-L2 expression in both the melanoma cell lines assessed. Contrary to these results, a third methyltransferase inhibitor, RG108, did not alter PD-1 ligand expression. Whether increased doses would result in detectable increases in PD-L1 or PD-L2 remains to be investigated. Regardless, these data are provocative and raise several questions. For example, is the upregulation by DNA methyltransferase inhibitors through a related or independent pathway as that of HDAC inhibitors detailed in this study? Supporting a hypothesis of related mechanisms, methylated DNA is known to recruit histone deacetylases, thereby repressing transcription (38). Additionally, histone deacetylases inhibitors have been characterized to down-regulate DNA methyltransferase activity (39). Studies are underway to address these possibilities.

In this study, all cell lines and primary melanomas evaluated showed upregulation of PD-L1 in response to class I HDAC inhibition. Furthermore, all cell lines had a detectable PD-L1 expression. Therefore, it remains to be investigated whether basal expression of PD-L1 is necessary for the described upregulation. As HDAC inhibition may only increase the transcriptional accessibility of the PD-L1 promoter, reliance on already functional transcriptional machinery (e.g. STATs) allowing basal expression may be necessary for the

observed upregulation. Of the cell lines investigated, WM793 expressed minimal PD-L1 surface protein in the basal state and had the lowest basal amount of histone 3 acetylation at the PD-L1 gene. While this basal acetylation and expression was minimally above background, HDAC inhibition still upregulated expression. The B78H1 line had high PD-L1 expression, but had comparatively less upregulation of PD-L1 with HDAC inhibition compared to B16F10. It is postulated that this may be the result of several previously described dysfunctional gene transcripts in B78H1 (40). These observations raise the question of what differences regulating the basal expression of PD-L1 exist in different melanomas. While beyond the scope of this study, these questions are important to address in the future for a more complete understanding of the basic biology of immune-tumor interactions and the obvious clinical implications, and are currently being explored.

While these results demonstrated the ability of HDAC inhibition to upregulate PD-1 ligand expression in melanoma, the particular HDAC(s) governing the expression of these molecules remains to be fully elucidated. While LBH589 is a pan-HDAC inhibitor, with a broad specificity against all HDACs, mocetinostat (MGCD0103) and etinostat (MS275) are class I HDAC inhibitors, most potent against HDACs 1/2/3/11 and HDACs 1/3, respectively (41, 42). These agents all upregulated PD-1 ligand expression, whereas HDAC6 and HDAC8 specific inhibitors could not. These data would suggest that HDAC1, HDAC2, HDAC3 or potentially a combination of these epigenetic regulators are responsible for maintaining the histone acetylation status of the PD-L1 and PD-L2 genes, thereby regulating the expression of these molecules. Indeed, these three class I HDACs are largely localized to the nucleus; the remaining classical HDACs can be found in the cytoplasm (22). Based on this knowledge, it is hypothesized that HDAC1, HDAC2 and/or HDAC3 control the histone acetylation of these genes, thereby regulating, in part, their transcriptional activity. This hypothesis is compatible with the data presented in this study, but remains to be further tested. Indeed, future identification of the specifics of which HDAC and possibly other epigenetic modifiers may allow for the use of selective agents to better alter the immune response, not just in malignancy, but autoimmunity.

Clinically, expression of PD-L1 in tumors by immunohistochemical staining is associated with patient response to PD-1 blockade (43). This likely results from PD-L1 expression reflecting an active immune response in the tumor microenvironment. Lack of expression of PD-L1 by tumors may result from a lack of pro-inflammatory immune infiltrate, and consequently a lack of T-cells generating cytokines that influence PD-L1 expression. Indeed, PD-L1 and PD-L2 expression is upregulated in response to the pro-inflammatory cytokines IFN- $\gamma$  and TNF (44). Under this model, the upregulation of PD-1 ligands by HDAC inhibition independent of increased immune infiltration, represents an undesirable effect amongst the previously demonstrated immune enhancing effects (e.g. increased MHC and antigen expression) of HDAC inhibitors. Consequently, the blockade of this pathway may increase the efficacy of HDAC inhibitor treatment as an immunotherapeutic strategy.

