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

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ABSTRACT
Entinostat, a class I-selective histone deacetylase inhibitor, has shown promising activity in ENCORE 301, a randomized, placebo-controlled, phase II trial of exemestane with or without entinostat in women with locally recurrent or metastatic estrogen receptor-positive breast cancer progressing on a nonsteroidal aromatase inhibitor. ENCORE 301 showed an 8.3-mo improvement in median overall survival among patients who received entinostat. We investigated the impact of entinostat on immune subsets with CD40, HLA-DR, and immune checkpoint receptor expression analyses in 34 patient blood samples from ENCORE 301. We found that entinostat significantly decreased granulocytic and monocytic MDSCs at cycle 1 day 15. MDSC CD40 was significantly downregulated by entinostat. A significant increase in HLA-DR expression on CD14+ monocytes by entinostat was observed. Entinostat did not impact T-cell subsets or T-cell immune checkpoint receptor expression. Our findings suggest that a significant interplay between this epigenetic regimen and host immune homeostatic mechanisms may impact therapeutic outcome.

Introduction
The interaction between anticancer drugs and the host immune system has been implicated in response to therapy.1 Although the antitumor effects of histone deacetylase inhibitors (HDACi) have been studied, the impact of HDACi on cancer patient systemic immunity remains unclear.2 A recent preclinical study has shown that an intact immune system is required for the anticancer activities of HDACi, suggesting that immunomodulatory effects of HDACi play a pivotal role in antitumor effects.3

Entinostat is a class 1 isoform-selective HDACi with favorable safety and efficacy profiles, currently in phase III clinical development in breast cancer.4-6 Prior preclinical studies of the impact of entinostat on immune subsets have yielded differing results, perhaps influenced by differences in the systems studied. In vitro treatment of human T-cells with entinostat has been reported to increase the percentage of regulatory T-cells (Tregs) among CD4+ T-cells7 and entinostat increased the percentage of Tregs among CD4+ T-cells in peripheral blood and lymph nodes of rats in vivo.8 In contrast to these results, entinostat downregulated Foxp3 expression, decreased the percent of Tregs among CD4+ T-cells, and blocked Treg immunosuppressive function without affecting T-effector cells in syngeneic mouse renal cell and prostate tumor models.9 Entinostat had synergistic effects with an adenoviral vector expressing human dopachrome tautomerase (hDCT) and an oncolytic vesicular stomatitis virus expressing hDCT in a murine melanoma tumor model.10 In addition, Kim et al. reported that entinostat targets murine granulocytic myeloid-derived suppressor cells (MDSCs), which were shown to be responsible for resistance to immune checkpoint blockade with anti-programmed cell death-1 (anti-PD-1) and anti-cytotoxic T-lymphocyte-associated antigen-4 (anti-CTLA-4) antibodies. The immunomodulatory effect of entinostat on granulocytic MDSCs, when combined with anti-PD-1/anti-CTLA-4 antibodies, led to frequent cures in two murine tumor models unresponsive to anti-CTLA-4/anti-PD-1 antibody therapy: large primary tumors derived from modestly immunogenic CT26 murine colon cancer cells; and primary and metastatic tumors derived from 4T1 cells, a poorly immunogenic, highly metastatic murine breast cancer.11,12 However, the impact of entinostat on immune subsets in breast cancer patients, or indeed, the impact of any epigenetic therapy on myeloid immune subsets in cancer patients, has not been reported.

Entinostat has demonstrated promising clinical activity in ENCORE 301, a randomized, placebo-controlled, phase II trial of exemestane plus entinostat (EE) versus
exametane plus placebo (EP) in postmenopausal women with locally recurrent or metastatic estrogen receptor (ER)-positive breast cancer that has progressed on nonsteroidal aromatase inhibitors. ENCORE 301 met the primary progression-free survival (PFS) endpoint and showed an 8.3-month improvement in the overall survival (OS) exploratory endpoint for the EE arm. Based on these results, entinostat was granted FDA Breakthrough Therapy designation in 2013, and a randomized phase III trial of exemestane with or without entinostat in hormone receptor-positive advanced breast cancer, based on the design of ENCORE 301, is currently ongoing (NCT02115282).

