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Entinostat neutralizes myeloid derived suppressor cells and enhances the antitumor effect of PD-1 inhibition in murine models of lung and renal cell carcinoma

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Abstract

Purpose—Recent advances in immunotherapy highlight the antitumor effects of immune-checkpoint inhibition despite a relatively limited subset of patients receiving clinical benefit. The selective class I histone deacetylase inhibitor (HDACi) entinostat has been reported to have immunomodulatory activity including targeting of immune suppressor cells in the tumor microenvironment. Thus, we decided to assess whether entinostat could enhance anti-PD-1 treatment and investigate those alterations in the immunosuppressive tumor microenvironment that contribute to the combined anti-tumor activity.

Experimental design—We utilized syngeneic mouse models of lung (LLC) and renal cell (RENCA) carcinoma, and assessed immune correlates, tumor growth and survival following treatment with entinostat (5 or 10 mg/kg, P.O.) and a PD-1 inhibitor (10 and 20 mg/kg, s.c.).

Results—Entinostat enhanced the antitumor effect of PD-1 inhibition in two syngeneic mouse tumor models by reducing tumor growth and increasing survival. Entinostat inhibited the

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immunosuppressive function of both PMN- and M-MDSC populations. Analysis of MDSC response to entinostat revealed significantly reduced arginase-1, iNOS and COX-2 levels, suggesting potential mechanisms for the altered function. We also observed significant alterations in cytokine/chemokine release *in vivo* with a shift from an immunosuppressive to a tumor suppressive microenvironment.

Conclusions—Our results demonstrate that entinostat enhances the antitumor effect of PD-1 targeting through functional inhibition of MDSCs, and a transition away from an immune suppressive tumor microenvironment. These data provide a mechanistic rationale for the clinical testing and potential markers of response of this novel combination in solid tumor patients.

Keywords

Renal cell carcinoma; lung carcinoma; histone deacetylase inhibition; myeloid derived suppressor cells; program cell death protein 1 inhibition

INTRODUCTION

Momentum in the field of cancer immunotherapy has been significantly accelerating as several, durable and effective cancer treatments based on promoting an anti-tumor immune response are now available for patients with solid tumors, including renal cell carcinoma (RCC) and non-small cell lung cancer (NSCLC). The PD-1/PD-L1 axis plays a central role in the immune evasion capability of tumor cells by hampering the antitumor activity of cytotoxic T cells. Blocking these immune checkpoints, which have been reported as being aberrantly expressed on solid tumors such as RCC and NSCLC, has recently led to the approval of nivolumab, pembrolizumab, and atezolizumab in these diseases (1,2) (3,4). However, despite these significant clinical advances, the clinical benefit of immunotherapies for RCC and NSCLC are restricted to a subset of patients. Tumor escape due to immune tolerance and an immunosuppressive tumor microenvironment (TME) represents a major obstacle in maximizing the full clinical potential of immune-checkpoint inhibitors both in the indications where they have shown activity but also in expanding to tumor types where these agents have not been as effective.

Entinostat, is an oral, class I-specific HDACi, shown to disrupt the dynamic interactions between the tumor microenvironment and host immune surveillance (5,6). Tumor cells classically avoid immune surveillance by releasing a plethora of immune-suppressive factors and chemo-attractants enabling tumor-promoting inflammation (7). HDAC inhibitors can increase immunogenicity of tumor cells by activating expression of tumor antigen, antigen presentation, and co-stimulation molecules in tumor cells (6,8). Additionally, our group has shown that entinostat synergistically enhances IL-2 immunotherapy in the RENCA model by inhibiting the function of immunosuppressive T regulatory (Treg) cells through acetylation of the STAT3 transcription factor (5).

Myeloid derived suppressor cells (MDSCs) represent a highly immunosuppressive population of tumor infiltrating immature myeloid cells. These cells contribute to tumor immune escape by inhibiting cytotoxic T cell proliferation and driving T regulatory cell induction (3,9,10). Recent studies have reported that *in vitro* pan-HDAC inhibition may

influence MDSCs to a more differentiated status of macrophage or dendritic cell (8,11). Alternatively, another study treating bone marrow precursor cells with pan-HDAC inhibitors resulted in the expansion of monocytic MDSC populations (12). Interestingly, a combination of demethylating agent and HDAC inhibitor enhanced the anti-tumor effect of combined PD-1 and CTLA4 inhibition in colon and breast cancer models, and was associated with decreased MDSCs (Kim et al PNAS 2014).

In this study, our aim was to further understand the mechanistic basis for how entinostat targeting of MDSCs and alteration of the immunosuppressive tumor microenvironment leads to enhanced immune-checkpoint inhibitor anti-tumor activity. We conducted preclinical studies of entinostat in combination with anti-PD-1 antibody treatment in two syngeneic mouse models, RENCA and Lewis lung carcinoma (LLC), and assessed the effect of single agents and the combination on immune relevant endpoints. Our *in vivo* results, which we subsequently confirmed using *in vitro* mechanism based assays, demonstrate that the significant anti-tumor activity of entinostat combined with anti-PD-1 is associated with a direct impact of entinostat on blocking the function of immunosuppressive tumor infiltrating MDSCs. Our characterization of entinostat mediated changes in enzymes, cytokines, chemokines and other growth factors associated with an immunosuppressive TME offer multiple candidates to serve as potential obtained from ongoing clinical trials. We believe our data further support the importance of targeting MDSC function to enhance immune checkpoint blockade, and significantly advance the mechanistic rationale for the clinical testing of entinostat combined with PD-1/PD-L1 targeted therapies.

MATERIALS AND METHODS

Cell lines

The RENCA-Luc murine renal cell carcinoma cell line, purchased from American Type Culture Collection (National Cancer Institute), was stably transfected with a luciferase reporter in the Pili laboratory. Cells were cultured using RPMI 1640 (Corning) with 10% fetal bovine serum (Corning) and 1% Pen/Strep (Life Technologies). Cells were incubated in an incubator maintained at 37°C and 5% CO₂. 75–80% confluent cells were harvested for orthotopic injection into the kidney of Balb/c mice using 0.25% Trypsin (Corning) and suspended in a 1:1 ratio of matrigel (Corning) and DPBS (Gibco). LLC lung carcinoma and CT26 colon carcinoma were obtained from ATCC and cultured in DMEM (Corning Incorporated) supplemented with 10% FBS (Atlanta Biologicals, Inc.) and 1% antibiotics (Thermo Fisher Scientific Inc.). The cells were harvested using 0.25% Trypsin (Thermo Fisher Scientific Inc.), suspended in DPBS (Corning) as 200 µL containing 5×10⁵ cells, and injected s.c. into the mice. After tumors were established, the mice were randomized into 2 groups and orally treated with 10 mg/kg of entinostat daily. The J774M cell line was kindly provided by Georgia Cancer Center (Dr. Kebin Liu) and cultured with DMEM (Corning) media with 10% fetal bovine serum (Corning) and 1% Pen/Strep (Life Technologies). Cells were incubated in a 37°C and 5% CO₂. 70–80% confluent cells were harvested using a cell scraper and passaged as suggested for the parent cell line via ATCC guidelines.

HPC (hematopoietic progenitor cells) culture

Lineage negative cells were purified from C57BL/6 naïve bone marrow cells using lineage cell depletion kit (Miltenyi). Naïve bone marrow cells were subsequently treated with biotinylated anti-lineage antibody and anti-biotin microbeads, and pass through the MACS column according to the manufacture's instruction. Lineage negative cells were cultured in RPMI (Corning Incorporated) supplemented with 10% FBS, 1% antibiotics, and 50 μ M 2-mercaptoethanol (Thermo Fisher Scientific Inc.) with 20 ng/mL of recombinant GM-CSF (Invitrogen), at 50,000 cells/well in 24-well plates. Tumor explant supernatants (TES) were obtained by culturing small pieces of EL4 tumors with complete RPMI media for 24 hours. On Day 1, TES or media with DMSO or entinostat solution was added into the wells at 10% to get the final concentration of 100 or 500 nM entinostat. On Day 3, the half of culture supernatant was exchanged to fresh media supplemented with 20 ng/mL of GM-CSF with or without 10% TES. On Day 6, the cells were collected and analyzed by flow cytometric analysis, or used for functional analysis.

