Published in final edited form as:

Cancer Immunol Res. 2018 December ; 6(12): 1561–1577. doi:10.1158/2326-6066.CIR-18-0070.

Entinostat converts immune-resistant breast and pancreatic cancers into checkpoint-responsive tumors by reprogramming tumor-infiltrating MDSCs

Brian J. Christmas¹, Christine I. Rafie¹, Alexander C. Hopkins¹, Blake A. Scott¹, Hayley S. Ma¹, Kayla A. Cruz¹, Skylar Woolman¹, Todd D. Armstrong¹, Roisin M. Connolly², Nilo A. Azad^{1,2}, Elizabeth M. Jaffee^{1,2}, and Evanthia T. Roussos Torres^{1,2}

¹Viragh Center for Pancreatic Clinical Research and Care, Bloomberg Kimmel Institute for Immunotherapy, University School of Medicine, Baltimore, MD 21205, USA

²Department of Oncology, and the Sidney Kimmel Cancer Center at Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

Abstract

Immune checkpoint inhibition (ICI) has revolutionized treatment in cancers that are naturally immunogenic by enabling infiltration of T cells into the tumor microenvironment (TME) and promoting cytotoxic signaling pathways. Tumors possessing complex immunosuppressive TME's such as breast and pancreatic cancers present unique therapeutic obstacles as response rates to ICI remain low. Such tumors often recruit myeloid-derived suppressor cells (MDSCs) whose functioning prohibits both T-cell activation and infiltration. We attempted to sensitize these tumors to ICI using epigenetic modulation to target MDSC trafficking and function to foster a less immunosuppressive TME. We showed that combining a histone deacetylase inhibitor, entinostat (ENT), with anti-PD-1, anti-CTLA-4, or both, significantly improved tumor-free survival in both the HER2/neu transgenic breast cancer and the Panc02 metastatic pancreatic cancer mouse models. Using flow cytometry, gene expression profiling, and *ex vivo* functional assays, we characterized populations of tumor-infiltrating lymphocytes (TILs) and MDSCs, as well as their functional capabilities. We showed that addition of ENT to checkpoint inhibition led to significantly decreased suppression by granulocytic-MDSCs in the TME of both tumor types. We also demonstrated an increase in activated granzyme-B-producing CD8⁺ T effector cells in mice

Corresponding author: Evanthia Roussos Torres, MD/PhD, Johns Hopkins University Hospital, 1650 Orleans Street, Bunting Blaustein Cancer Research Building, 4M07, Baltimore, MD 21287, 410-955-2957 (phone), 410-614-8216 (Fax), ertorres@jhu.edu. Authors' Contributions:

Conception and design: Christmas, Brian J., Connolly, Roisin M., Azad, Nilo, Jaffee, Elizabeth M., Roussos Torres, Evanthia T.

Development of methodology: Christmas, Brian J., Jaffee, Elizabeth M., Roussos Torres, Evanthia T.

Acquisition of data (provided animals, acquired and managed animals, provided facilities, etc.): Christmas, Brian J., Rafie, Christine, Scott, Blake A., Ma, Hayley S., Cruz, Kayla, Woolman, Skylar, Armstrong, Todd D., Roussos Torres, Evanthia T.

Analysis and interpretation of data (e.g. statistical analysis, biostatistics, computational analysis): Christmas, Brian J., Hopkins, Alexander, Roussos Torres, Evanthia T.

Writing, review, and/or revision of the manuscript: Christmas, Brian J., Jaffee, Elizabeth M., Roussos Torres, Evanthia T.

Administrative, technical or material support (i.e. reporting or organizing data, constructing databases): Christmas, Brian J., Rafie, Christine, Hopkins, Alexander, Scott, Blake A., Ma, Hayley S., Armstrong, Todd D., Connolly, Roisin M., Azad, Nilo, Jaffee, Elizabeth M., Roussos Torres, Evanthia T.

Conflict of interest disclosure statement:

Through a licensing agreement with JHU, Dr. Jaffee has the potential to receive royalties from Aduro for a human GVAX vaccine. Dr. Jaffee receives research funding from Aduro Biotech and Bristol-Myers Squibb.

treated with combination therapy. Gene expression profiling of both MDSCs and TILs identified significant changes in immune-related pathways. In summary, addition of ENT to ICI significantly altered infiltration and function of innate immune cells, allowing for a more robust adaptive immune response. These findings provide a rationale for combination therapy in patients with immune-resistant tumors, including breast and pancreatic cancers.

Introduction:

Immunotherapy has changed the standard of care for some patients with advanced cancers and has great promise to prevent recurrence and prolong survival due to the long-term memory capabilities of the adaptive immune system. Although the presence of tumor-infiltrating lymphocytes (TILs) and evidence of immune activation have been associated with improved outcomes in HER2⁺ and triple-negative breast cancers (TNBC) (1,2), only modest response rates have been observed with use of single-agent immune checkpoint inhibitors (ICIs) (3–10). Preclinical studies have focused on understanding these therapies in TNBC models (11) but have yet to thoroughly examine other subtypes of breast cancer. It has also been previously reported that antibodies blocking programmed cell death protein-1 (anti-PD-1) can significantly improve the activity of anti-HER2 therapies, but we have yet to understand if there is any added benefit of other ICIs (12). Similarly, single-agent ICIs have been shown to be ineffective in the clinical setting in metastatic pancreatic ductal adenocarcinomas (PDAC) (13,14). However, efficacy has been seen using combinatorial approaches in preclinical models. It has been shown that priming the tumor with a vaccine sensitizes PDAC to anti-PD-1 therapy by inducing the infiltration of cytotoxic CD8⁺ T cells (15). In PDAC patients, a similar vaccine was reported to induce TILs (15). Improved efficacy of both anti-PD-1 and anti-CTLA-4 was achieved in murine models of PDAC by reprogramming immunosuppressive tumor-associated macrophages (TAMs) (16). Taken together, these studies indicate the possibility to sensitize traditionally non-immunogenic tumors to ICI therapy if TILs can be recruited and induced and/or immunosuppressive factors reduced within the tumor microenvironment (TME).

The natural resistance of breast and pancreatic tumors to single-agent ICI therapy is due to a lack of infiltrating T cells and an abundance of suppressive immune cells such as MDSCs. Specifically, granulocytic (G-MDSCs) and monocytic MDSCs (M-MDSCs) collectively inhibit T-cell function by depleting L-arginine through arginase-1 (Arg-1) expression and by inducing PD-L1 expression and the recruitment of regulatory T cells (T_{regs}) (16–19). High numbers of tumor-infiltrating myeloid cells correlates with early or local metastatic relapse (11). Previous studies have also shown changes in both circulating and tumor-infiltrating G-MDSCs in response to ICIs as a potential mechanism inhibiting antitumor responses (11,20,21). Thus, targeting and strategic reprogramming of these myeloid responses may overcome the immunosuppressive microenvironment and lead to an enhanced response to immune therapy through improved T-cell infiltration and function.

Studies using epigenetic modulatory drugs have shown promise in decreasing the infiltration and inhibitory capabilities of immunosuppressive cells. ENT ablates MDSC-mediated immunosuppressive activity when combined with ICIs in *in vivo* models of lung and renal

cell cancer (21). Tomita et al. reported decreased circulating G-MDSCs and M-MDSCs in clinical samples of breast cancer patients treated with ENT in combination with an aromatase inhibitor compared to placebo combined with aromatase inhibitor (22). Taken together, these data suggest that ENT can alter suppressive myeloid populations in multiple tumor types and synergize with immunotherapies to slow tumor growth and progression.

Our work explored the impact of ENT on the HER2⁺ breast and PDAC TMEs in immunocompetent mice. Combination therapy of ENT with anti-PD-1 or anti-CTLA-4 significantly improved survival compared to either agent alone or combined ICIs. ENT increased tumor infiltration by G-MDSCs and changed the polarity of their immunosuppressive ability to a nonfunctional phenotype. Flow cytometry and gene expression profiling of treated tumors demonstrated altered expression of functional molecules and specific genetic pathways involved in proliferation and motility. Our observations suggest that G-MDSCs can exhibit multiple phenotypes and support that a possible mechanism by which ENT allows ICIs to augment the infiltration and cytotoxicity of cancer-killing T cells is through the ablation of the immunosuppressive function of these infiltrating myeloid cells.

Materials and Methods:

Mice and cell lines

All animal studies were approved by Institutional Review Board of Johns Hopkins University. Animals were kept in pathogen-free conditions and were treated in accordance with institutional and American Association of Laboratory Animal Committee policies. The neu-N mice were originally from W. Muller McMaster University, Hamilton, Ontario, Canada. Colonies were renewed yearly from Jackson labs and bred in house by brother/sister mating. For studies of metastatic pancreatic cancer, we used male C57BL/6J (The Jackson Laboratories, stock #000664) mice. The T cell receptor (TCR) transgenic mice are specific for the immunodominant HER2/neu epitope neu₄₂₀₋₄₂₉ and were made in house at the JHU Transgenic Core Laboratory as previously described (23). These mice were bred in house by brother/sister mating, and each animal was assessed for expression of the desired neu-specific V β region via staining with the H-2D^d rat HER2/neu₄₂₀₋₄₂₉ PDSLRDLSVF tetramer (NIH Tetramer Core Facility).

All cell lines were regularly tested for Mycoplasma every three months in accordance to laboratory policy. NT2.5 cells were derived from spontaneous mammary tumors growing in female neu-N mice. *In vitro* cell lines were established and authenticated as previously described (24,25). Culture conditions for NT2.5 cells were as follows: 37°C, 5% CO₂ in RPMI 1640 (Gibco, cat. 11875-093) supplemented with 20% fetal bovine serum (Gemini, cat. 100-106), 1.2% HEPES buffer (Gibco, cat. 15630-080), 1% L-glutamine (Gibco, cat. 25030-081), 1% MEM non-essential amino acids (Gibco, cat. 11140-050), 0.5% penicillin streptomycin (Gibco, cat. 15140-122), 1% sodium pyruvate (Sigma, cat. S8636), 0.2% insulin (NovoLog, cat. U-100), 0.02% gentamicin (Sigma, cat. G1397). Panc02 is a murine pancreatic tumor cell line with ductal morphology derived from a methylcholanthrene-treated C57B1/6 mouse and authenticated as previously described (26,27). Culture conditions for Panc02 cells were as follows: 37°C, 10% CO₂ in DMEM (Gibco, cat. 11965-

084) supplemented with 10% fetal bovine serum (Gemini, cat. 100–106), 1% L-glutamine (Gibco, cat. 25030–081), 0.5% penicillin streptomycin (Gibco, cat. 15140–122). The T2D^q cells are a fibroblast cell line established via transfection with murine H-2D^q as previously described (28). Culture conditions for T2D^q cells are as follows: 37°C, 5% CO₂ in RPMI supplemented with 10% fetal bovine serum (Gibco, cat. 100–106), 1% L-glutamine (Gibco, cat. 25030–081), 1% sodium pyruvate (Gibco, cat. 11965–084), 1% MEM non-essential amino acids (Gibco, cat. 11140–050), 0.5% penicillin streptomycin (Gibco, cat. 15140–122), 0.0007% Hygromycin B (Roche, cat. 10843555001). T2D^q cells were cultured from frozen stocks and passaged three times before use.

