An Intact Immune System Is Required for the Anticancer Activities of Histone Deacetylase Inhibitors

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Abstract

Cell-intrinsic effects such as induction of apoptosis and/or inhibition of cell proliferation have been proposed as the major antitumor responses to histone deacetylase inhibitors (HDACi). These compounds can also mediate immune-modulatory effects that may contribute to their anticancer activities. However, HDACi can also induce anti-inflammatory, and potentially immunosuppressive, outcomes. We therefore sought to clarify the role of the immune system in mediating the efficacy of HDACi in a physiologic setting, using preclinical, syngeneic murine models of hematologic malignancies and solid tumors. We showed an intact immune system was required for the robust anticancer effects of the HDACi vorinostat and panobinostat against a colon adenocarcinoma and two aggressive models of leukemia/lymphoma. Importantly, although HDACi-treated immunocompromised mice bearing established lymphoma succumbed to disease significantly earlier than tumor bearing, HDACi-treated wild-type (WT) mice, treatment with the conventional chemotherapeutic etoposide equivalently enhanced the survival of both strains. IFN-γ and tumor cell signaling through IFN-γR were particularly important for the anticancer effects of HDACi, and vorinostat and IFN-γ acted in concert to enhance the immunogenicity of tumor cells. Furthermore, we show that a combination of vorinostat with α-galactosylceramide (α-GalCer), an IFN-γ–inducing agent, was significantly more potent against established lymphoma than vorinostat treatment alone. Intriguingly, B cells, but not natural killer cells or CD8+ T cells, were implicated as effectors of the vorinostat antitumor immune response. Together, our data suggest HDACi are immunostimulatory during cancer treatment and that combinatorial therapeutic regimes with immunotherapies should be considered in the clinic. Cancer Res; 73(24); 1–12. ©2013 AACR.

Introduction

Histone deacetylase inhibitors (HDACi) are U.S. Food and Drug Administration–approved agents used for the treatment of hematologic malignancies (1, 2). HDACi can induce a range of tumor cell-intrinsic biologic responses such as induction of apoptosis, senescence, differentiation, or inhibition of cell-cycle progression that could account for their antitumor activities. Indeed, using preclinical models, we, and others, have previously demonstrated that induction of tumor cell apoptosis is obligate for HDACi to reduce tumor burden in vivo and enhance the survival of tumor-bearing mice (3, 4). In addition to direct effects on tumor cell growth and survival, the therapeutic effects of HDACi may also involve effects on the host immune and vascular systems (5). For example, treatment of tumor cells with HDACi can enhance expression of putative tumor antigens (6), major histocompatibility complex (MHC) class I and II molecules and associated machinery (7, 8), costimulatory molecules (9) and natural killer (NK) cell-activating ligands (10, 11), and enhance phagocytosis by dendritic cells (DC; ref. 12). This could augment the immunogenicity and antigen presenting capacity of tumor cells and increase their susceptibility to killing by cytotoxic lymphocytes (CTL). Indeed, HDACi have been shown to greatly enhance the sensitivity of tumors to immunotherapy. This combinatorial approach has proven highly successful against a range of malignancies in vivo suggesting HDACi may also engage and mediate an antitumor immune response during cancer-related therapy (12–14).

Paradoxically, it is possible that HDACi treatment may also diminish the function of antitumor immune effector cells. There is evidence to suggest the cytolytic functions of NK cells are repressed in patients with cutaneous T-cell lymphoma treated with vorinostat (15) and Schmudde and colleagues (16) demonstrated the activation of naïve lymphocytes by tumor antigens, but not the effector functions of activated lymphocytes, was abrogated by HDACi. HDACi are also thought

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to have anti-inflammatory properties and can reduce the bacterial lipopolysaccharide (LPS)–induced production of proinflammatory cytokines by human peripheral blood mononuclear cells and in mice (17). In addition to their direct anti-inflammatory actions, HDACi can moderate immune function via induction of Foxp3+ Tregs (regulatory T cells; refs. 14, 18) and suppression of CD4+ T-cell responses to T-cell receptor activation (19). Furthermore, preclinical and clinical trials have been initiated using HDACi to treat autoimmune and inflammatory conditions such as systemic lupus erythematosus (20, 21) and colitis (18).