Tumor, bone marrow, and spleen cells isolation

Live tumor sections were isolated from tumors, cut into small pieces, and digested with an enzyme cocktail solution from the mouse tumor dissociation kit (Miltenyi Biotec – 130-096-730). Tumors were incubated with the enzyme cocktail for 30 minutes at 37°C with agitation. The enzyme reaction was arrested using PBS, cells were spun at 300g, 4°C for 7 minutes, re-suspended in PBS and mashed through a 70 μ M cell strainer. Cells from these tumors were either used for flow cytometry analysis or further processed and used for functional analyses. Whole spleens and bone marrow were harvested from mice, and processed into single cell suspensions. Cells were then washed, lysed with red blood cell (RBC) lysis buffer (Affymetrix 00-4333-57) or using ammonium chloride lysis buffer, and cultured in RPMI medium with 10% FBS, Pen (100units/ml)-Strep (100mg/ml), 1mM sodium pyruvate, 100mM non-essential amino-acids, 2mM L-Glutamine, 55 μ M BME, with anti-CD3 (eBioscience 16-0031-85) and anti-CD28 (eBioscience 5012503) antibodies for approximately 24 hours. CD8⁺ T cells were then isolated using a CD8a⁺ T cell isolation kit from Miltenyi Biotec (130-104-075), stained with carboxyfluorescein succinimidyl ester (CFSE) (NC9759757), per the manufacturer's protocol, and co-cultured with MDSCs as described below.

MDSC Isolation and T cell suppression assay

MDSCs were isolated from tumors using Miltenyi Biotec's Myeloid Derived Suppressor Cell Isolation Kit (130-094-538) and co-cultured with isolated CD8⁺ T cells in serially diluted concentrations. T cells (1×10^5 ; isolated with a CD8a⁺ T cell isolation kit; Miltenyi Biotec) were cultured in plates with varying numbers of either PMN-MDSCs or M-MDSCs isolated from RENCA tumors for 16–18 hours. T cells isolate in the listed method were co-cultured with entinostat treated J774M cells for 68–72 hours. Cells were then harvested, stained and analyzed via FACS analysis.

In vivo treatment

All procedures were performed and approved in strict accordance with the Institutional Animal Care and Use Committee (IACUC) at Roswell Park Cancer Institute, Indiana University School of Medicine, Wistar Institute, and with the NIH Guide for the Care and Use of Laboratory Animal guidelines. Female five- to six- week old Balb/c and C57BL/6 mice (Charles River Labs) were maintained in a temperature controlled room with a 12/12 hour light/dark schedule and food provided *ad libitum*. 70–80% confluent RENCA-Luc cells were harvested using 0.25% Trypsin (Corning) and suspended in a 1:1 ratio of matrigel (Corning) and HBSS (Gibco), 10ul containing 1×10^4 cells was injected under the renal capsule. One week post-injection preliminary bioluminescent imaging, mice were randomized into four groups: control, entinostat, anti-PD-1, or a combination of entinostat and anti-PD-1. Mouse tumors were serially imaged using a bioluminescent IVIS imaging machine. LLC tumor-bearing mice were randomized into 4 groups (n = 5) on Day 11 and orally treated with vehicle (1% DMSO) or entinostat solution (10 mg/kg) every day. Anti-PD-1 antibody (clone RMP1-14, Bio X Cell) or isotype control antibody (clone 2A3, Rat IgG2a, Bio X Cell) was i.p. treated on Days 11, 14, 18 and 21. Mice in the treatment groups were treated orally with entinostat 5mg/kg 5 days/week, and with PD-1 inhibitor 10mg/kg or 20mg/kg (2nd survival study) I.P. from BioXCell, or a combination treatment regimen.

Cell staining and flow cytometry

Splenocytes, tumor cell suspensions, and peripheral blood cells were washed, blocked with Fc Block (anti-mouse CD16/32 mAb; BD Biosciences) at 4°C for 15 minutes, and stained with fluorescence conjugated antibodies against surface markers CD45(clone 30-F11), CD11b (clone M1/70), CD11c, Gr1 (clone RB6-8C5), Ly6C (clone AL-21), Ly6G (clone 1A8), F480 (clone BM8), I-Ab, I-Ad, CD8a (clone 53-6.7), CD4 (clone RM4-5) antibodies purchased from BioLegend, eBioscience or BD Biosciences. Cells were then fixed in Fixation/Permeabilization buffer (eBioscience) and stained with antibodies against intracellular proteins, including FoxP3 (NRRF-30) and Granzyme B (clone GB11). The antibodies were purchased from BD Biosciences, Biolegend, and R&D Systems and used in staining. Lineage antibody cocktail was purchased from eBioscience. Anti-mouse CCR2 antibody was purchased from R&D Systems, Inc. Stained cells and isotype-control-stained cells, were assayed using a LSRII, LSR4 or Fortessa flow cytometer (BD Biosciences). Data analysis was performed using the FlowJo (FlowJo LLC, Tree Star) and/or ModFit LT 4.1 software.

Proteome Profile

Tumor tissue was homogenized in PBS containing protease inhibitors. Following homogenization, Triton X-100 was added to a final concentration of 1%, frozen at –80C, thawed, centrifuged as 10,000g for 5 minutes, quantified and assayed according to the manufacturer’s protocol. Blood samples were collected from mice in each cohort, allowed to clot for 2 hours at room temperature, centrifuged at 2000g for 15 minutes. Serum samples were frozen at –80°C until time of analysis at which time they were run according to manufacturer’s protocol. All samples were processed and run on R&D Systems mouse XL

cytokine array kit (Ary028). Analyses were performed using HLIImage++ QuickSpots Tool (Western Vision Software) and GraphPad Prism7.

Immunoprecipitation and Western Blot Analysis

J774M cells were treated with entinostat at 0.5 μ M, 0.75 μ M, or 1 μ M for 6 hours and harvested in non-denaturing lysis buffer. Cells were subjected to immunoprecipitation per the manufacturer's protocol – Abcam 206996. STAT3 antibody was applied to 350ug of lysate in micro-centrifuge tubes and placed on a rotary mixer overnight at 4°C. The next day, protein A/G Sepharose® beads were applied and the mixture was placed on the rotary mixer for one hour at 4°C. Following pull-down of STAT3 samples were subjected to gel electrophoresis on 4–20% precast mini-protean polyacrylamide gels (Bio-Rad) and transferred onto nitrocellulose membranes. Proteins of interest were detected using acetylated lysine (1:1000, Cell Signaling 9441S) and STAT3 (1:1000, Cell Signaling 12640S). After incubation with conjugated secondary antibodies (Bio-Rad), membranes were exposed to chemiluminescence according to manufacturer's instructions (Thermo Fisher Scientific) and exposed to film. Quantitative measurements were performed using ImageJ and GraphPad (Prism 7) software.