Neu-N *in vivo* studies

The neu-N model is a syngeneic model where NT2.5 cells derived from a spontaneous mammary tumor were implanted via injection of 5×10^4 cells into the mammary fat pad of 7–8 week old female neu-N mice (24). NT2.5 cells were cultured from frozen stocks and passaged twice before injection. Tumors were allowed to seed for 3 days prior to initiating treatment with various drug combinations as described below. Untreated mice developed palpable tumors within 1 week.

PDAC hemi-splenectomy *in vivo* studies

To study metastatic PDAC, we used a hemi-splenectomy model using 7–8 week old syngeneic male C57BL/6J mice. This involved giving an intrasplenic injection of 2×10^6 of the pancreatic adenocarcinoma cell line (Panc02), as previously described (29–31). Panc02 cells were cultured from frozen stocks and passaged twice before injection. Metastases were allowed to establish for 7 days prior to starting treatment with various drug combinations as described below.

Rationale for mouse model selection

We selected the Panc02 hemi-splenectomy model in order to recapitulate late stage, metastatic disease, as this is most commonly how patients present. It should be noted that the Panc02 cell line was derived from a chemically induced tumor and contains a high mutational burden. Other groups circumvent this issue by using the genetically engineered KPC PDAC model, which results in pancreatic tumors that are more genetically and pathologically similar human PDAC (30,32,33). While the KPC model more accurately recapitulates primary disease in humans, these animals take a minimum 16 weeks to develop locally invasive PDAC and develop salivary tumors before metastases occur. These tumors grow large and often prohibit maintenance of mice long enough to study natural metastases (30). We recognize the shortcomings of using the Panc02 cell line, which is why we paired the findings in this model with those in an extremely tolerant and non-immunogenic breast cancer model (31). Future work with the KPC model could yield informative data to further translate the outcomes from preclinical models to human disease.

Drug dosing and selection

The ENT dosing solution (5 mg/kg) was made by suspending solid ENT (generously provided by Syndax Pharmaceuticals) in 0.5% methylcellulose via sonication and given by

oral gavage daily for three weeks as described in Fig. 1a. To imitate oral gavage in control groups, 0.5% methylcellulose was given to vehicle mice, as well as mice receiving only antibody therapy, during the three weeks of ENT dosing. Mice received anti-PD-1 and/or anti-CTLA-4 twice a week for three weeks as stated in Fig. 1a. All studies in neu-N model were performed with and without an anti-HER2 antibody to mimic treatment with and without trastuzumab. Anti-HER2 antibody was given once a week for three weeks as described in Fig. 1a. Following initial three weeks of treatment, as described above, maintenance dosing was continued with antibodies every other week. During maintenance therapy, animals receive two doses of anti-PD-1 and/or anti-CTLA-4 as well as one dose of anti-HER2 every other week as shown in Fig. 1a. Dosage of antibodies was as follows: anti-PD-1, 100 µg/mouse via intraperitoneal injection (i.p.); anti-CTLA-4, 100 µg/mouse i.p.; anti-HER2, 100 µg/mouse i.p. Monoclonal antibodies were obtained from BioXcell [anti-PD-1 (clone RPM1-14), anti-CTLA-4 (clone 9H-10), and anti-HER2 (clone 7.16.4)] and all were diluted to 0.5 mg/mL in PBS. Isotypes were used to treat vehicle mice and were also obtained from BioXcell [anti-PD-1 isotype: rat IgG2a (clone 2A3); anti-CTLA-4 isotype: polyclonal Syrian Hamster IgG; anti-HER2 isotype: mouse IgG2a (clone C1.18.4)]. All isotype antibodies were also diluted in PBS to 0.5 mg/mL. Dosages of each antibody were based off of prior studies (11). For mice receiving dual checkpoint antibody therapy, anti-CTLA-4 was given alone twice prior to addition of anti-PD-1, given that anti-CTLA-4 has been described as a T-cell repertoire-expanding agent, whereas anti-PD-1 has been determined to expand antigen-experienced T cells and help prevent the exhaustion of activated T cells (34).

For preliminary experiments using the demethylating agent SGI-110 (Astex) and the histone deacetylase inhibitor ACY-1215 (Acetylon), the PDAC hemi-splenectomy described above was used. Murine GVAX was prepared by irradiating a 1:1 mixture of Panc02 and the GM-CSF-secreting bystander line B78H1 as previously described (27,35). A low dose of cyclophosphamide (100 mg/kg) was given four days post-surgery and GVAX was administered five days post-surgery sub-cutaneously into three limbs of the mice. SGI-110 was administered subcutaneously into the flank of the animal at 24.4 mg/kg once a week over the course of three weeks while ACY-1215 was administered i.p. at 50 mg/kg daily. Following dosing of ACY-1215 and GVAX, survival was monitored until 120 days post-surgery.

Rationale for use of entinostat

It has previously been shown that inhibition of class I HDACs targeted by ENT improves TLR signaling and cytokine production in myeloid cells (36), as well as cytokine signaling in T cells (37). Therefore, we hypothesized that use of ENT acted on these cell types to help sensitive non-immunogenic tumors to ICIs. Although we showed that ENT did improve efficacy of ICIs in the neu-N and PDAC models, the mechanism appeared to be largely dependent on inhibiting the ability of MDSCs to produce immunosuppressive factors, specifically arginase-1 (Arg-1). Although a specific class I HDAC has not been identified as a regulator of Arg-1, a few studies illustrate a potential mechanism for the regulation of *Arg1* transcription involving HDAC2. It has previously been shown that a complex of Tet2 and HDAC2 is required for regulating the transcription of various inflammatory genes in

macrophages, such as *Il6* and *Tnfa*, and this regulation can be abrogated with HDAC2 siRNA, as well as entinostat *in vitro* (38). Pan et al. showed that myeloid-specific knockout of Tet2 resulted in decreased Arg-1 expression in TAMs, as well as decreased ability to suppress T cell proliferation (39). Taken together, these studies suggest Arg-1 could be regulated in a similar way as IL6, where Tet2 recruits HDAC2 to the arginase-1 promoter to regulate gene transcription. Rationale for addition of ICIs to ENT was also provided by two promising ongoing clinical trials that combine nivolumab, ipilimumab and ENT (NCT02453620, NCT03250273). However, mechanisms of action of these drug combinations remain elusive, and thus, these preclinical studies were designed to inform rational design of clinical correlates for these trials and elucidate mechanisms behind potential anti-tumor responses.

Survival and tumor growth assays

Following injection of either Panc02 or NT2.5 cells, mice were treated for three weeks as depicted in Fig. 1a, and then received maintenance therapy until succumbing to disease or requiring sacrifice due to tumor burden (up to 2 cm³). In the neu-N model, mice were examined for palpable tumors starting one-week post tumor cell challenge, and subsequently measured by calipers ($\pm$ 0.01 mm) twice weekly. Survival experiments were continued until mice were euthanized according to protocol due to tumor burden (neu-N model) or the development of ascites (PDAC model).

Tumor dissociation

To obtain single-cell suspensions from breast tumors, tumors were harvested, weighed, diced, and then dissociated using a tumor dissociation kit (Miltenyi Biotec, cat. 130–096-730) and the OctoDissociator (Miltenyi Biotec) per the manufacturer's instructions. The 37C_m_TDK_2 program was used to dissociate tumors per the manufacturer's instructions. Samples were filtered using a 40 μ m cell strainer and red blood cells were lysed using ACK lysis buffer (Quality Biological, cat. 118–156-721). The resulting single-cell suspensions were used for subsequent isolation of specific immune cell types or flow cytometry as described below. Blood from neu-N mice was obtained via retro-orbital bleeds as per protocol. Red blood cells were lysed using ACK lysis buffer (Quality Biological, cat. 118–156-721). Isolated cells were analyzed via flow cytometry.

To obtain single-cell suspensions of livers containing metastases in the PDAC model, livers were harvested and processed by mashing the liver through 100 μ m and 40 μ m cell strainers as previously described (35). Red blood cells were lysed using ACK lysis buffer (Quality Biological, cat. 118–156-721), and liver pellets were resuspended in 40% Percoll (GE Healthcare Life Sciences, cat. 17–0891-01) and underlayered with 80% Percoll. The immune cell layer was then removed and analyzed via flow cytometry.

Immune cell isolation

G-MDSCs were isolated from single-cell suspensions from tumors following tumor dissociation using Miltenyi Biotec's Myeloid-Derived Suppressor Cell Isolation Kit (cat. 130–094-538) according to the manufacturer's protocol. Ly6G⁺ cells were positively selected to isolate G-MDSCs from Ly6G⁺ M-MDSCs and were passed through LS columns

(Miltenyi Biotec, cat. 130–042–401) twice to increase purity. Eluted G-MDSCs were then used for downstream assays described below.

CD8⁺ neu-specific T cells were negatively isolated from spleens of the TCR transgenic mice described above by mashing spleens through 100 µm cell strainers. Red blood cells were lysed using ACK lysis buffer (Quality Biological, cat. 118–156–721), and CD8⁺ T cells were isolated from spleen pellets using the EasySep Mouse CD8⁺ Isolation Kit (StemCell, cat. 19853) per the manufacturer's instructions. CD8⁺ T cells were then used for the *ex vivo* suppression assay described below.

Flow cytometry

Isolated single-cell suspensions were washed and then incubated for 30 minutes with Live/Dead Near-IR (ThermoFisher, cat. L10119) according to the manufacturer's protocol, followed by a 30-minute incubation with the appropriate flow cytometry antibodies (Supplementary Table S1). For samples being analyzed for cytokine expression, single-cell suspensions obtained from tumor dissociations were plated at 37°C with anti-CD3/CD28 beads (ThermoFisher, cat. 11453D) overnight at a bead-to-cell ratio of 1:1 per the manufacturer's instructions for T-cell activation. A protein transport inhibitor cocktail (eBioscience, cat. 00–4980–03) was introduced at 1x concentration during the last 4–6 hours of stimulation. The samples were harvested after 16 hours. For samples being stained for intracellular markers, cells were fixed and permeabilized (Transcription Factor Fixation/Permeabilization kit, eBioscience, cat. 00–5523–00) and then incubated with the appropriate antibodies for 30 minutes. Samples were run on a CytoFLEX or Gallios (Beckman Coulter) cytometer and analyzed using FlowJo (FlowJo LLC) or Kaluza (Beckman Coulter).

Flow cytometry gating strategies

G- and M-MDSCs were identified by gating out debris from bulk tumor based on forward scatter and side scatter. Single cells were then gated by forward scatter-area and forward scatter-height. Dead cells were then gated out of the bulk tumor cell population. T cells and mature macrophages were gated out by selecting the CD3 and F4/80 double negative population, respectively. CD11b⁺ cells were then gated, and G- and M-MDSCs were differentiated using Ly6G vs. Ly6C. G-MDSCs were defined as Ly6G⁺ Ly6C^{lo}, and M-MDSCs were defined as Ly6G[–] Ly6C⁺. See Supplementary Fig. S1a.

