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Modulation of Antigen Presenting Cells by HDAC inhibitors: implications in autoimmunity and cancer

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Abstract

HDAC inhibitors are being increasingly used in the treatment of diverse conditions from autoimmunity to cancer. In this context, HDACi have been ascribed many immunomodulatory effects, assigning a novel and promising role to these drugs. This review will summarize the current observations arising from both pre-clinical and clinical studies in these pathological conditions. However, it is left to be explained how a single agent can have both pro- and anti-inflammatory effects in either, physiological or pathological conditions. This question is explored in greater detail by focusing on the effects of HDACi on antigen presenting cells, key regulators of immune activation. In particular, HDACi modulation of molecules involved in antigen processing and presentation, as well as costimulatory, adhesion molecules and cytokines will be discussed in the context of both, professional and non-professional antigen presenting cells. Professional antigen presenting cells encompass classic immune cells; however, it is increasingly evident that other somatic cells, including cancer cells, are not immunologically inert and can display functions similar to professional APCs, a challenging feature that needs to be explored as a potential target. In this way, professional and non-professional APCs can regulate their particular microenvironmental niche, affecting either a pro-or anti-inflammatory milieu.

Introduction

The immune system plays an essential role in protecting the host from foreign (e.g., bacteria and viruses) and native (e.g. tumor cells) insults. There are two arms of the immune system, innate and adaptive. Innate immunity is rapid and non-antigen specific, responding to pathogen-associated molecular patterns (PAMPs), such as LPS. The adaptive arm response is delayed, antigen specific, and has memory to respond toward recurrent stimuli. Sitting at the crossroads between these two arms is a variety of cells known as professional antigen

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presenting cells (APCs). Macrophages, dendritic cells, and B-cells fall within this category. These cells are involved in shaping the ultimate response by the adaptive arm, whether it be tolerance or immunity, and if it is the latter, humoral or cell-mediated. Inappropriate or persistent activation of the immune system can lead to a plethora of autoimmune conditions, while inactivity can lead to persistent infections and tumor development. Examples of these two edges will be discussed in the present review.

It is becoming increasingly evident that other somatic cells, including cancer cells, are not immunologically passive. Indeed, these cells can act as APCs and in this review will be referred to as non-professional APCs. Both professional and non-professional APCs provide for specific immune activation through the presentation of peptides in the context of the MHC classes I and II. Furthermore, these two groups of APCs can express a variety of costimulatory and inhibitory surface molecules, as well as secrete pro- or anti-inflammatory cytokines. Regulation of the aforementioned occurs on multiple levels depending on the microenvironment as well as the tissue involved. Among the regulatory pathways, there is growing interest in epigenetic control with the observation that an increasing armamentarium of epigenetic modifiers such as HDAC inhibitors (HDACi) and DNA methyltransferase inhibitors (DNMTi) have displayed immunological effects. Additionally, selective drugs targeting specific epigenetic modifiers are being developed for more tailored therapies and to diminish undesirable side effects.

This review will discuss HDACi that have shown pre-clinical and clinical efficacy in the treatment of both autoimmune conditions and cancer. Particular interest will be given to effects that HDACi have on various components involved in antigen processing and presentation in MHC, herein known as the antigen presentation machinery (APM). Moreover, potential explanations for the paradoxical pro- and anti-inflammatory effects of HDACi will be explored in the context of the mechanistic roles of specific HDACs.

Histone Deacetylases

In eukaryotic cells, DNA is wrapped around small positively charged proteins known as histones to form the basic chromatin building block, the nucleosome¹. The conformation of nucleosomes is highly dynamic, mainly due to variations in the interaction between histones and DNA. Modulation therein comprises one mechanism of epigenetics; that is, regulation of DNA expression which does not alter the coding sequence. Important to mention are the modifications generated by a heterogeneous group of proteins known as histone “writers”². These writers have distinct enzymatic activities conferring upon histones a variety of post-translational modifications including methylation, ubiquitination, phosphorylation, proline-isomerization, ADPribosylation or modifying them by other means such as citrullination or proteolytic cleavage³. Some of these chemical modifications are dynamic and reversible processes mediated by two antagonistic sets of enzymes that attach or remove a chemical group in a site-specific manner.

One of the most studied histone modifications is the acetylation of lysine at the N-terminal tails of histones. In an unmodified state, the highly positive N-terminal end of histones provides for a more compacted structure through interactions with the negatively charged

DNA backbone. This generates an obstacle for the binding of transcription factors and the recruitment of other proteins that need to read the “writing pattern” on nucleosomes to exert their transcriptional functions. In this context, acetylation of histones neutralizes these positive charges, promoting a relaxed nucleosome conformation and allowing the binding of various proteins including transcription factors. These acetyl modifications are introduced by Histone Acetyltransferases (HATs), a heterogeneous group of proteins often found in multi-protein co-activation complexes that can be selectively recruited to particular DNA sequences⁴. Conversely, acetyl modifications can be removed by Histone Deacetylases (HDACs). The eighteen HDACs identified in humans are subdivided in two families: the classical HDAC family of zinc dependent metalloproteins, composed of classes I, II and IV; and the NAD⁺-dependent Class III sirtuin family of HDACs, which are not within the scope of this review. Class I HDACs (HDAC1, 2, 3 and 8) are most closely related to the yeast deacetylase RPD3. Class II HDACs encompass HDAC4, 5, 6, 7, 9, and 10, sharing homology with the yeast deacetylase HDA1. Finally, the newest HDAC identified, HDAC11, is the sole member of Class IV, and does not share homology with either RPD3 or HDA1^{5, 6}. Table I summarizes some of the current knowledge regarding zinc dependant HDACs.