Thus, the effects of HDACi on the function of the immune system seem to be diverse and context-dependent. However, very few of these studies have demonstrated the importance of immune modulation in dictating the antitumor effects and therapeutic outcome of HDACi as a monotherapy in a physiologic setting. To address this, we used preclinical, syngeneic mouse models of cancer to show that the immune system was required for the comprehensive antitumor effects of vorinostat and panobinostat. Striking roles for IFN-γ and tumor cell–restricted IFN-γR signaling were observed in vivo, and vorinostat and IFN-γ acted in concert to enhance the immunogenicity of tumor cells. Treatment of lymphoma with vorinostat in combination with the IFN-γ–inducing agent (α-galactosylceramide, α-GalCer) was significantly more efficacious than vorinostat alone, exemplifying the important role for IFN-γ. Finally, an intriguing role for B cells, but not CD8+ T cells or NK cells, was also demonstrated, highlighting a novel mechanism of immune engagement by HDACi during the treatment of cancer.

**Materials and Methods**

**In vivo tumor models**

Mice (6–12 weeks of age) were housed under specific pathogen-free conditions with food and water freely available according to the Peter MacCallum Cancer Centre Animal Experimental Ethics Committee Guidelines. Wild-type C57Bl/6 (WT) and μMT−/−, C57Bl/6 (μMT−/−) mice were purchased from The Walter and Eliza Hall Institute of Medical Research and Rag-2−/−, e-γ-chain−/−, C57Bl/6 (Rag-2γ−/−), IFN-γ−/−, C57Bl/6 (IFN-γ−/−), and IFN-γR−/−, C57Bl/6 (IFN-γR−/−) mice were bred in-house at the Peter MacCallum Cancer Centre (East Melbourne, Australia). The C57Bl/6-derived MC38 colon adenocarcinoma line (kindly provided by Jeff Schlom, NIH, Bethesda, MD) was maintained as described previously (12). The C57Bl/6-derived Eμ-myc leukemia/lymphoma 4242 and 299 lines (derived in-house at Peter MacCallum Cancer Centre) were maintained as described previously (3). MC38 cells (1 × 10⁶) were injected subcutaneously into the right flank of C57Bl/6 mice. Therapy was initiated when tumor sizes reached 9 mm² (~4 days after inoculation) and tumor growth was measured every 2 to 3 days. Eμ-myc 4242, MSCV–IRES–GFPP, 299 MSCV–IRES–GFPP, and 4242 MSCV–IRES–GFPP/mgrAIC (dnIFN-γR8) cells (1 × 10⁵–5 × 10⁵) were injected i.v. into the tail vein of WT, Rag-2γ−/−, IFN-γ−/−, IFN-γR−/−, and μMT−/− mice. Therapy was initiated when more than 1% GFP+ cells were detected in the peripheral blood (~7–14 days after inoculation), and mice were cycled when encumbered by disease. Mice were treated with vorinostat (with or without α-GalCer), panobinostat, etoposide, or anti-IFN-γ mAb (H-22 clone) as indicated.

**In situ apoptosis assay and histologic assessment**

MC38 tumors from WT and Rag-2γ−/− mice resected 4 to 24 hours after treatment with vorinostat or vehicle, or spleens from vorinostat- and vehicle control–treated WT and Rag-2γ−/− mice bearing established Eμ-myc lymphoma resected at endpoint were fixed in 4% buffered formalin and paraffin embedded, and sections were cut. MC38 tumor sections were interrogated for in situ apoptosis detected using the Apoptag Peroxidase In Situ Apoptosis Detection Kit (Chemicon International; as per the manufacturer’s instructions) and quantified with MetaMorph Microscopy Automation and Image Analysis Software (Molecular Devices). Splenic sections were examined for tumor burden by hematoxylin and eosin staining.

**Flow cytometry for tumor burden and intracellular IFN-γ**

Tumor burden was assessed in peripheral blood and/or spleen of Eμ-myc lymphoma–bearing WT and Rag-2γ−/− mice by gating on live, GFP+B220+ cells, and compared with B220+ CD19+ B cells (anti-B220 RA3-6B2, anti-CD19 1D3; both eBiosciences). Intracellular staining for IFN-γ (XMG1.2; eBiosciences) was performed on peripheral blood following surface staining for B-cell markers, using the BD Cytofix/Cytoperm Kit (BD Biosciences) and compared with isotype control (Rat IgG1 and eBRG1; eBiosciences). After staining, cells were washed twice and resuspended in PBS containing 2% fetal calf serum and Fluoro-Gold (Fluorochrome, LLC), and acquired by flow cytometry.

