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


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 coinhibitory 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 (HDAC) 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 antitumor immune responses. Here, we show that class I HDAC 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 with 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.

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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 (OS) 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 response rates of 10% to 15% and 30% to 40% when blocking CTLA-4 or PD-1, respectively (4, 5). These results led to the FDA approval of the 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 regulates 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 T-cell engagement, such as ZAP-70, PKC-θ, 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 allows 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% to 45% (5, 17). The 2-year survival rate of treatment-refractory patients receiving nivolumab was 43% (18). In clinical trials targeting PD-L1, its blockade resulted in objective response rates 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 multiprotein 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 romidepsin have been FDA approved for the treatment of cutaneous T-cell lymphoma, and belinostat was recently approved for the treatment of peripheral T-cell lymphoma and panobinostat for multiple myeloma. In addition, several HDAC inhibitors are in mid-to-late-phase clinical trials, including studies of 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 immunologic changes in malignant cells. These changes include increased differentiation antigen expression, enhanced MHC class I and II surface expression, and increases in other immunologically relevant costimulatory molecules such as CD80 and CD86 (23–25). These changes lead to augmented antitumor 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 improved 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 and Methods

Cell lines and patient samples

The human melanoma cell lines WM793, WM983A, WM35, WM1366, and the murine cell line B16F10 were purchased from the ATCC. Mel-624 and Mel-888 were kindly provided by Dr. Shari Pilon-Thomas, and SkM621 by Dr. Keiran Smalley at H. Lee Moffitt Cancer Center (Tampa, FL). Human cell lines were authenticated using short tandem repeat profiling within 6 months before article submission. Melanoma patient samples were obtained from surgical biopsies (clinical trial MCC15375; IRB approved protocol #106509; H. Lee Moffitt Cancer Center) and provided by Dr. Amod Sarnaik (H. Lee Moffitt Cancer Center). All cells were cultured in RPMI-1640, supplemented with 10% FBS, non-essential amino acids, penicillin, streptavidin, amphotericin B, and Mycozap.

Mouse models

C57BL/6 mice were purchased from NCI Laboratories and Charles River Laboratories, and housed at H. Lee Moffitt Cancer Center animal facility. For in vivo studies, mice were s.c. inoculated with 1 × 10⁷ B16F10 melanoma cells. Assessment of tumor growth and survival was performed after i.p. administration of 15 mg/kg of LBH589 three times a week (Monday, Wednesday, and Friday) alone or in combination with 3 mg/kg of PD-1 blocking antibody from BioXCell twice weekly (Tuesday and Thursday), for 3 weeks. Treatments started 7 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 (width² × length)/2. For analysis of PD-L1 and PD-L2 expression in vivo, mice were treated three times with 15 mg/kg of LBH589 or dextrose 5% 10 days after B16F10 inoculation. Mice were euthanized and tumors were harvested within 2 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. MGCD0103, MS275, PXD101, PCI34051, and ACY1215 were purchased from Selleck Chemicals. DMSO-reconstituted HDAC inhibitors aliquots were stored at −80°C. For in vitro studies, stocks were diluted to final concentration immediately before 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, 2 mM EDTA, 2% FBS). Cells were stained with phycoerythrin, fluorescein isothiocyanate, or allophycocyanin-conjugated antibodies from eBioscience against PD-L1 and PD-L2, for 30 minutes at 4°C. Cells were then washed, resuspended in FACS buffer containing DAPI (50 ng/mL), and immediately acquired using an LSR II flow cytometer from BD Biosciences. 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 and Novusbio, respectively. Intracellular staining was performed using the transcription factor staining buffer set from eBioscience, according to the manufacturer’s instructions. Analyses were performed using FlowJo software.