Quantitative real-time PCR

mRNA was extracted from J774M cells that were treated \pm entinostat using standard Trizol protocols or using the total RNA extraction kit (Omega Bio-tek). RNA concentration and purity were determined through measurement of A260/280 ratios with a Synergy Hi Multi-Mode reader. cDNA was prepared using the iScript kit (Bio-Rad) or the cDNA reverse transcriptase kit (Applied Biosystems) and qPCR was performed in triplicate for each sample using SYBR Master Mixture (Bio-Rad or Applied Biosystems). Samples were run on an Applied Biosystems 7900HT fast real time PCR system. Sequence Detection Systems software v2.3 was used to identify the cycle threshold (C_t) values and to generate gene expression curves. Data were normalized to Gapdh expression and fold change was calculated. The primers used for target genes were: Gapdh 5' - AACTTTGGCATTGTGGAAGG-3' & 5' -ACACATTGGGGGTAGGAACA-3'; iNOS, 5' - AACGGAGAACGTTGGATTTG-3' and 5' -CAGCACAAGGGGTTTTCTTC-3'; Arg1, 5' - GCTGTCTTCCCAAGAGTTGGG-3' and 5' -ATGGAAGAGACCTTCAGCTAC-3'; COX2, 5' -CCAGCACTTCACCCATCAGTT-3' and 5' - ACCCAGTCTCGCTTATGA-3'; Actb, 5' -ATGGAGGGGAATACAGCCC-3' and 5' - TTCTTTGCAGCTCCTTCGTT-3'. Expressions of the different genes were normalized to *Gapdh* or *Actb*. Relative expression was calculated using the 2^{-C_t} method.

NO production

Ly6G+ cells from HPC culture were cocultured with naïve whole spleen cells and 0.1 μ g/mL of anti-CD3 and CD28 antibodies for 24 hours. Nitrite concentration in culture supernatant was measured using Griess Reagent System (Promega).

Arginase activity

Ly6G⁺ cells from spleen cells or HPC culture were lysed with the lysis buffer of Arginase activity assay kit (abcam). Arginase activity of the cell lysates was measured using Arginase activity assay kit. The arginase activity was normalized using the protein concentration which was detected by the Bradford dye-binding method using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Inc.).

PEG₂ Production Assay

Ly6G⁺ cells from HPC culture were cultured at 2×10^6 cells/mL in RPMI complete media with 20 ng/mL of GM-CSF for 20 hours. PGE₂ concentration in supernatant was measured using PGE₂ ELISA Kit (Invitrogen).

ROS staining

To determine the intracellular levels of ROS, spleen cells, tumor cells, and HPC culture cells were stained with antibodies against cell surface antigen and then incubated with DCFDA (Invitrogen) at 37°C for 30 min. The cells were analyzed by flow cytometry and the mean fluorescence intensity of the ROS-reactive dichlorofluorescein was analyzed using FlowJo software.

Statistical analysis

All statistical analyses were performed using GraphPad Prism7 software for Windows. Analysis of survival was conducted using the Kaplan-Meier method. Differences in treatment group survivals were assessed with the log-rank test. All other statistical analyses in this study were performed between experimental groups using the Student's T test with Welch's correction. A p-value <0.05 was considered to be statistically significant.

RESULTS

Entinostat enhances the antitumor effect of PD-1 inhibition in syngeneic mouse cancer models

We tested entinostat in combination with a mouse checkpoint inhibitor anti-PD-1 antibody in RENCA and LLC syngeneic mouse models of cancer. Both these models have been shown to attract highly immunosuppressive MDSCs to the TME, which play a key role in suppression of anti-tumor T cell activity (13,14). Balb/c or C57BL/6 mice were inoculated orthotopically with luciferin tagged RENCA cells or subcutaneously with LLC tumor cells, respectively. RENCA tumor bearing mice were randomized based on bioluminescent readouts or tumor volume and separated into four groups: control, entinostat (5mg/kg), anti-PD-1 (10mg/kg), or the combination of entinostat and anti-PD-1. Treatment with entinostat alone resulted in significant inhibition of tumor growth across the studied models (RENCA: 52.6% growth inhibition; entinostat vs. control: $p = 0.0015$), while anti-PD-1 alone only moderately reduced tumor growth (35.05% growth inhibition; anti-PD-1 vs. control: $p = 0.0768$) (Figure 1A–C). The combination of entinostat and anti-PD-1 treatment was most effective in enhancing tumor growth inhibition compared to the control and each of the

single treatment groups (83.3% reduction; combination vs. vehicle: $p < 0.0001$; combination vs. entinostat: $p = 0.0115$; combination vs. anti-PD-1: $p = 0.0076$) (Figure 1A–C).

Clinically, anti-PD-1 immunotherapy has shown prolonged stabilization of disease in approximately 40% of patients with RCC and lung cancer (1,2,15) leading to improvements in overall survival in these indications. Following the previous study in the RENCA model demonstrating tumor growth inhibition, we examined the survival outcome for the entinostat and anti-PD-1 antibody combination. Using the doses previously described we observed a significant increase in survival for the combination treatment (combination vs. anti-PD-1: $p = 0.0012$; combination vs. control: $p = 0.0009$) (Figure 1D). Our results showed prolonged survival in the anti-PD-1 group and an enhanced effect in the combination treated cohort also with increased dose of the PD-1 inhibitor (combination vs. control: $p = 0.0471$; combination vs. entinostat: $p = 0.0372$) (Figure 1E). To expand these observations to a different mouse model, we used LLC tumor-bearing mice. At the selected dose (10 mg/kg) entinostat as a single agent caused a modest but significant decrease in tumor growth. Similar decreases were observed in mice treated with PD-1 antibody alone. However, the combination of entinostat and anti-PD-1 resulted in significant reductions in tumor growth (Figure 1F). The results of these studies demonstrate that the combination of entinostat with anti-PD-1 inhibits tumor growth in different tumor models.

Enhanced anti-PD-1 immunotherapy is associated with increased antitumor immune responses and decreased presence of immunosuppressive cell populations

The PD-1/PD-L1 axis plays a central role in assisting tumor evasion of immune surveillance *via* promotion of activated T cell apoptosis (1,16,17). Additionally, HDACi treatment has been shown to alter the tumor microenvironment by reducing Treg cell activity and enhancing CD8 T cell infiltration (18,19). To determine whether the inhibition of tumor growth resulting from the entinostat/anti-PD-1 combination treatment was associated with an enhanced immune response, we examined the circulating and tumor infiltrating lymphoid and myeloid populations. End-point blood and tumor samples were collected from RENCA tumor-bearing mice and subjected to immunofluorescence staining and FACS analysis. We observed an increased Treg (CD4⁺FoxP3⁺) presence in the blood and no significant difference in the TME, consistent with our previous report (5). However, consistent with our previous results (5), entinostat treatment resulted in a significant decrease in the protein levels of FoxP3 in the circulating CD4⁺FoxP3⁺ cell subtype, as represented by the MFI (mean fluorescence intensity) (Figure 2A). The combination group, while also showing a significant reduction in circulating FoxP3 protein levels as compared to the control group, did not show reduced FoxP3 levels relative to entinostat alone (Figure 2A). In the TME we did observe a significant reduction in the MFI of CD4⁺FoxP3⁺ cells, suggesting an inhibition of Treg function (20,21) in response to combination treatment (Figure 2B).

CD8⁺ T cells are affected by the PD-1/PD-L1 axis and are crucial to tumor surveillance. When the PD-1/PD-L1 checkpoint axis is blocked, there is often an increase in T_H1 cell function and tumor infiltration (22,23). We found that the combination effect of entinostat and anti-PD-1 immunotherapy resulted in a significant increase in tumor infiltrating CD8⁺ T cells (control vs. combination: $p = 0.0352$) (Figure 2C, **left panel**). Similarly, we observed a

statistically significant increase in the CD8⁺ T cell – T regulatory cell ratio suggesting the generation of a less immunosuppressive environment (control vs. combination: $p = 0.0218$) (Figure 2C, **right panel**).