**Analysis of cellular signaling pathways by Western blot analysis**

Western blotting was carried out on protein lysates prepared from Eμ-myc lymphoma cells (1 × 10⁶) cultured in the presence of 0.5 μmol/L vorinostat and/or 100 U/mL IFN-γ (Merck Millipore) or dimethyl sulfoxide (DMSO) control in complete media for 1 to 6 hours, or MC38 tumors removed from WT and Rag-2γ−/− mice resected 4 hours after treatment with vorinostat or vehicle, following standard Western blotting techniques. Specific proteins were detected on the membrane using anti-mouse STAT1, pSTAT1, AchH4K5, and AchH3 polyclonal antibodies (Upstate Signaling Solutions) then reprobed for anti-mouse β-actin (AC-74; Sigma) as a loading control.

**Quantitative PCR for IFN-γ expression**

Naïve B cells were isolated from the spleen of WT mice using CD45R (B220) MicroBeads (Miltenyi) and cultured in complete media with 100 U/mL IFN-γ, 2 ng/mL interleukin (IL)-12, 20 ng/mL IL-18, and 25 μg/mL LPS in the presence of 0.5 μmol/L vorinostat or DMSO vehicle for 72 hours. Total RNA was isolated from cell pellets by TRIzol extraction (Invitrogen), 300 ng RNA was reversed transcribed with M-MLV RT
An intact immune system is required for the sustained anticancer effects of vorinostat against an aggressive lymphoma

Our laboratory has previously demonstrated that HDACi are highly efficacious against primary B-cell lymphomas arising from Eμ-myc transgenic mice (3, 4, 23). Therefore, we used the Eμ-myc model to confirm the observation that HDACi required an intact immune system for sustained therapeutic efficacy. As expected, the treatment of WT mice bearing established Eμ-myc tumors with vorinostat significantly enhanced survival compared with control treatment (Fig. 2A and Supplementary Table S1). Although vorinostat also significantly enhanced the survival of Rag-2γc−/− mice transplanted with the same Eμ-myc tumor used in WT mice, vorinostat-treated, tumor-bearing Rag-2γc−/− mice succumbed to lymphoma significantly earlier than vorinostat-treated, tumor-bearing WT mice (Fig. 2A and Supplementary Table S1). Consistent with results using the MC38 solid tumor line (Fig. 1B and C), the acute, direct antitumor activity of vorinostat was not diminished in Rag-2γc−/− mice as the percentage of GFP+ tumor cells was significantly reduced in the spleen of both WT and Rag-2γc−/− mice after 5 days treatment (Fig. 2B). At the time of sacrifice, however, the spleen weight of control- and vorinostat-treated Rag-2γc−/− mice was equivalent, suggesting vorinostat could not sustainably reduce tumor expansion as was observed in WT mice (Fig. 2C). Histologic assessment confirmed this finding, as vorinostat treatment reduced tumor burden and restored normal splenic architecture in WT mice, but not in Rag-2γc−/− mice (Fig. 2D). Rag-2γc−/− mice did not suffer from overt drug toxicity as no significant weight loss was recorded over the treatment period for either strain of mouse (Supplementary Fig. S2A). Eμ-myc cells did not develop resistance to vorinostat throughout the course of treatment as cells harvested from vorinostat-treated WT and Rag-2γc−/− mice at sacrifice remained sensitive to vorinostat-induced apoptosis upon re-treatment ex vivo (Supplementary Fig. S2B). Together, these data suggest Rag-2γc−/− mice succumbed to lymphoma despite vorinostat treatment due to lack of a functional immune system.