Originally described as histone modifiers, HDACs have been demonstrated to target a variety of other proteins involved in diverse cellular processes⁷. Interestingly, there are a large number of transcription factors that are regulated by acetylation/deacetylation, positioning HDACs and HDACi as indirect regulators of gene control. For example, deacetylation of p53⁸, STAT3⁹, GATA1¹⁰, and E2F1¹¹ have been reported to diminish their ability to bind DNA. Conversely, deacetylation enhances the binding of YY1¹², HMG¹³ and p65¹⁴ to DNA. Additionally, the acetylation status of many proteins has been described as a regulatory mechanism to control their capacity to interact with other proteins, as observed with the acetylation of STAT3⁹ and the estrogen receptor¹⁵. Moreover, the effect of protein acetylation has been reported to regulate the transcriptional activity and protein stabilization of several transcription factors, including p53⁸, androgen receptor¹⁶, and E2F¹⁷. In addition to transcription factors, other classes of non-histone proteins are regulated by HDACs. The best characterized are the molecular chaperone Hsp90¹⁸ and the cytoskeletal protein α -tubulin, both regulated by HDAC6¹⁹. The deacetylation of non-histone proteins by HDACs is opening new perspectives for the rational use of HDACi. Perhaps, the canonical role of these drugs as epigenetic modifiers must be revisited to consider them global regulators of cellular functions. In this context, multiple groups are working to characterize the “acetylome”²⁰. One recent screen identified over 3600 acetylation sites on 1750 proteins involved in diverse cellular processes from DNA regulation to RNA splicing and protein synthesis and degradation²¹. Undoubtedly, the knowledge generated by this data will improve our understanding of the heterogeneous participation of HDACs in cellular processes beyond their classic role in transcriptional regulation.

Histone Deacetylase Inhibitors

HDACi are a heterogeneous group of chemical compounds with the ability to inhibit the enzymatic activity of HDACs. Their classification is based mainly on their chemical structure and fall into six different groups; short-chain fatty acids (e.g., valproic acid),

hydroxamates (e.g., TSA, SAHA), benzamides (e.g., MS-275), cyclic tetrapeptides (e.g., depsipeptide), electrophilic ketones (e.g., trifluoromethylketone), and miscellaneous compounds (e.g., MGCD0103). The inhibitory effect of a majority of HDACi is due to the interaction of the drug with the active zinc site at the catalytic pocket of HDACs, as determined by crystallographic studies of HDACs/HDACi (Table I). Interestingly, many of these compounds were used in the clinic prior to the knowledge of their mechanism, treating various neurologic conditions such as epilepsy since the 19th century. In the 1970's, Leder et al. observed an antiproliferative effect in erythroleukemic cells²². However, it wasn't until years later that Yoshida and colleagues found that TSA treatment of various cell lines, including mammary gland tumor cells, resulted in the inhibition of HDACs as evidenced by increased histone acetylation²³.

Although a significant number of HDACi have been designed or discovered, only twelve have progressed to clinical trials for use as a single agent or in combination with other drugs and/or radiation therapy²⁴. Table II lists the active-non-recruiting HDACi clinical trials (NCI records) either alone or in combination with other drugs in a variety of malignancies and diseases. Some of the HDACi currently in use are Belinostat (PXD101), Givinostat (ITF2357), Panobinostat (LBH589), Vorinostat (SAHA, MK0683), Valproic acid, Romidepsin (FK2288, FR901228), CHR 2845, and JNJ-26481585.

Although many of these trials are in cancer, where the desire would be to boost immunity against tumor cells, HDACi are also being used for the treatment of autoimmune conditions where the goal is to suppress inappropriate responses against self. Interestingly, in Phase I and Phase II clinical trials of some HDACi (e.g. Valproic acid, SAHA, MS-275, and ITF2357), treated patients developed severe infections even in the absence of neutropenia, emphasizing the immunosuppressive activities of HDACi²⁵⁻²⁷.

It is interesting to note that pan-HDACi do not result in drastic global genetic changes, with only about 2% of the genome being affected. In one study, TSA treatment modified the expression of 1594 genes, from which 772 were found to have been down-regulated and 822 were up-regulated²⁸. Another point to mention is that HDACi may have different effects depending on the dose. For example, lower doses (0.25 and 1 μ M) of TSA treatment blocked the production of cytokines in macrophages, while higher doses (20 μ M) induced apoptosis in a time dependent manner²⁹.

The oftentimes paradoxical effects of HDACi exerting both pro- and anti-inflammatory effects may stem from the fact that a majority of HDACi in clinical use are pan-HDACi in that they inhibit all three classes of zinc-dependent HDACs. Undoubtedly, mechanistic insights into these contradictory effects in cancer, autoimmunity, and other pathologies will aid the development of more targeted therapeutic designs to regulate specific targets and pathways. It is important to recognize that the use of HDACi in the treatment of cancer is affecting not only the proliferation and survivability of cancer cells, but also many other pathways, including those related to immunological networks.

Immunologic Effects of HDACi in Cancer

Disturbances in the physiological patterns of DNA methylation and histone modifications can contribute to the initiation and progression of malignancies³⁰. Therefore, epigenetic modifiers may offer therapeutic potential. As of 2011, the U.S Food and Drug Administration (FDA) have only approved the use of two pan-HDACi, Vorinostat (Zolinza)³¹ and Romidepsin (Istodax)³², for the treatment of refractory cutaneous T-Cell lymphoma (CTCL).

Inhibiting HDACs promotes several biological changes in cells. Some common consequences include the induction of apoptosis through extrinsic (death receptor) and intrinsic (mitochondrial) pathways, cell cycle arrest, and induction of differentiation, as well as anti-angiogenic, anti-invasive and immunomodulatory effects^{33, 34}. Interestingly, these compounds demonstrate some selectivity for transformed cells, with normal cells being more resistant to HDACi³⁵. However, there is no consensus about common cellular mechanisms ruled by the action of all HDACs, with some investigators proposing that the effect over cell proliferation and apoptosis being mainly mediated by repression of Class I HDACs^{36, 37}. Contrarily, recent evidence suggests that genetic or pharmacological inhibition of some Class II and IV HDACs have the capacity to inhibit the proliferation of cancer cells³⁸. An example is HDAC6 as a regulator proliferation in some leukemia and lymphoma cells through effects on the aggresome, microtubule stability and function, and intracellular trafficking^{39, 40}.