To understand the immunomodulatory activity of entinostat in breast cancer patients, we performed comprehensive analyses of peripheral immune subsets including T-cell subsets with immune checkpoint receptor analysis, monocytocytes with HLA-DR analysis, and assessment of four populations of MDSCs including CD40 expression analysis using available peripheral blood mononuclear cells (PBMCs) from 34 patients enrolled in ENCORE 301. The clinical outcomes of these 34 patients, including PFS (EE median 4.9 mo vs. EP 1.8 mo; HR 0.56) and OS (EE median 28.1 mo vs. EP 20.3 mo; HR 0.62) were consistent with the original intention-to-treat population.6

Here, we show a significant decrease in specific populations of MDSCs and modulation of MDSC CD40 expression shortly after the initiation of entinostat (cycle 1 day 15 (C1D15)) on a schedule of daily exemestane and once weekly entinostat compared to patients treated with daily EP. In addition, entinostat increased HLA-DR expression on CD14+ monocytes in breast cancer patients. These favorable immunomodulatory effects in myeloid subsets may contribute to the observed clinical activity of entinostat in ENCORE 301.6 Our studies may provide new insights into the immunomodulatory action of entinostat in cancer patients and support the preclinical rationale for combination therapy with immune checkpoint blockade.11

Results

Entinostat increases HLA-DR expression on CD14+ monocytes in breast cancer patients

PBMCs from 34 postmenopausal women with locally recurrent or metastatic breast cancer treated with EP or EE were available for analysis (EP, n = 14; EE, n = 20). CD14+HLA-DRlow/neg monocytes have been shown to associate with poor survival in renal cell carcinoma and mediate chemotherapy-resistance in lymphoma.12-14 HDACi in vitro have been shown to upregulate HLA molecules including HLA-DR and alter the HLA-DR peptideome of cells.2,15-18 We have demonstrated an upregulation of HLA-DR on Tregs post-therapy in a phase II trial of the pan-HDAC inhibitor belinostat in thymic epithelial malignancies.19 However, the impact of HDACi on HLA-DR expression on circulating monocytes in cancer patients has not been reported. We analyzed HLA-DR expression levels of CD14+ monocytes in PBMCs from ENCORE 301 by multiparameter flow cytometry. The gating strategy is shown in Fig. 1A. The level of CD14+HLA-DRhigh monocytes as a percentage of CD45+ cells significantly increased after two doses of entinostat (at C1D15) in the EE cohort compared to the EP cohort (Fig. 1B and Table 1; median percentage change from baseline to C1D15, EE +34.08% vs. EP −11.38%; p = 0.0004). In addition, HLA-DR expression on the total CD14+ monocyte population significantly increased in the EE cohort compared to the EP cohort (Fig. 1C and Table 1; median percentage change from baseline to C1D15, EE +16.26% vs. EP −4.74%; p = 0.015). The levels of CD14+ monocytes and CD14+HLA-DRlow/neg monocytes did not show a significant difference between the EE and EP cohorts (Table 1). We also studied the impact of entinostat on HLA-DR expression in CD14+ monocytes in vitro. Entinostat increased the expression of HLA-DR on CD14+ monocytes (Fig. 1D, p = 0.008). These results suggest that the addition of entinostat to exemestane treatment in breast cancer patients has the ability to increase HLA-DR expression on CD14+ monocytes and increase the subset of CD14+HLA-DRhigh monocytes within 2 weeks of initiating therapy.