Of note, we found that staining with CD45 in combination with more specific markers, such as CD3 and CD11b, did not further exclude non-immune cell types from the final gated populations compared to single staining with CD3 or CD11b. For example, there were very few cells that were positive for CD3 or CD11b and negative for CD45. Given these findings, we did not use CD45 in our final FACS panels. See Supplementary Fig. S1b.

In order to characterize the CD8⁺ T cells, debris was gated out of bulk tumor based on forward scatter and side scatter. Dead cells were then gated out of the bulk tumor population and lymphocytes were subsequently selected as CD3⁺ and then gated by CD4 vs. CD8. To determine the activation state of CD8⁺ T cells, CD8⁺ cells were then gated by CD44 vs. CD62L, as well as CD8 vs. granzyme-B. The exhaustion state of CD8⁺ T cells was determined by gating CD8⁺ cells by PD-1 vs. Lag3. In order to characterize the CD4⁺ T

cells, debris was gated out of bulk tumor based on forward scatter and side scatter. Dead cells were then gated out of the bulk tumor population and lymphocytes were subsequently selected as CD3⁺ and then selected as CD4⁺. See Supplementary Fig. S2a.

Ex vivo suppression assay

Isolated G-MDSCs were co-cultured with peptide-specific stimulated T cells and quantified for T-cell proliferation. G-MDSCs were isolated from tumors following tumor dissociation as described above. Eluted G-MDSCs were co-cultured with 1×10^5 CFSE-labeled CD8⁺ neu-specific T cells isolated from untreated neu-specific TCR-transgenic mice (23) at varying ratios (1:1 – 1:8 MDSC:T cells). Prior to plating, T-2D^q antigen presenting cells (APCs) were pulsed with either R-neu_{420–429} or control peptide (NP) 0.4 µg peptide for 4 hours; both purchased from the Johns Hopkins University Synthesis and Sequencing facility at greater than 95% purity. The APCs were simultaneously added to the cocultured CD8⁺ T cells and MDSCs at a ratio of 2:1 APCs:T cells, and plates were incubated at 37°C for a total of 48 hours. Cells were then harvested, stained With Live/Dead NIR (ThermoFisher, cat. L10119), as well as CD8 and Thy1.2 and analyzed via flow cytometric analysis as described above. Dilutions of initial CFSE were indications of T-cell divisions, where fewer divisions indicated greater suppressive G-MDSC activity. All antibodies used are listed in Supplementary Table S1.

Due to the small number of infiltrating M-MDSCs, we were unable to isolate sufficient numbers of these cells to perform a suppression assay. We attempted using flow cytometry as an alternative method of assessing M-MDSCs. However, the sorted M-MDSCs had poor viability, which had -confounding effects on this assay.

Arginase assay

Arginase activity was measured colorimetrically *ex vivo* using Abcam's Arginase Activity Assay Kit (cat. ab180877). G-MDSCs were isolated from tumors and processed as described above and plated per manufacturer's instructions. In short, cells were lysed with kit's lysis buffer at 1×10^6 /1mL, and plated in varying dilutions (30k or 60k cells per well) in duplicate in a flat-bottom, low-retention plate carefully to avoid bubble formation. Target samples were incubated for 20 minutes at 37°C with H₂O₂ substrate solution, while background wells were incubated with additional buffer. Standards were prepared per kit instructions, and the enzymatic reaction mixture was prepared and added to all wells. Raw absorbance values were immediately obtained over a 30-minute period using a plate reader (Molecular Devices SpectraMax M3) at OD=570nm at 37°C. Arginase Activity Units were then calculated from raw absorbance values. $OD = (OD2 - OD_{bg2}) - (OD1 - OD_{bg1})$ was used to obtain the nmol of H₂O₂ generated by arginase, collected from a standard curve of known H₂O₂ concentrations. Arginase activity is calculated as $(B / T * V) * D$ in units/mL, where B is amount of H₂O₂ from standard curve (nmol), V is the sample volume added into reaction well (µL), D is sample dilution factor. One unit of Arginase activity refers to the amount of arginase that will generate 1.0 nmol of H₂O₂ per minute at pH 8 at 37°C.

Gene expression profiling

RNA was extracted from whole tumors or G-MDSCs isolated from tumors using the Qiagen RNeasy extraction kit (cat. 74104). Using a NanoDrop ND-1000, RNA quality and quantity were determined (samples with A260/280 < 1.8 were excluded). Gene expression was measured on the NanoString nCounter Analysis System (NanoString Technologies). The NanoString PanCancer Immune CodeSet (cat. XT-CSO-MIP1–12) was used to perform the nCounter Gene Expression Assay for whole tumor samples, whereas the Mouse Myeloid Innate Immunity CodeSet (cat. XT-CSO-MII2–12) was used to perform nCounter Gene Expression Assay for isolated G-MDSCs. Quantification of target mRNA in each sample was performed by detection within the nCounter Digital Analyzer. The data obtained from the PanCancer Immune CodeSet were deposited on the public database GEO under the accession number GSE121031. The data obtained from the Mouse Myeloid Innate Immunity CodeSet were deposited on the public database GEO under the accession number GSE121030.

Gene expression analysis

Data from the NanoString nCounter system was normalized to the internal positive controls and housekeeping genes using the recommended settings in the nSolver software normalization module (NanoString Technologies). Normalized data was exported and differential expression analysis was performed using a linear model method with the limma package (40) for the R programming language. To identify pathways which were differentially expressed in each treatment, the gage package (41) was used to test for differentially expressed KEGG pathways. Pathways of interest were visualized using the pathview package (42).

QC plots for NanoString cell typing

The QC plots show the correlation between genes included in the assay that are used to call specific cell types. nSolver uses expression data from *Lag3*, *Cd244*, *Eomes*, and *Ptger4* to call the “Exhausted CD8” set and expression data from *Cd6*, *Cd3d*, *Cd3e*, *Sh2d1a*, *Trat1*, and *Cd3g* to call the “T-cell” set. These plots report how well the expression of the genes within each cell type set correlate with each other. In our data from the PanCancer Immune panel, the algorithm to call cell types uses *Cd3g* and *Cd3d* for “T cells” and *Lag3* for “Exhausted CD8” cells. Due to the inherent variability between animals, more than the recommended 4 biological replicates are likely necessary to prevent the nSolver algorithm from discarding genes used to make cell type calls. See Supplementary Fig. S2b, S2c.

Quantative-PCR (qPCR)

Reverse transcription of mRNA and subsequent qPCR was performed on RNA isolates to validate expression changes seen in the NanoString analysis. RNA extracted from isolated G-MDSCs and whole tumor (Qiagen RNeasy kit, cat. 74104) were normalized to 10 ng/μL, and 1 μg of total RNA was used for reverse transcription. The SuperScript VILO cDNA Synthesis Kit (Invitrogen #11754050) was used following the manufacturer’s instructions. qPCR of subsequent cDNA was performed using gene specific proprietary primer and probe constructs for selected genes obtained from TaqMan (ThermoFisher, see individual product

numbers per gene), and used with TaqMan Universal Master Mix II, no UNG (Applied Biosystems #4440040) following manufacturer's instructions. qPCR was performed on the StepOnePlus real-time PCR system (Applied Biosystems). To validate the ERBB pathway, *Tgfa* (Mm00446231_m1) and *Egf* (Mm004438696_m1) were checked; for the mTOR pathway, *Tgfa* (Mm00446231_m1) and *Frizzled4* (Mm00433382_m1) were checked; for the VEGF pathway, *Vegfa* (Mm01281449_m1) and *Cox2* (Mm03294838_g1) were checked. For the Leukocyte transendothelial migration pathway, *Pik3cg* (Mm00445038_m1) and *Vcam1* (Mm01320970_m1) were checked; for the osteoclast differentiation pathway, *Infγ* (Mm01168134_m1) and *Il1b* (Mm00434228_m1) were checked. *18s* (Mm04277571_s1) was used as a control, and *Stat3* (Mm01219775_m1) and *Arg1* (Mm00475988_m1) were also checked. The 2-step real-time PCR cycling conditions used were 95 °C for 20 s, 40 cycles of 95 °C for 3 s, and then 60 °C for 30 s. Gene expression was quantified from raw data by calculating mean CT values from the three replicates and comparing the CT values of each gene to the 18s reference gene. Fold changes were quantified as 2^{-CT} and plotted against corresponding NanoString fold change values.

Western blots

G-MDSCs were isolated from whole tumor of treated animals using Miltenyi Biotec's MDSC Isolation Kit and pooled by treatment group. Samples being used for phospho-STAT3 analysis were subsequently stimulated with IL6 (20 ng/μL) for 25 minutes. Samples were lysed in RIPA buffer with added 1μM DTT, 1μM PMSF, and 1:100 protease/phosphatase inhibitor cocktail (Cell Signaling #5872S) and quantified by BCA (Pierce, #23225). 125 μg of protein was run in 4–12% Bis-Tris gels under denaturing conditions. The LiCor Odyssey developing and imaging system was used, and the following primary antibodies were diluted in Odyssey Blocking Buffer (TBS) plus 0.2% Tween® 20 at specified concentrations: Abcam anti-interferon gamma rabbit (1:1000), Abcam anti-VEGFA rabbit (1:1000), Abcam anti-EGF (1:1000), Cell Signaling TNFα Rabbit monoclonal antibody (mAb) (1:1000), Cell Signaling PI3 Kinase p110γ Rabbit mAb (1:1000), Cell Signaling Stat3 Rabbit mAb (1:2000), Cell Signaling Phospho-Stat3 (Tyr705) Rabbit mAb (1:2000). Membranes were blocked with Odyssey blocking buffer TBS for 1hr and then incubated with primary antibody solution overnight at 4°C with gentle shaking. Membranes were washed with 1X TBS-T and subsequently incubated with the following secondary antibodies: 680RD Donkey anti-Chicken IgG (1:10,000), 800CW Donkey anti-Rabbit IgG (1:10,000) in Odyssey Blocking Buffer (TBS) plus 0.2% Tween® 20. Membranes were protected from light during incubation with secondary mixture for one hour at room temperature with gentle shaking. Membranes were subsequently washed with 1X TBS-T and imaged with the Odyssey® imaging system. Images were analyzed using ImageJ.