Alterations in immunologic components have been shown to contribute to the development of cancer. For example, severe combined immunodeficiency (SCID) mice are more susceptible to chemical induced carcinogenesis, and immunosuppressed individuals, whether genetic or iatrogenic, have an increased risk of malignancy⁴¹. The importance of immunity has been elegantly explained by the immunoediting hypothesis proposed by Robert Schreiber's group⁴². They propose that cancer initiation and progression can be explained under the "classic clonal selection model" suggesting that tumors develop when a select population is able to escape the immunosurveillance machinery and therefore form, and become, the majority of the tumor population^{43,44}. In the context of the antitumor activity of HDACi, although there is evidence for direct cytotoxicity, many studies point to a critical immune contribution. Immune modulation by HDACi occurs at multiple levels. On the tumor side, enhanced expression of receptors for immune recognition has been observed. These include MHC molecules and stress-related ligands, MIC-A and MIC-B, which ligate to NKG2D on CD8+ cytotoxic T-lymphocytes (CTLs) and natural killer cells⁴⁵. Mechanistically, treatment of HDACi led to reduced recruitment of HDAC1 to the promoter of MIC-A and -B. Furthermore, Murakami et al. reported an upregulation of the death receptor Fas in HDACi treated murine melanoma, which upon ligation to Fas-L expressed on CTLs results in induction of apoptosis in the tumor cell⁴⁶. The importance of the immune system has been underscored by experiments demonstrating that the combination treatment with HDACi LAQ824 and adoptive transfer of antigen specific T-cells were required for antitumor activity⁴⁷. In another study, increased perforin expression, a pore forming protein involved in cytotoxicity, was noticed in the T-cell compartment after HDACi treatment⁴⁶. Conversely, depletion of CTLs or knocking out perforin resulted in abrogation of response

and tumor progression similar to untreated controls⁴⁸. Although there is a clinical bias for HDACi use for hematologic malignancies, multiple pre-clinical studies have demonstrated efficacy in solid tumors, including melanoma, lung, breast, colon, and kidney cancers⁴⁸.

In spite of these findings, there are multiple reports demonstrating an immunosuppressive effect of HDACi. For instance, in DCs, HDACi have been recognized to block the differentiation of IL-17 producing T-cells⁴⁹ as well as inhibit DC-stimulated allogeneic T-cell proliferation and Th1 polarization^{50, 51}. These effects would be undesirable in the context of cancer, but the same anti-inflammatory qualities could be applied in the field of autoimmunity, which will be reviewed in the following section.

Immunologic Effects of HDACi in Autoimmunity

There are about 80 different known autoimmune diseases affecting about 5% of the Western world⁵². Epigenetics modifiers such as HDACi and DNMTi have been documented to have regulatory effects on the immunobiology of cells in several inflammatory and autoimmune diseases⁵³. Therefore, they have been applied in pre-clinical models of multiple inflammatory diseases such as rheumatoid arthritis (RA) and juvenile idiopathic arthritis (JIA)⁵⁴⁻⁵⁶, systemic lupus erythematosus (SLE)⁵⁷, chronic obstructive pulmonary disease (COPD)⁵⁸, multiple sclerosis (MS)⁵⁹, systemic sclerosis⁶⁰, psoriasis⁶¹, ulcerative colitis (UC)⁶², Crohn's disease (CD)⁶³, and muscular dystrophy⁶⁴. These studies have demonstrated effects both on T-cells as well as APCs. More specifically, various studies have demonstrated enhanced production of anti-inflammatory molecules by APCs and augmented Treg generation. In many of these diseases, alterations in specific HDACs have been observed, providing rationale for developing more selective HDACi (Table I).

These observations, as well as the documented anti-inflammatory effects of HDACi in cancer cells⁶⁵, support the use of epigenetic modifiers to target cellular pathways involved in the pathogenesis of autoimmune diseases^{66, 67}. In SLE for example, epigenetic modifications affect a considerable number of genes⁶⁸. The majority of these susceptible genes are involved in central processes controlling immune cell development and behavior, such as lymphocyte differentiation (PDCD1, IKZF1, IKZF3), apoptosis (PARP, CRP, ATG5), cytokine signaling (STAT4, IRF5, TNFSF4), and complement activation (C1q, C2, C24a, C4b)^{69, 70}. Many of these observations have been validated by studies using HDACi. For example, TSA has been used in the setting of SLE resulting in reversal of the atypical expression of inflammatory genes in isolate T helper cells⁷¹. Moreover, in a RA rat model, systemic TSA treatment has also been shown to alleviate the disease by up-regulation of CDK inhibitors (p16^{INK4a} and p21^{WAF1/Cip1}), as well as the inhibition of inflammatory cytokines IL-1, IL-6, and TNF- α in diseased joints⁷². Blockade of HDAC enzymatic activity can also transform the immunopathology of RA and other immune-mediated inflammatory diseases through epigenetic dependent or independent processes by influencing the dynamics of molecular and cellular pathways^{29, 73}.

Taken together, the contradictory outcomes (i.e., anti- and pro-inflammatory effects) suggest a more complex scenario for understanding the mechanistic action of HDACi, and encourages the use of selective drugs targeting specific HDACs to elucidate potentially

unique roles, and more importantly, avoid undesirable side effects. Indeed, there is a growing body of literature concerning the role of individual HDACs in regulating various immune components. Interestingly, HDACi treatment and mechanistic studies point to the regulation of qualities in APCs. These cells are an important component of the immune system, sitting at the crossroads between immunity and tolerance. Therefore, a strategy such as HDACi, which manipulates these cells, could be exploited in the context of both cancer and autoimmunity, and might explain at least in part the contradictory HDACi effects outlined above. The remainder of this review will summarize and attempt to explain the multitude of effects by HDACi with this concept in mind.

Components of the Antigen Presentation Machinery

Antigen presentation lies at the gateway to adaptive immunity. As previously mentioned, during the initial steps, professional APCs are constantly surveying the environment and taking up samples of the extracellular space via endocytic pathways. These antigens are then processed and presented by MHC II, or MHC I through cross presentation. The microenvironment in which these antigens are encountered dictates whether the APC will be pro- or anti-inflammatory. Such programming of signaling will then be conveyed to T-cells through the APC repertoire of costimulatory/inhibitory ligands and cytokine production, thereby shaping the immune response, whether activating or inhibitory (i.e., peripheral tolerance). There is a growing body of evidence that other somatic cells, not classically considered APCs, are not immunologically inert and can in fact act as non-professional APCs. In addition to constitutive expression of MHC I, non-professional APCs have been shown to express MHC II, costimulatory/inhibitory ligands, and secrete cytokines. Often, these APC-like functions are acquired upon certain stimuli. The following sections will discuss the effects of various HDACi on augmenting APC-like function, and also explore potential mechanisms in an attempt to explain the role of HDACi in the modulation of antigen presentation in the context of MHC, costimulatory molecules, and cytokine production.