Entinostat decreases monocytic and granulocytic MDSCs in breast cancer patients

Recently, Kim et al. showed that entinostat acts directly on murine MDSCs and decreases the number of granulocytic MDSCs in combination with immune checkpoint blockade in two tumor-bearing murine models.11 MDSCs, a heterogeneous population of myeloid cells at different stages of cell differentiation, play a key role in tumor immune escape.20-22 All murine MDSCs express Gr1 and CD11b.21,23 There is no human analog of Gr1, and CD11b is not lineage specific.20,23 In the absence of a specific marker, human MDSCs are analyzed phenotypically by the presence and absence of several haematopoietic markers. Thus, we studied the immunomodulatory effects of entinostat on four MDSC phenotypes described in the literature, here termed granulocytic MDSCs, Lin- MDSCs, monocytic MDSCs, and immature MDSCs (Fig. 2A; detailed phenotypic markers for MDSC phenotypes are shown in Materials and Methods). Interestingly, we observed a significant reduction in monocytic MDSCs (Fig. 2B; median percentage change from baseline to C1D15, EE −62.33% vs. EP +1.97%, p = 0.002) and granulocytic MDSCs (Fig. 2C; median percentage change from baseline to C1D15, EE −34.53% vs. EP +3.82%, p = 0.029) at C1D15 in the EE cohort compared to the EP cohort. Entinostat did not alter the levels of Lin− MDSCs or immature MDSCs (Table 1). These results suggest that entinostat targets specific populations of human MDSCs (monocytic and granulocytic MDSCs) in breast cancer patients.

We investigated the effect of entinostat on in vitro-generated MDSCs. Entinostat decreased the number of in vitro-generated monocytic MDSCs and granulocytic MDSCs (Fig. 3C; monocytic MDSCs, p = 0.004; granulocytic MDSCs, p = 0.004). MDSCs without cytokine induction were also decreased by entinostat (data not shown). In addition, we found entinostat is selectively toxic to MDSCs in vitro. Percentages of dead cells in monocytic
Figure 1. Entinostat increases HLA-DR expression on CD14+ monocytes in breast cancer patients. (A) Gating strategy for analysis of CD14+ monocytes (left panel), CD14+HLA-DRhi monocytes (red box, right upper panel), and CD14+HLA-DRlow/neg monocytes (blue box, right lower panel) in PBMCs of breast cancer patients. Initially gated on single viable CD45 cells. (B) Change of percentage CD14+HLA-DRhi monocytes among single viable CD45+ PBMCs from baseline to C1D15 in exemestane (EP) arm (n = 14) and exemestane + entinostat (EE) arm (n = 20). The level of CD14+HLA-DRhi monocytes was significantly increased in the EE arm compared to the EP arm (p = 0.0004). (C) Change of HLA-DR expression (median fluorescence intensity, MFI) on CD14+ monocytes from baseline to C1D15 in the EP arm (n = 14) and EE arm (n = 20). The level of HLA-DR expression on CD14+ monocytes was significantly increased in the EE arm compared to the EP arm (p = 0.015). (D) HLA-DR expression on CD14+ monocytes in vitro. Fresh PBMCs were cultured with DMSO or entinostat (0.5 μM) for 2 d. Left panel shows a representative histogram of HLA-DR expression on CD14+ monocytes cultured with DMSO (blue histogram) or with entinostat (red histogram). Black histogram shows isotype control. Right panel shows the difference of HLA-DR expression levels on CD14+ monocytes cultured with DMSO or entinostat. Each line represents a different healthy donor (n = 8, p = 0.008). Median fluorescence intensity, MFI.
MDSCs and granulocytic MDSCs were increased by entinostat (monocytic MDSCs, $p = 0.016$; granulocytic MDSCs, $p = 0.016$), although the percentage of dead cells in lineage cells (CD3$^+$, CD19$^+$, or CD56$^+$) was not increased (Fig. 3D).

