Modulation of antitumor immunity with histone deacetylase inhibitors

Tyler R McCaw¹, Troy D Randall¹, Andres Forero² & Donald J Buchsbaum^{*,3}

¹Department of Medicine, Division of Clinical Immunology & Rheumatology, University of Alabama at Birmingham, Birmingham, AL, USA, 35233

²Department of Medicine, Division of Hematology & Oncology, University of Alabama at Birmingham, Birmingham, AL, USA, 35233

³Department of Radiation Oncology, University of Alabama at Birmingham, Birmingham, AL, USA, 35233

* Author for correspondence: Tel.: +1 205 934 7077; djb@uab.edu

Histone deacetylase inhibitors possess a broad array of antitumor activities; however, their net impact on the evolving antitumor immune response is highly dependent on the inhibitors used and the histone deacetylases they target. Herein, we sequentially focus on each stage of the antitumor immune response – from dendritic cell activation and migration, antigen uptake and presentation, T-cell activation and differentiation and the enactment of antitumor effector functions within the tumor microenvironment. In particular, we will discuss how various inhibitors have different effects depending on cellular activation, experimental design and specific histone deacetylases being targeted – and how these changes impact the outcome of an antitumor immune response. At last, we consider the impact these inhibitors may have on T-cell exhaustion and implications for combination with other immunomodulating therapies.

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Gene expression is largely regulated at the level of chromatin structure, which is controlled in part by interactions between histones, transcription factors and other DNA-binding proteins. The functions of histones in particular are regulated by post-translational modifications, such as acetylation and methylation, often referred to as epigenetic marks. For example, acetylation of the ϵ -amino group on lysine residues at the N-terminal tails of histones is generally associated with an open chromatin structure and increased transcription [1]. Histone acetyltransferases and histone deacetylases (HDACs) control the balance of histone acetylation and thereby help regulate chromatin structure and transcription through nucleosome dynamics [2].

The ability of HDACs to deacetylate histones and thereby alter chromatin structure and transcription is generally regarded as their primary activity. However, these enzymes also remove acetyl groups from over 1750 nonhistone proteins. The nonhistone targets of HDACs are commonly constituents of large, macromolecular complexes that are involved in a range of cellular processes, including chromatin remodeling, nuclear transport, cell cycling, signaling and apoptosis pathways [3]. In addition, the activities of HDACs can indirectly promote gene-specific DNA methylation [4], another epigenetic mechanism for controlling gene expression. As a result, HDACs are involved in impressively complex molecular networks that regulate cellular activity and transcription, both positively and negatively.

To date, 18 HDACs are classified into four groups based on sequence homology with yeast enzymes that have similar activities. Class I includes HDACs 1, 2, 3 and 8. Class II HDACs are subdivided into class IIa (HDACs 4, 5, 7 and 9) and class IIb (HDACs 6 and 10). Class III HDACs are members of the sirtuin family – classified according to their homology with yeast Sir2 protein. At last, HDAC11 is the sole member of class IV. The enzymatic activities of class I, II and IV HDACs are derived from a charge-relay system with a Zn^{2+} ion in the active site, whereas class III sirtuins possess NAD^{+} -dependent enzymatic activity. HDAC inhibitors target class I, II and IV HDACs by intercalating into the active site-containing pocket and blocking catalytic activity [5]. Numerous HDAC inhibitors

Table 1. Histone deacetylase specificities of inhibitors discussed in this review.

HDACi	HDAC specificity	Ref.
<i>HDAC inhibitors discussed</i>		
ACY-738	6	[94]
BML-210	1, 2, 3	[105]
Bufexamac	1, 6	[95]
Chidamide [†]	1, 2, 3, 10	[40]
Dacinostat (LAQ824)	class I, class II	[96]
Entinostat (MS275)	1, 2, 3, 11	[97]
EX-527	Sirt1	[98]
Givinostat (ITF2357)	class I, class II	[99]
Nicotinamide (NAM) [†]	class III	[100]
Panobinostat (LBH589) [†]	class I, class II, class IV	[101]
Romidepsin (depsipeptide, FK228) [†]	class I	[102]
Sodium butyrate [†]	class I, class IIa	[100]
Trichostatin A (TSA)	class I, class II	[100]
Tubacin	6	[103]
Tubastatin A	6	[104]
Valproic acid (VPA) [†]	1, 2, 3, 4, 5, 7	[106]
Vorinostat (SAHA) [†]	class I, class II	[9]

[†]Denotes compounds currently approved for clinical use with indications including: cutaneous T-cell lymphoma (romidepsin and vorinostat), peripheral T-cell lymphoma (chidamide approved in China, romidepsin), multiple myeloma (panobinostat) and nonmalignant indications (nicotinamide, sodium butyrate and valproic acid). Consensus on exact isoforms inhibited is lacking, but the interested reader is directed to nuanced discussions of HDAC inhibitor specificities [107–109].

HDACi: Histone deacetylase inhibitor.

are now available with a range of specificities and potencies [6] and the inhibitory specificities of those discussed herein are listed in [Table 1](#).