In addition to FoxP3⁺ Tregs, there are multiple immunosuppressive myeloid cells recruited to the tumor microenvironment including tumor associated macrophages (TAMs) and myeloid derived suppressor cells (MDSCs). Upon migration of immature myeloid cells to the tumor these cells are often primed to become TAMs in response to chemokine and cytokine release from the tumor cells. These cells are marked by the pan-macrophage marker F4/80 in combination with CD45⁺CD11b⁺ markers. We observed a significant reduction in TAMs in the combination group as compared to control and entinostat alone (combination vs. control: $p = 0.0272$; combination vs. entinostat: $p = 0.009$) in the RENCA model. Entinostat alone compared to control caused a modest and not statistically significant reduction in TAMs.(Figure 2D). Taken together, these results suggest that entinostat combined with anti-PD-1 immunotherapy significantly alters the cellular milieu in the TME to reflect a change towards immune responsiveness.

Myeloid derived suppressor cell function is impaired by entinostat treatment

Myeloid derived suppressor cells (MDSCs) develop from immature myeloid cells and contribute to the immune suppressive TME by inhibiting anti-tumor T cell immune responses. MDSCs are present in two phenotypically defined sub-populations: granulocytic Ly6G⁺Ly6C^{low} (PMN-MDSCs) and monocytic Ly6C⁺Ly6G⁻ (M-MDSCs) MDSCs (24). Recent data suggests that phenotype is an important descriptor of MDSCs, but not a sufficient criterion for defining and identifying MDSCs, which requires additional functional characterization of the cells (25). The tumor attracts MDSCs, monocytes, and immature myeloid cells *via* release of chemo-attractants, such as CCL2, CCL5, CCL7, & CXCL12 (8,25–28). Once in the TME, MDSCs have been shown to promote resistance to CD8⁺ T cell cells and enhance the immune escape of the tumor *via* multiple mechanisms including upregulation of surface PD-L1, production of immunosuppressive cytokines (IL-10, TGF- β) & Treg attracting cytokines (CCL4, CCL5), elevation of arginase 1 (Arg1) & iNOS (26,27,29).

Treatment with entinostat alone caused an increase in phenotypically defined PMN-MDSC and M-MDSC within tumors, while anti-PD-1 single agent treatment led to a reduction in the PMN-MDSC populations (Figure 3A–B, Supplemental Figure 1A). The slight increase in MDSC populations observed in the entinostat group was amplified in the combination group (Figure 3B). We also observed a significant increase in accumulation of splenic PMN-MDSC ($p = 0.0053$) and M-MDSCs ($p = 0.0063$) in the entinostat treated LLC model (Figure 3C) and PMN-MDSCs ($p = 0.0441$) in the combination treated RENCA model (Figure 3D). Additionally, we observed increases in the bone marrow MDSCs in LLC tumor-bearing mice (Supplemental Figure 1B). Similar effects of entinostat on up-regulation of the population of PMN-MDSC in bone marrow, spleen, and tumors was observed in another tumor model – CT26 colon carcinoma (Supplemental Figure 1C).

As an increase in immunosuppressive MDSCs was not consistent with the anti-tumor activity that was observed with either entinostat alone or in combination with anti-PD-1, the

functional activity of accumulating MDSCs was examined. We tested *ex vivo* the ability of tumor associated MDSCs to suppress proliferation of antigen-specific and non-specific (CD3/CD28 stimulated) CD8⁺ T cells from tumor-free mice. PMN-MDSC and M-MDSC from tumors of untreated mice showed potent suppressive activity (Figure 3E–G). In sharp contrast, MDSCs isolated from tumors of entinostat-treated or entinostat/anti-PD-1 treated mice had poor suppressive activity as demonstrated by an inability to inhibit proliferation of stimulated CD8⁺ T cells (Figure 3E–G). MDSCs from the spleen of LLC tumor-bearing mice also showed a reduced capacity for inhibition of T cell proliferation in the entinostat treated cohort (Supplemental Figure 2A). Further examination of the CD8⁺ T cells from these co-culture experiments revealed increased Granzyme B production by approximately 40% compared to the control group, in which MDSCs from an untreated tumor-bearing mouse were co-cultured with pre-stimulated CD8⁺ T cells (*p-value* = 0.01 for each condition and MDSC: T cell ratio). (Figure 3H, Supplemental Figure 2B). These data indicate that although entinostat alone, and when combined with anti-PD-1, increased intra-tumor numbers of PMN-MDSC and M-MDSC, that these cells have lost the majority of their immunosuppressive activity.

Entinostat treatment of MDSC-like cell line J774M and LLC isolated MDSCs reveals potential mechanistic targets

The J774M cell line, kindly supplied by the Kebin Liu laboratory at Georgia Cancer Center, Augusta University, has recently been characterized as a stable MDSC-like cell line (30,31). To validate these findings for the purposes of our experiments, we stained these cells for Ly6C and Ly6G to confirm the MDSC-like status of the cells. As shown in Figure 4A the subpopulation ratio of these cells closely resembles what is found in the RENCA TME. Of the CD45⁺CD11b⁺Gr1⁺ populations, ~90% of the cells are PMN-MDSC (Ly6G⁺) and ~10% of the cells are M-MDSC (Ly6C⁺) (32). Following validation of the cell phenotype, we studied the effect of entinostat on the functional alteration of the immunosuppressive capacity of these cells. We treated the cells for up to 48 hours with concentrations ranging from 0.01 – 5 μ M of entinostat with no significant impact on J774M cell proliferation or viability (Supplemental Figure 3). Co-culture of entinostat treated J774M cells with pre-activated CD8⁺ T cells for 68–72 hours revealed a significant increase in CD8⁺ T cell proliferation nearing that of CD8⁺ T cells alone (Figure 4B–C). Our previous work with entinostat treatment of FoxP3⁺ Tregs demonstrated that inhibition of class 1 HDACs led to acetylation of STAT3 and subsequent inhibition of STAT3 phosphorylation and activation. To determine if entinostat utilized a similar mechanism of STAT3 inhibition in MDSCs, J774M cells were treated for 6 hours with 0.5 μ M, 0.75 μ M, or 1 μ M of entinostat. *Via* immunoprecipitation of STAT3 and Western blot probing for acetylated lysine, we noted that 0.5 μ M of entinostat treatment induced STAT3 acetylation without altering the total presence of STAT3 protein (Figure 4D).

MDSCs have been shown to express high levels of arginase 1 (ARG1), COX-2 and iNOS which are key mediators of the immunosuppressive activity of MDSCs. Further investigation of the J774M cells revealed a significant inhibition of arginase-1 (Arg1) expression in the entinostat treated cells as compared to the untreated cells (control vs. 0.5 μ M entinostat: *p* = 0.003; control vs. 1 μ M entinostat: *p* = 0.0041; control vs. 2 μ M entinostat: *p* = 0.0043)

(Figure 4E). Arg1 allows for induction of cell cycle arrest in the cytotoxic T cell population *via* Arg1 conversion of circulating L-arginine pools to urea and L-ornithine, thus reducing the presence of extra-cellular L-arginine, which is necessary for cytotoxic T cell survival (10,28). Consistent with the effects of entinostat on J774 MDSCs, further characterization of MDSCs isolated from entinostat treated LLC tumor-bearing mice indicated significantly reduced expression of *nos2* and *cox2* genes (Figure 4F). Entinostat did not affect the level of reactive oxygen species (ROS) in MDSCs (Figure 4G). These data indicate that entinostat may directly target STAT3 signaling to impair the T cell inhibiting activity of MDSCs, through down-regulation of arginase 1, iNOS or Cox2.