**Entinostat decreases CD40 expression on MDSCs in breast cancer patients**

In preclinical models, the costimulatory receptor CD40 has been shown to play a key role in MDSC immunosuppressive function. However, the role of CD40 on MDSCs in cancer patients remains unclear and the impact of entinostat on MDSC CD40 has not been reported. Thus, we examined the immunomodulatory effect of entinostat on MDSC CD40 expression levels by examining four MDSC phenotypic panels. We found that CD40 expression in the EE cohort was significantly downregulated in monocytic MDSCs (median percentage change from baseline to C1D15, EE $-17.18\%$ vs. EP $+1.57\%$, $p = 0.011$), Lin$^-$ MDSCs (EE $-15.38\%$ vs. EP $+3.37\%$, $p = 0.02$), and immature MDSCs (EE $-8.51\%$ vs. EP $+9.00\%$, $p = 0.007$) compared to CD40 on MDSCs in the EP cohort (Fig. 3A and Table 1). Among the four panels, only granulocytic MDSCs did not show a significant CD40 decrease in the EE cohort (EE $-0.72\%$ vs. EP $+10.52\%$, $p = 0.22$) (Fig. 3B), indicating that entinostat decreases the expression of CD40 on MDSCs in breast cancer patients and that this effect may target specific MDSC populations.

**Entinostat does not impact immune checkpoint receptor expression in T-cell subsets of breast cancer patients**

The impact of entinostat on T-cell subsets or their immune checkpoint receptor expression in cancer patients has not been reported. Thus, we studied the impact of entinostat on the levels of CD8$^+$ T-cells, Foxp3$^+$ CD4$^+$ T-cells, and Tregs. We also analyzed the levels of immune checkpoint receptors PD-1, CTLA-4, and T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), on T-cell subsets in the EP and the EE arms (gating strategy and representative expression of immune checkpoint receptors in T-cell subsets are shown in Fig. 4). Entinostat did not have a significant impact on either the levels of T-cell subsets (data not shown) including Tregs, or their immune checkpoint receptor expression (Fig. S1).

**Discussion**

Recently, our findings in ENCORE 301, a randomized, placebo-controlled, phase II trial of EE vs. EP in women with locally recurrent or metastatic ER+ breast cancer progressing on a nonsteroidal aromatase inhibitor showed that entinostat added to exemestane is generally well tolerated and associated with prolonged OS. Importantly, we also demonstrated that global protein hyperacetylation in PBMCs was associated with longer PFS (HR 0.32; 95% CI, 0.13 to 0.79) at C1D15, suggesting that entinostat may directly target peripheral immune cells and that immunomodulatory effects of entinostat might be implicated in response to therapy in breast cancer patients enrolled on ENCORE 301. To elucidate the impact of entinostat on systemic immunity in cancer patients, we evaluated PBMCs obtained from the ENCORE 301 trial. In the 34 patients whose PBMCs were assessed in the current correlative immune biomarker study, the clinical outcome for PFS (EE median 4.9 mo vs. EP 1.8 mo; HR 0.56) and OS (EE median 28.1 mo vs. EP 20.3 mo; HR 0.62) as well as baseline demographics were consistent with the intention-to-treat population.

MDSCs play a key role in tumor immune escape by inhibiting effector T-cell proliferation and Th1 cytokine secretion, and by stimulating the recruitment and proliferation of Tregs. MDSCs have been shown to promote cancer cell proliferation, tumor angiogenesis, epithelial-mesenchymal transition and dissemination, and high MDSC levels have been shown to correlate with poor OS of cancer patients. Therapies to target MDSCs and studies focused on combining a therapy targeting MDSCs with a T-cell-directed immunotherapy are emerging. However, human MDSCs are complex and difficult to characterize due to the absence of lineage-specific antigens, in contrast to murine MDSCs, which are characterized based on the expression of Gr1 in combination with CD11b.