Many tumors have globally altered DNA methylation patterns as well as increased HDAC expression, mechanisms used to epigenetically silence tumor-suppressor and immune-stimulatory genes and thereby promote immune evasion and tumor outgrowth [7,8]. In recognition of this phenomenon, HDAC inhibitors were initially pursued as anticancer drugs with the hope that they would induce cellular differentiation, growth arrest and apoptosis in transformed cells [9]. Indeed, the single agent efficacy of these agents in hematologic malignancies can in part be ascribed to alterations thereof [10,11]. More generally, HDAC inhibitors exert antitumor effects through a number of mechanisms, not limited to chromatin remodeling, which have been detailed elsewhere [12]. Briefly, altered acetylation status of various nonhistone proteins, including p53, BCL2 and members of the JAK/STAT pathway, contribute to observed antitumor effects [13]. However, it is now apparent that many of the functional outcomes following treatment with HDAC inhibitors in preclinical cancer models are, at least in part, contingent upon an intact immune system, as T-cell depletion or experiments in immunocompromised mice have abrogated their antitumor effects.

The generation of an effective antitumor immune response requires antigen uptake, processing and presentation to T cells by professional antigen-presenting cells (APCs) in secondary lymphoid organs, activation of naive T cells and trafficking of activated T cells into the tumor for enactment of effector functions [14]. HDAC inhibitors are capable of influencing each of these steps; however, the effect of HDAC inhibition on antitumor immunity and the mechanisms involved are often very different, depending on the inhibitor, the HDACs that are targeted and the experimental systems employed. In this review, we will clarify some of these issues, focusing on the potential of HDAC inhibitors to modulate antitumor T-cell responses at each stage of the immune response.

Dendritic cell activation

Professional APCs – dendritic cells (DCs), macrophages and B cells – are necessary for the activation and differentiation of naive T cells. Among these, DCs are the most pivotal, bridging innate and adaptive immunity by sensing danger signals and microbial products and activating T cells accordingly [15,16]. Activated DCs present peptide antigens in major histocompatibility class I (MHC I) or class II (MHC II) molecules, which are recognized

by antigen-specific CD8 and CD4 T cells, respectively. Optimal T-cell activation also requires costimulation, which is provided by the activation-induced expression of ligands, such as CD80 and CD86, which engage costimulatory receptors on T cells. Cytokines secreted by APCs and other cells provide additional signals to T cells and guide their differentiation toward different effector fates. Thus, the activities of antigen-presenting DCs directly regulate the magnitude and effector functions of T cells.

Resting DCs reside in peripheral tissues. Following activation, they increase expression of CCR7 and migrate to the draining lymph node and activate T cells – a process that can be impaired by inhibitors of class I HDACs as well as pan-HDAC inhibitors (Figure 1). For example, the HDAC inhibitors entinostat and valproic acid (VPA) impair poly-I:C-stimulated DC activation *in vitro*, as observed by decreases in nuclear RelB, IRF-3 and IRF-8 [17]. VPA also impairs the chemotaxis of DCs to the CCR7 ligand, CCL19 [17] and reduces the surface expression of CCR7 on plasmacytoid DCs [18]. In fact, a variety of class-specific and pan-HDAC inhibitors decrease the expression of costimulatory molecules (CD40, CD80, CD83 and CD86) and Th1 polarizing cytokines (IL-6, IL-12 and TNF- α) by DCs following Toll-like receptor-mediated activation [17–22], suggesting that DC-mediated T-cell activation will likely also be impaired.

Consistent with this idea, the pan-HDAC inhibitor, panobinostat, reduces antigen uptake, expression of costimulatory molecules and production of Th1 polarizing inflammatory cytokines by poly-I:C-stimulated DCs, which in turn impairs the differentiation of IFN- γ -producing T cells [19]. Similarly, the HDAC inhibitors, vorinostat and givinostat, reduce the T-cell stimulatory capacity of DCs as a consequence of increased expression of indoleamine 2,3-dioxygenase (IDO), both *in vitro* and in the context of graft versus host disease [21]. Interestingly, in co-cultures of ovalbumin-pulsed DCs and ovalbumin-specific CD4 T cells, the expression of Th1-polarizing cytokines and costimulatory molecules is decreased following treatment with dacinostat, whereas the costimulatory signals associated with Th2 polarization remain unaffected, suggesting that the observed changes in DC activation may skew CD4 T cells toward Th2 differentiation [20]. Notably, Th2 responses are associated with normal wound repair, which would likely promote tumor growth.