HDACi Modulation of MHC I

MHC I is a key sensor of cell-intrinsic aberrations, including viral infection and oncogenic transformation. As such, it provides a beacon for CTL recognition and clearance. This molecule is expressed in all nucleated cells, with decreased expression on hematopoietic stem cells, and absent on red blood cells. MHC I peptides are derived from normal protein turnover. Structurally, MHC I is composed an α -chain and β 2-microglobulin. In humans, there are three α -chain variants, known as human leukocyte antigens (HLA) -A, -B, and -C encoded on chromosome 6. These proteins are known as H-2K, D, and L in mouse. The α -chain is synthesized in the lumen of the endoplasmic reticulum (ER) and stabilized by calnexin until β 2-microglobulin binds (Fig. 1). Next, calnexin is replaced by calreticulin, which then becomes a complex with tapasin, transporters associated with antigen processing (TAP) -1 and -2, and Erp57. Calreticulin stabilizes the MHC I heterodimer until a peptide is loaded, while tapasin links this complex with TAP-1 and -2, ATP Binding cassettes responsible for translocating peptides from the cytosol into the ER lumen. Once peptides are in the ER, further editing can occur by ER-associated amino peptidase. Erp57 is responsible

for opening up MHC I to facilitate peptide binding. Once peptide binds, the complex dissociates leaving just the α -chain, β 2-microglobulin, and peptide. This complex is then transported and presented on the surface of the cell. Peptides which bind in the cleft of MHC I must possess particular anchor sequences, normally hydrophobic or basic amino acids. These peptides are generated in the modified proteosomes, which contain subunits known as large multifunctional peptidase (LMP) -2 and -7 involved in generating peptides which have a higher chance to bind. Moreover, TAP-1 and TAP-2 have also shown preference for translocating appropriate peptides.

Interestingly many of the aforementioned components are located on the same chromosome in close proximity to the MHC locus and are regulated by similar promoters⁷⁴. Evolutionarily, this linkage may have arisen to provide for a rapid and coordinated induction of this machinery. Indeed, similar stimuli, such as IFN- γ , can induce the whole cluster of genes. Furthermore, the class II major histocompatibility complex transactivator (CIITA), involved in MHC II regulation, can also facilitate IFN- γ induction of MHC Class I⁷⁵. Therefore, epigenetic changes in master stimuli, such as IFN- γ , will affect the expression of the whole cluster of genes, suggesting that epigenetic regulation could also work as an indirect mechanism to control the antigen presentation through the modulation of these master stimuli.

MHC I on professional APCs are also utilized in cross-presentation for CTL activation⁷⁶. This is important in light of the fact that as cells die, whether due to infection by viruses or during normal turnover of transformed cells, proteins are released into the extracellular space. These are then taken up by professional APCs and, as covered in the following section, classically presented in the context of MHC II, which would then elicit an inappropriate extracellular, antibody response. Furthermore, if a virus does not infect APCs, how would these viral antigens be presented in MHC I? To address these issues, professional APCs have developed an alternative pathway whereby peptides within the endocytic vesicle can be translocated into the cytosol via sec61. The peptides can then be transported into the ER and presented in MHC I just as a normal intracellular peptide would be as outlined above (Fig. 1).

As is evident from the aforementioned, MHC I is regulated by a diverse array of cellular processes including transcriptional regulation, protein degradation, transport into endoplasmic reticulum, and ultimately, transport to and display on the cell surface in the context of MHC I. Indeed, many components have been shown to be affected by HDACi treatment, either positively or negatively²⁸. In this context, pan-HDACi treatment has predominantly been demonstrated to upregulate surface MHC I expression, as well as multiple components of the APM including TAP-1, TAP-2, tapasin, calnexin, calreticulin, ERp57, LMP2, and LMP7^{45, 77, 78}. The observation that a single treatment resulted in the expression of multiple APM components suggests regulation at the gene level. That is, either through direct regulation of the epigenetic signature at the promoter, or through modulation of an upstream target, for example CIITA.

Interestingly, some studies have shown the opposite, inhibitory effect on antigen presentation. These studies utilized similar HDACi, TSA or sodium butyrate. One report

demonstrated decreased expression of genes coding for proteasome catalytic proteins and tapasins in a gene array⁷⁹, while another demonstrated a downregulation of HLA after LBH589 treatment⁸⁰. The reasons for these discrepancies are not clear and need to be addressed in future investigations, perhaps by using selective HDACi.

In one study, although a slight upregulation in overall HLA-A2 expression was observed, surface MHC expression of hTERT peptides, an attractive tumor antigen, was decreased leading to impaired susceptibility to be killed by hTERT-specific T-cells. This was in the face of increased hTERT mRNA after HDACi treatment⁷⁹. This observation brings up the key question of whether HDACi-mediated upregulation of MHC I actually leads to enhanced immune recognition. Many of the previous studies demonstrating this to be the case used the artificial precept of peptide loading onto HDACi-treated cells. Therefore, the specific peptide, for example chicken ovalbumin, with high affinity for the MHC I cleft would competitively replace the existing peptide on the cell surface.

Indeed, Celis et al. have recently demonstrated that even though IFN- γ induces upregulation of MHC I, this did not correlate with enhanced immune recognition. In contrast, they observed epitope spreading, that is, a relative reduction in expression of specific tumor peptides, leading to impaired recognition by antigen specific T-cells⁸¹. Therefore, it would be important to more clearly determine whether this is the case in HDACi-treated cells.

Mechanistically, the effect of HDACi on MHC I can be explained by modulation of specific HDACs. For example, HDAC1, 2, and 8 have been demonstrated to form part of a co-repressor complex to downregulate MHC I in cervical cancer cells⁷⁸. Furthermore, HDACs have been shown to be involved in silencing of cancer testis antigens such as MAGE⁸². Induction of such CTAs has been of interest in inducing specific antitumor immunity. In addition, HDAC6/Hsp90 was shown to be involved in regulating peptide stability, leading to enhanced presentation of viral peptides in MHC I and, ultimately, immune recognition and clearance⁸³.