Because of the phenotypic complexity of human MDSCs, few studies have analyzed more than one subset in cancer...
patients on clinical trial\textsuperscript{23,28}. In the current study, we analyzed four phenotypes of MDSCs and found that entinostat distinctively modulates each phenotype. Human MDSCs are characterized by multiple phenotypic markers including CD11b, CD14, and CD33, and the absence of lineage markers and HLA-DR\textsuperscript{20,25,29}. In this study, granulocytic MDSCs, monocytic MDSCs, and immature MDSCs were termed based on the literature\textsuperscript{20,25}. Immune-suppressive function of these MDSC phenotypes has been reported\textsuperscript{20,25}. As we show in Fig. 2A, Lin\textsuperscript{−} MDSCs contain both monocytic MDSCs and immature MDSC populations. The monocyte differentiation antigen CD14 has been used to define monocytic MDSCs.\textsuperscript{20,25,29}
Although CD15 is often used to identify granulocytic MDSCs, Zea et al. reported that CD14+CD11b− cells produced arginase and also expressed CD15, but were negative for CD80, CD83, CD86, MHC II, and CD11a.30 The CD14+CD11b−CD33− cells had a polymorphonuclear granulocyte morphology. In addition, increased CD14+CD11b+CD33+ granulocytic MDSCs have been shown to associate with poor clinical outcome.20,25 Thus, we defined CD14+CD11b+CD33+ cells as granulocytic MDSCs. In the current study, the immature MDSC population was defined using the same phenotypic markers (CD14−, CD11b+, CD33+) as granulocytic MDSCs in addition to lineage markers (CD3, CD19, CD56) and HLA-DR. Lineage markers and HLA-DR were employed for immature MDSC immunophenotyping to perform more detailed MDSC phenotypic analyses. However, our data demonstrated that the granulocytic MDSC level significantly decreased in the EE cohort but immature MDSCs did not, suggesting granulocytic MDSCs (CD14−CD11b+CD33+ cells) may reflect the entinostat effect more than immature MDSCs (Lin−HLA-DR−CD11b+CD33+CD14− cells) in breast cancer patients.
We demonstrate that two once-weekly doses of entinostat in combination with exemestane decreased the levels of monocytic and granulocytic MDSCs, in contrast to exemestane alone. Our finding of granulocytic MDSC reduction by entinostat in cancer patients is consistent with a recent preclinical study demonstrating that addition of entinostat to isolated MDSCs from 4T1 tumor-bearing mice resulted in a selective decrease in granulocytic MDSCs.11 In this murine model, granulocytic MDSCs were shown to be responsible for resistance to immune checkpoint blockade with anti-CTLA-1 or anti-PD-1 antibody, and the reduction of granulocytic MDSCs by entinostat was essential to cure of murine 4T1 breast cancer.11 These data demonstrating entinostat targeting of MDSCs in an in vivo murine model, together with our data in cancer patients may facilitate combination of this epigenetic modulator with immunotherapy.

The costimulatory receptor CD40 is expressed on monocytes, dendritic cells, B-cells, and MDSCs.24,31,32 In tumor-bearing murine models, CD40 expression on MDSCs plays a critical role in MDSC-mediated immune suppression and is required for Treg induction.24 A recent study has demonstrated higher levels of CD40-expressing MDSCs in cancer patients compared to healthy donors.32 The nonclinical pan-HDAC inhibitor, trichostatin A, has been shown to upregulate CD40 expression on human and murine tumor cell lines in vitro.15 However, the impact of entinostat, or any HDAC inhibitor in clinical trial on MDSC CD40 has not been reported. In the present study, entinostat significantly decreased CD40 expression levels in three MDSC populations, suggesting entinostat not only decreased the number of MDSCs, but also the immunosuppressive function of MDSCs, and that the impact of entinostat on MDSCs may be associated with enhanced antitumor immunity and
improved survival in patients treated with entinostat. Further studies are required to investigate the epigenetic immunomodulatory mechanisms of entinostat as well as the functional role of CD40 on MDSCs in cancer patients.