In contrast to the activities of class I-specific and pan-HDAC inhibitors, inhibition of the class IIb HDAC6 using tubastatin A impairs the production of the immune-suppressive cytokine, IL-10, by DCs and macrophages in an ovalbumin-specific CD4 T-cell adoptive transfer model. This effect is mediated through disruption of the HDAC6-STAT3 complex, leading to a decrease in phosphorylated STAT3, even though acetylation status is not affected. Impaired STAT3 signaling, in turn, increases ovalbumin-specific CD4 T-cell production of IFN- γ [23]. Interestingly, the activity of HDAC11 appears to oppose this function, as overexpression of HDAC11 in primary and RAW264.7 macrophages impairs binding of STAT3 to the IL-10 promoter, thereby reducing IL-10 production and improving DC-mediated CD4 T-cell activation. Conversely, APCs lacking HDAC11 activity showed enhanced IL-10 expression as well as a decrement in IL-12 production [24].

Taken together, these data suggest that the effect of HDAC inhibitors on DC function depends strongly upon the class of HDACs targeted and that some inhibitors can bias the T-cell-stimulatory function of DCs toward either Th1 or Th2 polarization. The activities of HDAC inhibitors also depend on the activation state of the responding DCs - resting DCs are more susceptible to the effects of HDAC inhibitors than previously-activated DCs, suggesting that the existing epigenetic framework of DCs at the time of HDAC inhibitor exposure dictates the extent to which HDAC inhibition impairs immune priming functions, especially in the case of class I-specific and pan-HDAC inhibitors (detailed in Table 2).

T-cell activation

Following antigen encounter, naive T cells dramatically alter their cellular programming to differentiate into effector T cells, a process dominated by metabolic shift from oxidative phosphorylation to aerobic glycolysis [25]. Perturbations in this process can mitigate effector functions, delimiting their ability to control tumor growth [26]. MYC, a critical mediator of recently-activated T-cell metabolic reprogramming, is swiftly upregulated following T cell receptor (TCR) engagement [27], leading to T-cell growth and clonal expansion [28]. However, activation-induced proliferation and IL-2 production are significantly impaired when peripheral blood leukocytes or purified T cells are activated in the presence of the HDAC inhibitors trichostatin A (TSA) or romidepsin [29], an observation that correlates with a strong reduction in MYC expression. Similarly, MYC mRNA and protein expression are reduced in T-cell acute lymphoblastic leukemia (T-ALL) cell lines and patient samples when cultured with the broad-spectrum inhibitor vorinostat [30]. In fact, inhibitors like vorinostat and romidepsin not only reduce MYC expression, they also increase the expression of its antagonists, MXI1, MAD and MLX [31]. Thus, some HDAC inhibitors suppress

Figure 1. Schematic representing the various points in the priming and activation of antitumor immune responses that can be modulated by histone deacetylase inhibition.

Beginning with antigen uptake and DC maturation through trafficking to lymphatic tissues and interactions with naive and/or resting T cells, HDAC inhibitors can negatively impact priming of tumor-specific T cells. However, once activated, HDAC inhibition can potentiate T-cell responses, reducing activation-induced cell death for increased overall survival, increasing T-cell trafficking to the tumor site and boosting intratumoral effector T-cell functions like cytokine production and tumor cell killing. HDAC inhibitors can also directly impact tumor cells for increased expression of damage-associated molecular patterns, which signal largely through toll-like receptors to activate immune cells, and major histocompatibility and costimulatory molecules. The net effect of HDAC inhibitors will also depend on the balance between pro- and anti-Treg activities within the tumor microenvironment. DC: Dendritic cell; HDAC: Histone deacetylase.

metabolic changes critical for activation and differentiation of naive T cells into fully functioning effector cells (Figure 1).

Consistent with the idea that HDAC inhibitors impair TCR signaling, the HDAC inhibitor TSA reduces the accumulation of nuclear NFκB following T-cell activation, ultimately leading to poor expression of critical molecules like IL-2, IL-2Rβ, ICAM-1, LFA-1, CD28, CD40L and CD69 [32]. Some of these effects can likely be attributed to HDAC1 and HDAC2, as developing T cells fail to mature properly in the thymus of HDAC1 and HDAC2 double knockout mice (but not either one individually), due to impaired TCR signaling [33].

Table 2. Studies, experimental designs and findings involving the role of histone deacetylase inhibition on dendritic cell functions.