Therapeutically, combining the HDAC inhibitor LBH589 with PD-1 blockade *in vivo* enhanced anti-tumor activity compared to either agent alone resulting in a delay in tumor progression and an increase in overall survival in murine melanoma. Previously it was shown that LBH589 as a single agent increases the survival time of B16F10 tumor bearing,

immunocompetent mice. However, this effect was lost on immunodeficient mice (23). A similar increase in survival by LBH589 in immunocompetent as a single agent was seen in this study. However, in the experiments presented herein using the B16F10 model, PD-1 blockade as a single agent did not prolong survival compared to control treatments. Curran et al. showed that PD-1 blockade had minimal effects in murine B16F10 models unless combined with vaccination, a result reminiscent of that seen with murine CTLA-4 blockade (45, 46). Intriguingly, the efficacy of PD-1 blockade was enhanced with LBH589 treatment without the need for vaccination. This may be the result of the ability of HDAC inhibitors to augment MHC and differentiation antigen expression in melanoma, achieving a superior ability to activate T-cells (23).

Collectively, these results add to the growing body of data demonstrating that HDAC inhibitors may alter the immune landscape of malignant cells, through changes in expression of co-stimulatory molecules, MHC and tumor antigens, as well as cytokine production by tumor cells. Likewise, these data further highlight the intimate connection between epigenetics and immune regulation. While the results of this study raise many questions about the basic biology of PD-1 ligand expression in melanoma, they also demonstrate clinical relevance for the immune effects of HDAC inhibitors and provide a rationale for the clinical evaluation of PD-1 blockade in combination with HDAC inhibition.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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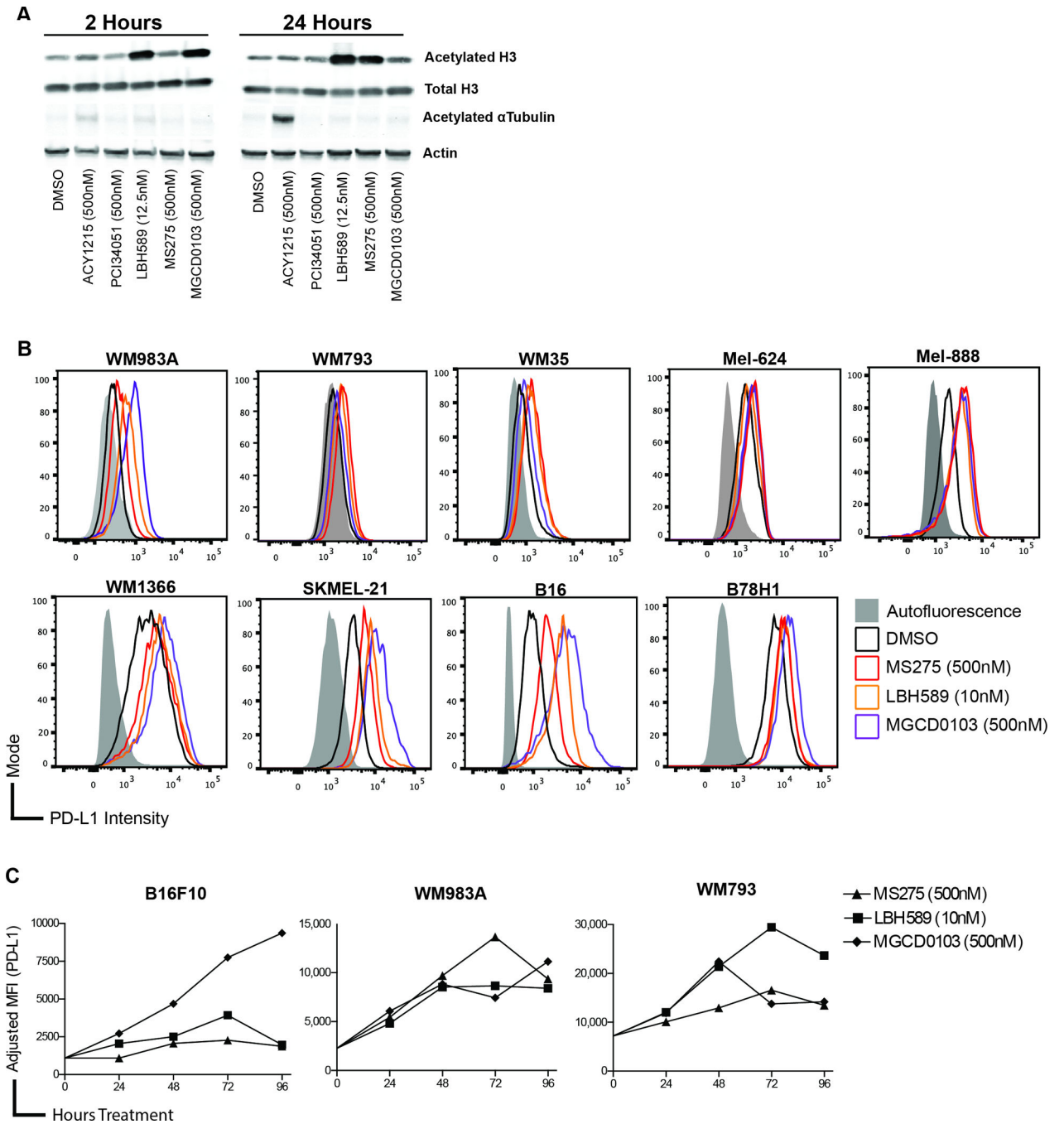
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**Figure 1. HDAC inhibitors upregulate PD-L1 in melanoma**