In the current study, we also studied the direct impact of entinostat on PBMCs. We observed a similar effect of entinostat on HLA-DR expression on CD14<sup>+</sup> monocytes and MDSCs in vitro as the results obtained from breast cancer patient samples from ENCORE 301, suggesting a direct immunomodulatory effect of entinostat in vivo. We also found that entinostat is selectively toxic to granulocytic and monocytic MDSCs in vitro. However, entinostat did not decrease MDSC CD40 in vitro although, in cancer patients, monocytic MDSC CD40 was significantly decreased in the EE cohort. We consider that the discrepancy in the MDSC CD40 result between in vitro experiments and in cancer patients might occur because the conditions of in vitro cultured PBMCs from healthy donors may differ from the in vivo immune status in patients pre- and post-therapy, especially considering that there appears to be an improvement in the immune dysregulation of malignancy in response to entinostat, and patient PBMCs reflect interactions between host immune cells and the tumor microenvironment. These conditions could not be reproduced in in vitro-cultured PBMCs from healthy donors. Taken together, our data from ENCORE 301 and in vitro experiments suggest that entinostat has direct immunomodulatory effects resulting in increased HLA-DR expression on CD14<sup>+</sup> monocytes and decreased MDSCs in cancer patients. However, the results demonstrating decreased MDSC CD40 in breast cancer patients treated with entinostat might be an indirect effect through the impact of entinostat on the tumor microenvironment or on tumor cells that secrete several critical regulators associated with MDSC induction in vivo.

CD14<sup>+</sup>HLA-DR<sup>lo/neg</sup> monocytes have been demonstrated to have immunosuppressive activity and are characterized by decreased capacity for antigen presentation and impaired ability to differentiate into mature dendritic cells. Recently, an elevation in the number of CD14<sup>+</sup>HLA-DR<sup>lo/neg</sup> monocytes has been reported in patients with various malignancies, including breast cancer. However, the mechanisms underlying the increase in CD14<sup>+</sup>HLA-DR<sup>lo/neg</sup> monocytes are not fully understood. Further studies are needed to elucidate the role of CD14<sup>+</sup>HLA-DR<sup>lo/neg</sup> monocytes in the immune response to entinostat and to identify potential therapeutic targets for improving patient outcomes.
monocytes has been shown to correlate with poor survival in cancer patients.\textsuperscript{12,13} These observations suggest that it may be important to maintain HLA-DR levels on CD14$^{+}$ monocytes for effective host antitumor immunity. HDACi have been shown to upregulate HLA expression in cancer cell lines and macrophages.\textsuperscript{2,3,34} Thus, we considered that entinostat might change HLA-DR expression levels on circulating CD14$^{+}$ monocytes of cancer patients. In this study, we found a significant increase of CD14$^{+}$HLA-DR$^{+}$ monocytes in patients treated with entinostat, suggesting epigenetic immunomodulation of circulating CD14$^{+}$ monocytes by entinostat may have increased systemic immunocompetent monocytes, and that this may have contributed to the improved survival of breast cancer patients on the EE arm of ENCORE 301.

Pan-HDACi or class II HDACi have been shown to promote murine Treg expansion and Treg immunosuppressive function.\textsuperscript{7-30} In contrast to these results, recently, we have shown that 24-h continuous intravenous infusion of the pan-HDACi belinostat significantly decreased Tregs in patients with thymic epithelial tumors ($p = 0.0009$).\textsuperscript{40} In preclinical studies, the class I-selective HDACi entinostat has been reported to either activate or inhibit Tregs.\textsuperscript{2,7-10,39}

In this study, we did not observe a statistically significant impact of entinostat in combination with exemestane versus EP on the levels of T-cell subsets including Tregs, or on the level of expression of immune checkpoint receptors. It remains unclear whether longer exposure to entinostat may be required to modulate T-cell subsets in cancer patients. However, we previously demonstrated a significant correlation between protein lysine hyperacetylation and PFS in the same ENCORE 301 samples studied here.\textsuperscript{6}