HDACi	Dose	<i>In vitro/vivo</i>	Model	Host	Effects	Ref.
Impact of HDAC inhibition on DC maturation, function & relevant gene expression						
Entinostat	1–10 ng/ml	<i>In vitro</i>	Poly(I-C) stimulation	Human	Down: CD40, CD80, CD83, TNF- α , IL-6, IL-10, IL-12, CCL19 migration	[17]
VPA	10–100 μ g/ml				Down: CD40, CD80, CD83, CCL19 migration, nuclear RelB, IRF-3, IRF-8	
VPA	0.1–0.5 mM	<i>In vitro</i>	Healthy donor PBMC CpG stimulation	Human	Up: IL-10 producing CD4 T cells Down: CD80, CD83, CD86, CD40, ICOSL, PD-L1, CCR7, IFN α , TNF- α , IL-6	[18]
Panobinostat	2.5–20 nM	<i>In vitro</i>	Poly(I-C) or LPS stimulation	Human	Down: CD40, CD83, IL-6, IL-10, IL-12, IL-23, TNF- α , MHCI, RelB, antigen uptake, T-cell IFN- γ production	[19]
LAQ824	10–80 nM	<i>In vitro</i>	LPS stimulation OVA and DO11.10	Both	Down: MCP-1, MCP-2, MCP-3, CCL15, CCL23, CCR1, CCR5, CD38, SPHK1, CD40, SLAM, IL-12, IL-15, EBI3, ITAC, MIG, IP10, T-cell IFN- γ production, DC-induced Th1 migration	[20]
SAHA	100 nM–5 μ M	Both	Experimental GVHD	Both	Down: CD40, CD80, CD86, TNF- α , IL-6, IL-12, nonactivated T-cell proliferation, T-cell IFN- γ production Up: IDO	[21]
ITF2357	10–100 nM (5 mg/kg)				Down: TNF- α , nonactivated T-cell proliferation Up: IDO	
Romidepsin	50 ng/ml	<i>In vitro</i>	TLR7/8 agonist (007)	Human	Down: CD80, IL-12	[22]
Tubastatin A	5 μ M (25 mg/kg)	Both	HDAC6 KO OVA and OT-II	Mouse	Down: IL-10	[23]

Parenttheses indicate *in vivo* doses.
DC: Dendritic cell; GVHD: Graft versus host disease; HDACi: Histone deacetylase inhibitor; IDO: Indoleamine 2,3-dioxygenase; LPS: Lipopolysaccharide; PBMC: Peripheral blood mononuclear cell; TLR: Toll-like receptor; VPA: Valproic acid.

Table 3. Studies, experimental designs and findings involving the effect of histone deacetylase inhibition on naive and resting T-cell activation.

HDACi	Dose	<i>In vitro/vivo</i>	Model	Host	Effects	Ref.
Impact of HDAC inhibition on T-cell activation						
SAHA	1 μ M	<i>In vitro</i>	DND-41 cell line Primary T-ALL cell lines	Human	Down: MYC	[30]
SAHA	2.5 μ M	<i>In vitro</i>	CEM cell culture	Human	Down: MYC, I κ B α Up: MAD, MXI1, MLX, TIEG, ID2, ID3, GADD45b, TNF, REL α	[31]
Romidepsin	-					
Romidepsin	50 ng/ml	<i>In vitro</i>	CTCL	Human	Up: CD4 and CD8 T-cell apoptosis	[22]
Romidepsin	1–100 ng/ml	<i>In vitro</i>	Healthy donor PBLs Jurkat	Human	Down: T-cell proliferation, CD11a, CD25, CD54, CD154, IL-2, MYC	[29]
TSA	10–1000 ng/ml					
TSA	5–100 nM	<i>In vitro</i>	Naive T-cell stimulation with aCD3/aCD28 or PMA/I, 20 h	Mouse	Down: IL-2, IL-2R α , IL-2R β , LFA-1, ICAM-1, CD3 ϵ , CD28, CD40L, CD69, nuclear NF κ B Up: ROS-mediated apoptosis	[32]

CTCL: Cutaneous T-cell lymphoma; HDACi: Histone deacetylase inhibitor; PBL: Peripheral blood leukocyte; ROS: Reactive oxygen species; T-ALL: T-cell acute lymphoblastic leukemia; TSA: Trichostatin A.

Abortive TCR signaling often leads to T-cell apoptosis. Not surprisingly, therefore, HDAC inhibitors like TSA can trigger growth arrest and reactive oxygen-mediated apoptosis in naive T cells at concentrations as low as 5 nM. Similarly, vorinostat and romidepsin promote the expression of various components in the TGF- β pathway (TIEG, ID2, ID3 and GADD45b) in human T-ALL cell lines, which may also contribute to growth arrest and apoptosis [31]. Consistent with this idea, activation of the TGF- β -Smad3 pathway impairs CD28-mediated CD4 T-cell growth and proliferation [34]. These observations are also in agreement with empirical evidence from patients with cutaneous T-cell lymphoma treated with romidepsin, as frequencies of circulating CD4 and CD8 T cells are reduced *in vivo* and T cells cultured with romidepsin *ex vivo* showed increased apoptosis [22]. Taken together, these data suggest that HDAC inhibitors profoundly impact T-cell development, the maintenance of the naive T-cell compartment and important T-cell activation pathways (detailed in Table 3), all of which are likely to impact

Table 4. Studies, experimental designs and findings involving the effect of histone deacetylase inhibition on effector T-cell functions.