Western blot analysis

Cells were lysed with lysis buffer (1% SDS, 4M Urea, 100 mM diithiothreitol in 100 mM/L Tris) and sonicated on ice for 15 minutes of alternated on/off 30-second pulses. Lysates were mixed 5:1 with gel loading buffer (0.2% [weight/volume]) bromophenol blue, 200 mM/L DTT, and 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°C. Antibodies against
acetylated histone 3, total histone 3, acetylated α-tubulin and β-actin were purchased from Cell Signaling Technology. 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 and colleagues (28), adjusted for the number of cells for each immunoprecipitation, and substituted with a concentration of 0.5 mmol/L EGTA for buffers containing this reagent. Briefly, 5 × 10⁶ cells were treated for 2 hours with LBH589 12.5 nmol/L or 0.5 mmol/L EGTA for buffers containing this reagent. Brie

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Thermo Fisher Scientific were used for each immunoprecipitation. After overnight antibody incubation, reactions were incubated for 2 hours at 4°C with 50 μL of protein A/G plus beads from Santa Cruz Biotechnology. DNA purification was done by using the MiniElute PCR Purification Kit from Qiagen, following the manufacturer’s instructions. Evaluation of the ChIP was performed by SYBRGreen-based quantitative real-time PCR from Bio-Rad Laboratories using a Bio-Rad CFX96 PCR instrument. ChIP primers were designed using NCBI-Blast and covered 1800 base pairs upstream the start codon of PDL-1 and PDL-2 human genes. Amplicons were between 60 and 150 base pairs. Primers were as follows:

**PD-L1 promoter region:**

Fw 5′-GGCAAATTCGGTTCCTTA-3′, Rv 5′-TCTTCTCTA-GATGGGCTGGAT-3′,

Fw 5′-GCTGCGCCAACAACCTATT-3′, Rv 5′-TTTGGCAG- GACGATGGAG-3′,

Fw 5′-CTAGAAGGTCGCGGCGGAT-3′, Rv 5′-GGCCCAA- GATGCAGACGAT-3′,

Fw 5′-ATGGGTCTGCTGACTTTT-3′, Rv 5′-GGCGTCGCC- CCTTCTGATAA-3′,

Fw 5′-CCGGGAGCGGCTTCTGATAA-3′, Rv 5′-AACCCCAA- CATTGCAACGGC-3′,

Fw 5′-ACGAAAGAATCTCCGCCCCAT-3′, Rv 5′-CCCAAGG- CAGCAAATCAGC-3′,

Fw 5′-AGACGAGGGCTCCTACAC-3′, Rv 5′-ATGGGTCTG- CACTGCCTCC-3′,

Fw 5′-GTAGGCGCGCTTCTGCTCC-3′, Rv 5′-GTGTAGA- GACCCCTCCTGCT-3′,

Fw 5′-TAGGGGGCAGTAGACCCAAT-3′, Rv 5′-CAAAACT- GAATCGGTCCTGG-3′;

**PD-L2 promoter region:**

Fw 5′-CTGGCCACACGACTAAGACA-3′, Rv 5′-CTTCCCCCAT- GTCCCCCTGAG-3′,

Fw 5′-GGCAAGGAGAAAGATGAG-3′, Rv 5′-CCGCCACGT- TACCTCCAGG-3′,

Fw 5′-TGCGCTGATTTTGTGCG-3′, Rv 5′-ATGAGGACT- TGCCACAGC-3′,

Fw 5′-AAGGGCTGGCCATCCTTCCTC-3′, Rv 5′-TCTGGGCGA- GCAGAACATT-3′.

Quantitative real-time PCR

Cells were lysed by Trizol from Invitrogen. 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 were assessed by Bio-Rad CFX software and calculated by the formula 2^(-A Ct) for relative mRNA expression. Correlation of mRNA levels with PD-L1 surface expression and gene acetylation was calculated by the formula 2^(-A Ct), adjusted by ×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′-CTCCTGAGGAAAAAACCATACACG-3′, Rv 5′-GATG- GTCCGCCAAATTACCA-3′;

18S: Fw 5′-GTAAACCGTGTAACCCCCATT-3′, Rv 5′-CCATCCA- ATCGGTAGTACG-3′.