Preclinical evidence\textsuperscript{11} in concert with the clinical data presented here suggests that entinostat may primarily modulate immunity by its impact on MDSCs and monocytes. These observations suggest that entinostat may positively influence antitumor immunity via immune subtypes that differ from the primary targets of immune checkpoint inhibitors.\textsuperscript{41-44} and thus these observations support combination of this epigenetic therapy with checkpoint blockade (Fig. 5).\textsuperscript{2,11} Further studies of the immunomodulatory activity of entinostat are being performed in the on-going phase Ib/II study of entinostat in combination with the anti-PD-1 antibody pembrolizumab in non-small cell lung cancer and melanoma (NCT02437136).

In conclusion, our findings from ENCORE 301, a randomized, placebo-controlled, phase II trial of exemestane with or without entinostat provide the first evidence of HDACi-mediated reduction of MDSCs and increase of immunocompetent CD14$^{+}$HLA-DR$^{hi}$ monocytes in cancer patients. These observations of positive immunomodulatory activity associated with the addition of entinostat to aromatase inhibitor therapy may in part explain the improved OS observed in breast cancer patients treated with entinostat combined with exemestane compared to patients treated with exemestane alone in ENCORE 301, and may also provide a rationale for combination studies of entinostat with immune checkpoint blockade.

### Materials and methods

#### Patients and clinical samples

Peripheral blood samples were from the subset of 49 patients (27 EE and 22 EP) analyzed for protein acetylation in ENCORE 301, a randomized, placebo-controlled, phase II trial of EE vs. EP in postmenopausal women with locally recurrent or metastatic ER-positive breast cancer progressed on nonsteroidal aromatase inhibitor.\textsuperscript{8} Treatment consisting of exemestane 25 mg by mouth once daily plus placebo or entinostat 5 mg by mouth once weekly continued until progressive disease or unacceptable toxicity. The detailed study design has been described.\textsuperscript{6} Samples were collected on cycle 1 day 1 (C1D1; pre-treatment) and C1D15. Of the 49 patients analyzed previously, both C1D1 and C1D15 samples were available for immune subset analysis from 34 patients (20 EE and 14 EP). The clinical outcome for PFS (EE median 4.9 mo versus EP 1.8 mo; HR 0.56) and OS (EE median 28.1 mo vs. EP 20.3 mo; HR 0.62) in the 34 patients as well as baseline demographics were consistent with the intention-to-treat population reported recently.\textsuperscript{6} Whole blood samples were collected in cell preparation tubes with sodium citrate (BD Vacutainer CPT Tubes, BD Biosciences, San Jose, CA, USA). PBMCs were obtained by centrifugation and viably frozen until analysis.