(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 alpha-tubulin and  $\beta$ -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 PD-L1 expression was evaluated. (C) 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



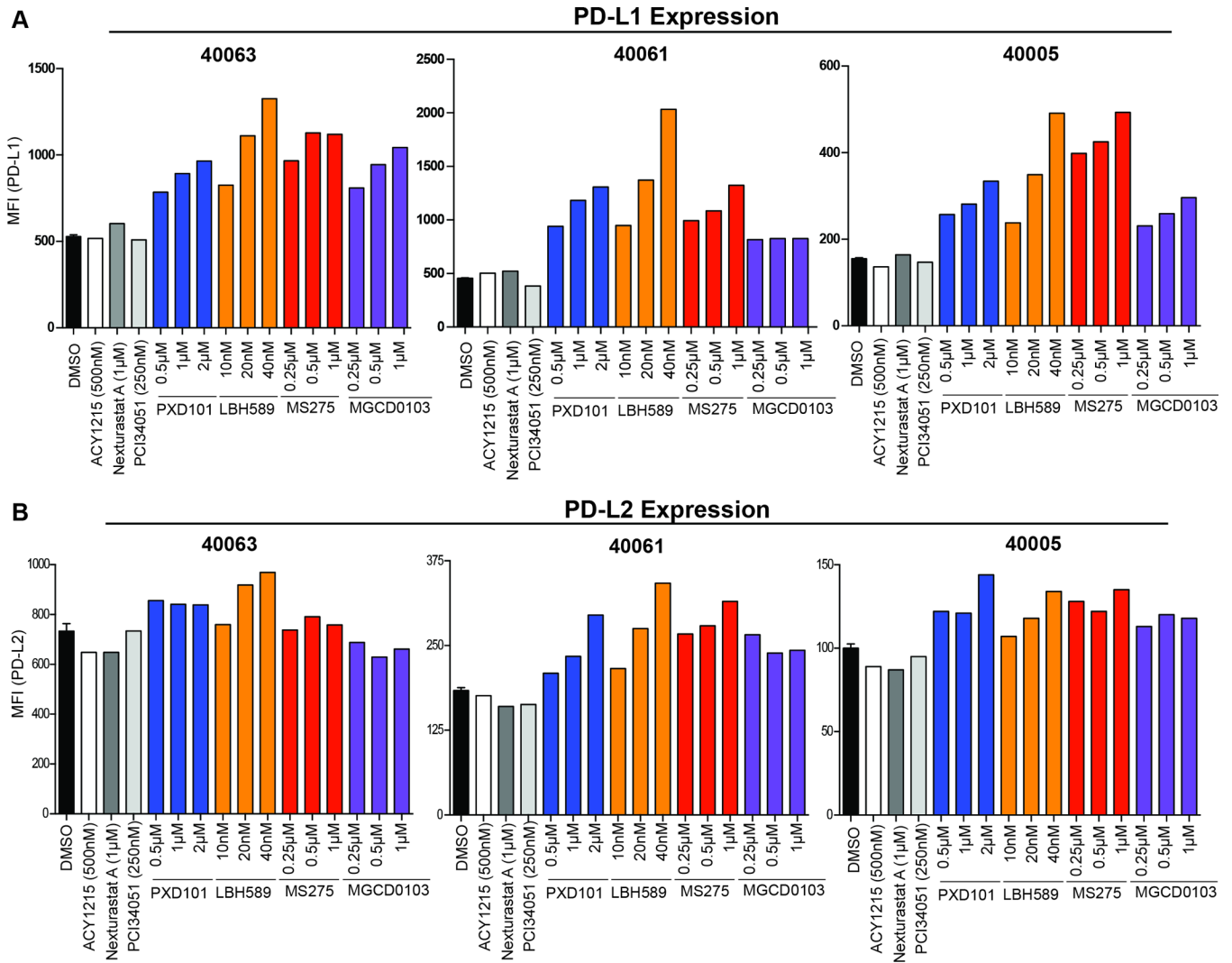
hours prior to evaluation of PD-L1. Expression of DMSO-treated cells was graphed as zero hour treatment. All values are graphed as mean fluorescence intensity (MFI) with autofluorescence values subtracted. Results shown are representative of 2–3 independent experiments.