Statistical analyses

For survival data, results were plotted using a Kaplan-Meier curve and statistical significance was determined via a log-rank test, (n = 10 mice/group). For tumor growth data, statistical significance was determined using a two-way analysis of variance (ANOVA) (n = 10 mice/group). For all flow and arginase data, mice were sacrificed during the third week of treatment. For dot plots, each dot represents one mouse and each bar represents mean ±

SEM. (n = 3–5 mice/group). Significance was determined by a one-way ANOVA with Tukey's multiple comparisons test. All experiments were repeated at least 3 times. All statistical analysis listed here were performed using GraphPad Prism v7.00. Statistically significant p values are abbreviated as follows: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Results:

Baseline expression of immune checkpoints on bulk tumor and immune cells

Previous studies have examined the expression of PD-L1 as a predictor of response and show varied correlation between protein expression and response (8,43,44). Thus, we evaluated changes in expression of PD-1, PD-L1, and CTLA-4 via flow cytometry in bulk tumors, as well as on intratumoral CD8⁺ T cells, CD4⁺ T cells, and MDSCs (cell types determined by gating strategies outlined in methods section) to determine if changes in expression following treatment with ENT alone validates a rationale for combining ICIs with ENT. We found no significant changes in expression of these checkpoint molecules on bulk tumor, as well as on intratumoral CD8⁺ T cells, CD4⁺ T cells, and MDSCs, with the exception of PD-1 expression on CD8⁺ T cells, which showed an increase in expression (Supplementary Fig. S3a-d). Thus, we considered that increased expression of checkpoint molecules within the TME may not be predictive of response to ICIs in our models. However, various groups have previously shown synergy between HDAC inhibition and various immunotherapies, including checkpoint inhibition, which does provide rationale for addition of these checkpoint inhibitors to ENT as completed in our study (11,21,36,37,45). Specifically, previous studies showed that in the 4T1 breast cancer model, 5-azacytidine plus ENT decreased infiltration of MDSCs into the tumor and concluded that it was ENT driving this effect (11). We also performed a comparison of the HDACis ENT and ACY-1215 as well as a demethylating agent (SGI-110) in the murine PDAC model. We determined only ENT significantly improved survival over other drugs (Supplementary Fig. S4a). Thus, we chose to move forward using ENT given its efficacy in both tumor models. We substituted anti-PD-1 for GVAX as our immunotherapy, which yielded the same survival benefit when combined with ENT (Fig. 1b, Supplementary Fig. S4a). Thus, moving forward we used checkpoint inhibitors as our immunotherapy instead of GVAX.

ENT priming sensitizes tumors to checkpoint inhibition and improves survival

We hypothesized that ENT would sensitize naturally immune-resistant tumors, which often fail to recruit T cells into their TME, to ICIs. To test this hypothesis, we evaluated ENT given in combination with anti-PD-1, anti-CTLA-4, or a combination of the three drugs in neu-N/HER2⁺ breast cancer and Panc02 metastatic PDAC models. A final dosing schedule is shown in Fig. 1a. In the PDAC model, ENT+anti-PD-1, ENT+anti-CTLA-4, ENT+both ICIs, and anti-PD-1+anti-CTLA-4 significantly improved survival over treatment with vehicle or ENT alone (Fig. 1b). In the neu-N model, treatment with anti-HER2 significantly improves survival and inhibits tumor growth (Fig. 1c, 1d), consistent with studies showing efficacy in patients treated with HER-directed therapy (46). Combination ENT+anti-PD-1+anti-HER2, ENT+anti-CTLA-4+anti-HER2, or ENT+anti-HER2+both ICIs significantly improved survival compared to vehicle or ENT alone in the neu-N breast tumor

model (Fig. 1c). Combination immunotherapy alone did not improve survival when compared to vehicle or ENT (Fig. 1c). The combination of ENT+both ICIs in the absence of anti-HER2 significantly improved survival (Supplementary Fig. S4b). Future studies will focus on the specific contribution of anti-HER2 to these therapies. However, for the remainder of the experiments presented herein, addition of anti-HER2 served to prove that the results were similarly efficacious (with and without it) to better inform the use of ENT +ICIs in patients receiving ongoing anti-HER2 therapy. Combination therapies with ENT +single ICIs led to a significant decrease in tumor burden in the neu-N model, both with and without anti-HER2 (Fig. 1c, Supplementary Fig. S4c)

Combined therapy shifts the TME from an M-MDSC to a G-MDSC dominated milieu

Given the improved antitumor immune responses observed in the mouse tumors, we next asked if ENT+ICIs were affecting MDSCs, as previous studies demonstrate decreased recruitment of MDSCs into the TME following similar therapies (11). To evaluate changes in MDSC populations, breast tumors and PDAC liver metastases were harvested following a full course of treatment. Immune cells were isolated and were characterized as G- and M-MDSCs via flow cytometry (Fig. 2a, Supplementary Fig. S1a). Analysis showed that treatment with ENT in combination with anti-PD-1 or anti-CTLA-4 shifted the MDSC population to be dominated by the less immunosuppressive G-MDSCs (20) (Fig. 2b, 2c). Single-agent treatment with ENT significantly increased G-MDSCs in PDAC hepatic metastases (Fig. 2c), resembling the increase trends observed in the periphery of tumor-bearing neu-N mice (Supplementary Fig. S5a, S5b). However, the addition of ICIs was required to observe significant G-MDSC induction in the primary breast tumors. The observed increase in G-MDSCs was not the result of a redistribution between the proportions of myeloid cells since treatment of mice with combination therapies significantly increased the absolute number of G-MDSCs adjusted to tumor or liver weight (Supplementary Fig. S5c, S5d). Treatment with ICIs alone did not alter MDSC infiltration into the TME compared to vehicle, and no significant difference in infiltration by M-MDSCs in either model was seen (Fig. 2d, 2e).

ENT alters G-MDSC function, rendering them less immune suppressive

Because we observed increased recruitment of G-MDSCs into tumors of mice in the setting of decreased tumor burden, we evaluated the function of these cells. Traditionally, production of Arg-1 by MDSCs is used as a marker of their immunosuppressive capability (21). Thus, we performed immunosuppression assays and determined Arg-1 production both *ex vivo* and by flow cytometry. Data from immunosuppression assays revealed that the ability of G-MDSCs to suppress T-cell proliferation is inhibited in mice receiving ENT (Fig. 3a). Arg-1 protein expression was evaluated in both G- and M-MDSCs using a colorimetric arginase activity assay in the neu-N model (Fig. 3b, 3c) and by flow cytometry in the neu-N and PDAC models (Supplementary Fig. S6a-d). Results from both assays indicated that G- and M-MDSCs isolated from ENT+ICI-treated mice produced significantly less Arg-1. Taken together, these data suggest that the impaired immunosuppressive function of tumor-infiltrating G-MDSCs is, at least in part, due to decreased Arg-1 production.

Evaluation of PD-L1 was also investigated as a measure of MDSC suppressive cell function. We analyzed the surface expression of PD-L1 on intratumoral G-MDSCs by flow cytometry. PD-L1 expression was significantly reduced on G-MDSCs with ENT+ICI treatment (Supplementary Fig. S6e, S6f). This suggested that treatment of G-MDSCs with ENT reduced their ability to inhibit T-cell activity by diminishing availability of the PD-L1/PD-1 T-cell inhibitory pathway.

ENT+ICI therapy significantly alters the signaling pathways involved in myeloid function

ENT alters the global acetylation state of cells (47), but its effect on subpopulations of myeloid cells has not been thoroughly studied. To identify possible mechanisms for their impaired function, we utilized NanoString transcript profiling to evaluate broad changes in the transcriptome of isolated G-MDSCs induced by treatment. Treatment with ENT and subsequently anti-CTLA-4 and/or anti-PD-1 significantly altered the expression of many genes important to G-MDSC's function (Fig. 4a). Further evaluation revealed relevant pathways that were significantly up- or downregulated and contained significantly altered genes that drove the differential regulation of these pathways (Fig. 4b, 4c, Supplementary Fig. S7a, S7b). We narrowed our focus to certain pathways and genes that may be more specifically responsible for the observed MDSC phenotypes and only report treatment combinations that showed significant changes across all biological replicates (Fig. 4b, 4c). Our data reveal several observations. First, treatment with ENT alone and ENT+anti-PD-1 decreased expression of genes in both the ErbB and VEGF signaling pathways compared to vehicle-treated mice (Fig. 4b). However, expression was increased in both pathways in mice treated with ENT+anti-CTLA-4 (Fig. 4c). Second, treatment with ENT+anti-PD-1 downregulated the mTOR pathway (Fig. 4b), whereas ENT+anti-CTLA-4 increased overall expression of the pathway (Fig. 4c). Fig. 4d depicts the VEGF pathway, highlighting key genes altered due to treatment with ENT compared to vehicle. Fourth, significant changes due to ENT+ICI treatment in pathways involving adhesion and motility suggested that treatment with ENT+ICI increased G-MDSC trafficking, which may explain the observed increased infiltration of G-MDSCs (Supplementary Fig. S7a, S7b). Fifth, changes in pathways controlling different hormone production, apoptosis, and MAPK signaling were observed (Supplementary Fig. S7a, S7b). Finally, we found over 100 significantly differentially expressed genes independent of the aforementioned pathways. Key genes of interest are listed (Supplementary Fig. S7c). Expression of significantly altered pathways and genes of interest were validated using quantitative-PCR (qPCR) (Supplementary Fig. S8). We also validated these findings by investigating changes in protein expression via western blot. EGF expression was analyzed to represent the ErbB pathway, VEGFA for the VEGF pathway, and TNF α for the mTOR pathway. Protein expression of STAT3, a key gene of interest, was evaluated along with activation of the STAT3 protein via phosphorylation at Tyr705. These data show that although the protein expression changes observed in G-MDSCs isolated from treated mice did not correlate with changes seen at the mRNA level (Supplementary Fig. S9a, S9b, Supplementary Fig. S7c), a decrease in phospho-STAT3 was observed in treated mice (Supplementary Fig. S9a, S9b), indicating a decrease in STAT3 activity. Changes in protein expression of EGF, VEGFA, and TNF α correlated with mRNA expression in groups treated with ENT+anti-CTLA-4 compared to ENT-treated animals (Supplementary Fig. S9c). Changes in protein expression of EGF correlated with mRNA

expression in groups treated with ENT compared to vehicle-treated animals (Supplementary Fig. S9d). However, changes in EGF protein expression did not correlate with mRNA expression in the ENT+anti-PD-1 group compared to vehicle-treated animals (Supplementary Fig. S7c, S9e). No correlation between changes in VEGFA protein expression and mRNA expression in either the ENT or ENT+anti-PD-1 groups was seen (Supplementary Fig. S9d, S9e), as well as for TNF α expression in the ENT+anti-PD-1 group (Supplementary Fig. S9e). Future work will focus on changes in expression of both genes and proteins within these pathways to determine if they play a mechanistic role in altered G-MDSC function.

ENT+ICIs promote infiltration of effector CD8⁺ T cells independent of T_{reg} infiltration

After determining that ENT+ICIs inhibited the immunosuppressive function of G-MDSCs, we sought to establish whether this change was sufficient to promote infiltration of functioning CD8⁺ T cells into the TME (36). Cells were isolated from tumors and changes within the T-cell populations were evaluated using flow cytometry. We observed increased infiltration of CD8⁺ T effector cells and decreased naïve and memory CD8⁺ T cells when ENT was combined with anti-PD-1, anti-CTLA-4, or both (Fig. 5a, 5b). Evaluation of the proliferative capacity of infiltrating CD8⁺ T cells within the PDAC TME showed increased proliferation with the addition of ICIs, determined by Ki67 staining (Supplementary Fig. S10a). PDAC mice treated with ENT+anti-CTLA-4 also showed a significant increase in granzyme-B-producing CD8⁺ T cells. A pattern suggesting an increase in granzyme-B-producing CD8⁺ T cells was also observed in the anti-HER2+ENT+anti-CTLA-4-treated neu-N mice. The addition of anti-PD-1 reduced this effect in both tumor types (Fig. 5c, 5d). IFN γ production by CD8⁺ T cells was also measured. However, combination-treated mice only showed a trending increase in both models (Supplementary Fig. S10b, S10c). These data indicate that the combination of ENT with ICIs induced infiltration of effector CD8⁺ T cells with cytotoxic capabilities.