Anticancer activities of HDACi, but not etoposide, are mediated by the immune system

We extended our discovery that sustained anticancer effects of vorinostat required an intact host immune system by using a second, genetically distinct Eμ-myc lymphoma and a second HDACi, panobinostat. Consistent with Fig. 2A, vorinostat did prolong the survival of Rag-2γc−/− mice bearing a genetically different Eμ-myc lymphoma compared with the control-treated group (Fig. 3A and Supplementary Table S1). However, tumor-bearing Rag-2γc−/− mice again succumbed to disease significantly earlier than tumor-bearing WT mice despite vorinostat treatment (Fig. 3A and Supplementary Table S1). Also, in agreement with our previous publication (4), WT mice bearing established Eμ-myc lymphoma responded successfully to panobinostat and survival was significantly enhanced compared with control treatment (Fig. 3B and Supplementary Table S1). Similarly, Rag-2γc−/− mice bearing established Eμ-myc lymphoma treated with panobinostat survived significantly longer than control-treated Rag-2γc−/− mice.

Results

An intact immune system is required for the robust anticancer activities of vorinostat against a colon carcinoma

We previously demonstrated that vorinostat treatment of MC38 colon adenocarcinoma cells significantly enhanced phagocytosis by DCs (12). To further determine whether vorinostat enhanced the immunogenicity of MC38 tumor cells, we assessed markers of immunogenic cell death (22) throughout the course of treatment as cells harvested from both vorinostat-treated WT and Rag-2γc−/− mice (lacking all lymphocytes; ref. 24) bearing established MC38 tumors were treated with vorinostat and tumor growth was monitored. As expected, vorinostat treatment significantly delayed tumor growth in WT mice (Fig. 1A). Strikingly, the antitumor effect of vorinostat was lost in Rag-2γc−/− mice (Fig. 1A). This was not due to a failure of vorinostat to inhibit HDACi in the immunodeficient mice despite faster growth of MC38 tumors (Fig. 1B). Lysine 5 on histone 4 (H4K5) was hyperacetylated in vorinostat-treated MC38 tumors extracted from both WT and Rag-2γc−/− mice in comparison with the relevant control-treated tumors from each genotype, also confirming appropriate penetration of HDACi into the tumor mass in both immune-compromised and -competent mice (Fig. 1B). Similarly, TUNEL staining revealed tumor cell apoptosis in tumors from both vorinostat-treated WT and Rag-2γc−/− mice (Fig. 1C and D). These data suggest an intact immune system is required to mediate the long-term, complete anticancer effects of vorinostat against solid malignancies.

IFN-γ detection

Serum was extracted from mouse blood samples and interrogated for IFN-γ using the BD Cytometric Cytokine Bead Array mouse IFN-γ Flex Set and Cell Signaling Master Buffer Set (BD Biosciences) as per the manufacturer’s instructions.

Statistical analysis

Statistical significance was assessed using Prism software (GraphPad Software) and calculated by the Mann–Whitney test (nonparametric data) or a log-rank sum test (for overall survival), and a P value of more than 0.05 was always considered statistically significant.

(Primega), and quantitative PCR was performed on 10 ng cDNA using Brilliant II SYBR Green (Agilent Technologies) with primers specific to mouse IFN-γ (F: 5’-GCAACAGCAAGGCAGAAAAG-3’, R: 5’-AGCTCATGTAGCTTGCG-3’) and L32 (F: 5’-TTCTGTGTTCCAATGCTAAG-3’, R: 5’-TGTGAGCGATTCAGCAC-3’).
mice; however, Rag-2γ−/− mice succumbed to lymphoma during treatment with panobinostat, significantly earlier than panobinostat-treated WT mice (Fig. 3B and Supplementary Table S1). Importantly, Rag-2γ−/− mice bearing established Eμ-myc lymphoma had a sustained response to etoposide that was equivalent to that of etoposide-treated, tumor-bearing WT mice (Fig. 3C and Supplementary Table S1). Together, these data suggest that an intact immune system is required for prolonged therapeutic effects mediated by at least two HDACi against different primary Eμ-myc lymphomas and a genetically distinct solid organ malignancy.