HDACi	Dose	<i>In vitro/vivo</i>	Model	Host	Effects	Ref.
<i>Impact of HDAC inhibition on effector T cells & relevant gene expression</i>						
Romidepsin	(2 mg/kg)	<i>In vivo</i>	LKR	Mouse	Up: T-cell cxcl5, ccl10, CD4 tumor infiltration	[63]
TSA	(1–3 μ M/kg)	<i>In vivo</i>	B16F0, EL4	Mouse	Down: FasL, CD4+ T-cell activation induced cell death Up: CD44, IL-2, IFN- γ , TNF- α	[36]
Sodium butyrate	10–100 nM	<i>In vitro</i>	A1.1	Mouse	Down: CD4 T-cell activation induced cell death, FasL, NFAT1, Egr2	
Panobinostat	0.1–100 nM	<i>In vitro</i>	PBMC from melanoma, myeloma and breast cancer patients	Human	Up: T-cell apoptosis Up/down: pSTAT1, 3, 5, 6, pAKT, pERK1/2, CyD3	[35]
Chidamide	3.1–400 nM (12.5–50 mg/kg)	<i>In vitro</i>	Healthy donor PBMCs T-cell lymphoma WBCs	Human	Up: cd3z, cd3e, cd16, cx3cr1, fasl, gzma, gzmh, gzmK, perforin, il7r, klrg1, nkg2d	[40]
Entinostat	(5 mg/kg/day)	<i>In vivo</i>	RENCA	Mouse	Up: Tumor cell lysis Down: Treg numbers	[37]
Entinostat	0.5 μ M (5–20 mg/kg/day)	Both	RENCA	Mouse	Up: CD8 IFN- γ production, STAT3 acetylation Down: Treg FoxP3 MFI and suppression of T-cell proliferation	[38]

Parentheses indicate *in vivo* doses.
 HDACi: Histone deacetylase inhibitor; PBMC: Peripheral blood mononuclear cell; TSA: Trichostatin A.

antitumor T-cell responses.

Antitumor activity of effector T cells

Despite the apparently negative effects of HDAC inhibitors on developing and concurrently activated T cells, the impact of HDAC inhibitors on previously-activated effector T cells is more variable. For example, lymphocytes obtained from the peripheral blood of melanoma, myeloma or breast cancer patients as well as healthy donors were less sensitive to the cytostatic and cytotoxic effects of panobinostat when they were activated with anti-CD3 and IL-2 prior to the addition of the inhibitor [35]. Similarly, both TSA and sodium butyrate prevent FasL-driven activation-induced cell death of CD4 T cells, increase tumor-infiltrating CD4 T cells and reduce tumor growth in mouse models of lymphoma and melanoma, due to the suppression of NFAT1-mediated FasL expression on activated CD4 T cells, but not CD8 T cells [36]. Thus, HDAC inhibitors may promote or suppress T-cell responses, depending on the activation state of T cells and on the context of their activation.

Tumor-oriented cytolytic activity is typically ascribed to CD8 effector T cells, which kill through several mechanisms, most notably perforin and granzyme secretion. In concert with CD4 T cells, cytotoxic CD8 T cells also produce IFN- γ and TNF- α , cytokines that activate local APCs and make tumor cells more immunogenic by enhancing MHC expression, activating the immunoproteasome and directly promoting tumor cytotoxicity. Thus, the modulation of CD8 T-cell responses with HDAC inhibitors is a critically important consideration. For example, the combination of entinostat and IL-2 elicits dramatic increases in IFN- γ production and CD8 T-cell cytotoxicity in a mouse model of renal cancer [37,38]. This combination has more recently been tested in a Phase I/II clinical trial for the treatment of metastatic clear cell renal cell carcinoma, showing improved response rates and longer progression free survival, effects that correlated with fewer regulatory T cells as well as increased tumor-infiltrating CD8 T cells in responders [39]. Similarly, chidamide, which inhibits HDACs 1, 2, 3 and 10, increases the cytolytic activity of human PBMCs *ex vivo* [40]. In contrast to these predominantly class I inhibitors, selective inhibition of HDAC6 may impair the cytotoxic capacity of CD8 T cells, as tumor-specific CD8 T cells from mice treated with tubastatin A (HDAC6-specific inhibitor) and HDAC6 KO mice showed reduced lytic capacity, possibly as a consequence of improper intracellular trafficking and exocytosis of perforin [41]. Accordingly, class I HDAC inhibition appears to augment T-cell function (when given after initial activation), while class II inhibition may impede T-cell effector functions and antitumor immunity (findings detailed in Table 4).

Tumor antigen presentation & immunogenicity

Increasing tumor immunogenicity is perhaps the best-studied consequence of HDAC inhibition with respect to antitumor immunity. Indeed, much of the benefit conferred by HDAC inhibitors may be contingent upon their

ability to alter the tumor microenvironment (TME). First, both class I- and pan-specific HDAC inhibitors increase the expression of MHC I antigen processing pathway and promote re-expression of MHC I molecules on the surface of tumor cells, leading to enhanced recognition and lysis by cytotoxic CD8 T cells in a variety of cancer types [42–45]. HDAC inhibitors also promote the expression of the MHC II antigen processing and presentation pathway, leading to CD4 T-cell activation [43,45–47].