Although the CD8⁺ T cells appeared to exhibit improved antitumor activity, we wanted to characterize the ability of treatment to promote T-cell activation versus exhaustion. We evaluated the expression of various surface markers in CD8⁺ T cells from tumors in both models, and first observed significantly increased expression of PD-1 (Supplementary Fig. S11a, S11b). Because PD-1 can be a sign of activation as well as exhaustion, we evaluated the co-expression of a second marker Lag3. Co-expression of these molecules is indicative of exhausted CD8⁺ T cells, whereas the absence of Lag3 on PD-1⁺ CD8⁺ T cells is indicative of activated T cells (48,49). Our results only showed a significant increase in PD-1⁺Lag3⁻CD8⁺ T cells infiltrating tumors from both models (Fig. 5e, 5f). We noted a pattern of non-significant increases of exhausted PD-1⁺Lag3⁺CD8⁺ T cells (Supplementary Fig. S11c, S11d). These data support that most CD8⁺ T cells infiltrating tumors in response to ENT+ICIs were activated and not exhausted. We also checked the co-expression of Tim3 and CTLA-4 with PD-1 as additional markers of exhaustion (48,49) and found similar trends. Analysis of transcription factor expression revealed a 50% increase in terminally exhausted CD8⁺ T cells in mice treated with ENT+anti-PD-1+anti-CTLA-4 in both neu-N tumors and PDAC metastases (Supplementary Fig. S11e, S11f). These are also the treatment groups with the highest proliferative capacity. This analysis showed no significant change in

progenitor exhausted CD8⁺ T cells in the neu-N model and a 35–60% increase in the PDAC model (Supplementary Fig. 11g, 11h). Taken together, these data support that treatment generally induces an activated T-cell state.

Previous studies revealed that ENT+interleukin-2 induces a decrease in tumor burden, which correlates with an increase in effector T cells and a reduction in tumor-infiltrating regulatory T cells (T_{regs}) (33). Because T_{regs} have also been described as a major immunosuppressive component of the TME (50), we used flow cytometry to determine if ENT altered T_{reg} infiltration. However, we only observed a trending decrease in T_{regs} in both models (Fig. 6a, 6b), which was not statistically significant.

ENT + ICIs significantly alters gene expression profiles in immune signaling pathways

Our data showed that ENT broadly impacted multiple populations of immune cells within the TME. Thus, we performed general immune transcriptome profiling on whole tumors isolated from the neu-N model using a PanCancer immune-profiling gene panel for the NanoString platform. Numerous significant changes were found in multiple genes and pathways (Fig. 7a). Specifically, two pathways were found to be significantly altered (listed in the table in Fig. 7b). We only report treatment combinations that showed significant changes. One of these pathways, leukocyte trans-endothelial migration, was upregulated, suggesting a mechanism by which leukocytes may be able to traffic into the TME and contribute to an antitumor response in mice treated with ENT+anti-CTLA-4 (Fig. 7b). The osteoclast differentiation pathway was significantly upregulated in tumors from mice treated with ENT+anti-CTLA-4 vs. ENT alone (Fig. 7b). Many of the genes involved in this pathway were also involved in pathways that contribute to the regulation of adaptive immunity. Our experiments also identified 20 individual genes significantly altered in cells from whole tumors treated with ENT+anti-CTLA-4 and ENT+anti-CTLA-4+anti-PD-1 (Supplementary Table S2). Detailed investigation of these genes is necessary to determine how other immune compartments are being affected. Use of NanoString enables evaluation of immune cell subsets and related pathways. However, none of the immune cell subsets were significantly altered in this evaluation. Fig. 7c demonstrates an increase in gene expression profiles that suggest both “T cells” (determined by expression of *Cd3g* and *Cd3d*) and “exhausted CD8” (determined by expression of *Lag3*) showed a trending increase in treated groups, corroborating findings from flow cytometry (Fig. 5). QC plots depicting the correlations of the genes used to call these cell types are included in Supplementary Fig. S2b, S2c. Platforms for more directed approaches to investigation of gene expression profiling are still evolving and can be limited in feasibility by large cohort animal studies, as seen with our findings using the NanoString platform. Future studies will evaluate gene expression at the single-cell level to work around the inherent biological variability associated with animal studies.

Discussion

We demonstrated in the neu-N and PDAC models that mice treated with the combination of ENT+ICIs exhibited significantly improved survival compared to mice treated with either ENT or ICIs alone. We also showed that the efficacy of ENT+anti-CTLA-4 treatment was

associated with a significant increase in tumor-infiltrating G-MDSCs that exhibited impaired immunosuppressive function. We also identified changes in important signaling pathways that converged to control and regulate myeloid immunosuppressive activities, which suggest novel mechanisms responsible for the observed MDSC dysfunction in tumors. ENT was primarily responsible for significantly affecting genes controlling myeloid function, such as those within the VEGF and ErbB pathways, and may have also acted to prime CD8⁺ T cells by increasing their expression of PD-1, although the addition of anti-PD-1 further augmented these changes, resulting in altered signaling in the mTOR pathway. The combination of ENT+anti-CTLA-4 induced changes in expression of downstream signaling molecules, at which the three pathways converged, leading to a similar outcome on myeloid suppression. These data showed that it was the addition of ICIs to ENT that led to improved infiltration of activated CD8⁺ T effector cells and increased cytotoxic characteristics. Examination of gene expression changes in whole tumors revealed that addition of anti-PD-1 and/or anti-CTLA-4 affected T-cell trafficking. Taken together, these data elucidate a suggested mechanism by which ENT alters the function of myeloid cells and the addition of ICIs to ENT further alters the myeloid compartment and improves T-cell responses. This mechanistic understanding delineates the important effects of each individual drug on specific immune compartments within the TME. These data could be used for developing scientifically driven combinations of epigenetic- and immune-based therapies.

Various studies have shown that ENT can reduce tumor burden and alter MDSC infiltration, facilitating increased efficacy of single agent ICIs in other murine models of cancer (11,21,36,37,45). These data support the therapeutic strategy of using agents that target multiple immune compartments within the TME, where ENT inhibits the immunosuppressive myeloid populations and ICIs allow for the activation and recruitment of T cells in order to induce a robust antitumor response. We showed that whereas ENT+anti-PD-1+anti-CTLA-4 was efficacious, it did not significantly improve survival over ENT+anti-PD-1 or ENT+anti-CTLA-4. A previously proposed mechanism for improved survival is that addition of ENT to anti-PD-1 leads to decreased immunosuppressive capacity of tumor-infiltrating MDSCs and allows for increased infiltration of cytotoxic T cells (21). We not only confirmed these findings in breast and PDAC models but also expanded upon them by showing a decrease in Arg-1 protein activity and production (21). Given that our data were generated entirely *ex vivo*, it supports changes in Arg-1 protein production as a significant mechanism underlying the MDSC impairment reported. In further support of this proposed mechanism, our gene expression profiling of isolated G-MDSCs allowed us to identify changes specific to the G-MDSC transcriptome in key signaling pathways such as VEGF, mTOR, and ErbB. Changes in Arg-1 and PD-L1 protein expression have not been analyzed in human MDSCs treated with ENT. However, a clinical trial has analyzed changes in MDSC populations as well as changes in CD40. CD40 has previously been described to be necessary in human MDSC-mediated T-cell suppression (51). Tomita et al. show that treatment of breast cancer patients with ENT significantly decreases CD40 expression (22), suggesting ENT may also induce immunosuppressive dysfunction in human MDSCs.

The most impactful finding of our work is the identification of changes in gene expression responsible for the impaired immunosuppressive functions of G-MDSCs that are driven by

ENT. First, we observed downregulation of genes within the VEGF signaling pathway in isolated G-MDSCs in ENT or ENT+anti-PD-1-treated versus vehicle-treated mice. Previous studies showed that elimination of VEGF can abrogate Arg-1 protein expression in intratumoral MDSCs and subsequently their ability to suppress T-cell proliferation (52), highlighting the importance of VEGF signaling in immunosuppressive functions of MDSCs. Second, decreased expression of genes in the ErbB signaling pathway were seen in isolated G-MDSCs with ENT or ENT+anti-PD-1 treatment. This may also contribute to the decrease in suppressive nature of these cells because EGFR-deficient macrophages are known to produce less immunosuppressive factors, including Arg-1 (53). Taken together, ENT appears to be the treatment driving these observed changes, indicating that it may be inducing MDSC dysfunction by decreasing signaling within both pathways (Fig. 7d). Third, treatment with ENT+anti-PD-1 significantly decreased expression of genes within the mTOR pathway. It has previously been shown that inhibition of mTORC1 can disrupt the immunosuppressive abilities of myeloid cells, including Arg-1 protein expression (54,55). Therefore, it is likely that PD-1 inhibition also contributes to the observed MDSC dysfunction through decreased mTOR signaling (Fig. 7d). We evaluated protein expression of representative proteins for each pathway in order to attempt to validate the observed changes in gene expression of each pathway. Although some of these observed gene expression changes correlated with similar changes in protein expression, others did not. This was expected given that post-transcriptional modifications often cause discrepancies between levels of message and protein. The reported changes in overall pathways were not determined by changes in expression of single genes, rather the cumulative change of all genes in the pathway. Thus, expression of individual genes within a pathway were not representative of the overall reported pathway changes. Key genes directly affecting MDSC function will need to be identified before further protein analysis is pursued.