#### Flow cytometric analysis

Multiparameter flow cytometric analysis was performed on PBMCs as described previously.\textsuperscript{40,45,46} Briefly, cells were incubated with Fc receptor blocking agent (Miltenyi Biotec, Bergisch Gladbach, DE) and stained with monoclonal antibodies for 20 min at 4 °C in a darkened room. The following immunophenotypic markers were used to define four populations of MDSCs: CD14$^{-}$CD11b$^{+}$CD33$^{+}$, granulocytic MDSC; lineage (CD3, CD19, CD56)$^{-}$HLA-DR$^{-}$CD11b$^{+}$CD33$^{+}$, Lin$^{-}$ MDSC; Lin$^{-}$HLA-DR$^{-}$CD11b$^{+}$CD33$^{+}$CD14$^{-}$, monocytic MDSC; Lin$^{-}$HLA-DR$^{-}$CD11b$^{+}$CD33$^{+}$CD14$^{-}$, immature MDSC. Each MDSC phenotype was also evaluated for CD40 expression. For CD8$^{+}$ T-cells, Foxp3-negative CD4$^{+}$ T-cells, and Tregs, the following immunophenotypic markers were used: CD4$^{+}$CD8$^{+}$, CD8$^{+}$ T-cells; CD8$^{+}$Foxp3$^{-}$, Foxp3$^{+}$CD4$^{+}$ T-cells; CD8$^{+}$CD25$^{hi}$Foxp3$^{+}$, Tregs. CD8$^{+}$ T-cells, Foxp3$^{+}$CD4$^{+}$ T-cells, and Tregs were evaluated for levels of expression of immune checkpoint receptors (CTLA-4, PD-1, and TIM-3). CD14$^{+}$ monocytes were evaluated for HLA-DR expression, and the levels of CD14$^{+}$HLA-DR$^{hi}$ monocytes and CD14$^{+}$HLA-DR$^{low/neg}$ monocytes were evaluated as described previously.\textsuperscript{47} The following monoclonal antibodies were used (all from BioLegend, San Diego, CA, USA): for MDSC analysis, Alexa Fluor 647-CD3 clone OX13, Alexa Fluor 647-CD56 clone MEM-188, Alexa Fluor 647-CD19 clone HIB19, PE-Cy7-HLA-DR clone L243, PerCP-CD14 clone HCD14, Alexa Fluor 488-CD11b clone ICRF44, PE-CD33 clone WM53, and Pacific Blue-CD40 clone 5C3. For T-cell subset analysis, Pacific Blue-CD8$^{+}$ clone SK1, PE-Cy7-CD4$^{+}$ clone RAPA-T4, PE-Cy7-CD25 clone BC96, Alexa Fluor 488-Foxp3 clone 206D, APC-Cy7-PD-1 clone EH12.2H7, PE-CTLA-4 clone L3D10, and APC-TIM-3 clone F38-2E2 were used. For monocyte analyses,
PerCP-CD14 clone HCD14 and PE-HLA-DR clone LN3 were used. For analysis of Foxp3 expression, cells were fixed and permeabilized using Fix/Perm buffer (eBioscience), and then labeled with anti-Foxp3 antibody as described previously.40,46,48 Matched isotype controls were used for each antibody to establish the gates. Live cells were discriminated by means of LIVE/DEAD Fixable Aqua Dead Cell Stain (Life Technologies, Grand Island, NY, USA) and dead cells were excluded from all analyses. Anti-CD45+ antibody, APC/Cy7-CD45 clone HI30, was used to identify haematopoietic cells and CD45-negative cells were excluded from all analyses. All flow cytometric analyses were performed using a MACSQuant Analyzer (Miltenyi Biotec). Flow cytometric data for distinct parameters were quantified either as the percentage of cells among viable CD45+ single cells or the median fluorescence intensity (MFI), as indicated. Data were analyzed using FlowJo software (FlowJo LLC., Ashland, OR, USA). Flow cytometric analyses were performed under blinded conditions.

**In vitro assay**

PBMCs of healthy donors were isolated from buffy coats by Ficoll (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) density gradient separation. Fresh PBMCs (2 x 10^6/well) were cultured with DMSO or entinostat (0.5 mM, Sigma-Aldrich, St. Louis, MO, USA) and GM-CSF (10 ng/mL; R&D Systems, Minneapolis, MN, USA) to induce human MDSCs in vitro as described previously.49 Differences in variables between DMSO and entinostat treatments were evaluated by a Wilcoxon signed-rank test.

**Statistical analysis**

The distribution of the percentage change from baseline for the myeloid subsets evaluated was summarized separately for each treatment arm using conventional summary statistics (mean, standard deviation, median, and range). Box plots were used to depict the distributions graphically. Differences between treatment arms were evaluated using the Wilcoxon rank-sum test. p-values were assessed using a two-sided significance level of 5%. No adjustment was made for multiple testing. The statistical analysis was performed using SAS software (version 9.2).

**Disclosure of potential conflicts of interest**

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