HDACi Modulation of MHC II

In contrast to intracellular protein presentation by MHC I, MHC II is traditionally involved in presenting peptides from antigens located in the extracellular space. MHC II in turn binds to cognate CD4 T-cells leading to their activation. Like MHC I and other membrane bound proteins, Class II is assembled in the ER. To prevent endogenous peptides from binding to MHC II in the ER, another molecule, the invariant chain (Ii), is present which blocks the cleft. As MHC II is being assembled, it also associates with calnexin; however, once the MHC II/Ii complex is complete, calnexin dissociates. The MHC II complex now leaves the ER and traffics to the plasma membrane (Figure 1). The sequence of events leading to peptide presentation, whether peptide is loaded prior to surface expression or the MHC II/Ii complex is presented on the surface and then subsequently re-internalized before peptide loading, is still unclear. Whatever the case may be, extracellular material which has been endocytosed is processed after fusion with the lysosome by a family of cathepsins, predominantly S and L. The phagolysosome then fuses with the membrane-bound vesicle containing MHC II. The cathepsins sequentially cleave Ii to ultimately leave a fragment

known as the Class II –associated invariant chain peptide (CLIP). A third component, HLA-DM, associates with MHC II when it is associated with CLIP. HLA-DM stabilizes the complex in the acidic environment of the phagolysosome and is also involved in peptide loading by displacing CLIP and also any other weakly bound peptides. Finally, after achieving high affinity peptide binding in the cleft, MHC II is presented on the cell surface.

As mentioned previously, MHC II is constitutively expressed on professional APCs (e.g., B-cells, DCs, Macrophages) and thymic epithelial cells. However, it is also induced on a variety of other cells, including cancer cells. In this context, it is important to mention the role of IFN- γ as a key stimulus in inducing MHC II in various cells. TSA has been shown to enhance IFN- γ mediated upregulation of MHC II and collagen repression^{84, 85}.

Mechanistically, TSA treatment appears to stabilize RFX5, a component of the enhanceosome leading to H3 acetylation within the MHC II promoter⁸⁴.

Both pan- and Class I-selective HDACi have been demonstrated to induce upregulation of MHC II⁷⁷. Furthermore, HDACi treatment of plasma cells can convert them into potent APCs capable of activating CD4+ T-cells⁸⁶. Two key mechanisms have been shown to regulate MHC II, the enhanceosome and CIITA, the latter considered the master transcriptional regulator⁸⁷. It is also important to note that there exist three isoforms of CIITA, which demonstrate differential activation in tissue- and stimulus-specific fashions. For our purposes, pIV-CIITA is IFN- γ inducible in nonprofessional antigen presenting cells, while the pI-CIITA and pIII-CIITA regulate constitutive activation in DCs and B-cells, respectively⁸⁸. Moreover, pII-CIITA contains response elements for IFN- γ and other transcription factors, including STAT1, which in turn has been shown to be regulated by HDACs⁸². Additionally, it has been reported that CIITA can be regulated by acetylation. Specifically, this protein can be acetylated by CBP and PCAF and deacetylated by HDAC2⁸⁵, but also can physically interact with HDAC1⁸⁴. Mechanistically, the deacetylation of CIITA by HDAC2 promotes its degradation in macrophages⁸⁹. Indeed, HDAC1 and 2 have been found to repress MHC II in various tumors⁸². HDAC1 and 2 also work downstream of CIITA, being present within a corepressor complex with mSin3a at the promoter region of MHC II at the steady state⁹⁰. Furthermore Blimp-1, a master regulator of B-cell differentiation, may recruit a co-repressor complex containing HDACs to the CIITA promoter⁸⁶. Given the immunologic importance of MHC II, it is interesting to note that TSA is able to induce MHC II in a CIITA, IFN- γ independent fashion, though the mechanism has not been clearly defined^{90, 91}.

Effect of HDACi on molecules important for antigen presentation

Costimulatory and Adhesion Molecules

Optimal antigen presentation requires the involvement of secondary, co-stimulatory signals as well as adhesion molecules. Indeed, MHC-TCR ligation alone without costimulation induces anergy⁹². CD80 and CD86 are two costimulatory molecules part of the B7 family. These two ligate CD28 on T-cells, leading to downstream activation of IL-2 production. CD40 is also involved in immune activation, but on the side of the APC. CD40L on T-cells binds to and activates the APCs leading to expression of microbicidal products in macrophages and inducing antibody production in B-cells. It has been shown that HDACi

affect the expression of CD80, CD86, and CD40 on both tumor and immune cells, both in positive and negative manners; however, a majority evidencing a positive effect. Furthermore, there are reports of differential upregulation depending on state of maturity. For example, CD86 was upregulated on immature, but not mature *in vitro* generated myeloid derived DCs. In this context, an understanding of the mechanism of HDACi on either up- or down- regulation of these costimulatory molecules can be exploited in the context of both autoimmunity and cancer.

Additionally, it has been shown that HDACi can modulate the expression of adhesion molecules. For example, ICAM-1 is upregulated following HDACi treatment on several tumors, including tumor endothelial cells⁸⁶. ICAM-1 binds to LFA-1 on T-cells and is involved in strengthening the immune synapse. At the same time, HDAC inhibition, through targeting HDAC6 in T-cells, could improve immune synapse organization⁹³. Therefore, it is important to remember that systemic HDACi therapy could have disparate effect on a variety of cell types which could lead to antagonistic or synergistic effects.

Cytokines

Even with proper recognition, adhesion, and costimulation, a third component is required to dictate the characteristics of the resulting immune response. Cytokines are involved not only in the initial induction to shape the ultimate immune response, but also during an ongoing reaction to moderate and even shutdown the response. Cytokines can direct the differentiation of effector cells into appropriate responders (e.g., Th1 vs Th2), and activate or suppress APCs. For example, IFN- γ has been demonstrated as a key modulator of the APC function by increasing expression of MHC and costimulatory molecules. In this context, HDACs and HDACi have been described as key regulators of cytokine expression, and therefore, modulate immune function.