Gene expression within the mTOR, ErbB, and VEGF pathways were upregulated with ENT+anti-CTLA-4 treatment, although this treatment also inhibited MDSC function similar to the combination of ENT+anti-PD-1. We hypothesize that these differential gene expression changes across treatment groups may converge at the transcription factor *Stat3*, which is downstream of all of the pathways we have mentioned (53,56). STAT3 is known to be essential in MDSC immunosuppressive function (57) and has previously been shown to be specifically targeted by ENT in MDSCs in *in vitro* systems (21,58–60). G-MDSCs from mice treated with ENT+anti-CTLA-4 showed a significant decrease in *Stat3* expression, whereas significant downregulation in upstream activators were observed in the ENT or ENT+anti-PD-1 treated groups. This suggests that the ultimate change in regulation of *Stat3* occurs either directly or indirectly depending on ICI treatment but leads to the same effect on G-MDSC function. The ENT+anti-CTLA-4 treated mice also exhibited a significant decrease in the expression of *Vegfa*, which has been shown to be a target of STAT3 transcriptional activity (61–63). Despite the increase in signaling intermediates in this pathway, a decrease in *Vegfa* expression, and subsequent VEGFR2 signaling, could be caused by less STAT3 activity. This could help explain the observed MDSC dysfunction with ENT+anti-CTLA-4, as inhibition of VEGFA signaling can prevent Arg-1 production (Fig. 7d) (52). G-MDSCs from ENT+anti-CTLA-4 treated mice did show lower expression of *Arg1* compared to the mice receiving ENT+anti-PD-1, further indicating that inhibition

of STAT3 signaling may be one of the driving mechanisms in the observed MDSC dysfunction. Taken together, we hypothesize that the mTOR, ErbB, and VEGF signaling pathways converge at *Stat3* to drive immunosuppressive functions of MDSCs. This hypothesis requires significantly more investigation but given previous studies linking treatment with ENT to STAT3 activity (21) and linking STAT3 to the suppressive function of Arg-1 (57), this seems like a plausible explanation for our observations. Analysis of protein expression of STAT3 from neu-N mouse model did not follow the same trends as those observed at the mRNA level. However, investigation of phospho-STAT3 in isolated G-MDSCs showed a decrease in STAT3 phosphorylation in all treatment groups compared to vehicle, indicating decreased STAT3 activity may play a role in the observed MDSC dysfunction in treated animals. It has been shown that HDAC2 is important in the regulation of inflammatory genes in myeloid cells (39), suggesting class I HDACs may play a role in the regulation of Arg-1 (38). Lastly, it should be noted that preliminary work using the HDAC6 inhibitor ACY-1215 did not improve survival when combined with immunotherapy, suggesting class II HDACs may not play a pivotal role in the functional capabilities of MDSCs. This is an area where future studies can aim to delineate the roles of class I and II HDACs in MDSC function as well as *Stat3* regulation.

Whereas modulation of MDSC function seemed primarily responsible for the improved survival observed with ENT+ICIs in these models, the addition of ICIs also significantly improved the infiltration and cytotoxic ability of CD8⁺ T effector cells within the TME. We identified significant upregulation of the leukocyte trans-endothelial migration pathway, which likely aids in the infiltration of TILs, and the addition of anti-CTLA-4 to ENT drives this observed change. The 20 genes that were found to be significantly affected by treatment with ENT+anti-CTLA-4 seemed to be expressed in a wide range of immune cell types. This suggests cell types, such as dendritic cells and natural killer cells, may also be secondarily affected by ICIs but are not primarily responsible for improved survival in response to treatments in these tumor models. The observed increase in infiltration of activated T cells may also be explained by the induction of expression of immunogenic non-coding long terminal repeats (LTRs) in tumor cells. A study by Brocks et al. shows that treatment with both a DNMTi and an HDACi leads to activation of these LTR elements (64). In the future, we will examine whether treatment with ENT without DNMTi can induce a biologically relevant amount of LTR expression *in vivo*.

In summary, these data support the use of ENT as a method of sensitizing non-immunogenic tumors to ICI therapy. ENT primed the MDSC compartment of the TME to permit T-cell infiltration into tumors thereby making them available for activation by ICI therapy. ICI therapy may also contribute to reprogramming MDSCs in favor of T-cell activity. These data have provided a deeper understanding of how to improve response to ICIs in patients with breast and pancreatic cancers. Specifically, they are informing the laboratory correlates for our clinical trials evaluating ENT+combined ICIs in advanced solid tumors with a dose expansion in advanced breast cancer (NCT02453620), as well as ENT+nivolumab in patients with unresectable PDAC (NCT03250273). The preclinical work presented here will provide the necessary scientific evidence to create rational combinations of immune therapies in future clinical trials to maximize efficacy of these promising treatments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

We would like to thank all members of the Jaffee lab for help throughout the course of these experiments. We would like to thank Sudipto Ganguly from Dr. Drew Pardoll's lab for his guidance using NanoString technology. We thank Dr. Elana Fertig for her input regarding revisions and her thorough review of the manuscript. Additionally, we would like to thank Syndax Pharmaceuticals for supplying the entinostat used in these experiments. We would also like to thank Michael Palien for his expert artistic skills used to create the cartoon in Fig 7.

Financial Support:

Dr. Jaffee's lab members are supported through NIH RO1CA184926, Stand Up 2 Cancer – Lustgarten Foundation Pancreatic Cancer Convergence Dream Team Translational Research Grant (Grant Number: SU2C-AACR-DT14–14); as well as Stand Up To Cancer which is a program of the Entertainment Industry Foundation administered by the American Association for Cancer Research; a gastrointestinal cancers Spore grant—NIH/NCI P50CA062924; the Lustgarten Foundation's Research Investigator's Award Program. Dr. Jaffee and Roussos Torres also acknowledge funding from the Broccoli Foundation, The Bloomberg–Kimmel Institute for Cancer Immunotherapy, and The Skip Viragh Center for Pancreas Cancer Clinical Research and Patient Care, and The Commonwealth Foundation for Cancer Research. Dr. Roussos Torres is funded through the MacMillan Pathway to Independence Fellowship as well as the Training Grant (5T32 CA009071–34) “Molecular Targets for Cancer Detection & Treatment”.

References:

1. Denkert C, von Minckwitz G, Darb-Esfahani S, Lederer B, Heppner BI, Weber KE, et al. Tumour-infiltrating lymphocytes and prognosis in different subtypes of breast cancer: a pooled analysis of 3771 patients treated with neoadjuvant therapy. *Lancet Oncol* [Internet]. 2017 [cited 2017 Dec 18]; Available from: <http://linkinghub.elsevier.com/retrieve/pii/S147020451730904X>
2. Solinas C, Gombos A, Latifyan S, Piccart-Gebhart M, Kok M, Buisseret L. Targeting immune checkpoints in breast cancer: an update of early results. *ESMO Open* [Internet]. 2017 [cited 2017 Dec 18];2:e000255 Available from: <http://www.ncbi.nlm.nih.gov/pubmed/29177095>
3. Nanda R, Chow LQM, Dees EC, Berger R, Gupta S, Geva R, et al. Pembrolizumab in Patients With Advanced Triple-Negative Breast Cancer: Phase Ib KEYNOTE-012 Study. *J Clin Oncol* [Internet]. 2016 [cited 2017 Dec 18];34:2460–7. Available from: <http://ascopubs.org/doi/10.1200/JCO.2015.64.8931>
4. Dirix LY, Takacs I, Jerusalem G, Nikolinakos P, Arkenau H-T, Forero-Torres A, et al. Avelumab, an anti-PD-L1 antibody, in patients with locally advanced or metastatic breast cancer: a phase 1b JAVELIN Solid Tumor study Breast Cancer Res Treat [Internet]. Springer US; [cited 2017 Dec 18]; 167 Available from: <http://link.springer.com/10.1007/s10549-017-4537-5>
5. Schmid P, Cruz C BF. Atezolizumab in metastatic TNBC (mTNBC): Long-term clinical outcomes and biomarker analyses. 2017 AACR Annual Meeting Abstract #2986; 2017.
6. Rugo HS, Delord J-P, S-a I et al. Preliminary efficacy and safety of pembrolizumab (MK-3475) in patients with PD-L1-positive, estrogen receptor-positive (ER+)/HER2 negative advanced breast cancer enrolled in KEYNOTE-028. 2015 San Antonio Breast Cancer Symposium. Abstract S5–07; 2015.
7. Tolaney S, Savulsky C, Aktan G, Xing D, Almonte A, Karantza V, et al. Abstract P5–15-02: Phase 1b/2 study to evaluate eribulin mesylate in combination with pembrolizumab in patients with metastatic triple-negative breast cancer Cancer Res [Internet]. American Association for Cancer Research; 2017 [cited 2017 Dec 18];77:P5–15-02–P5–15-02. Available from: <http://cancerres.aacrjournals.org/lookup/doi/10.1158/1538-7445.SABCS16-P5-15-02>
8. Adams S, Schmid P, Rugo HS et al. Phase 2 study of pembrolizumab (pembro) monotherapy for previously treated metastatic triple-negative breast cancer (mTNBC): KEYNOTE-086 cohort A. *Journal of Clinical Oncology*. 35, no. 15_suppl (May 2017) 1008–1008.; 2017.

9. Adams S, Loi S, Toppmeyer D et al. Phase 2 study of pembrolizumab as first-line therapy for PD-L1-positive metastatic triple-negative breast cancer (mTNBC): Preliminary data from KEYNOTE-086 cohort B. *Journal of Clinical Oncology*. 35, no. 15_suppl (5 2017) 1088–1088.;
10. SABCS 2017: Combination of Pembrolizumab and Trastuzumab Shows Early Promise for Patients With Trastuzumab-Resistant Breast Cancer - The ASCO Post [Internet]. [cited 2018 Feb 2]. Available from: <http://www.ascopost.com/News/58327>
11. Kim K, Skora AD, Li Z, Liu Q, Tam AJ, Blosser RL, et al. Eradication of metastatic mouse cancers resistant to immune checkpoint blockade by suppression of myeloid-derived cells. *Proc Natl Acad Sci U S A* [Internet]. 2014;111:11774–9. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/25071169>
12. Stagg J, Loi S, Divisekera U, Ngiow SF, Duret H, Yagita H, et al. Anti-ErbB-2 mAb therapy requires type I and II interferons and synergizes with anti-PD-1 or anti-CD137 mAb therapy *Proc Natl Acad Sci U S A* [Internet]. National Academy of Sciences; 2011 [cited 2017 Dec 19]; 108:7142–7. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/21482773>
13. Brahmer JR, Tykodi SS, Chow LQM, Hwu W-J, Topalian SL, Hwu P, et al. Safety and Activity of Anti-PD-L1 Antibody in Patients with Advanced Cancer. *N Engl J Med* [Internet]. 2012;366:2455–65. Available from: <http://www.nejm.org/doi/abs/10.1056/NEJMoa1200694>
14. Royal RE, Levy C, Turner K, Mathur A, Hughes M, Kammula US, et al. Phase 2 trial of single agent Ipilimumab (anti-CTLA-4) for locally advanced or metastatic pancreatic adenocarcinoma. *J Immunother*. 2010;33:828–33. [PubMed: 20842054]
15. Lutz ER, Wu AA, Bigelow E, Sharma R, Mo G, Soares K, et al. Immunotherapy Converts Nonimmunogenic Pancreatic Tumors into Immunogenic Foci of Immune Regulation. *Cancer Immunol Res* [Internet]. 2014 [cited 2018 Jan 16];2:616–31. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/24942756>
16. Bronte V, Serafini P, Mazzoni A, Segal DM, Zanovello P. L-arginine metabolism in myeloid cells controls T-lymphocyte functions. *Trends Immunol* [Internet]. 2003;24:302–6. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/12810105>
17. Kusmartsev S, Nagaraj S, Gabrilovich DI. Tumor-associated CD8+ T cell tolerance induced by bone marrow-derived immature myeloid cells. *J Immunol* [Internet]. 2005;175:4583–92. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/16177103>
18. Kamran N, Kadiyala P, Saxena M, Candolfi M, Li Y, Moreno-Ayala MA, et al. Immunosuppressive Myeloid Cells' Blockade in the Glioma Microenvironment Enhances the Efficacy of Immune-Stimulatory Gene Therapy. *Mol Ther* [Internet]. 2017;25:232–48. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/28129117>
19. Yang R, Cai Z, Zhang Y, Yutzy WH th, Roby KF, Roden RB. CD80 in immune suppression by mouse ovarian carcinoma-associated Gr-1+CD11b+ myeloid cells. *Cancer Res* [Internet]. 2006;66:6807–15. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/16818658>
20. Marvel D, Gabrilovich DI. Myeloid-derived suppressor cells in the tumor microenvironment: expect the unexpected. *J Clin Invest* [Internet]. 2015;125:3356–64. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/26168215>
21. Orillion A, Hashimoto A, Damayanti N, Shen L, Adelaiye-Ogala R, Arisa S, et al. Entinostat Neutralizes Myeloid-Derived Suppressor Cells and Enhances the Antitumor Effect of PD-1 Inhibition in Murine Models of Lung and Renal Cell Carcinoma. *Clin Cancer Res* [Internet]. 2017;23:5187–201. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/28698201>
22. Tomita Y, Lee M-J, Lee S, Tomita S, Chumsri S, Cruickshank S, et al. The interplay of epigenetic therapy and immunity in locally recurrent or metastatic estrogen receptor-positive breast cancer: Correlative analysis of ENCORE 301, a randomized, placebo-controlled phase II trial of exemestane with or without entinostat. *Oncoimmunology* [Internet]. 2016 [cited 2018 Jan 6]; 5:e1219008 Available from: <https://www.tandfonline.com/doi/full/10.1080/2162402X.2016.1219008>
23. Weiss VL, Lee TH, Song H, Kouo TS, Black CM, Sgouros G, et al. Trafficking of High Avidity HER-2/neu-Specific T Cells into HER-2/neu-Expressing Tumors after Depletion of Effector/Memory-Like Regulatory T Cells Kassiotis G, editor. *PLoS One* [Internet]. Public Library of Science; 2012 [cited 2018 Jan 16];7:e31962 Available from: <http://dx.plos.org/10.1371/journal.pone.0031962>