**IFN-γ has a key role in mediating anticancer effects of vorinostat**

Our previous study (26) led us to the hypothesis that IFN-γ was required for prolonged HDACi-mediated antitumor responses. Indeed, although the survival of IFN-γ−/− mice bearing established Eμ-myc lymphoma was significantly enhanced by treatment with vorinostat compared with control treatment, tumor-bearing, vorinostat-treated IFN-γ−/− mice succumbed to lymphoma significantly earlier than tumor-bearing vorinostat-treated WT mice (Fig. 4A and Supplementary Table S1). Surprisingly, however, IFN-γR−/− mice were significantly more resistant to growth of Eμ-myc lymphomas following transplant (Fig. 4B, Supplementary Fig. S3A and Table S1). Furthermore, vorinostat was significantly more efficacious against tumors established in IFN-γR−/− mice compared with WT mice (Fig. 4B and Supplementary Table S1). We sought to determine why IFN-γR−/− mice were inherently more resistant to growth of transplanted Eμ-myc tumors compared with WT mice. First, established Eμ-myc cells were found to express the IFN-γR at similar levels in transplanted
WT and IFN-γR−/− mice, and expression was relatively unchanged at the cessation of either control or vorinostat treatment, suggesting resistance to lymphoma was not mediated by an anti-IFN-γR immune response in IFN-γR−/− mice (Supplementary Fig. S4B). We hypothesised that IFN-γR−/− mice have inherently elevated IFN-γ levels. Indeed, naive and lymphoma-bearing IFN-γR−/− mice had significantly higher levels of serum IFN-γ than WT mice (Fig. 5A and B, respectively). Furthermore, when lymphoma-bearing IFN-γR−/− mice were treated with an IFN-γ-inducing agent (α-GalCer-pulsed tumor cell vaccine; ref. 26), IFN-γR−/− mice produced significantly higher levels of serum IFN-γ than equivalently treated, lymphoma-bearing WT mice (Fig. 5B). The role of IFN-γ in delaying progression of Eμ-myc lymphoma was confirmed when IFN-γR−/− mice treated with a neutralizing anti-IFN-γ monoclonal antibody (mAb) succumbed to transplanted Eμ-myc lymphoma at a similar time to WT mice, which was significantly earlier than control IgG (CIgG)–treated IFN-γR−/−
Intact immune system is required for sustained anticancer effect of HDACi against genetically distinct lymphomas. WT and Rag-2γ−/− mice were inoculated intravenously with Eμ-myc 4242 or 299 lymphoma (as indicated) and treatment began when tumor was established (∼7–14 days after inoculation, respectively). A, overall survival of mice (299) treated as described in Fig. 2A is shown (n ≥ 8 mice/group); *, P < 0.0001 (vorinostat-treated groups). B, overall survival of mice (4242) treated with 20 mg/kg panobinostat i.p. daily (5 days on per 2 days off) for 3 weeks, or vehicle control is shown (n ≥ 8 mice per group); *, P < 0.0001 (panobinostat-treated groups). C, overall survival of mice (4242) treated with 30 mg/kg etoposide i.p. on days 1, 3, 5, and 8 after therapy initiation, or vehicle control is shown (n ≥ 14 mice per group); n.s., nonsignificant (etoposide-treated groups). Data shown are representative of at least two independent experiments.

mice (Fig. 5C). Investigation into the IFN-γ signaling pathway of Eμ-myc cells revealed that not only is STAT1 robustly phosphorylated upon treatment with IFN-γ (as expected given the high expression of IFN-γR; Supplementary Fig. S4B), but that vorinostat sensitized tumor cells to IFN-γ leading to higher levels of phosphorylated STAT1 (pSTAT1; Fig. 5D). The activity of vorinostat, as confirmed by hyperacetylation of histone H3, did not alter induction of pSTAT1 by IFN-γ or expression of STAT1, thus confirming that, in Eμ-myc cells at least, IFN-γ signaling occurs independently of HDAC activity. Together, these data suggest that IFN-γ is required for the prolonged antitumor response of vorinostat, and that IFN-γ exerts its effects directly on Eμ-myc cells.

**Functional tumor cell IFN-γR signaling is required for robust anticancer effects of HDACi**

We next aimed to confirm the role of IFN-γ and IFN-γR tumor cell signaling in tumor-bearing immunocompetent mice during vorinostat treatment. A dominant-negative form of the IFN-γR (dnIFN-γR) with a mutated intracellular signaling domain that inhibits signal transduction through the WT receptor (27) was expressed in Eμ-myc cells (Supplementary Fig. S4A). Signaling downstream of the IFN-γR was completely suppressed in dnIFN-γR-expressing Eμ-myc cells, as STAT1 was not phosphorylated in response to IFN-γ treatment, despite equivalent expression of STAT1 compared with empty vector–expressing Eμ-myc cells (Supplementary Fig. S4A). Vorinostat induced hyperacetylation of histone H3 in both cell types (Supplementary Fig. S4B). IFN-γ has been shown to both inhibit (28) and enhance (29) proliferation of tumor cells, and has been shown to induce tumor cell apoptosis (30). However, treatment of Eμ-myc cells with IFN-γ in vitro did not inhibit proliferation (Supplementary Fig. S4C), nor induce apoptosis (Supplementary Fig. S4D) regardless of addition of...
vorfynostat. Similarly, expression of the dnIFN-γ did not alter proliferation of Eμ-myc cells (Supplementary Fig. S4E), nor influence sensitivity to vorinostat (Supplementary Fig. S4F). IFN-γ may also enhance tumor cell immunogenicity (31, 32). Indeed, treatment of Eμ-myc cells with IFN-γ and vorinostat enhanced the expression of MHC class I and II above levels induced by each agent alone in a manner dependent on functional IFN-γR signaling (Supplementary Fig. S5). The importance of tumor cell–restricted IFN-γR signaling for sustained in vivo responses to vorinostat was investigated. Treatment of mice bearing dnIFN-γR Eμ-myc lymphoma with vorinostat significantly enhanced survival compared with control treatment; however, vorinostat-treated mice bearing dnIFN-γR Eμ-myc lymphoma succumbed to lymphoma significantly earlier than vorinostat-treated mice bearing empty vector Eμ-myc lymphoma (Fig. 5E). These data demonstrate that tumor cell signaling through the IFN-γR is necessary for vorinostat to mediate a robust antitumor response in vivo.

Combination of vorinostat and IFN-γ-inducing immunotherapy is highly efficacious in the treatment of established lymphoma

We rationalized that a combination of vorinostat with an inducer of IFN-γ may provide greater therapeutic effects than single-agent treatment. We therefore used α-GalCer, an agent that is currently under clinical investigation as an anticancer immunotherapy and vaccine adjuvant (33). α-GalCer treatment of WT mice bearing established Eμ-myc lymphoma significantly enhanced serum IFN-γ levels compared with control treatment, regardless of vorinostat (Fig. 6A). After 14 days of therapy, tumor growth was arrested in vorinostat-treated mice compared with both control- and α-GalCer–treated mice. However, treatment with the combination of α-GalCer and vorinostat reduced tumor burden to undetectable levels in the peripheral blood (Fig. 6B). Furthermore, treatment of tumor-bearing mice with the combination therapy achieved a significant survival advantage compared with vorinostat treatment alone (Fig. 6C). The fact that α-GalCer alone had no impact on tumor growth or mouse survival, along with Fig. 5D, suggests that the treatment of established tumors with HDACi may sensitize tumor cells to the anticancer effects of IFN-γ. Furthermore, these data suggest that the administration of HDACi concurrently with acute induction of IFN-γ is better able to control tumor growth than HDACi alone and is a viable option for enhancing the therapeutic efficacy of HDACi.

B cells play a role in mediating the antitumor effects of vorinostat

Given the role of IFN-γ during vorinostat treatment and the enhanced immunogenicity of tumor cells by IFN-γ and vorinostat, we hypothesised CTLs may be a key effector cell of the antitumor immune response. Thus, WT mice bearing established Eμ-myc lymphoma were depleted of specific lymphocyte subsets and treated with vorinostat. However, despite efficient depletion from peripheral blood (Supplementary Fig. S6), survival of mice lacking functional CD8+ T cells bearing Eμ-myc lymphoma was equivalent to that of tumor-bearing ClgG–depleted WT mice following vorinostat
treatment (Supplementary Fig. S7A). Similarly, vorinostat sustained survival of tumor-bearing WT mice depleted of NK cells equivalently to that of CIGG-depleted tumor-bearing WT mice (Supplementary Fig. S7D). Codepletion of CD8 and CD4+ T cells or CD8a+ T cells and NK cells did not alter survival compared with depletion of individual subsets (Supplementary Fig. S7C and S7E), together suggesting CTLs do not play a role in mediating the anticancer effects of vorinostat. In support, survival of mice lacking perforin (perforin−/−) bearing Eμ-myc lymphoma was equivalent to that of WT mice following vorinostat treatment (Supplementary Fig. S7F).

Given these results, we investigated the role of another potential effector lymphocyte, B cells. Interestingly, despite vorinostat significantly enhancing survival of B-cell deficient mice (μMT−/−; mice; ref. 34) bearing Eμ-myc tumors compared with control, vorinostat-treated, tumor-bearing μMT−/− succumbed to lymphoma significantly earlier than vorinostat-treated, tumor-bearing WT mice (Fig. 7A). These data suggest that B cells may have an important role in mediating the antitumor effects of vorinostat. Consistent with this observation, although the tumorigenic Eμ-myc B lymphoma cells were depleted upon treatment with vorinostat in vivo, the normal B-cell compartment was not affected (Fig. 7B). Furthermore, stimulation of naive murine B cells for 72 hours in vitro with known inducers of IFN-γ (35) concurrently with vorinostat treatment significantly enhanced the expression of IFN-γ mRNA compared with control-treated, stimulated B cells (Fig. 7C). Naive B cells did not produce IFN-γ, nor did vorinostat treated, unstimulated B cells at the same time point (data not shown), demonstrating the requirement for immune engagement concomitant with vorinostat treatment for enhanced IFN-γ production. Finally, analysis of B cells from the peripheral blood of WT mice bearing Eμ-myc lymphoma following vorinostat treatment revealed significantly higher levels of intracellular IFN-γ compared with mice receiving control treatment (Fig. 7D), suggesting B cells are active producers of IFN-γ in vivo. Together, these data demonstrate a striking role for B cells in mediating the vorinostat antitumor immune response, and suggest these cells may produce IFN-γ in response to vorinostat treatment.

Discussion

It had previously been suggested that HDACi may inhibit antitumor immunity (15, 16) and that HDACi possess immunosuppressive properties (17, 18, 20, 21). Herein, we have used...
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sophisticated mouse models and genetic and biologic tools to conclusively demonstrate that although HDACi have some early intrinsic and direct cytotoxic effects on tumor cells, an intact immune system is required for vorinostat and panobinostat to induce sustained anticancer responses against solid and hematologic tumors. These data complement our previous work demonstrating the combination of HDACi with immunotherapy is highly efficacious against solid malignancies (12), and the work of others demonstrating the production of IFN-γ by CD8+ T cells in mice following injection with HDACi-treated tumor cells (9).

Our data provide the first demonstration that HDACi elicit antitumor immunity in vivo. The remarkable capacity of chemotherapeutics to stimulate antitumor immunity has recently been described. Certain drugs such as doxorubicin, daunorubicin, and gemcitabine can mediate a potent CD8+ T-cell response by inducing immunogenic cell death (also demonstrated herein following vorinostat treatment), upregulating tumor antigens, or increasing cross presentation of tumor antigen, respectively (22, 36, 37). Other drugs, such as etoposide, are considered nonimmunogenic (38), as confirmed herein. We were unable to identify distinct immune cell subset that was singularly important for sustained antitumor effects of HDACi. However, our studies did demonstrate that B cells, rather than CTLs described in the studies above, play a very important functional role in mediating potent responses to vorinostat. A precise role for B cells in mediating tumor eradication has not been well documented, but there is evidence that effector B cells can be directly cytotoxic to tumor cells (39, 40), and produce IFN-γ and other stimulatory cytokines (41) as well as tumor-specific antibodies that would putatively enhance host antitumor responses through...
antibody-dependent cellular cytotoxicity (39, 40). Our data suggest the key role of B cells is to produce IFN-γ. We observed a strong induction of MHC class I on Eμ-myc cells following IFN-γ and vorinostat treatment, which may indicate a role for CD4+ T cells in mediating a humoral antitumor response. Any potential functional interplay between CD4+ T cells and B cells, as well as the role for additional supporting cells such as NK cells, γδT cells, macrophages, and DCs, in our system requires further assessment.

The importance of host-derived IFN-γ in mediating a prolonged therapeutic response to vorinostat has not previously been reported. Although the induction of IFN-γ by chemotherapeutics during anticancer treatment has been demonstrated previously, (37, 38), the requirement for IFN-γ for therapeutic efficacy has only been demonstrated for doxorubicin (42) and HDACi as shown herein. The role of IFN-γ in protecting the host from cancer development has long been recognized, and IFN-γ can directly influence tumor cell immunogenicity (31, 32), proliferation (28, 29), and death (30). It seems that during the treatment of Eμ-myc lymphoma, vorinostat, and IFN-γ act in concert to enhance tumor cell immunogenicity, demonstrated by upregulation of MHC expression and an abrogated vorinostat response in vivo using Eμ-myc cells defective in IFN-γ signaling. Loss-of-function mutations in the IFN-γ signaling pathway are a common mechanism by which tumor cells “escape” the immune system (32) and Kaplan and colleagues found 25% of human lung adenocarcinoma cells were resistant to IFN-γ due to mutations in components of the IFN-γ signaling pathway such as JAK1, JAK2, and IFN-γR1 α chain (31).

As well as genetic tumor “escape” mechanisms, epigenetic mechanisms are also prevalent (43), particularly in the repression of MHC molecules. Upon stimulation with IFN-γ, histone H3 and H4 at the MHC class II locus are acetylated, leading to chromatin remodeling and gene expression (44). Expression of the CIITA transactivator, essential for MHC class II expression, is repressed during the development of B cells to plasma cells by the removal of activating histone acetylation marks and subsequent chromatin remodeling (45). It seems that tumor cells may harness these epigenetic modifications to shutdown expression of immunogenic molecules and evade antitumor immunity (46), as the treatment of IFN-γ-insensitive tumor cells with HDACi can enhance expression of MHC class I, II, and the costimulatory molecule CD40 (7). Indeed, we demonstrate that IFN-γ induction alone (by α-GalCer) did not elicit antitumor activity against Eμ-myc lymphoma; however, in combination with vorinostat, survival of tumor-bearing mice was significantly enhanced, suggesting HDACi treatment may sensitize tumor cells to IFN-γ.

There is evidence supporting a biologically important interplay between HDACi and IFN-γ. HDACi are closely associated with the regulation of STAT-independent, IFN-inducible genes at the transcriptional level (44, 45). Nuclear STAT1 and 2 coprecipitate with HDAC1 and 2 (47) suggesting gene transcription by STAT 1/2 is influenced by either direct STAT acetylation or by recruitment of HDACs to STAT DNA binding sites (47, 48). Moreover, cytoplasmic, inactive STAT1 can be directly acetylated at lysine residues 410 and/or 413 by HDAC3 (49). The acetylation of STAT1 is thought to act as a “timer,” inhibiting activity of IFN-induced STAT1 by restricting nuclear translocation, thereby promoting restoration of a resting cellular state (49). However, the regulation of STAT1 activity by acetylation is currently under debate as Antunes and colleagues were unable to detect acetylation of STAT1 either in a resting state, or following HDACi or IFN treatment (50). The phosphorylation of STAT1 also dictates its activity, and we demonstrated that IFN-γ signaling induced phosphorylation of STAT1 at the tyrosine residue 701 (Tyr701) in Eμ-myc tumor cells. Our data also demonstrated phosphorylation of STAT1 not only occurred independently of HDAC activity, but was also enhanced following vorinostat treatment. The phosphorylation of STAT1 independent of HDAC activity has been demonstrated by some (47, 48, 50), however, others suggest STAT1 cannot be phosphorylated in the presence of HDAC inhibition due to the concomitant inhibition of the phosphorylation/acetylation “switch” (occurring at Tyr701; refs. 23, 49).

Our study provides direct experimental evidence demonstrating the functional importance of the host immune system in mediating sustained antitumor responses to HDACi. We have identified key immune cells (B cells) and effector molecules (IFN-γ) that play important roles in significantly augmented HDACi efficacy in preclinical models. Moreover, using α-GalCer we have identified a novel combination strategy to exploit the functional interplay between IFN-γ and HDACi that was well tolerated and therapeutically advantageous.

Disclosure of Potential Conflicts of Interest

R.W. Johnstone has received commercial research grant and has honoraria from speakers’ bureau from Novartis. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

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