The effect of HDACi on cytokine molecules is even more muddled, with inhibition of both pro- and anti- inflammatory cytokines. With regard to enhancement of cytokine production, it is interesting that few reports demonstrate augmented anti-inflammatory cytokine secretion. One study demonstrated that TSA-treated T-cells had upregulated TGF- β 1 in a microarray; however, this observation was not further validated. Also, a study in regulatory T-cells demonstrated upregulation of the IL-10 mRNA; however, this was not observed in effector T-cells⁹⁴. Again, there was no evidence provided for augmented secretion. TGF- β and IL-10 are involved in polarizing T-cells to suppressive Tregs and Th2 to combat extracellular pathogens, respectively. Other cytokines involved in T-cell polarization are also modulated by HDACi. Important to mention is the inhibition of IL-12 and IL-23 by TSA treatment. These cytokines, produced mainly by professional APCs, are key regulators of Th1 and Th17 responses to intracellular and extracellular pathogens^{95, 96}.

IL-6, TNF- α , MIP-2, and IL-8 have all been demonstrated to be increased after HDACi treatment⁸². These pro-inflammatory cytokines have implications in autoimmunity as well as tumor progression. Neutralization of TNF- α has been an effective treatment for rheumatoid arthritis, and IL-6 and IL-8 have been implicated in the inflammation-associated tumor progression. The macrophage inflammatory protein 2 (MIP-2), is a chemoattractant for neutrophils. In other studies, many of these same cytokines as well as IL-12p70, IL-23,

IFN- γ , and cox-2 have all been inhibited by HDACi following TLR stimulation⁸⁰. With respect to anti-inflammatory cytokines, HDACi treatment has been shown to inhibit IL-10⁹⁷ and TGF- β ⁹⁸. Indolamine Dioxygenase (IDO) has also been shown to be a target of pan-HDACi. Interestingly, this anti-inflammatory molecule has been shown to be both upregulated and downregulated in two similar models^{51, 80}.

In light of these contradictory observations, it is important to note that several findings have pointed to specific HDAC regulation of individual pro- and anti-inflammatory cytokines in both positive and negative fashions. For example, IFN- β , an anti-inflammatory cytokine with therapeutic potential in the treatment of multiple sclerosis⁹⁹, is inhibited by HDAC1 and HDAC8, but enhanced by HDAC6¹⁰⁰. Therefore, it is conceivable that HDAC1 or HDAC8 inhibition could lead to augmented IFN- β secretion. The idea that HDACi have not recapitulated the effects found in these mechanistic studies suggests that targeted HDAC inhibition may provide the added benefit of enabling directed, fine tuning of the cytokine milieu¹⁰¹.

These conflicting effects may be due to the diversity, and sometimes opposing roles, of specific HDACs. We have recently observed that this relationship exists between HDAC6 and HDAC11 over IL-10 gene transcription in macrophages. Overexpression of HDAC6 in a murine macrophage cell line, RAW264.7, enhances, while overexpression of HDAC11 suppresses IL-10 gene transcription and protein production following LPS stimulation. The converse was observed upon targeted disruption of HDAC6 or HDAC11 using specific shRNA. Mechanistically, these two HDACs were found to be recruited to the IL-10 promoter at the same time and to the same locus, suggesting a direct transcriptional control. It remains to be explained how a HDAC, a classic transcriptional repressor, could activate the transcription of a target gene. Moreover, we have found that HDAC6 and HDAC11 exist in the same complex. It is possible that even in close proximity to the gene promoter; these HDACs could have non-histone effects and maybe act as deacetylases for transcription factors. The direct regulation of HDAC6 and HDAC11 on the IL-10 promoter also has implications in the antigen presenting capabilities of macrophages and, moreover, modulating the immune response mediated by T-cells. These findings represent a novel molecular target to potentially influence immune activation versus immune tolerance, a critical decision with significant implications in cancer immunotherapy, transplantation and autoimmunity.

Further explanation for the paradoxical role of HDACi on cytokines can be gleaned from the differential effects on a non-histone target, NF- κ B. This transcription factor has pleiotropic targets and effects, regulating cell survival, proliferation, and cytokine production. Indeed, NF- κ B is critical in toll like receptor signaling, a sensor for PAMPs such as LPS and CpG. Specific HDACs have been shown to have both positive and negative regulatory roles on this pathway. Pan-HDACi have been shown to enhance NF- κ B acetylation, leading to diminished binding of the negative regulator I κ B, and augmented nuclear translocation and activation of NF- κ B target genes. This effect may be due, at least in part, to inhibition of HDAC3, which has been shown to deacetylate the NF- κ B subunit RelA, leading to significantly diminished binding to the negative regulatory molecule I κ B α ¹⁰². Conversely, HDACi have been shown to stabilize an inhibitor of NF- κ B, I κ B, by preventing

proteasome-mediated degradation. More globally, sodium butyrate inhibited the expression of b-type subunits of the proteasome, again leading to persistence of I κ B α ¹⁰³. In line with this last observation, specific HDACs have been shown to have a negative regulatory role on the NF- κ B signaling pathway. For example, the expression of the RelB subunit was diminished after HDACi treatment⁸⁰. Also, HDAC1 inhibits TLR-induced cytokine secretion, and HDAC6 promotes MyD88 degradation, a key proximal component in the signaling of various TLRs⁸⁹.

Another potential target of regulation by HDACi is STAT3. This protein has been demonstrated to be deregulated in cancer, contributing to tumor survival as well as immune evasion¹⁰⁴. Mechanistically, STAT3 has been implicated in immune tolerance through direct transcriptional induction of IL-10. Indeed inhibition of STAT3 in macrophages partially restores the function of tolerized T-cells to produce IL-2 and IFN- γ ¹⁰⁵. In turn, HDAC1 and HDAC3 have both been shown to regulate STAT3 either through direct deacetylation, leading to nuclear export and therefore cessation of STAT3 activity¹⁰⁶, or by providing a scaffold for PP2A phosphatase to dephosphorylate STAT3 and again stopping signaling¹⁰⁷.

Conclusions

It is evident that HDACi have pleiotropic effects that depend not only on the tissue/cell, but also the context and dosing. In fact, varying any of these factors can result in opposing effects as intimated above. For example, CD83, a marker for professional APC activation was inhibited by the pan-HDACi LBH589 in myeloid DCs⁸⁰ while TSA upregulated this same molecule on B-cells⁹¹. There are many potential explanations for these discrepancies.