24. Reilly RT, Gottlieb MB, Ercolini AM, Machiels JP, Kane CE, Okoye FI, et al. HER-2/neu is a tumor rejection target in tolerized HER-2/neu transgenic mice. *Cancer Res* [Internet]. 2000 [cited 2018 Jan 25];60:3569–76. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/10910070>
25. Mace K, Mayhew E, Mihich E, Ehrke MJ, Lei RY, Weintraub D, et al. Alterations in murine host defense functions by adriamycin or liposome-encapsulated adriamycin. *Cancer Res* [Internet]. American Association for Cancer Research; 1988 [cited 2018 Jan 9];48:130–6. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/3257160>
26. Corbett TH, Roberts BJ, Leopold WR, Peckham JC, Wilkoff LJ, Griswold DP, et al. Induction and chemotherapeutic response of two transplantable ductal adenocarcinomas of the pancreas in C57BL/6 mice. *Cancer Res* [Internet]. American Association for Cancer Research; 1984 [cited 2018 Aug 2];44:717–26. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/6692374>
27. Leao IC, Ganesan P, Armstrong TD, Jaffee EM. Effective depletion of regulatory T cells allows the recruitment of mesothelin-specific CD8 T cells to the antitumor immune response against a mesothelin-expressing mouse pancreatic adenocarcinoma. *Clin Transl Sci* [Internet]. Wiley-Blackwell; 2008 [cited 2018 Aug 2];1:228–39. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/20357913>
28. Ercolini AM, Machiels J-PH, Chen YC, Slansky JE, Giedlen M, Reilly RT, et al. Identification and characterization of the immunodominant rat HER-2/neu MHC class I epitope presented by spontaneous mammary tumors from HER-2/neu-transgenic mice. *J Immunol* [Internet]. 2003 [cited 2018 Oct 5];170:4273–80. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/12682262>
29. Soares KC, Foley K, Olino K, Leubner A, Mayo SC, Jain A, et al. A preclinical murine model of hepatic metastases. *J Vis Exp* [Internet]. 2014 [cited 2018 Jan 6];51677 Available from: <http://www.jove.com/video/51677/a-preclinical-murine-model-of-hepatic-metastases>
30. Lee JW, Komar CA, Bengsch F, Graham K, Beatty GL. Genetically Engineered Mouse Models of Pancreatic Cancer: The KPC Model (*LSL-Kras^{G12D/+};LSL-Trp53^{R172H/+};Pdx-1-Cre*), Its Variants, and Their Application in Immuno-oncology Drug Discovery *Curr Protoc Pharmacol* [Internet]. Hoboken, NJ, USA: John Wiley & Sons, Inc.; 2016 [cited 2018 Apr 2]. page 14.39.1–14.39.20. Available from: <http://doi.wiley.com/10.1002/cpph.2>
31. Foote JB, Kok M, Leatherman JM, Armstrong TD, Marcinkowski BC, Ojalvo LS, et al. A STING Agonist Given with OX40 Receptor and PD-L1 Modulators Primes Immunity and Reduces Tumor Growth in Tolerized Mice. [cited 2018 Feb 12]; Available from: <http://cancerimmunolres.aacrjournals.org.ezp.welch.jhmi.edu/content/canimm/5/6/468.full.pdf>
32. Mueller S, Engleitner T, Maresch R, Zukowska M, Lange S, Kaltenbacher T, et al. Evolutionary routes and KRAS dosage define pancreatic cancer phenotypes. *Nature* [Internet]. Nature Publishing Group; 2018 [cited 2018 Jul 18];554:62–8. Available from: <http://www.nature.com/doifinder/10.1038/nature25459>
33. Hingorani SR, Wang L, Multani AS, Combs C, Deramaudt TB, Hruban RH, et al. Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. *Cancer Cell* [Internet]. Cell Press; 2005 [cited 2018 May 21];7:469–83. Available from: <https://www.sciencedirect.com/science/article/pii/S1535610805001285?via%3Dihub>
34. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer* [Internet]. 2012 [cited 2018 Jan 6];12:252–64. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/22437870>
35. Soares KC, Rucki AA, Wu AA, Olino K, Xiao Q, Chai Y, et al. PD-1/PD-L1 blockade together with vaccine therapy facilitates effector T cell infiltration into pancreatic tumors. *J Immunother* [Internet]. 2015 [cited 2018 Jan 6];38:1–11. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4258151/pdf/nihms-636576.pdf>
36. Shakespear MR, Halili MA, Irvine KM, Fairlie DP, Sweet MJ. Histone deacetylases as regulators of inflammation and immunity. *Trends Immunol* [Internet]. 2011 [cited 2018 Apr 2];32:335–43. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/21570914>
37. Klampfer L, Huang J, Swaby L-A, Augenlicht L. Requirement of Histone Deacetylase Activity for Signaling by STAT1. *J Biol Chem* [Internet]. 2004 [cited 2018 Apr 2];279:30358–68. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/15123634>

38. Zhang Q, Zhao K, Shen Q, Han Y, Gu Y, Li X, et al. Tet2 is required to resolve inflammation by recruiting Hdac2 to specifically repress IL-6. *Nature* [Internet]. 2015 [cited 2018 Jan 12];525:389–93. Available from: <http://www.nature.com/articles/nature15252>
39. Pan W, Zhu S, Qu K, Meeth K, Cheng J, He K, et al. The DNA Methylcytosine Dioxygenase Tet2 Sustains Immunosuppressive Function of Tumor-Infiltrating Myeloid Cells to Promote Melanoma Progression. *Immunity* [Internet]. 2017 [cited 2018 Jan 12];47:284–297.e5. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/28813659>
40. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* [Internet]. 2015 [cited 2018 Jan 18];43:e47–e47. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/25605792>
41. Luo W, Friedman MS, Shedden K, Hankenson KD, Woolf PJ. GAGE: generally applicable gene set enrichment for pathway analysis. *BMC Bioinformatics* [Internet]. 2009 [cited 2018 Jan 18];10:161 Available from: <http://www.ncbi.nlm.nih.gov/pubmed/19473525>
42. Luo W, Brouwer C. Pathview: an R/Bioconductor package for pathway-based data integration and visualization. *Bioinformatics* [Internet]. 2013 [cited 2018 Jan 18];29:1830–1. Available from: <https://academic.oup.com/bioinformatics/article-lookup/doi/10.1093/bioinformatics/btt285>
43. Garon EB, Rizvi NA, Hui R, Leighl N, Balmanoukian AS, Eder JP, et al. Pembrolizumab for the Treatment of Non–Small-Cell Lung Cancer. *N Engl J Med* [Internet]. Massachusetts Medical Society; 2015 [cited 2018 Jul 19];372:2018–28. Available from: <http://www.nejm.org/doi/10.1056/NEJMoa1501824>
44. Thompson RH, Kuntz SM, Leibovich BC, Dong H, Lohse CM, Webster WS, et al. Tumor B7-H1 Is Associated with Poor Prognosis in Renal Cell Carcinoma Patients with Long-term Follow-up. *Cancer Res* [Internet]. 2006 [cited 2018 Apr 2];66:3381–5. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/16585157>
45. Shen L, Ciesielski M, Ramakrishnan S, Miles KM, Ellis L, Sotomayor P, et al. Class I histone deacetylase inhibitor entinostat suppresses regulatory T cells and enhances immunotherapies in renal and prostate cancer models Ling MT, editor. *PLoS One* [Internet]. 2012 [cited 2018 Jan 6];7:e30815 Available from: <http://dx.plos.org/10.1371/journal.pone.0030815>
46. Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* [Internet]. 2001 [cited 2018 Jun 25];344:783–92. Available from: <http://www.nejm.org/doi/abs/10.1056/NEJM200103153441101>
47. Falkenberg KJ, Johnstone RW. Histone deacetylases and their inhibitors in cancer, neurological diseases and immune disorders. *Nat Rev Drug Discov* [Internet]. Nature Publishing Group; 2014 [cited 2018 Oct 5];13:673–91. Available from: <http://www.nature.com/articles/nrd4360>
48. Baitsch L, Legat A, Barba L, Fuertes Marraco SA, Rivals J-P, Baumgaertner P, et al. Extended Co-Expression of Inhibitory Receptors by Human CD8 T-Cells Depending on Differentiation, Antigen-Specificity and Anatomical Localization Ashour HM, editor. *PLoS One* [Internet]. Public Library of Science; 2012 [cited 2018 Jan 26];7:e30852 Available from: <http://dx.plos.org/10.1371/journal.pone.0030852>
49. Blackburn SD, Shin H, Haining WN, Zou T, Workman CJ, Polley A, et al. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol* [Internet]. 2009 [cited 2018 Jan 26];10:29–37. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/19043418>
50. Shimizu K, Iyoda T, Okada M, Yamasaki S, Fujii S. Immune suppression and reversal of the suppressive tumor microenvironment. *Int Immunol* [Internet]. Oxford University Press; 2018 [cited 2018 Oct 5];30:445–55. Available from: <https://academic.oup.com/intimm/article/30/10/445/5043264>
51. Pan P-Y, Ma G, Weber KJ, Ozao-Choy J, Wang G, Yin B, et al. Immune stimulatory receptor CD40 is required for T-cell suppression and T regulatory cell activation mediated by myeloid-derived suppressor cells in cancer. *Cancer Res* [Internet]. 2010 [cited 2018 May 14];70:99–108. Available from: <http://cancerres.aacrjournals.org/cgi/doi/10.1158/0008-5472.CAN-09-1882>
52. Horikawa N, Abiko K, Matsumura N, Hamanishi J, Baba T, Yamaguchi K, et al. Expression of Vascular Endothelial Growth Factor in Ovarian Cancer Inhibits Tumor Immunity through the