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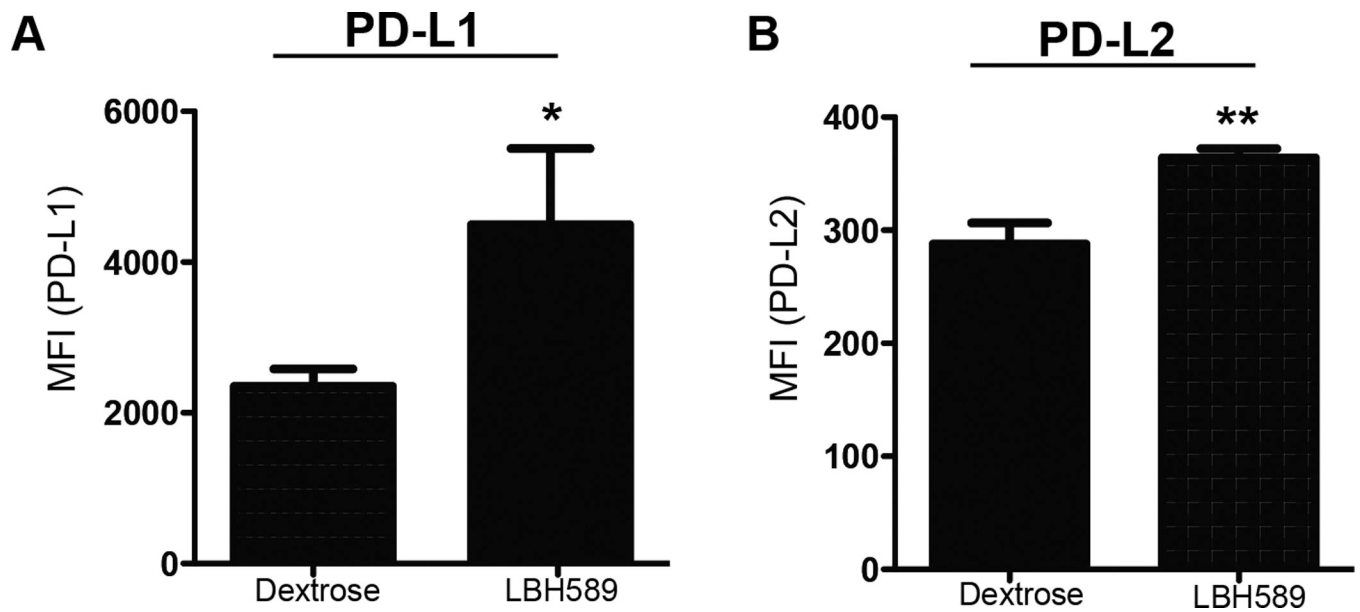
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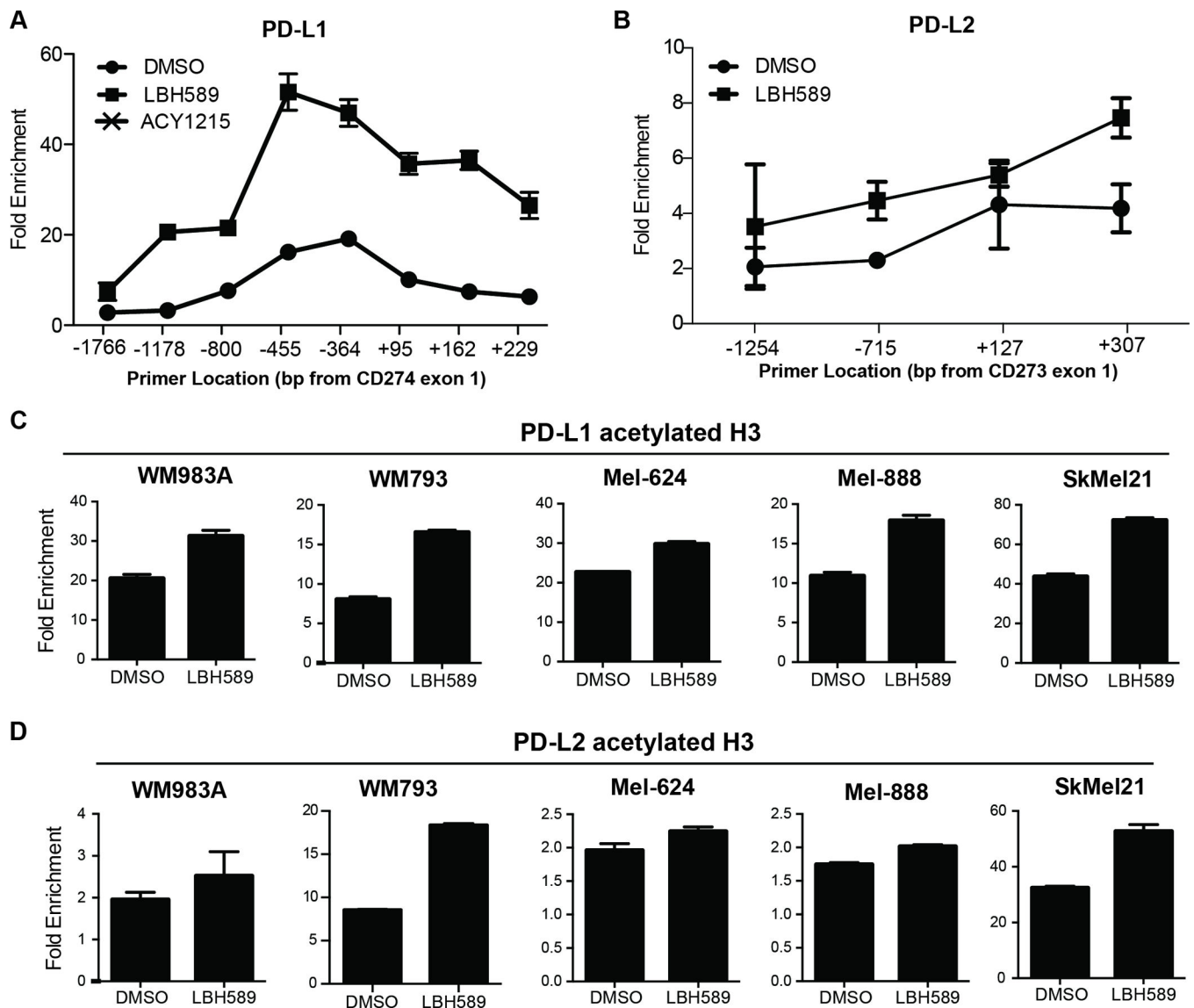
**Figure 2. Inhibition of class I HDACs increases PD-L1 and PD-L2 expression in patient melanomas in a dose dependent manner**