Effect of entinostat on differentiation and function of MDSC *in vitro*

Previous data has demonstrated that class 1 HDACs may play a role in the differentiation of myeloid progenitor cells to MDSCs, dendritic cells (DCs) and macrophage. To evaluate the effect of entinostat on MDSC differentiation, enriched bone marrow hematopoietic progenitor cells (HPC) from tumor-free mice were cultured with GM-CSF and tumor explants supernatant (TES) for 6 days in the presence of different concentrations of entinostat. In the absence of TES, entinostat caused a significant decrease in the differentiation of dendritic cells (DCs), without affecting macrophages and Ly6C^{hi} monocytes. In contrast, the presence of Ly6G⁺ granulocytes was significantly increased (Figure 5A). Entinostat-inducible up-regulation of granulocytic differentiation was observed during HPC differentiation in the presence of TES. No effect on macrophages and DCs was seen. However, the presence of monocytes was significantly decreased (Figure 5B). Thus, consistent with *in vivo* data, entinostat promoted differentiation of granulocytic cells. To assess the effect of entinostat on MDSC suppressive activity, Ly6G⁺ cells were isolated after a 6-day HPC culture with different concentrations of entinostat, GM-CSF, and TES, and used in an antigen-specific suppressive assay. PMN-MDSC derived in this manner had potent suppressive activity which was inhibited in a dose dependent manner by entinostat (Figure 5C). Similar to the results obtained *in vivo*, entinostat significantly reduced expression of genes involved in immune suppression in PMN-MDSC: *nos2*, *arg1*, and *cox2* (Figure 5D). Down-regulation of the expression of *nos2* was associated with decreased production of NO in entinostat-treated cells (Figure 5E). Reduced expression of *arg-1*, was associated with decreased Arginase activity (Figure 5F). MDSC inducing prostaglandin E2 (PEG2) was significantly reduced with entinostat treatment (Figure 5G). Thus, the effect of entinostat on PMN-MDSC generated from HPCs fully recapitulated the effect seen *in vivo*.

Entinostat treatment primes the tumor microenvironment for enhanced response to immunotherapy

To examine the effect of entinostat on the TME, we subjected tumor samples from control, entinostat and combination treated cohorts to a proteome profiler analysis (Ary028) which provided a readout of 111 chemokines and cytokines (Figure 6A–B, Supplemental Figures 4, 5). Within this pool, we observed a significant increase in MDSC associated trafficking/accumulation cytokines in both the entinostat and combination cohorts relative to the control IL-6: *p* 0.05 and VEGF: *p* 0.01 (Figure 6C). Additionally, we observed a significant decrease in MDSC activation cytokines G-CSF: *p* 0.001/0.01, GM-CSF: *p* 0.05/0.001, IL-1 β : *p* 0.001/0.01, & IL-10: *p* 0.001 in the TME of both the entinostat and

combination cohorts relative to the control (Figure 6B). We also noted a significant upregulation of anti-tumor chemokines and cytokines, which contribute to pro-MDSC inhibition (IL-1ra: $p = 0.01/0.0001$) (33) and innate anti-tumor response (IL-4: $p = 0.001/0.01$ & IL-12p40: $p = 0.01/0.05$) (Figure 6C). These results suggest that entinostat treatment is sufficient to alter the immune profile of the TME towards an anti-tumor status which may prime the TME to better respond to immunotherapeutic interventions, such as anti-PD-1 treatment. For these data on entinostat treatment priming the host immune system, we subjected serum samples from the control and entinostat treated mice to the same AryO28 proteome profiler. We observed significant decreases in multiple, circulating pro-tumor associated chemokines and cytokines between the control and entinostat treated cohorts (Figure 6D). Among these were a MDSC expansion regulator, adiponectin ($p = 0.01$); pro-tumor chemoattractant, angiopoietin-2 ($p = 0.0001$); inflammation promoting chitinase 3-like 1 ($p = 0.001$), CCL12 ($p = 0.01$), complement component C5 ($p = 0.01$), c-reactive protein ($p = 0.001$), IL-6 ($p = 0.0001$), pentraxins 2/3 ($p = 0.01$) & periostin ($p = 0.001$); T regulatory cell chemokine, CCL17 ($p = 0.001$), MDSC chemoattractants M-CSF ($p = 0.01$) and GM-CSF ($p = 0.01$); EMT/invasion matrix-metalloproteinases, MMP-2 ($p = 0.001$) & MMP-9 ($p = 0.0001$); CCL2 ($p = 0.01$) – MDSC attractant – inducing osteoprotegerin ($p = 0.001$); and leukocyte attractant VCAM ($p = 0.001$) (Figure 6D).

While we did not observe upregulation of anti-tumor cytokines/chemokines in the entinostat treated group, there was a significant upregulation of multiple cytokines/chemokines in the combination cohort suggesting that anti-PD-1 immunotherapy and entinostat work together to enhance the host immune system for improved immunotherapeutic responses (Figure 6E). We observed increases in the following anti-tumor related cytokines/chemokines: T cell attractants & anti-endothelial markers (CXCL9 ($p = 0.001$), CXCL10 ($p = 0.0001$)), tumor proliferation inhibitory cytokines (IL-4 ($p = 0.0001$) & IL-13 ($p = 0.0001$)), T cell chemoattractant (E-selectin ($p = 0.0001$)), and anti-tumor marker (IL-12p40 ($p = 0.0001$)) (Figure 6E). These results suggest that entinostat treatment alters the host environment and the TME in a manner that allows for enhancement of anti-PD-1 immunotherapy treatment.

DISCUSSION

The recent advancement in the therapeutic approach for patients with solid tumors, including RCC and NSCLC, has been remarkable, and driven forward in particular with the emergence of effective and generally well tolerated immunotherapies showing durable clinical benefit. We are still striving to have these advantageous therapies become effective for a larger population of patients as only ~20–30% of patients with RCC and NSCLC will have a durable response (1,2). Our study offers evidence supporting further development of the HDACi entinostat as an effective treatment to combine with immunotherapies.

In this study we tested the effect of combining a class I-selective HDAC inhibitor, entinostat, with anti-PD-1 immunotherapy in two syngeneic mouse models of RCC and NSCLC along with a series of *in vitro* experiments aimed at characterizing the basis for the enhanced anti-tumor activity observed for this combination. In addition to delayed tumor growth, we observed that entinostat and anti-PD-1 immunotherapy prolonged survival in our RCC model. Combination treatment reduced tumor associated FoxP3⁺ cells which had been

slightly elevated with anti-PD-1 treatment and significantly reduced the presence of FoxP3 protein in the cells suggesting greater anti-tumor activity. Treg inhibition due to entinostat treatment was accompanied by increased CD8⁺ infiltration into the TME and a subsequent increase in the CD8⁺ to Treg ratio. These findings support the hypothesis that entinostat in combination with immunotherapy treatment, rather than being directly cytotoxic to the tumor, has significant immunomodulatory activity (5,34).

Recent reports suggest that HDAC inhibition alters the cytokine release and may change the function of innate immune cell infiltrates in the tumor microenvironment (6,8,12,35). Interestingly, correlative studies performed in breast cancer patients receiving entinostat showed a decrease in both monocytic and granulocytic MDSCs (35). In our experimental conditions, we actually observed an increase in both monocytic and granulocytic subsets of the MDSC population. MDSC are well known inhibitors of T cell proliferation and activity. Recent reports suggest that HDAC may play a role in this suppressive function (6,10,12,22,24,26,28). Thus, our study shows that entinostat treatment has an inhibitory effect on PMN- and M-MDSC suppressive function both *in vivo* and *in vitro* – either with cells directly from the TME or using an MDSC-like cell line. Additional analysis of MDSCs treated with entinostat revealed a significant inhibition of arginase 1 activity and NO production as well as *cox2* expression suggesting potential mechanisms of action by which entinostat inhibits the function of the immunosuppressive MDSC populations. It is also intriguing that we observed a reduction of tumor infiltrating macrophages in the entinostat treated tumor suggesting a potential role of HDAC inhibition also on the innate immune response.