Statistical analysis

The statistical significance of PD-L1 and PD-L2 expression was determined by unpaired, two-tailed, Student t test. Difference in tumor growth was evaluated by one-way ANOVA, at the indicated time points. Mouse survival was assessed by the 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 R² values, and linear regression were performed. All statistical analyses were performed using GraphPad Prism 6.0 software. Findings with P values less than 0.05 were considered statistically significant.

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 α-tubulin is a surrogate for inhibition of HDAC6 (29). At 2 hours of treatment, both LBH589 (panobinostat) and MGCD0103 (more-tinostat) 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 α-tubulin. At 2 hours of treatment, LBH589 also resulted in a less profound, but observable increase in acetylated α-tubulin. Total histone 3 and β-actin were used 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 10 nmol/L LBH589, 500 nmol/L MS275, 500 nmol/L 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. Auto-fluorescence was determined by the use of fluorescence minus one control. In all cell lines tested, the three HDAC inhibitors upregulated surface expression of PD-L1, though to varying degrees (Fig. 1B and Table 1). Although expression was elevated above autofluorescence in all evaluated cell lines, varying degrees of basal PD-L1 expression were seen. In addition, doubling the dose

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 α-tubulin, and β-actin. B, indicated melanoma cell lines were treated with 500 nmol/L MS275 (red), 10 nmol/L LBH589 (orange), 500 nmol/L 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 500 nmol/L MS275 (triangles), 10 nmol/L LBH589 (squares), or 500 nmol/L MGCD0103 (diamonds) at 96, 72, 48, or 24 hours before 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 two to three independent experiments.
Upregulation of PD-L1 by HDAC inhibition is durable

of inhibitor used for LBH589, MS275, and MGCDD0103 further elevated the expression of PD-L1 (Supplementary Fig. S1).

In addition to evaluating the effects of HDAC inhibitors, the impact of DNA methylation inhibitors on melanoma PD-L1 expression was evaluated. At 48 hours of treatment with 5 μmol/L of azacitidine or 500 nmol/L of decitabine, both Mel-624 and Mel-888 experienced significant upregulation of PD-L1 and PD-L2 surface expression (Supplementary Fig. S2). However, 1 μmol/L of 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 MGCDD0103 at the indicated doses and harvested for analysis as described above. The mean fluorescent intensity (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, depending upon the inhibitor and the cell line. In addition, the degree of upregulation induced by the individual HDAC inhibitors differed. In B16F10, MS275 induced the least amount of upregulation, with LBH589 intermediate and MGCDD0103 the most robust. Indeed, MGCDD0103 treatment was still increasing at the 96-hour time point. In contrast with 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 (Supplementary Fig. S3; ref. 35). 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 and stained for PD-L1 and PD-L2; data were acquired by flow cytometry. For all cell lines, DMSO controls were run in triplicate. Autofluorescence MFI values were subtracted and adjusted MFI values graphed (Fig. 2). As before, PD-L1 expression was enhanced with LBH589, MS275, and MGCDD0103 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, Nexturtast A, and PC134051 produced no changes in PD-L1 expression at the concentrations evaluated. ACY1215 and Nexturtast A are isotype selective for HDAC6, while PC134051 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 1 HDAC1, HDAC2, and/or HDAC3. Similar to PD-L1, PD-L2 expression was also enhanced by LBH589, MS275, MGCDD0103, and PDX101, but not ACY1215, Nexturtast A, or PC134051 (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 s.c. injected with 10^5 B16F10 melanoma cells. Tumors were allowed to grow, and on day 10, once tumors became visible, treatment with LBH589 (15 mg/kg) or 5% dextrose control was given for 3 consecutive days. On the third day of treatment, tumors were excised, physically disassociated by repeated passage of tumors through a 70-μm sterile filter, and stained. Expression of PD-L1 (Fig. 3A) and PD-L2 (Fig. 3B) on viable, CD45^-/CD3^- cells was assessed by flow cytometry and reported as the average MFI ± the SEM. LBH589 treatment resulted in a significant increase of PD-L1 expression compared with 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 with 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 12.5 nmol/L LBH589 for 2 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 approximately 1,700 base pairs toward 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 expand upon these findings, additional melanoma cell lines were also evaluated by ChIP in a similar fashion as above (Fig. 4C). All melanomas