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) PD-L1 and (B) PD-L2. DMSO controls were run in triplicate. MFI values are graphed with autofluorescence values subtracted.

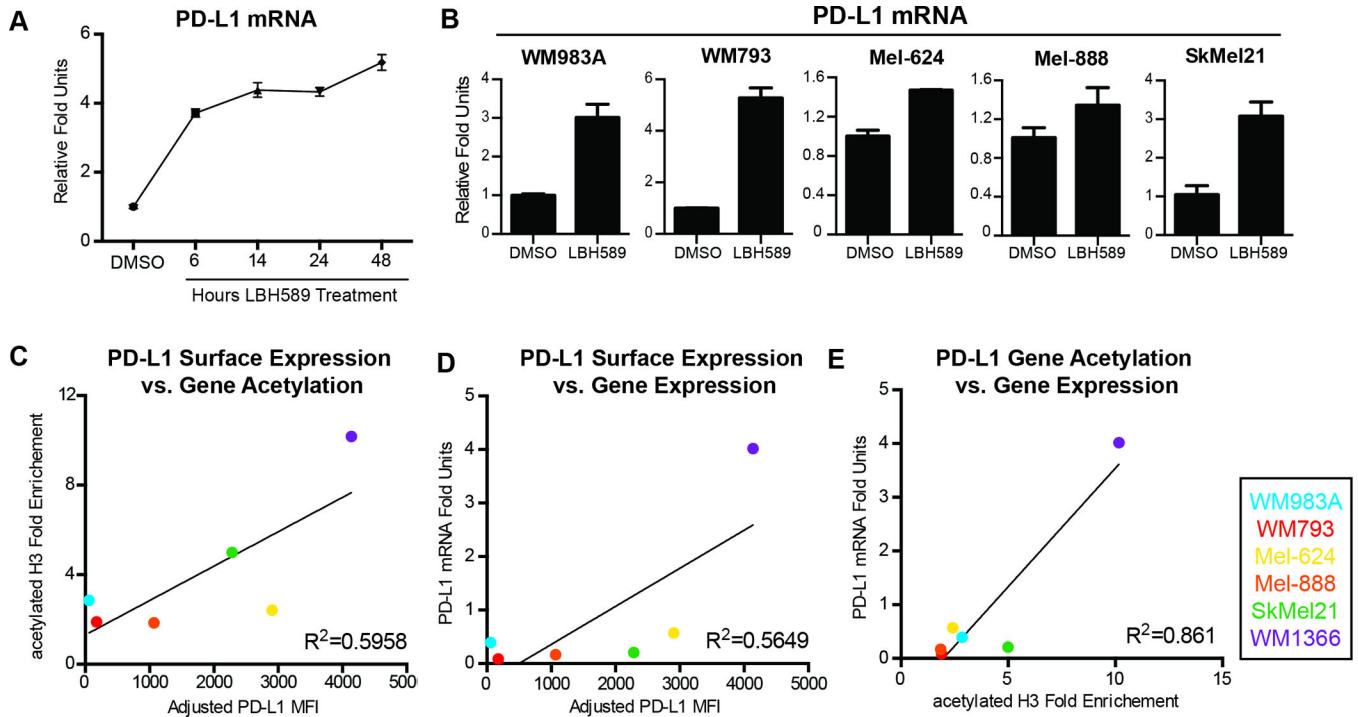


**Figure 3. HDAC inhibitors upregulate PD-L1 and PD-L2 expression *in vivo***

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) PD-L1 and (B) PD-L2 expression were evaluated by flow cytometry. \* $P < 0.05$ , \*\* $P < 0.01$ .