- Accumulation of Myeloid-Derived Suppressor Cells. Clin Cancer Res [Internet]. 2017 [cited 2018 Jan 12];23:587–99. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/27401249>
53. Wei J, Besner GE. M1 to M2 macrophage polarization in heparin-binding epidermal growth factor-like growth factor therapy for necrotizing enterocolitis. J Surg Res [Internet]. 2015 [cited 2018 Jan 12];197:126–38. Available from: <http://linkinghub.elsevier.com/retrieve/pii/S0022480415002917>
 54. Kaneda MM, Messer KS, Ralainirina N, Li H, Leem CJ, Gorjestani S, et al. Erratum: Corrigendum: PI3K γ is a molecular switch that controls immune suppression. Nature [Internet]. 2017 [cited 2018 Jan 12];542:124–124. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/27974794>
 55. Wu T, Zhao Y, Wang H, Li Y, Shao L, Wang R, et al. mTOR masters monocytic myeloid-derived suppressor cells in mice with allografts or tumors. Sci Rep [Internet]. 2016 [cited 2018 Jan 12]; 6:20250 Available from: <http://www.nature.com/articles/srep20250>
 56. Hardbower DM, Singh K, Asim M, Verriere TG, Olivares-Villagómez D, Barry DP, et al. EGFR regulates macrophage activation and function in bacterial infection. J Clin Invest [Internet]. 2016 [cited 2018 Jan 12];126:3296–312. Available from: <https://www.jci.org/articles/view/83585>
 57. Vasquez-Dunddel D, Pan F, Zeng Q, Gorbounov M, Albesiano E, Fu J, et al. STAT3 regulates arginase-I in myeloid-derived suppressor cells from cancer patients. J Clin Invest [Internet]. American Society for Clinical Investigation; 2013 [cited 2018 Feb 6];123:1580–9. Available from: <https://www.jci.org/articles/view/60083>
 58. Kumar V, Donthireddy L, Marvel D, Condamine T, Wang F, Lavilla-Alonso S, et al. Cancer-Associated Fibroblasts Neutralize the Anti-tumor Effect of CSF1 Receptor Blockade by Inducing PMN-MDSC Infiltration of Tumors. Cancer Cell [Internet]. Elsevier; 2017 [cited 2017 Dec 11]; 32:654–668.e5. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/29136508>
 59. Condamine T, Gabrilovich DI. Molecular mechanisms regulating myeloid-derived suppressor cell differentiation and function. Trends Immunol [Internet]. 2011 [cited 2018 Jan 12];32:19–25. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/21067974>
 60. Dodd KM, Yang J, Shen MH, Sampson JR, Tee AR. mTORC1 drives HIF-1 α and VEGF-A signalling via multiple mechanisms involving 4E-BP1, S6K1 and STAT3. Oncogene [Internet]. 2015 [cited 2018 Jan 12];34:2239–50. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/24931163>
 61. Wei D, Le X, Zheng L, Wang L, Frey JA, Gao AC, et al. Stat3 activation regulates the expression of vascular endothelial growth factor and human pancreatic cancer angiogenesis and metastasis. Oncogene [Internet]. Nature Publishing Group; 2003 [cited 2018 Jan 22];22:319–29. Available from: <http://www.nature.com/articles/1206122>
 62. Niu G, Wright KL, Huang M, Song L, Haura E, Turkson J, et al. Constitutive Stat3 activity up-regulates VEGF expression and tumor angiogenesis. Oncogene [Internet]. 2002 [cited 2018 Jan 22];21:2000–8. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/11960372>
 63. Wei L-H, Kuo M-L, Chen C-A, Chou C-H, Lai K-B, Lee C-N, et al. Interleukin-6 promotes cervical tumor growth by VEGF-dependent angiogenesis via a STAT3 pathway. Oncogene [Internet]. 2003 [cited 2018 Jan 22];22:1517–27. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/12629515>
 64. Brocks D, Schmidt CR, Daskalakis M, Jang HS, Shah NM, Li D, et al. DNMT and HDAC inhibitors induce cryptic transcription start sites encoded in long terminal repeats. Nat Genet [Internet]. 2017 [cited 2018 Jul 16];49:1052–60. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/28604729>

Figure 1: Addition of entinostat to checkpoint inhibition significantly improves survival and slows tumor progression.

(a) Timeline of study design. In the neu-N model, ENT treatment was started 3 days following tumor implantation. For the metastatic pancreatic cancer model, ENT treatment began 7 days after hemi-splenectomy and tumor implantation. In both models, ENT was given for 5 consecutive days each week over the course of 3 weeks. ICI antibody dosing was performed biweekly, whereas anti-HER2 was given weekly. For survival studies, mice continued to receive two doses of ICI antibody as well as one dose of anti-HER2 (neu-N

model only) every other week after the first three weeks of treatment. Mice did not continue to receive ENT after the first three weeks of treatment. This was considered the maintenance phase of treatment. For all immune infiltrate analyses, mice were sacrificed during the third week of treatment. **(b)** Survival of PDAC mice receiving ENT and ICIs. **(c)** Survival and **(d)** tumor growth of neu-N mice receiving ENT and ICIs with anti-HER2. Statistics for tumor growth were calculated at day 43. For survival data, results were plotted using a Kaplan-Meier curve and statistical significance was determined via a log-rank test; n=10 mice/group. For tumor growth data, statistical significance was determined using a two-way ANOVA; n=10 mice/group. All experiments were repeated at least 3 times. Statistically significant p values are abbreviated as follows: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 2: ENT+ICIs significantly increases infiltration of G-MDSCs into the tumor microenvironment.

(a) Gating strategy for identification of MDSCs by flow cytometry. G-MDSCs: CD3⁻F4/80⁻CD11b⁺Ly6G⁺Ly6C^{lo}; M-MDSCs: CD3⁻F4/80⁻CD11b⁺Ly6G⁻Ly6C⁺. (b) Changes in G-MDSC infiltration in breast tumors and (c) hepatic metastases of PDAC mice. (d) Changes in M-MDSC infiltration in breast tumors of neu-N mice and (e) in hepatic metastases of PDAC mice. In all experiments, cells were isolated from breast tumors and livers containing PDAC metastases, respectively. For all flow data, mice were sacrificed during the third week

of treatment. Each dot represents one mouse and each bar represents mean $\pm$ SEM; n=3–5 mice/group. Significance was determined by a one-way ANOVA with Tukey's multiple comparisons test. All experiments were repeated at least 3 times. Statistically significant p values are abbreviated as follows: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 3: ENT+ICI combination therapy inhibits immunosuppressive functions of MDSCs.

(a) Ly6G⁺ G-MDSCs isolated from breast tumors of individual animals were cocultured with naïve neu-specific CD8⁺ T cells and peptide-pulsed APCs. Proliferation was determined by CFSE staining. Each bar is an average of 3–4 mice/treatment group. Error bars indicate mean±SEM. Significance was determined by a one-way ANOVA with Tukey's multiple comparisons test. Changes in arginase activity of (b) G-MDSCs and (c) M-MDSCs isolated from treated breast tumors determined via colorimetric assay. For arginase data, mice were sacrificed during the third week of treatment. Each dot represents one mouse and each bar represents mean±SEM (n=3–5 mice/group). All experiments were repeated at least 3 times. Statistically significant p values are abbreviated as follows: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 4: HDACi+ICI combined therapy significantly alters the signaling pathway gene expression profiles involved in myeloid function.
(a) Heatmap of gene expression changes in all-treatment groups compared to vehicle treatment alone. Red indicates upregulation; blue indicates downregulation. (b) Tables listing selected significantly downregulated KEGG pathways and (c) significantly upregulated KEGG pathways, with the top 5 genes differentially regulated listed in last column. (d) Differentially regulated genes in the VEGF signaling pathway in mice treated with ENT vs. vehicle. Expression of genes listed in white boxes were measured but did not

show any fold change. Expression of genes in gray boxes were not measured. NanoString gene expression profiling was performed once with at least 4 mice/group. Statistically significant p values are abbreviated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

CD8⁺ T cells determined by co-expression of PD-1 and Lag3 in both the neu-N and (f) the PDAC model. For all flow data, mice were sacrificed during the third week of treatment. Each dot represents one mouse and each bar represents mean±SEM (n=3–5 mice/group). Significance was determined by a one-way ANOVA with Tukey's multiple comparisons test. All experiments were repeated at least 3 times. Statistically significant p values are abbreviated as follows: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 6: HDACi+ICI combination therapy causes reduction in T_{reg} infiltration.

Changes in T_{reg} infiltration in breast tumors of (a) neu-N mice and (b) hepatic metastases of PDAC mice receiving therapy. Cells were isolated from breast tumors and livers containing PDAC metastases, respectively. For all flow data, mice were sacrificed during the third week of treatment. Each dot represents one mouse and each bar represents mean $\pm$ SEM (n=3–5 mice/group). Significance was determined by a one-way ANOVA with Tukey's multiple comparisons test. All experiments were repeated at least 3 times. Statistically significant p values are abbreviated as follows: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

a) PanCancer Immune Panel of whole tumor

b) Pathways Upregulated

Treatment	KEGG Pathway	P-value	Most differentially expressed genes
ENT_vs_ENT + CTLA4	Leukocyte transendothelial migration	0.0383	Cdh5 ,Cxcr4, Nox2, Icam1, Itga4
	Osteoclast differentiation	0.0443	Btk, Chuk, Socs3, Socs1, Creb1

Figure 7: HDACi+ICI combination therapy significantly alters the leukocyte transendothelial migration pathway.
(a) Heatmap of gene expression changes comparing all treatment groups using the PanCancer Immune profiling gene set. Red indicates upregulation; blue indicates downregulation. (b) Table of significantly altered KEGG pathways. 5 most differentially expressed genes listed in last column. (c) Graphs depicting an increase in genes indicative of bulk T cells and exhausted T cells in combination-treated groups. Genes upregulated listed in red, and genes downregulated are in blue. Expression of genes listed in white boxes were

measured but did not show any fold change. Expression of genes in gray boxes were not measured by the assay. **(d)** Diagram showing proposed mechanism regulating decreased suppressive function of myeloid cells and increased infiltration of T cells as a result of treatment with ENT, anti-PD-1, and anti-CTLA-4. NanoString gene expression profiling was performed once with at least 4 mice/group. Statistically significant p values are abbreviated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript