An intact immune system is required for the anti-cancer activities of histone deacetylase inhibitors

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Running title
Anti-cancer activity of HDACi requires immune system

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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 anti-tumor responses to histone deacetylase inhibitors (HDACi). These compounds can also mediate immune-modulatory effects that may also contribute to their anti-cancer effects. 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 physiological setting, utilising pre-clinical, syngeneic murine models of haematological malignancies and solid tumors. We showed an intact immune system was required for the robust anti-cancer effects of the HDACi vorinostat and panobinostat against a colon adenocarcinoma and two aggressive models of leukemia/lymphoma. Importantly, while HDACi-treated immunocompromised mice bearing established lymphoma succumbed to disease significantly earlier than tumor-bearing, HDACi-treated wild type 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 anti-cancer 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-GalCer, an IFN-γ-inducing agent, was significantly more potent against established lymphoma than vorinostat treatment alone. Intriguingly, B cells, but not NK cells or CD8+ T cells, were implicated as effectors of the vorinostat anti-tumor immune response. Together our data suggests HDACi are immunostimulatory during cancer treatment and that combinatorial therapeutic regimes with immunotherapies should be considered in the clinic.
Introduction

Histone deacetylase inhibitors (HDACi) are FDA-approved agents used for the treatment of haematological malignancies (1, 2). HDACi can induce a range of tumor cell-intrinsic biological responses such as induction of apoptosis, senescence, differentiation or inhibition of cell cycle progression, that could account for their anti-tumor activities. Indeed, using pre-clinical 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), MHC class I and II molecules and associated machinery (7, 8), co-stimulatory molecules (9) and NK cell-activating ligands (10, 11), and enhance phagocytosis by DCs (12). This could augment the immunogenicity and antigen presenting capacity of tumor cells and increase their susceptibility to killing by cytotoxic lymphocytes (CTLs). 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 anti-tumor immune response during cancer therapy (12-14).

Paradoxically, it is possible that HDACi treatment may also diminish the function of anti-tumor immune effector cells. There is evidence to suggest the cytolytic functions of NK cells are repressed in cutaneous T cell lymphoma (CTCL) patients treated with vorinostat (15) and Schmudde et al (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 to have anti-inflammatory properties and can reduce the bacterial lipopolysaccharide (LPS)-induced production of pro-inflammatory cytokines by human peripheral blood mononuclear cells (PBMCs) and in mice (17). In addition to their direct anti-inflammatory actions, HDACi can moderate immune function via induction of Foxp3+ Treg (14, 18) and suppression of CD4+ T cell responses to TCR activation (19). Furthermore, pre-clinical and clinical trials have been initiated utilising HDACi to treat autoimmune and inflammatory conditions such as systemic lupus erythematosus [SLE; (20, 21)] and colitis (18).

Thus, the effects of HDACi on the function of the immune system appear to be diverse and context dependent. However, very few of these studies have demonstrated the importance of immune modulation in dictating the anti-tumor effects and therapeutic outcome of HDACi as a monotherapy in a physiological setting. To address this, we utilised pre-clinical, syngeneic mouse models of cancer to show that the immune system was required for the comprehensive anti-tumor 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 wks 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. C57Bl/6 and μMT-/-.C57Bl/6 mice were purchased from The Walter and Eliza Hall Institute of Medical Research and Rag-2–/- c-γ-chain-/-.C57Bl/6, IFN-γ−/-.C57Bl/6 and IFN-γR−/-.C57Bl/6 mice were bred in-house at the Peter MacCallum Cancer Centre. 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 (1x10⁶) were injected s.c. into the right flank of C57Bl/6 mice. Therapy was initiated when tumor sized reached 9 mm² (~4 d post inoculation) and tumor growth was measured every 2 – 3 d. Eμ-myc 4242 MSCV-IRES-GFP, 299 MSCV-IRES-GFP and 4242 MSCV-IRES-GFP/mgrΔIC (dnIFN-γR) cells (1 x 10⁴ – 5 x 10⁵) were injected i.v. into the tail vein of WT C57Bl/6, Rag-2–/- c-γ-chain-/-.C57Bl/6, IFN-γ−/-.C57Bl/6, IFN-γR−/-.C57Bl/6 and μMT−/-.C57Bl/6 mice. Therapy was initiated when > 1% GFP+ cells were detected in the peripheral blood (~7 – 14 d post inoculation), and mice were culled 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 histological assessment
MC38 tumors from WT and Rag-2γc−/− mice resected 4 - 24 h after treatment with vorinostat or vehicle, or spleens from vorinostat- and vehicle control-treated WT and Rag-2γc−/− mice bearing established Eμ-myc lymphoma resected at end-point 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-2γc−/− mice by gating on live, GFP+B220+ cells, and compared to 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 to isotype control (Rat IgG1, eBRG1, eBiosciences). After staining, cells were washed twice and resuspended in PBS containing 2% FCS and Fluoro-Gold™ (Fluorochrome, LLC), and acquired by flow cytometry.

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

Western blotting was carried out on protein lysates prepared from Eμ-myc lymphoma cells (1 x 10⁶) cultured in the presence of 0.5 μM vorinostat and/or 100 U/mL IFN-γ (Merck Millipore) or DMSO control in complete media for 1 – 6 h, or MC38 tumors...
removed from WT and Rag-2γc− mice resected 4 h after treatment with vorinostat or vehicle, following standard Western blotting techniques. Specific proteins detected on the membrane using anti-mouse STAT1, pSTAT1, AcH4K5 and AcH3 polyclonal antibodies (Upstate Signaling Solutions) then re-probed 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 IL-12, 20 ng/mL IL-18 and 25 μg/mL LPS in the presence of 0.5 μM vorinostat or DMSO vehicle for 72 h Total RNA was isolated from cell pellets by TRIzol® extraction (Invitrogen), 300 ng RNA was reversed transcribed with M-MLV RT (Promega), 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′-GCAACAGCAAGGCGAAAAAG-3′, R: 5′-AGCTCATTGAATGCTTGGCG-3′) and L32 (F: 5′-TTCTCGTCCACAATGTAAG-3′, R: 5′-TGTGAGCGATCTCACGAC-3′).

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 (non-parametric data), or a log-rank sum test (for overall survival), p-value > 0.05 was always considered statistically significant.

Results

An intact immune system is required for the robust anti-cancer 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) following exposure to vorinostat. Indeed, calreticulin was translocated to the surface of vorinostat-treated MC38 cells [as previously demonstrated on brain tumor cells by Sonneman et al (23)] and HMGB1 and ATP were released concurrently with the induction of apoptosis, but not in cells over-expressing the anti-apoptotic protein Bcl-2 (Supplementary Fig. S1A-D). Given this observation we next investigated whether the immune system played a role in mediating the anti-cancer effects of vorinostat in vivo. Wild type (WT) and Rag-2γc−/− mice [lacking all lymphocytes (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 anti-tumor effect of vorinostat was lost in Rag-2γc−/− mice (Fig. 1A). This was not due to a failure of vorinostat to inhibit HDACs in the immunodeficient mice despite faster growth of MC38 tumors. Lysine 5 on histone 4 (H4K5) was hyper-acetylated in vorinostat-treated MC38 tumors extracted from both WT and Rag-2γc−/− mice in comparison to 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, D). These data suggest an intact immune system is required to mediate the long-term, complete anti-cancer effects of vorinostat against solid malignancies.

**An intact immune system is required for the sustained anti-cancer 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-μc transgenic mice (3, 4, 25). Therefore we utilised the Eμ-μc 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-μc tumors with vorinostat significantly enhanced survival compared to control treatment (Fig. 2A and Supplementary Table S1). Although vorinostat also significantly enhanced the survival of Rag-2γc−/− mice transplanted with the same Eμ-μc 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 anti-tumor 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 both 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). Histological 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.

**Anti-cancer activities of HDACi, but not etoposide, are mediated by the immune system**

We extended our discovery that sustained anti-cancer effects of vorinostat required an intact host immune system by utilising a second, genetically distinct Eμ-myc lymphoma and a second HDACi, panobinostat. Consistent with Figure 2A, vorinostat did prolong the survival of Rag-2γc−/− mice bearing a genetically different Eμ-myc lymphoma compared to 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 to 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, however Rag-2γc−/− mice also succumbed to
lymphoma during treatment with panobinostat, and significantly earlier than panobinostat-treated WT mice (Fig. 3B and Supplementary Table S1). Importantly, Rag-2γc−/− mice bearing established Eμ-myc lymphoma had a sustained response to etoposide that was equivalent to that of etoposide-treated, tumor-bearing wild type 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 anti-cancer effects of vorinostat**

Our previous study (26) led us to the hypothesis that IFN-γ was required for prolonged HDACi-mediated anti-tumor responses. Indeed, while the survival of IFN-γ−/− mice bearing established Eμ-myc lymphoma was significantly enhanced by treatment with vorinostat compared to 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 Supplementary Table S1). Furthermore, vorinostat was significantly more efficacious against tumors established in IFN-γR−/− mice compared to 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 to WT mice. Firstly, 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 wild type 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 (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−/− 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. 4B), but that vorinostat sensitised tumor cells to IFN-γ leading to higher levels of phosphorylated STAT1 (pSTAT1; Fig. 5D). The activity of vorinostat, as confirmed by hyper-acetylation 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 anti-tumor 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 anti-cancer 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 wild type 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 to empty vector (EV)-expressing Eμ-myc cells (Supplementary Fig. S4A). Vorinostat induced hyper-acetylation of histone H3 in both cell types (Supplementary Fig. 4B). 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 vorinostat. 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 to control treatment, however, vorinostat-treated mice bearing dnIFN-γR Eμ-myc lymphoma succumbed to lymphoma significantly earlier than vorinostat-treated mice bearing EV Eμ-myc lymphoma (Fig. 5E). These data demonstrate that
tumor cell signaling through the IFN-γR is necessary for vorinostat to mediate a robust anti-tumor 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 anti-cancer immunotherapy and vaccine adjuvant (33). α-GalCer treatment of WT mice bearing established Eμ-myc lymphoma significantly enhanced serum IFN-γ levels compared to control treatment, regardless of vorinostat (Fig. 6A). After 14 days of therapy, tumor growth was arrested in vorinostat-treated mice compared to 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 to 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 anti-cancer 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 anti-tumor 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 anti-tumor immune response. Thus, WT mice bearing established Eμ-myc lymphoma were depleted 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 CIgG-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). Co-depletion of CD8 and CD4⁺ T cells, or CD8⁺ T cells and NK cells did not alter survival compared to depletion of individual subsets (Supplementary Fig. S7C and E), together suggesting CTLs do not play a role in mediating the anti-cancer 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 (34)] bearing established Eμ-myc tumors compared to 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 anti-tumor effects of vorinostat. Consistent with this observation, while 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 naïve
murine B cells for 72 h \textit{in vitro} with known inducers of IFN-\(\gamma\) (35) concurrently with vorinostat treatment significantly enhanced the expression of IFN-\(\gamma\) mRNA compared to control-treated, stimulated B cells (Fig. 7C). Naïve B cells did not produce IFN-\(\gamma\), 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-\(\gamma\) production. Finally, analysis of B cells from the peripheral blood of WT mice bearing E\(\mu\)-myc lymphoma following vorinostat treatment revealed significantly higher levels of intracellular IFN-\(\gamma\) compared to mice receiving control treatment (Fig. 7D), suggesting B cells are active producers of IFN-\(\gamma\) \textit{in vivo}. Together these data demonstrate a striking role for B cells in mediating the vorinostat anti-tumor immune response, and suggest these cells may produce IFN-\(\gamma\) in response to vorinostat treatment.

**Discussion**

It had previously been suggested that HDACi may inhibit anti-tumor immunity (15, 16) and that HDACi possess immunosuppressive properties (17, 18, 20, 21). Herein, we have used sophisticated mouse models and genetic and biological tools to conclusively demonstrate that while 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 anti-cancer responses against solid and haematological 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-\(\gamma\) by CD8+ T cells in mice following injection with HDACi-treated tumor cells (9).
Our data provides the first demonstration that HDACi elicit anti-tumor immunity in vivo. The remarkable capacity of chemotherapeutics to stimulate anti-tumor 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), upregulation of tumor antigen, or increasing cross presentation of tumor antigen, respectively (22, 36, 37). Other drugs, such as etoposide, are considered non-immunogenic (38), as confirmed herein. We were unable to identify distinct immune cell subset that was singularly important for sustained anti-tumor 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 anti-tumor responses through antibody-dependent cellular cytotoxicity (39, 40). Our data suggests the key role of B cells is to produce IFN-γ. We observed a strong induction of MHC class II on Eμ-myc cells following IFN-γ and vorinostat treatment, which may indicate a role for CD4+ T helper cells in mediating a humoral anti-tumor response. Any potential functional interplay between CD4+ T cells and B cells, as well as the role for additional supporting cells such as NKT 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. While the induction of IFN-γ by
chemotherapeutics during anti-cancer 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 appears 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 et al found 25% of human lung adenocarcinoma cell lines were resistant to IFN-γ due to mutations in components of the IFN-γ signaling pathway such as JAK1, JAK2 and IFN-γR 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 remodelling and gene expression (44) and expression of the CII transactivator (CIITA), 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 remodelling (45). It appears that tumor cells may harness these epigenetic modifications to shut down 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 co-stimulatory 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. HDACs are closely associated with the regulation of STAT-independent, IFN-inducible genes at the transcriptional level (44, 45), and 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 et al 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 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 (23, 49)].
Our study provides direct experimental evidence demonstrating the functional importance of the host immune system in mediating sustained anti-tumor 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 pre-clinical 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.

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Figure Legends

Figure 1. Intact immune system is required for anti-cancer effects of vorinostat against a colon carcinoma. (A) WT and Rag-2γc−/− mice bearing established s.c. MC38 colon carcinoma (>9mm²) were treated with 150 mg/kg vorinostat i.p. daily for 7 d, then 100 mg/kg for a further 7 d, or vehicle control treated, and tumor growth measured three times per week. Mean ± SEM of tumor size is shown (n = ≥6 mice per group), *p = <0.05 (between treatment groups at each time point). (B) Mice treated as
in A were sacrificed 4 h after the first dose of vorinostat or vehicle, and tumors harvested. Protein was extracted and interrogated by Western blotting for acetylated lysine 5 on histone H4 (AcH4K5), compared to the β-actin loading control. Each column refers to an individual mouse, representative of the group. (C) Mice treated as in A were sacrificed 24 h after the first dose of vorinostat or vehicle, and tumors harvested. Histological samples of each tumor were assessed for in situ apoptosis by TUNEL. Each row refers to an individual mouse, representative of the group. (D) The number of TUNEL positive cells (from histological samples in C) per field of view (x40 magnification; FOV) is shown. Horizontal bar represents mean ± SEM, *p = 0.0042, #p = 0.0008 (within genotypes). Data shown is representative of at least one - two independent experiments.

Figure 2. Vorinostat requires intact immune system for sustained anti-cancer effects against lymphoma. WT and Rag-2γc−/− mice were inoculated i.v. with Eμ-myc 4242 lymphoma and treatment began when tumor was established (~7 or ~14 d post inoculation respectively). (A) Overall survival of mice treated with 200mg/kg vorinostat i.p. daily for 7 d, then 150 mg/kg for a further 21 d, or vehicle control is shown (n = >24 mice per group), *p = <0.0001 (vorinostat-treated groups). (B) Spleens from mice described in A were harvested 12 h after the 5th dose of vorinostat or vehicle. Absolute number of live tumor cells (GFP+B220+viability dye) was assessed by flow cytometry. Mean ± SEM is shown (n = >4), *p = <0.0001 (within genotypes). (C) Individual spleen weights of mice described in A are shown and horizontal bar represents mean ± SEM, n.s. = not significant, *p = <0.0001 (within genotypes). (D) Histology of spleens described in C was assessed by hematoxylin and
eosin staining. Each panel is from an individual mouse and is representative of all in cohort.

**Figure 3. Intact immune system is required for sustained anti-cancer effect of HDACi against genetically distinct lymphomas.** WT and Rag-2γc−/− mice were inoculated i.v. with Eμ-myc 4242 or 299 lymphoma (as indicated) and treatment began when tumor was established (~7 or ~14 d post inoculation respectively). (A) Overall survival of mice (299) treated as described in Fig. 2A is shown (n = >8 mice per 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/two days off, then 15 mg/kg daily 5 days on/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.=non significant (etoposide-treated groups). Data shown is representative of at least two independent experiments.

**Figure 4. IFN-γ is required for therapeutic efficacy of vorinostat.** WT and IFN-γ−/− mice were inoculated i.v. with Eμ-myc 4242 lymphoma and vorinostat treatment (as described in Fig. 2A) began when tumor was established (~7 - 14 d post inoculation). (A) Overall survival of WT and IFN-γ−/− mice treated with vorinostat or vehicle is shown (n = >7 mice per group), *p = <0.0008 (vorinostat-treated groups). (B) Overall survival of WT and IFN-γR−/− mice (4242) treated with vorinostat or vehicle is shown (n = >10 mice per group), #p = 0.0001 and *p = 0.0002 (within treatment groups). Data shown is representative of at least two independent experiments.
Figure 5. Tumour cell IFN-γR signaling is required for therapeutic efficacy of vorinostat. WT and IFN-γR−/− mice were inoculated i.v. with Eμ-myc 299, 4242 or 4242 dnIFN-γR lymphoma (as indicated) and vorinostat treatment (as described in Fig. 2A) began when tumor was established (~7 - 14 d post inoculation). (A, B) Serum from WT and IFN-γR−/− mice was probed for IFN-γ by a flow cytometry-based cytokine bead array. (A) Individual IFN-γ serum levels from naïve mice are shown (n = 20 mice per group), *p = <0.0001. (B) Individual IFN-γ serum levels from tumour-bearing mice (299) treated with a vaccine of irradiated, αGalCer-loaded Eμ-myc 299 lymphoma cells (26), or vehicle control treated 5 days after inoculation with the same Eμ-myc lymphoma cells is shown (7 d post treatment). Horizontal bar represents mean ± SEM (n = >4 mice per group), *p = 0.0139 and #p = 0.0317 (within treatment groups). (C) WT and IFN-γR−/− mice (4242) were treated with an anti-IFN-γ neutralising mAb or CIgG on days -1 and 1 of tumor inoculation, then every 7 d until end-point. Overall survival is shown (n = >4 mice per group), *p = 0.01 (IFN-γR−/− groups). (D) Eμ-myc cells were treated with either 0.5 μM vorinostat, 100 U/mL IFN-γ, both or vehicle control-treated for 6 h in vitro and assessed for phosphorylated STAT-1 (pSTAT1), total STAT1 (STAT1) and acetylated histone H3 (AcH3) expression by Western blotting. Equivalent protein loading was confirmed by β-actin. (E) Overall survival of WT mice (4242 or dnIFN-γR) treated with vorinostat or vehicle is shown (n = >6 mice per group), *p = <0.0001 (vorinostat-treated groups). Data shown is representative of at least two independent experiments.

Figure 6. Combination of α-GalCer and vorinostat is significantly more potent than vorinostat alone. WT mice bearing established Eμ-myc 299 B cell lymphoma...
(~14 d post inoculation) were treated with vorinostat (as described in Fig. 2A), one dose of 2 µg α-GalCer i.p. on the first day of vorinostat treatment, vorinostat and α-GalCer at the same dose and schedule as for individual agents, or vehicle control-treated. (A) Serum was probed for IFN-γ by a flow cytometry-based cytokine bead array 16 h after the dose of treatment. Mean ± SEM of IFN-γ concentration is shown (n = >5 mice per group), *p = 0.0357 and n.s. = non significant (between treatment groups). (B) Peripheral tumor burden of mice was examined by flow cytometry on days -1 and 14 of therapy. Dot plots of tumor cells (GFP$^+$B220$^+$) shown are generated from one mouse per panel and are representative of the group (n = >5). Data shown is representative of at least two independent experiments.

Figure 7. B cells are required for the comprehensive anti-cancer effects of vorinostat. (A) WT and μMT$^{-/-}$ mice were inoculated i.v. with Eµ-myc 4242 and vorinostat treatment (as described in Fig. 2A) began when tumor was established (~7 - 14 d post inoculation). Overall survival of WT and μMT$^{-/-}$ mice treated with vorinostat or vehicle is shown (n = >11 mice per group), *p = 0.0344 (vorinostat-treated groups). (B) WT mice treated as in A were sacrificed after 7 d treatment and the percentage of live Eµ-myc 4242 tumor cells (GFP$^+$B220$^+$) and B cells (CD19$^+$B220$^+$) in the spleen was assessed by flow cytometry. Horizontal bar represents mean ± SEM (n = 9 mice per group), *p = <0.0001 and n.s. = non-significant (within cell types). (C) Naïve B cells were isolated from the spleen of WT mice and stimulated with inducers of IFN-γ in the presence of 0.5 µM vorinostat or vehicle for 72 h. IFN-γ expression was assessed by quantitative real-time PCR in comparison to naïve, unstimulated, untreated B cells following normalization to L32. Horizontal bar represents mean ± SEM, *p = 0.0238. (D) The peripheral blood of WT
mice treated as in A was examined for IFN-γ-expressing B cells on days 0 and 28 of vorinostat or vehicle treatment by intracellular staining and flow cytometry. The percentage of IFN-γ+ B cells (CD19+B220+) is shown, and the horizontal bar represents mean ± SEM (n=> mice per group), *p = 0.0060, n.s. = non-significant (within time points). Data shown is representative of at least one - two independent experiments.
Figure 1

A

Mean tumour size (mm$^2$) vs. Days post tumour inoculation for WT and Rag-2cγ$^{-/-}$ mice treated with Control or Vorinostat.

B

Western blot analysis of AcH4K5 and β-actin expression in WT and Rag-2cγ$^{-/-}$ mice treated with Control or Vorinostat. The bands are shown at 10 kDa and 42 kDa.

C

Micrographs showing TUNEL+ cells in WT and Rag-2cγ$^{-/-}$ mice treated with Control or Vorinostat. Scale bars are 200 μm and 50 μm.

D

Bar graph showing TUNEL+ cells per FOV for WT and Rag-2cγ$^{-/-}$ mice treated with Control or Vorinostat. The bars are marked with *, # for significant differences.
Figure 3

A. Vorinostat

- WT control
- WT drug
- Rag-2cγ^−/− control
- Rag-2cγ^−/− drug

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B. Panobinostat

- WT control
- WT drug
- Rag-2cγ^−/− control
- Rag-2cγ^−/− drug

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C. Etoposide

- WT control
- WT drug
- Rag-2cγ^−/− control
- Rag-2cγ^−/− drug

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Survival (%) vs. Days post tumor inoculation
Figure 6

A

IFN-γ (pg/mL)

0 1000

Cont α-GC Vor α-GC/Vor

Treatment

* n.s

B

Treatment day

-1 14

Cont α-GC Vor α-GC/Vor

C

Survival (%)

100

0 14 28 42 56

Days post tumor inoculation

Control Vor α-GC/Vor

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Figure 7

A) Survival (%)

B) Live cells (%)

C) IFN-γ expression (fold change)

D) IFN-γ+ B cells (%)

Days post tumour inoculation

Control

WT control

WT vorinostat

μMT-/- control

μMT-/- vorinostat

Survival (%)
An intact immune system is required for the anti-cancer activities of histone deacetylase inhibitors

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