Interestingly, HDAC inhibitors possessing activity against class I HDACs appear to be more potent at stimulating the MHC II antigen presentation pathway, which enhances CD4 T-cell activation and the expression of cytokines and costimulatory ligands; like IFN- γ , TNF- α , IL-2 and CD40L; that together activate cells in the TME and provide help to CD8 T cells [48,49]. Importantly, these compounds also increase expression of costimulatory proteins and adhesion molecules like CD80/86 [50], CD40 [45] and 4-1BB [51], all of which are involved in immune synapses between T cells and tumor cells. As a result, tumor cells act like APCs and more efficiently stimulate T cells. In fact, studies using murine breast tumors show that expression of these proteins can dramatically boost T-cell infiltration and effector functions (Figure 1) for superior antitumor immunity [52,53]. A similar link between the expression of MHC II on tumor cells and positive clinical outcomes is observed in patients with breast [54,55], ovarian [56], colorectal [57] or melanoma tumors [58].

Treatment with HDAC inhibitors also increases the susceptibility of tumor cells to other mechanisms of T-cell-mediated killing. For example, romidepsin promotes the expression of an active death-inducing signaling complex in hematopoietic malignancies, sensitizing tumor cells to TRAIL-mediated death through its receptors, DR4 and DR5 [59]. This effect is mediated by class I, but not class II, HDAC inhibitors and requires HDAC1 and HDAC2, but not HDAC3, HDAC6 or HDAC8 [60]. Similarly, the treatment of primary melanoma samples with the inhibitor vorinostat leads to DR5-dependent killing of tumor cells by CD8 T cells [61] and the treatment of human colon carcinoma cells with vorinostat promotes the expression of the death receptor Fas and enhanced Fas-dependent cytotoxicity by T cells [62].

In addition to promoting the expression of MHC molecules as well as costimulatory and death receptors by tumor cells, some HDAC inhibitors enhance T-cell migration to the tumor site (Figure 1). For example, treatment of lung cancer-bearing mice with romidepsin increases T-cell trafficking to tumor beds via increased expression of CCL5, CXCL9 and CXCL10 by tumor and stromal cells [63]. Interestingly, CXCL5 and CCL10 are also upregulated in tumor-infiltrating CD4 and CD8 T cells, which are indispensable for the antitumor activity [63]. Taken together, these results suggest that HDAC inhibition represents a powerful way to enhance the infiltration of tumors by T cells, increase the recognition of tumors by T cells and promote the susceptibility of tumor cells to T cell-mediated killing. Moreover, once IFN- γ -producing T cells are present and engaged, they will reinforce MHC expression, enhance tumor sensitivity to T-cell killing and recruit additional inflammatory cells that lead to tumor regression [53]. Thus, it is important to understand not only the direct effects of HDAC inhibitors on T cells, but also their indirect effects on T cells through modulating tumor cell phenotypes.

Regulatory T cells

Tumor outgrowth selectively occurs when adaptive immunity is subverted by an immunosuppressive TME. A suppressive TME often includes regulatory T cells (Tregs), which impair effector T-cell proliferation and function through a variety of mechanisms [64], including secretion of suppressive cytokines like IL-10, TGF- β and IL-35, consumption of environmental IL-2 and expression of inhibitory receptors like CTLA-4. Treg differentiation and function is dependent on the transcription factor FoxP3, which can be deacetylated by HDACs 1, 6, 7, 9 and SIRT1 (class III HDAC) [65–67]. Given that acetylation reduces the proteosomal degradation of FoxP3, inhibitors of these HDACs would be expected to increase FoxP3 expression and increase the Treg numbers or function. As a result, considerable attention is given to the impact of HDAC inhibitors on Tregs (Figure 1).

Consistent with the idea that certain HDAC inhibitors should increase Treg numbers and functions, administration of TSA to mice increases FoxP3 acetylation and binding to target loci; promotes the expression of FoxP3, CTLA-4, PD-1, GITR and IL-10 by Tregs; and enhances their suppressive activity [68]. HDAC-dependent mechanisms other than FoxP3 acetylation also promote Treg expansion and function. For example, the inhibition of HDAC6 by tubacin or tubastatin A increases HSP90 acetylation in Tregs, causing release of HSF-1 and subsequent upregulation of target genes involved in Treg function [69]. Moreover, knockout of HDAC9 leads to increased STAT5 acetylation and phosphorylation, which in turn upregulates the expression of Treg-associated genes. In fact, although HDAC6, HDAC9 and SIRT1 can all deacetylate FoxP3, each of these proteins regulates FoxP3

Table 5. Studies, experimental designs and findings involving the effect of histone deacetylase inhibition on regulatory T-cell number and function.