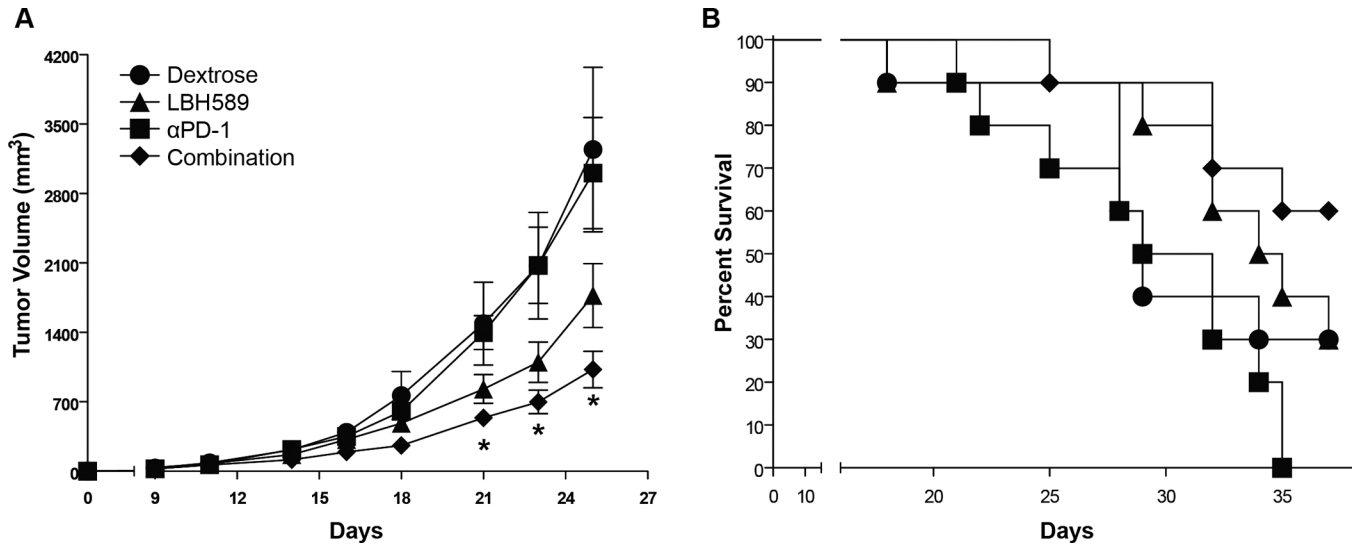


**Figure 4. HDAC inhibition increases histone acetylation at the PD-L1 and PD-L2 promoters**  
 Indicated melanoma cell lines were treated *in vitro* for two hours with 12.5nM LBH589 (squares) or DMSO control (circles). Cells were then fixed and chromatin immunoprecipitated for acetylated histone 3 or IgG control. DNA pull-down was quantified by qRT-PCR. Fold enrichment over corresponding IgG pull-down at the (A) PD-L1 and (B) PD-L2 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 PD-L1 and (D) +307 gene region of PD-L2. For all graphs, error bars are representative of technical replicates.



**Figure 5. PD-L1 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 PD-L1 expression. (B) Indicated cell lines were treated with DMSO or 12.5nM LBH589 for six hours and subsequently assessed by qRT-PCR for PD-L1 expression. For all graphs, error bars are representative of technical replicates. Correlations of (C) PD-L1 surface expression *versus* gene acetylation, (D) PD-L1 surface expression *versus* gene expression and (E) PD-L1 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 PD-L1 gene. Gene expression was determined by qRT-PCR and calculated as fold units relative to 18S endogenous ribosomal RNA. Flow cytometry analysis of PD-L1 surface expression was indicated as mean fluorescence intensity (MFI).



**Figure 6. Combining HDAC inhibition with PD-1 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), PD-1 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. \* $p < 0.05$ .

**Table 1**  
**PD-L1 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
<b>WM983A</b>	Autofluorescence	328	NA
	DMSO	386	NA
	LBH589 (10nM)	835	216%
	MS275 (500nM)	546	141%
	MGCD0103 (500nM)	1151	298%
<b>WM793</b>	Autofluorescence	1568	NA
	DMSO	1744	NA
	LBH589 (10nM)	2361	135%
	MS275 (500nM)	2730	157%
	MGCD0103 (500nM)	2108	121%
<b>B78H1</b>	Autofluorescence	651	NA
	DMSO	9044	NA
	LBH589 (10nM)	13839	153%
	MS275 (500nM)	13051	144%
	MGCD0103 (500nM)	18364	203%
<b>SkMel21</b>	Autofluorescence	1404	NA
	DMSO	3686	NA
	LBH589 (10nM)	10616	288%
	MS275 (500nM)	16508	448%
	MGCD0103 (500nM)	7171	195%
<b>WM35</b>	Autofluorescence	698	NA
	DMSO	1095	NA
	LBH589 (10nM)	1654	151%
	MS275 (500nM)	1693	155%
	MGCD0103 (500nM)	1261	115%
<b>WM1366</b>	Autofluorescence	648	NA
	DMSO	4788	NA
	LBH589 (10nM)	8184	171%
	MS275 (500nM)	6999	146%
	MGCD0103 (500nM)	10169	212%
<b>B16</b>	Autofluorescence	206	NA
	DMSO	1297	NA
	LBH589 (10nM)	4139	319%

Cell Line	Treatment	PDL1 MFI	% Change over DMSO
	MS275 (500nM)	2480	191%
	MGCD0103 (500nM)	7350	567%
<b>624</b>	Autofluorescence	1337	NA
	DMSO	4241	NA
	LBH589 (10nM)	6211	146%
	MS275 (500nM)	6446	152%
	MGCD0103 (500nM)	7142	168%
<b>888</b>	Autofluorescence	1027	NA
	DMSO	2097	NA
	LBH589 (10nM)	2999	143%
	MS275 (500nM)	3608	172%
	MGCD0103 (500nM)	3281	156%

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