Entinostat treatment has been associated with altered inflammatory responses via cytokine/chemokine release and trafficking (19,36,37). Such circulating proteins impact the function and response of the immune system to disease. The release of chemoattractants from the TME is necessary for immune infiltration and immune surveillance evasion (7,10,26,38). Through this complex interplay of communication signals tumor cells and their associated immune infiltrates avoid notice of the immune system (Supplemental Figure 6). Our study shows significant alterations in the communication between the tumor and host environments. Downregulation of immune infiltrate tracking, expansion, activation, and suppression proteins by entinostat suggest that class I HDAC inhibition plays a central role in this effect. Similarly, the upregulation of multiple anti-tumor proteins in the serum indicate that entinostat may prime the host environment to better respond to immunotherapy.

The treatment of RCC is rapidly evolving with the introduction of immuncheckpoint inhibitors. At the moment, the only approved drug targeting the PD-1/PDL1 axis is nivolumab in the second line setting but the results from two randomized phase III clinical trials with novel combinations of immune-checkpoint inhibitors are weighted and will likely change the standard of care for RCC in the first-line setting. Our positive results with entinostat and high-dose interleukin 2 (HD-IL2) in patients with RCC are also encouraging and confirm the clinical immunomodulation of this HDAC inhibitor (*Pili R et al ASCO 2016*). Thus, it is intriguing to speculate that the differential effect of HDAC inhibition on the immune system may be pleiotropic but perhaps also guided by the type of immunotherapy that is combined with. For example, it is possible that the effect on Treg and

MDSC may be different and have a different contribution depending whether we combine entinostat with either HD-IL2 or a PD-1 inhibitor. Furthermore, the different TME across different tumor types (either within RCC or other solid tumors) may differentially sensitize to this therapeutic approach. This hypothesis is clinically relevant as combinations of HDAC and immune-checkpoint inhibitors are being developed and tested in clinical trials.

In summary, our study suggests a novel mechanism by which HDAC inhibitor entinostat modulates the immune-suppressive TME resulting in an enhanced anti-tumor effect. Entinostat has potent immunomodulatory activity through inhibition of MDSC function that enhances anti-PD-1 induced anti-tumor response. These results have direct clinical implications in designing rational combination treatments for clinical trials. A phase I/II clinical trial of combinational HDAC inhibitor and anti-PD-1 has been initiated at our institute to determine the efficacy, objective response rate, and progression-free survival in RCC patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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TRANSLATIONAL RELEVANCE

Despite the significant progress achieved with PD-1/PD-L1 inhibitors in the treatment of solid tumors, the majority of patients still present with progressive disease following these agents. Resistance to immune checkpoint inhibitors may in part be mediated by an immunosuppressive tumor microenvironment which we have demonstrated can be altered with entinostat treatment in two different preclinical tumor models to enhance the anti-tumor efficacy of PD-1 targeted therapy. Therefore, we identified an effective combination strategy with anti-PD1 inhibition and entinostat that is being readily translated to patients with solid tumors, including lung and renal cell carcinoma.

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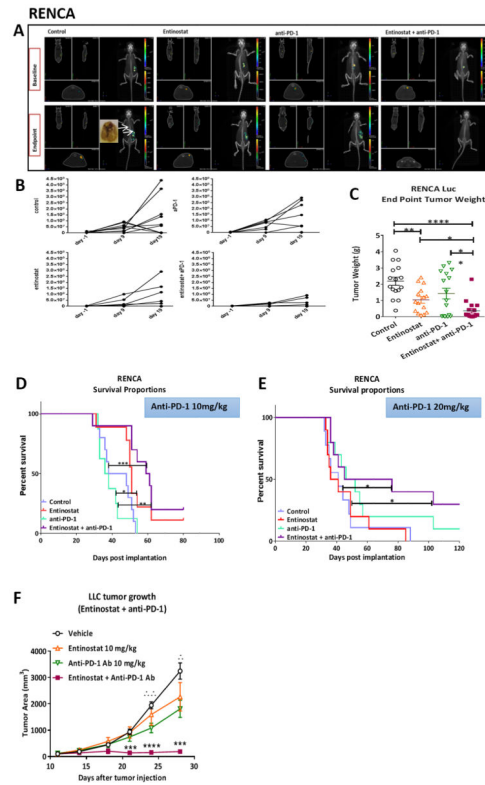


Figure 1. Entinostat improves immunotherapy in syngeneic models of mouse of renal cell and lung carcinoma

A–F RENCA model (**A**) Top: baseline bioluminescent imaging. Bottom: endpoint bioluminescent imaging. (**B**) Average Radiance [p/s/cm²/sr] of each mouse in control, entinostat, anti-PD-1, and entinostat + anti-PD-1 cohorts across the duration of the study. (**C**) Endpoint tumor weight in grams. (**D**) Survival study, with 10 mg/kg of anti-PD-1. (**E**) Survival study, with 20 mg/kg of anti-PD-1. Results are shown as mean ± SEM (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001). (**F**) The LLC tumor-bearing mice were orally treated with vehicle (1% DMSO) or entinostat solution (10 mg/kg) from Day 11 to Day 28. The mice were treated with anti-PD-1 antibody i.p. on Day 11, 14, 18 and 21. Results are indicated as mean ± SEM of n = 5 (**p < 0.01)

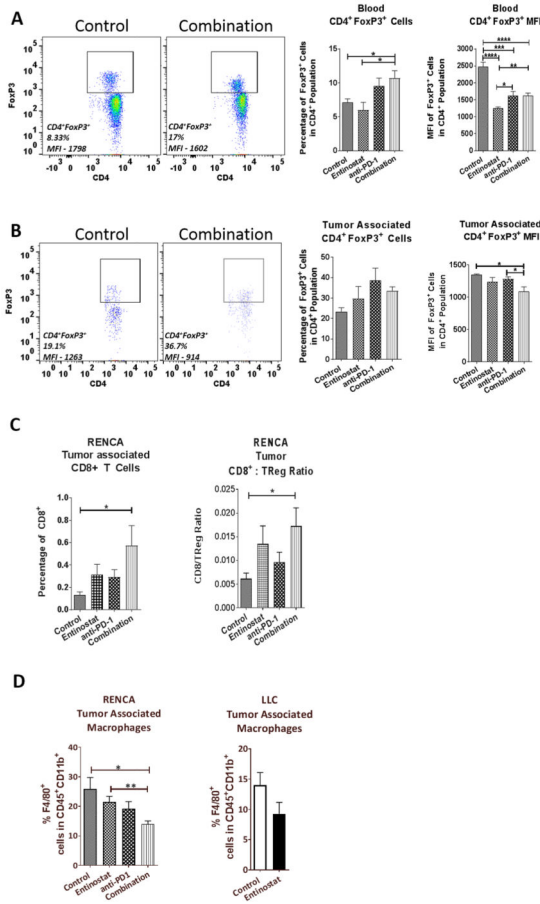


Figure 2. Entinostat modulates T cell and tumor associated macrophage response in the RENCA and LLC models

Blood and tumor samples were isolated from mice at the end of the study and processed for flow cytometry analysis. **(A)** Left: FACS analysis of blood shows the effect of vehicle and combination treatment on CD4 and FoxP3 levels. Right: Quantification of T regulatory cell presence in the blood and protein expression shown as mean fluorescence intensity (MFI). **(B)** Left: FACS analysis of tumor-cell suspensions from RENCA mice after control or entinostat treatment. Right: Quantification of T regulatory cell presence in the TME and protein expression shown as mean fluorescence intensity (MFI). **(C)** Quantitative FACS analysis of CD8⁺ T cell infiltrates into the TME. **(D)** Left: Quantitative FACS analysis results of tumor associate macrophage infiltration into the TME. n = 3–5 tumors/blood samples per cohort per panel. Right: LLC tumor-bearing mice were orally treated with vehicle (1% DMSO) or entinostat solution (10 mg/kg) for 2 weeks. Tumor cells were processed and analyzed by flow cytometric analysis. Results are shown as mean ± SEM (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001).

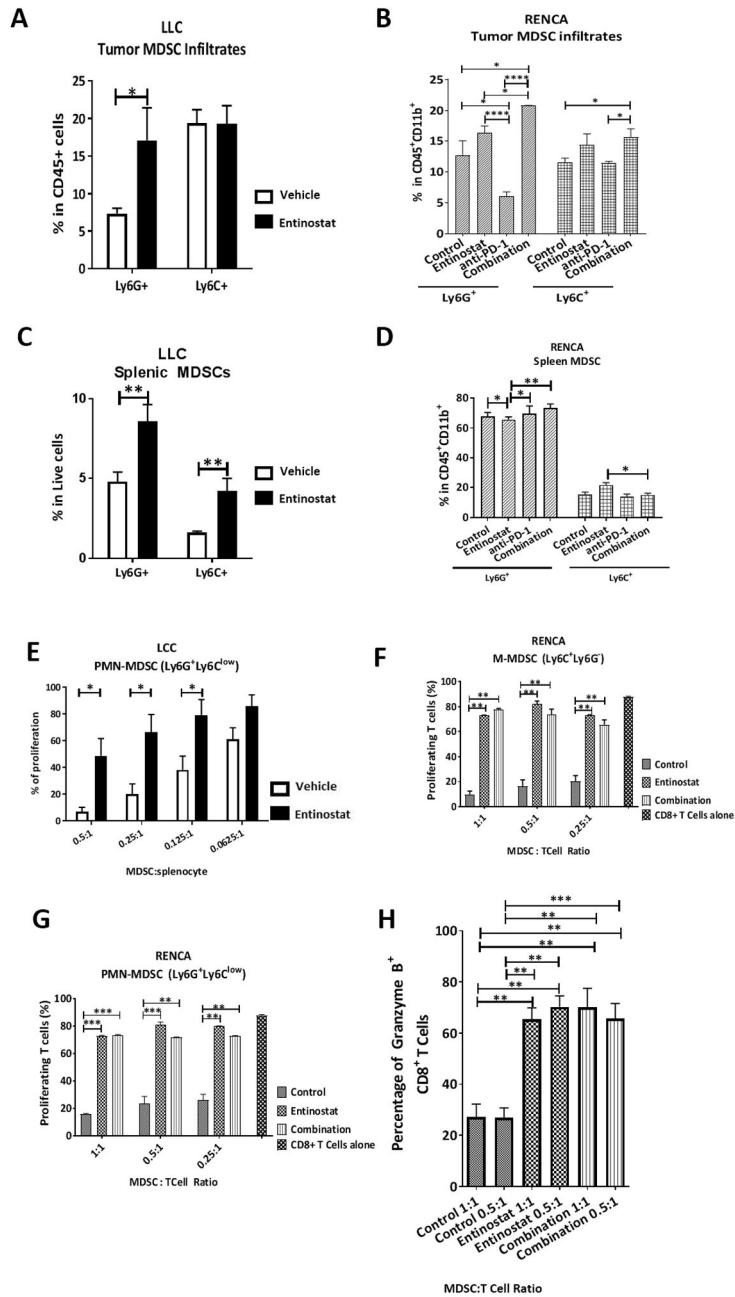


Figure 3. Entinostat inhibits the immunosuppressive capacity of MDSCs
(A,B) Cells with MDSC phenotype infiltrating LLC **(A)** (n=6–8) or RENCA tumors (n=3–5) in mice treated for 2–4 weeks with vehicle (1% DMSO) or entinostat. **(C, D)** Cells with MDSC phenotype in spleens of LLC tumor-bearing mice **(C)** or RENCA tumor-bearing mice **(D)**. **(E)** LLC Ly6G⁺ cells were enriched by MACS separation from tumor cells and cultured with splenocytes from PMEL mice and 0.1 mg/mL of gp100 peptide for 3 days. Cell proliferation was measured in triplicate using ³H-thymidine uptake. **(F)** M-MDSC and **(G)** PMN-MDSC cells isolated from the RENCA TME were co-cultured with CFSE tagged CD8⁺ T cells for 16–18 hours, at which time they were collected, stained with CD8 &

Granzyme B antibodies, and subjected to FACS analysis in triplicate for T cell proliferation (n = 3–5 tumors). (H) Quantitative representation of FACS analysis of cytotoxic CD8⁺ active protein Granzyme B from T cells which have been co-cultured with MDSCs from control, entinostat, or combination treated cohorts. Results are shown as mean ± SEM (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001).

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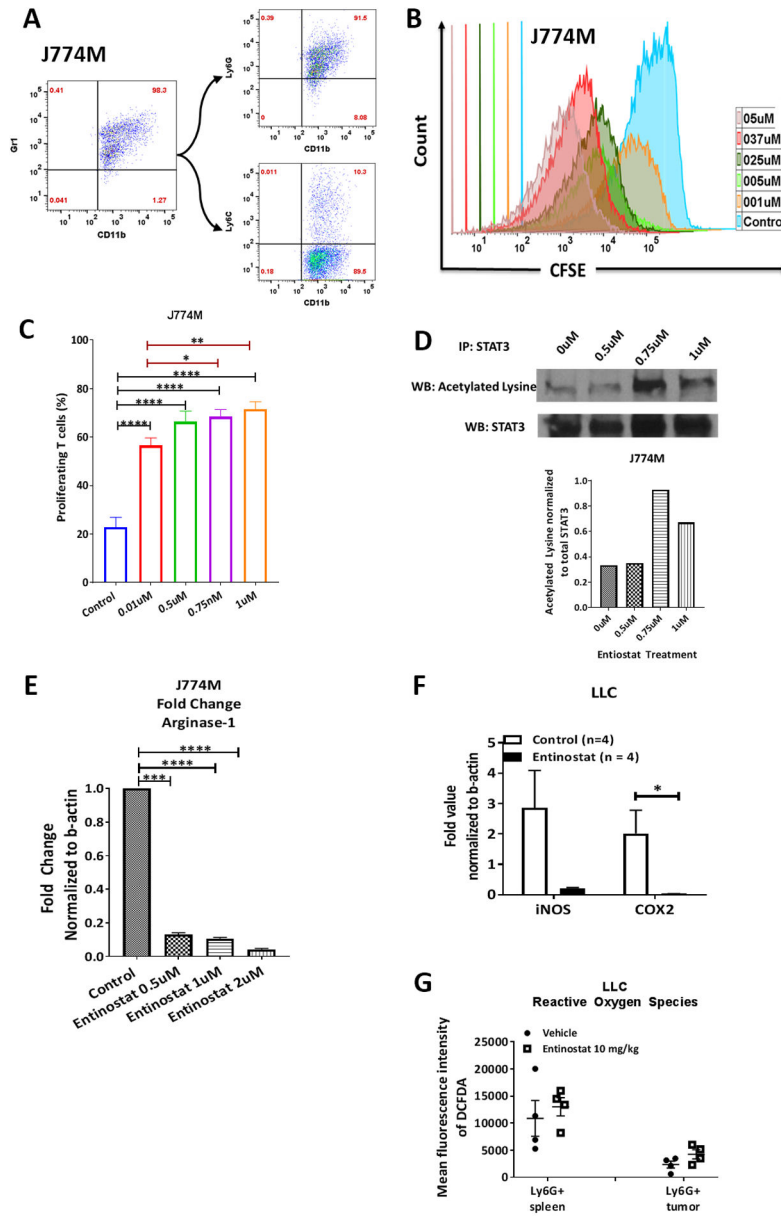


Figure 4. Entinostat diminishes inhibitory capabilities of MDSC-like cells revealing molecular modifications

(A) Characteristic FACS analysis of J774M cell line (B) CFSE fluorescent histograms of gated CD8⁺ T cells incubated with J77M cells at a ratio of 1:1. J774M cells were treated with increasing concentrations of entinostat – from right to left: control(untreated), 0.01μM, 0.05μM, 0.25μM, 0.37μM, 0.5μM. (C) Quantitative representation of B. bars show the mean percentage of proliferating CD8⁺ T cells (n = 3 technical replicates). This experiment was repeated 3 times independently. (D) Entinostat induces Stat3 acetylation in J774M MDSC-like cells. Cells were treated for 6 hours and then harvested for immunoprecipitation of STAT3 and Western blot staining for acetylated lysine and total STAT3. (E) Quantitative RT-PCR analysis indicates a significant decrease in key MDSC functional regulator Arginase-1 when J774M cells are treated with entinostat. (F) Total RNA was extracted from Ly6G+

cells which were enriched from spleen of LLC tumor bearing mice, and analyzed by qRT-PCR. (G) Spleen and tumor cells were stained with DCFDA and analyzed by flow. Results are shown as mean \pm SEM (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001).

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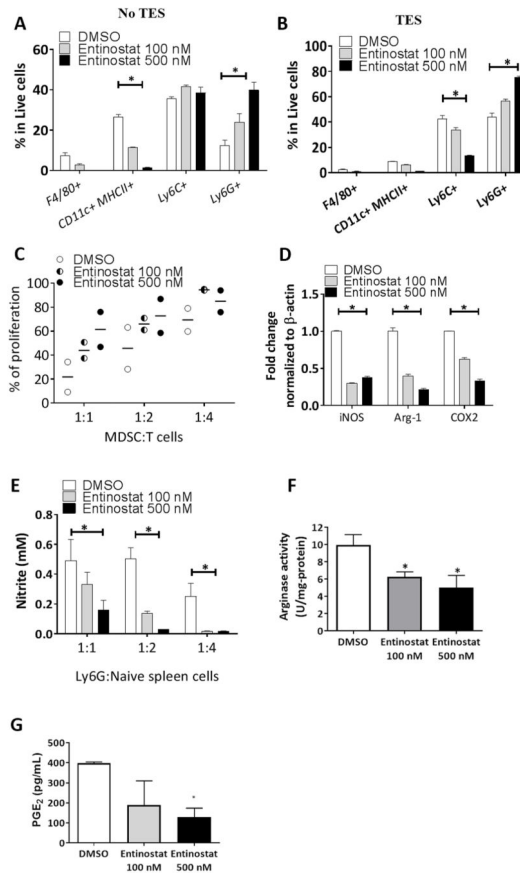


Figure 5. Effect of entinostat on factors involved in PMN-MDSC mediated suppression in Ly6G⁺ generated from HPC

(A-B) Lineage negative cells were enriched from bone marrow cells of naïve female C57BL/6 mice, and cultured in RPMI supplemented with 10% FBS, penicillin and streptomycin, and 20 ng/mL of GM-CSF at 50,000 cells/well in 24-well plates. On Day 1, TES or media with DMSO, 100 or 500 nM of entinostat was added into the wells at 10%. On Day 3, half of culture supernatant was exchanged to fresh media supplemented with 20 ng/mL of GM-CSF with or without TES. On Day 6, cells were collected and analyzed by flow cytometric analysis. Results represent mean \pm SEM of duplicate (* $p < 0.05$). (C) HPC cells from naïve bone marrow cells were cultured with 20 ng/mL of GM-CSF, 10% of TES, and DMSO or entinostat for 6 days. Ly6G⁺ cells were enriched by MACS separation and cocultured with splenocyte from PMEL mice and 0.1 mg/mL of gp100 peptide for 3 days. Cell proliferation was measured in triplicate using ³H-thymidine uptake. Results represent mean \pm SEM of 2 independent experiments. (D) HPC cells from naïve bone marrow cells were cultured with 20 ng/mL of GM-CSF, 10% of TES, and DMSO or entinostat for 6 days. Ly6G⁺ cells were enriched by MACS separation. RNA was extracted from Ly6G⁺ cells and analyzed by qRT-PCR. Data represent mean \pm SEM of triplicate, and statistically analyzed by Turkey's multiple comparisons test (* $p < 0.05$). (E) HPC from naïve bone marrow cells were cultured with 20 ng/mL of GM-CSF, 10% of TES, and DMSO or entinostat for 6 days. Ly6G⁺ cells were enriched by MACS separation and cocultured with naïve splenocytes and 0.1 mg/mL of anti-CD3 and anti-CD28 antibodies for 24 hours at 10⁵ cells/well. Nitrite

concentration in culture supernatant was analyzed by Griess Reagent System (Promega). Data represent mean \pm SEM of triplicate, and statistically analyzed by Turkey's multiple comparisons test (* $p < 0.05$). **(F)** Arginase activity (in vitro HPC). HPC from naïve bone marrow cells were cultured with 20 ng/mL of GM-CSF, 10% of TES, and DMSO or entinostat for 6 days. Ly6G+ cells were enriched by MACS separation and lysed with lysis buffer of Arginase activity assay kit (abcam). Arginase activity was measured using Arginase activity assay kit. Data represent mean \pm SEM of duplicated 2 independent experiments and statistically analyzed by t test (* $p < 0.05$). **(G)** PGE2 production in culture supernatant (in vitro HPC). HPC from naïve bone marrow cells were cultured with 20 ng/mL of GM-CSF, 10% of TES, and DMSO or entinostat for 6 days. Ly6G+ cells were enriched by MACS separation and cultured at 2×10^6 cells/mL in RPMI complete media with 20 ng/mL of GM-CSF for 20 hours. PGE2 concentration in supernatant was measured using PGE2 ELISA Kit (Invitrogen). Data represent mean \pm SEM of duplicate.

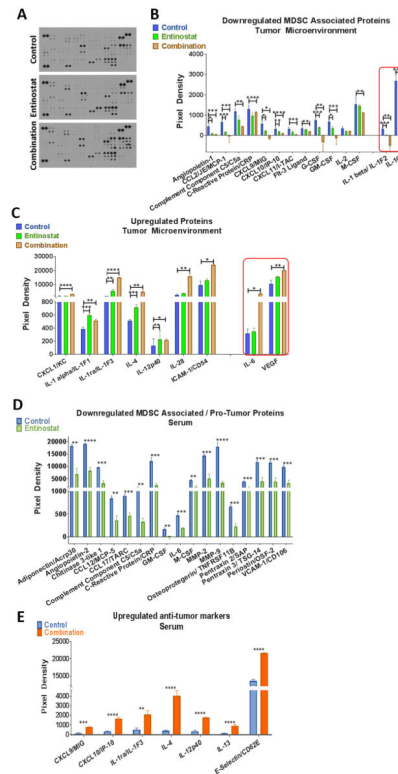


Figure 6. Treatment with entinostat significantly alters the highly immunosuppressive environment found in RENCA tumors

Tumor and blood samples collected from mice at the end of the study were processed and examined using the Proteome Profiler Mouse XL Cytokine array kit (Ary028). (A) Mouse cytokine/chemokine array results from tumor lysates in the control and entinostat treated cohorts (arrangement of the mouse cytokine/chemokine array can be found in the supplemental figures S.2) (n = 2 tumors/cohort & 3 data points per tumor). (B) Quantification of MDSC associated or pro-tumor cytokines/chemokines which were significantly downregulated in the presence of entinostat. (C) Quantification of anti-tumor chemokines/cytokines which were upregulated significantly in the presence of entinostat treatment. (D) Ary028 array results from serum samples of control and entinostat treated mice (n = 2 samples/cohort & 3 data points per tumor). (E) Array results comparing the control cohort with the combination cohort (n = 2 samples/cohort & 3 data points per tumor). Combination treatment significantly upregulated multiple anti-tumor associated chemokines and cytokines. Results are shown as mean \pm SEM (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001), statistics were calculated using multiple t tests, discovery was determined using the Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, with Q = 1%.