Intrinsic differences between pan-HDACi may provide some explanation. Although pan-HDACi have the potential to inhibit all zinc-dependent HDACs, they do so with differing kinetics, that is, the IC₅₀ for each individual HDAC may be slightly, or significantly different. Indeed the observation that HDACi have an immune effect at lower doses, while a higher dose is required for a direct anti-tumor effect (i.e., cell cycle arrest, apoptosis) may be due in part to a certain degree of selectivity at lower doses giving way to broad spectrum inhibition at higher doses. This observation lends credence to the idea that the contribution of individual HDACs is a key element to be considered in understanding the effects of HDACi. Using the CD83 example above, LBH589 and TSA could differentially inhibit distinct HDACs at lower doses, which have opposing regulatory roles on this activation marker.

Another, non-mutually exclusive reason lies in the difference in cell type. In this case, myeloid DCs are used in one instance while B-cells in the other. This brings up an important point regarding the epigenetic targeting of HDACs. Namely, HDACs do not contain intrinsic DNA binding activity. Therefore, they must rely on other cis-acting elements, which differ from cell to cell and upon the context. Indeed, these same HDACi have not been reported to have any modulatory effect on CD83 in solid tumors.

The context in which HDACi is given also dictates the response. For example, HDACi have been demonstrated to have both pro- and anti- inflammatory effects. Interestingly, work in

tumor models are biased towards the former, while that latter is reported in autoimmune literature. It is conceivable that the effect of HDACi is dependent on the micro-environmental niche upon specific antigen encounter. That is, the outcome depends on the predominant HDAC mediating the physiologic phenomenon. This is not unlike the oncogene addiction hypothesis in which some tumors are dependent on particular signaling pathways, and inhibition results in antitumor activity without significant deleterious effects on normal cells¹⁰⁸. Similarly, particular conditions may be addicted or dependent on a particular HDAC. Therefore, the effects of a pan-HDACi would be predominantly in antagonizing the status quo. As a result, under conditions where there is aberrant immune activation, such as autoimmunity, HDACi would portend an anti-inflammatory effect. The converse would be true where the immune system is suppressed, such as in cancer. Therefore, pan-HDACi are limited in their efficacy in that they may be most effective in tipping the balance from the extremes of pro- and anti-inflammation, thereby moderating the pathological condition, but not reversing it.

Another interesting observation that has important implications in the clinical use of HDACi is the timing of the treatment. Published work and unpublished observations from our lab and others, suggest differing results depending on whether HDACi is given before, with, or after a particular stimulus. For example, one group reported previously that pretreated macrophages with HDACi and then stimulated with LPS, result in an anti-inflammatory outcome¹⁰⁹. Contrarily, our group demonstrated a pro-inflammatory effect when HDACi is given concomitant with LPS. The reason for this difference again may depend on the predominant HDAC operative at the time of HDACi treatment.

This idea lends more credence to the development of more selective HDACi which would permit a more accurate and effective immune control. For example, in the context of HDAC6 and HDAC11 on IL-10, a pan-HDACi would theoretically inhibit both. Therefore, under conditions of high HDAC6 and high IL-10 production, treatment would result in decreased IL-10, by removing the metaphorical gas pedal. However, the pan-HDACi would also be removing the brake through inhibition of HDAC11. Furthermore, HDACs which serve as co-activators of pro-inflammatory cytokines would also be inhibited. In this context, an HDAC6 selective inhibitor would potentially be more effective, leading to targeted IL-10 inhibition without tempering other immunoregulatory pathways which could then enhance pro-inflammatory cytokine production.

Taken together, it is clear that HDACi hold great promise for altering the natural course of diseases, both autoimmune and cancer. Although immunologic effects of HDACi are often presented in the context of one disease state, autoimmunity and cancer lie on the same spectrum of immune dysfunction, albeit at opposite ends. Therefore, effects observed in one may be applicable to the other. However, for HDACi to be truly effective as a class, mechanistic studies are needed to tease out their contradictory effects, potentially leading to more selective inhibitors which may enhance the ability to more accurately fine tune the immune system and also demonstrate less toxicity and off target effects.

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Figure 1. MHC I and MHC II Processing

Table 1

HDACs Classification

Class	HDAC	Size (aa)	Deacetylase Domain (PF00850)	Protein Structure	Chromosome location	Complex	Knock-out Phenotype	Aberrations in Immune Diseases	Immunological targets
I	HDAC1 ¹¹⁰	482	5-384	-	1p34	Sin3, Nurd, Co-REST	Lethal before embryonic day 10.5 (e10.5) and display proliferation defects and general growth retardation ¹¹¹	RA ¹¹² , SLE ⁵⁷ , JIA ⁵⁵ , MS ¹¹³	IL-1 ¹¹⁴ , IL-2 ¹¹⁵ , IL-12 ¹¹⁶ , IL-5 ¹¹⁷ , INF- β ¹⁰⁰ , INF- γ ¹¹⁸ , IL-4 ¹¹⁹ , STAT5 ¹²⁰ , STAT3 ⁹ , MIC-A/B ¹²¹ , MHC I ⁷⁸
	HDAC2 ²²	488	23-319	3MAX (2.05Å) ¹²³	6q21	Sin3, Nurd, Co-REST	Lethal within the first 24 hours after birth with cardiac malformations ¹²⁴	RA ¹²⁵ , COPD ⁵⁸ , MD ¹²⁶	GM-CSF ¹²⁷ , INF- γ ¹¹⁸ , IL-4 ¹¹⁹ , STAT3 ⁹ , CITA ⁸⁵
	HDAC3 ²⁸	428	19-314	-	5q31	N-CoR	Lethal before e9.5 owing to defects in gastrulation ¹²⁹	SLE ⁵⁷ , SS ¹³⁰	IL-4 ¹¹⁹ , pro-IL-16 ¹³¹ , STAT3 ⁹ , STAT1 ¹³² , GATA1 ¹³³ , GATA2 ¹³⁴ , GATA3 ¹³⁵ , NF κ B ¹⁰²
	HDAC8 ³⁶	377	28-322	2V5W (2.00Å) ¹³⁷	Xq13	-	Perinatal lethality due to skull instability ¹³⁸	-	INF- β ¹⁰⁰
IIa	HDAC4 ³⁹	1084	671-992	2VQM (1.80Å) ¹⁴⁰	2q37.3	N-CoR, MEF2	Lethal within the first week of life owing to ectopic ossification of endochondral cartilage ¹⁴¹	MS ¹⁴²	GATA1 ¹³³
	HDAC5 ³⁹	1122	700-1022	-	17q21	N-CoR, MEF2	Non Lethal. Cardiac hypertrophy ¹⁴³	-	IL-8 ¹⁴⁴ , GATA1 ¹³³ , GATA3 ¹³⁵
	HDAC7 ⁴⁵	952	538-859	3COZ (2.10Å) ¹⁴⁶	12q13.1	N-CoR, Sin3, MEF2	Embryonic lethality. Endothelial cells missfunctions ¹⁴⁷	SLE ⁵⁷	IL-2 ¹⁴⁸ , FOXP3 ¹⁴⁸
	HDAC9 ⁴⁹	1011	651-972	-	7p21.1	N-CoR, MEF2	Non Lethal. Cardiac hypertrophy ¹⁵⁰	SLE ⁵⁷	IL-2 ¹⁴⁸ , FOXP3 ¹⁴⁸
IIb	HDAC6 ¹³⁹	1215	104-402 495-798	-	Xp11.23	HDAC11	Non Lethal ¹⁵¹	MS ¹¹³	INF- β ¹⁰⁰
	HDAC10 ¹⁵²	669	25-321	-	22q13.31	N-CoR	Non Lethal (Unpublished phenotype)	-	-
IV	HDAC11 ¹⁵³	347	30-318	-	3p25.1	HDAC6	Non Lethal (Unpublished phenotype)	-	IL-10 ¹⁰¹