HDACi	Dose	<i>In vitro/vivo</i>	Model	Host	Effects	Ref.
<i>Impact of HDAC inhibition on regulatory T cells & relevant genes</i>						
Entinostat	0.5 μ M (5–20 mg/kg)	Both	RENCA	Mouse	Up: CD8 IFN- γ production, STAT3 acetylation Down: FoxP3 MFI, Treg suppression of Teff proliferation	[38]
Entinostat	0.06 μ M	<i>In vitro</i>	Healthy donor	Human	Up: Treg suppression of Teff proliferation, CTLA-4 Down: Treg and Teff activation-induced proliferation	[70]
SAHA	0.2 μ M					
Sodium butyrate	200 μ M					
VPA	250 μ M					
Tubacin	1 μ M					
Bufexamac	1.25 μ M					
BML-210	2 μ M					
TSA	(1 mg/kg)	Both	C57BL/6	Mouse	Up: Treg suppression of Teff proliferation, Treg numbers, acetylated FoxP3, CTLA-4, PD1, GITR, IL-10	[68]
TSA	50–250 nM	<i>In vitro</i>	Human PBMC, skin T cells C57BL/6	Both	Up: FoxP3 expression and acetylation, Treg suppression of Teff proliferation	[66]
NAM	1–10 mM					
Tubacin	0.1 μ M (0.5 mg/kg/day)	Both	Multiple	Mouse	Up: CTLA-4, IL-10, GITR, PD-1, FoxP3, Treg suppression of Teff proliferation	[69]
Tubastatin A	20 nM (25 mg/kg/day)	Both	Multiple	Mouse	Up: Treg suppression of Teff proliferation, heat shock response, acetylated STAT5	[67]
Tubastatin A	(40 mg/kg/day)					
ACY-738	1 μ M					
Ex-527	5 μ M (1 mg/kg/day)					

Parentheses indicate *in vivo* doses.
HDACi: Histone deacetylase inhibitor; PBMC: Peripheral blood mononuclear cell; Teff: Effector T-cell; Treg: Regulatory T-cell; TSA: Trichostatin A; VPA: Valproic acid.

expression via different mechanisms [67]. Given the roles of HDAC6, HDAC9 and SIRT1 in FoxP3 expression, inhibitors targeting these molecules may impair, rather than promote antitumor immunity.

In contrast to class II and III inhibitors, class I inhibitors may actually decrease FoxP3 expression and impair the suppressive capacity of Tregs. For example, entinostat enhances the acetylation of STAT3, which impairs FoxP3 expression and Treg function [38]. Not all reports are consistent on this effect however, possibly due to important differences in experimental design. For instance, a wide range of structurally diverse HDAC inhibitors (BML-210, bufexamac, entinostat, vorinostat, sodium butyrate, VPA and tubacin), broadly suppress effector T-cell proliferation in co-cultures with Tregs, an effect that correlates with increased CTLA-4 expression [70]. However, this effect is notably diminished when Tregs are pretreated with inhibitors prior to co-culture with effector T cells [70]. Thus, for any particular inhibitor, it is essential to properly distinguish its effects on Tregs from its effects on effector T cells (detailed in Table 5). Given that FoxP3 gene transcription and FoxP3 protein acetylation are both dynamically regulated by multiple combinations of histone acetyltransferases and HDACs [65], it seems that therapeutic regimens involving HDAC inhibitors should be carefully designed to tip the balance of tumor-responsive T cells away from Tregs and toward effector T cells.

T-cell exhaustion

In addition to recognizing and killing pathogen-infected cells, it is now clear that T cells can recognize and eliminate malignantly transformed cells [71]. However, when immune responses fail to clear infections or tumors, repeated TCR signaling coupled with continued inflammation drives a cellular program of T-cell exhaustion [72]. T-cell exhaustion is characterized by the inability to express cytokines and effector molecules, as well as by the increased expression of inhibitory receptors [73], both of which are the consequence of epigenetic reprogramming [74,75]. The prototypic exhaustion marker, programmed death 1 (PD-1), is strongly expressed by exhausted T cells in a process mediated by alteration of epigenetic marks [76] and open chromatin regions [77]. These epigenetic changes enforce PD-1 expression and curb T-cell effector functions.

Regulation of T-cell effector function and exhaustion via epigenetic mechanisms begs the question: can HDAC inhibitors alter the cellular program driving T-cell exhaustion? The answer may be ‘not easily’, as the chromatin organizer Satb1 suppresses PD-1 expression by recruiting a nucleosome remodeling deacetylase (NuRD) complex, including HDACs 1 and 2, to the regulatory regions of the gene encoding PD-1 [78]. Thus, inhibitors of HDAC1 and HDAC2 would likely increase PD-1 expression. In addition, treatment of mouse and human melanoma cells with panobinostat leads to increased histone acetylation near the genes encoding PD-L1 and PD-L2, the ligands for PD-1, and promotes expression of these proteins [79]. Thus, HDAC inhibitors may increase PD-1 on T cells and upregulate its ligands on tumor cells, thereby rendering the TME even more suppressive. Encouragingly, treatment with the HDAC inhibitor VPA reverses functional exhaustion of CD8 T cells in a model of chronic viral infection, by increasing histone acetylation and promoting transcription of the genes encoding IFN- γ and IL-2, thereby leading to increased expression of IFN- γ and TNF- α protein [80]. Collectively, these studies demonstrate the complexity of epigenetic networks regulating T-cell exhaustion and argue for further investigation of HDAC inhibitors as a method of reducing T-cell exhaustion *in vivo*.