Figure 2.
Inhibition of class I HDACs increases PD-L1 and PD-L2 expressions 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 PD-L1 (A) and PD-L2 (B). DMSO controls were run in triplicate. MFI values are graphed with autofluorescence values subtracted.
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 12.5 nmol/L LBH589 for 6, 14, 24, or 48 hours. Expression of PD-L1 was elevated as early as 6 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 6 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 MF1 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. 5C–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 s.c. with B16F10 melanoma cells. Seven days later, 10 mice per group were treated with LBH589 (15 mg/kg), PD-1–blocking antibody (3 mg/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 (Supplementary Fig. S5E). 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 of the single-agent treatment groups achieved statistical significance, LBH589-treated mice showed a trend toward lower tumor volumes, in agreement with our previous publication (23). PD-1 blockade as a single agent achieved no discernible 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 with the PD-L1 gene, the PD-L2 gene had lower basal histone acetylation. This may explain low basal PD-L2 expression. Although LBH589 treatment increased acetylation levels in the PD-L2 gene, the increase was mild. Collectively, these data support a model in which HDAC inhibition increases chromatin relaxation, enhancing gene expression and resultant PD-1 ligand surface expression on melanoma cells.

Intriguingly, two 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 HDACs, thereby repressing transcription (38). In addition, HDACs inhibitors have been characterized to down-regulate DNA methyltransferase activity (39). Studies are under way 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 detectable PD-L1 expression. Therefore, whether basal expression of PD-L1 is necessary for the described upregulation remains to be investigated. 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. Although this basal acetylation and expression were 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 with 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. Although 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.

Although these results demonstrated the ability of HDAC inhibition to upregulate PD-L1 ligand expression in melanoma, the particular HDAC(s) governing the expression of these

Figure 4.
HDAC inhibition increases histone acetylation at the PD-L1 and PD-L2 promoters. Indicated melanoma cell lines were treated in vitro for 2 hours with 12.5 nmol/L LBH589 (squares) or DMSO control (circles). Cells were then fixed and chromatin immunoprecipitated for acetylated histone 3 or IgG control. DNA pulldown was quantified by qRT-PCR. Fold enrichment over corresponding IgG pulldown at the PD-L1 (A) and PD-L2 (B) 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 −455 gene region of PD-L1 (C) and +307 gene region of PD-L2 (D). For all graphs, error bars are representative of technical replicates.
molecules remain to be fully elucidated. Although 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, and 11 and HDACs 1 and 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). On the basis of 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 only in malignancy, but also in autoimmunity.

Clinically, expression of PD-L1 in tumors revealed 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 proinflammatory immune infiltrate, and consequently a lack of T cells generating cytokines that influence PD-L1 expression. Indeed, PD-L1 and PD-L2 expression are upregulated in response to the proinflammatory cytokines IFNγ and TNF (44). Under this model, the upregulation of PD-1 ligands by HDAC inhibition, independent of increased immune infiltration, represents an undesirable effect among 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 antitumor activity compared with either agent alone, resulting in a delay in tumor progression and an increase in OS 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 with LBH589 as a single agent in immunocompetent mice 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 with control treatments. Curran and colleagues (45) 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 (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...
Combining HDAC inhibition with PD-1 blockade in vivo results in delayed tumor growth and enhanced survival. C57BL/6 mice were inoculated s.c. with B16F10 melanoma. Seven days after inoculation, mice began treatment with LBH589 (15 mg/kg, triangles; Monday, Wednesday, and Friday), PD-1-blocking antibody (5 mg/kg, squares; Tuesday and Thursday), a combination of these agents (diamonds) or dextrose control (circles) for 3 weeks. A, tumor growth was measured; and B, survival monitored. The 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; *, \( P < 0.05 \).

landscape of malignant cells, through changes in expression of costimulatory 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. Although 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.

Disclosure of Potential Conflicts of Interest
J Weber has received honoraria from the speakers bureau and is a consultant/advisory board member for Bristol-Myers Squibb. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

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