SLE: Systemic Lupus Erythematosus, JIA: Juvenile Idiopathic Arthritis, COPD: Chronic Obstructive Pulmonary Disease, MS: Multiple Sclerosis, SS: Systemic Sclerosis

Table 2

On-going (active not recruiting) HDACi clinical trials

HDACi	Class Inhibitor	Type of Cancer	Phase	Comb. Therapy	NCI identifiers
CHR-2845	Class I	Hematological Disease, Lymphoid Malignancies	Phase I	None	NCT00820508
JNJ-26481585	Class I	Acute Myeloid Leukemia, Precursor Cell Lymphoblastic Leukemia-Lymphoma, Leukemia, Myelogenous, Chronic BCR-ABL Positive, Leukemia, Lymphocytic Chronic B-Cell	Phase I	None	NCT00676728
Romidepsin (Depsipeptide, FK228)	Class I	Peripheral T-cell Lymphoma	Phase II	Systemic Therapy	NCT00426764
Depsipeptide (NSC 630176)	Class I	Refractory Cutaneous T cell Lymphoma, Peripheral T Cell Lymphoma	Phase II	None	NCT00007345
Valporic acid	Class I Class IIa	Acute Myeloid Leukemia	Phase II	Decitabine	NCT00414310
Valporic acid	Class I Class IIa	Ovarian Cancer Solid Tumors	Phase I/II	Azacitidine Carboplatin	NCT00529022
Valproate	Class I Class IIa	Neuroectodermal Tumor Brain Metastases Advanced Cancer	Phase I	Etoposide	NCT00513162
Entinostat (SNDX-275) MS-275	Class I	Refractory Hodgkin's lymphoma	Phase II	None	NCT00866333
Entinostat (SNDX-275)	Class I	ER + Breast Cancer	Phase II	Exemestane	NCT00676663
Panobinostat (LBH589)	Class I, II, IV	Classical Hodgkin's Lymphoma	Phase III	Placebo	NCT01034163
Panobinostat (LBH589)	Class I, II, IV	Relapsed/Refractory Classical Hodgkin's Lymphoma	Phase II	None	NCT00742027
Panobinostat (LBH589)	Class I, II, IV	Multiple Myeloma	Phase I	None	NCT00532389
Panobinostat (LBH589)	Class I, II, IV	Primary Myelofibrosis, Post-Polycythemia Vera, Post-Essential Thrombocytopenia	Phase II	None	NCT00931762
Vorinostat	Class I, II, IV	Refractory Large B-Cell Lymphoma	Phase II	Bortezomib	NCT00703664
Vorinostat	Class I, II, IV	Stage IIIA Non-Small Cell Lung Cancer Stage IIIB Non-Small Cell Lung Cancer	Phase I/II	Paclitaxel Radiation Therapy	NCT00662311
Vorinostat	Class I, II, IV	Multiple Myeloma	Phase III	Bortezomib Placebo	NCT00773747
Vorinostat	Class I, II, IV	Leukemia, Myeloid, Acute Leukemia Promyelocytic, Acute Myelodysplastic Syndromes,	Phase I	Sorafenib	NCT00875745
Vorinostat (MK0683, SAHA)	Class I, II, IV	Relapsed or Refractory Multiple Myeloma	Phase I	Lenalidomide Dexamethasone	NCT00642954
Vorinostat	Class I, II, IV	Acute Myeloid Leukemia	Phase II	Gemtuzumab Ozogamicin	NCT00673153
Vorinostat	Class I, II, IV	Brain & Central System Tumor, Lymphoma, Unspecified Childhood Solid Tumor	Phase I	Bortezomib	NCT00994500
Vorinostat	Class I, II, IV	Sarcoma	Phase II	Bortezomib	NCT00937495
Vorinostat (MK0683)	Class I, II, IV	Relapsed or Refractory Multiple Myeloma	Phase II	Bortezomib Dexamethasone	NCT00773838
Vorinostat	Class I, II, IV	Carcinoma, Non-Small Cell Lung Cancer	Phase II	Bortezomib	NCT00937495

HDACi	Class Inhibitor	Type of Cancer	Phase	Comb. Therapy	NCI identifiers
Belinostat	Class I, II, IV	Cutaneous T-Cell Lymphoma	Phase II	None	NCT00274651
Belinostat (PXD101)	Class I, II, IV	Acute Myeloid Leukemia	Phase I/II	Idarubicin	NCT00878722

All active/non-recruiting HDACi trials as of August, 2011 www.clinicaltrials.gov