Combination with immunotherapies

Checkpoint blockade using anti-PD-1 or anti-CTLA-4 is capable of inducing durable remissions; however, only a minority of patients benefit substantially from these interventions [81,82]. The low frequency of responding patients may be due to the lack of an antitumor T-cell response prior to therapy. Therefore, the use of HDAC inhibitors to augment antitumor T-cell responses at multiple points may increase the frequency of patients responding to checkpoint blockade or other immunomodulatory treatments. In fact, combined treatment with entinostat and anti-PD-1 reduces tumor burden in murine models of melanoma [79] and lung cancer [63], in some cases by compromising the suppressive activities of myeloid-derived suppressor cells [83]. This approach is now being evaluated in clinical studies [84]. In a related approach, HDAC inhibitors (vorinostat or panobinostat) are also being combined with antibodies that stimulate CD40 and 4-1BB to enhance T-cell-mediated destruction of established solid tumors [85]. Thus, treatments that alleviate immune suppression or directly stimulate T-cell expansion appear to potentiate the effect of HDAC inhibitors.

Adoptive transfer of *ex vivo*-expanded tumor-specific T cells is another exciting avenue of tumor immunotherapy; however, this process often fails to achieve durable immunity due to limited long-term engraftment of the transferred T cells [86]. In this regard, treatment of B16 melanoma-bearing mice with dacinostat or panobinostat expands adoptively-transferred, tumor-specific CD8 T cells, increases the production of IFN- γ , TNF- α and IL-6, enhances the surface expression of OX40 and CD107a and impairs tumor growth [87,88]. Similarly, treatment of melanoma-bearing mice with romidepsin increased the response of adoptively transferred T cells following vaccination and promoted tumor killing [89]. Thus, HDAC inhibitors enhance the effects of adoptive T-cell therapies.

Conclusion

Our understanding of how HDAC inhibitors work has dramatically evolved from the relatively simple idea that they alter histone acetylation and regulate chromatin accessibility and transcription. We now know that these agents also affect the acetylation of many nonhistone proteins and alter the activities of elaborate regulatory networks that control cellular activation, migration, differentiation and apoptosis pathways – only partly via the regulation of gene expression. This complexity is clearly evident in the effect of various HDAC inhibitors on antitumor immune responses, which can be impacted positively or negatively at multiple stages, including DC activation and antigen presentation, T-cell differentiation and exhaustion, Treg suppressor function and tumor cell immunogenicity (Figure 1). For each of these examples, the identity of the targeted HDACs and the timing of their inhibition are critical to experimental outcomes, as resting and activated cells (DCs, effector T cells, Tregs and tumor cells) have profoundly different epigenetic frameworks and respond very differently to various inhibitors.

Future perspective

Epigenetic modulation is becoming an increasingly prevalent approach to cancer therapy; however, like traditional treatment regimens and newer checkpoint blockade antibodies, epigenetic modifiers are unlikely to be of clinical value in solid tumors as a monotherapy. Therefore, the continued progress of this field necessitates a more comprehensive understanding of how not just HDAC inhibitors but also other epigenetic modifying agents, like bromodomain and extraterminal (BET) inhibitors and DNA methyltransferase (DNMT) inhibitors, and combinations thereof impact the immune network, rather than single cell types in isolation. For example, combined

HDAC and BET inhibition was recently found to boost tumor-infiltrating T-cell effector function, while impairing those of regulatory T cells for improved tumor control, demonstrating the benefit of multimodal epigenome manipulation [90]. Combinations of epigenetic modifiers and the timing of administration relative to immune priming must be carefully considered, though, because while BET inhibitors can reduce intratumoral PD-L1 expression [91], they have also been demonstrated to suppress T-cell effector functions, in part through decreasing expression of MYC and NF- κ B [92]. Conversely, DNMT inhibitors can augment T-cell functions and lead to a concomitant increase in inhibitory molecules like PD-L1 [93]. Accordingly, future investigations should focus on how specific epigenetic modifiers and other immune modulating agents, each with orthogonal mechanisms of action, can be used in combination (perhaps in sequence) to directly or indirectly (via manipulating the TME) fine-tune antitumor immune networks.

Executive summary

Histone deacetylase inhibitors impair T-cell activation

- Antigen uptake, maturation and trafficking to lymphatic tissues by dendritic cells are impaired by histone deacetylase (HDAC) inhibition, which likely decreases the amplitude of antitumor T-cell responses.
- The effect of HDAC inhibition appears to be highly dependent on cellular activation state, as previously activated T and dendritic cells are less susceptible than unactivated counterparts.

Histone deacetylase inhibitors can potentiate intratumoral T-cell effector functions

- HDAC inhibition may increase T-cell cytotoxicity, while also augmenting efficacy of other immunotherapies, like checkpoint blockade, stimulatory antibodies and adoptive cellular transfer.
- HDAC inhibition can reduce the suppressive nature of the tumor microenvironment by impairing regulatory T cells, while also upregulating costimulatory and antigen presenting molecules directly on tumor cells.

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