HDAC Inhibition and Graft Versus Host Disease

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Histone deacetylase (HDAC) inhibitors are currently used clinically as anticancer drugs. Recent data have demonstrated that some of these drugs have potent antiinflammatory or immunomodulatory effects at noncytotoxic doses. The immunomodulatory effects have shown potential for therapeutic benefit after allogeneic bone marrow transplantation in several experimental models of graft versus host disease (GVHD). These effects, at least in part, result from the ability of HDAC inhibitors (HDACi) to suppress the function of host antigen presenting cells such as dendritic cells (DC). HDACi reduce the dendritic cell (DC) responses, in part, by enhancing the expression of indoleamine 2,3-dioxygenase (IDO) in a signal transducer and activator of transcription-3 (STAT-3) dependent manner. They also alter the function of other immune cells such as T regulatory cells and natural killer (NK) cells, which also play important roles in the biology of GVHD. Based on these observations, a clinical trial has been launched to evaluate the impact of HDAC inhibitors on clinical GVHD. The experimental, mechanistic studies along with the brief preliminary observations from the ongoing clinical trial are discussed in this review.

INTRODUCTION

Allogeneic hematopoietic cell transplantation (HCT) is an important therapeutic option for a variety of malignant and nonmalignant conditions. The therapeutic potential of allogeneic HCT relies on the graft-versus-leukemia (GVL) effect, which eradicates residual malignant cells by immunologic mechanisms (1). However, graft versus host disease (GVHD) remains the most frequent and serious complication following allogeneic HCT and limits the broader application of this important therapy. GVHD results from immunologically mediated injury to host tissues (2,3). Consequently, GVHD and GVL reactivity are tightly linked (4). As the number of allogeneic HCT continues to increase, a greater understanding of the pathogenesis of GVHD is being made that may lead to

the development of more effective therapies and treatment strategies.

The pathophysiology of GVHD is known to involve donor T-cell interactions with host antigen presenting cells (APCs) and the subsequent induction of proinflammatory cytokines and cellular effectors that cause target organ damage (5). Because host APCs are critical for induction of GVHD by priming donor CD4^{+} and CD8^{+} T, targeting host APCs may be a promising strategy to prevent GVHD (6). Clinical observations also support the role of APCs in the development of GVHD and the attractiveness of an approach that targets the role APCs play (7, 8).

Acetylation of histones represents one of several epigenetic modifications (9,10). Altering gene expression through chromatin modifications induced by acetyla-

tion and deacetylation of histone tails has gained wide attention (11). Histone deacetylase inhibitors (HDACi) cause reversible inhibition of HDAC enzymes, remodel chromatin, regulate gene expression (12) and have shown efficacy *in vitro* and *in vivo* as antitumor agents (13–16). Phase I/II clinical trials have demonstrated that HDAC inhibition is well tolerated and suberoylanilide hydroxamic acid (SAHA) or vorinostat is now a Food and Drug Administration (FDA) approved drug (15,16). The immunomodulatory effects of HDAC inhibitors, however, have been largely unrecognized until recently. Burgeoning evidence demonstrates that these agents have potent antiinflammatory effects at noncytotoxic doses and concentration (17,18).

In this review, we discuss the clinical features and pathophysiology of GVHD briefly and discuss the exciting and novel observations pertaining to the immunoregulatory effects of HDACi on GVHD. We summarize our current knowledge of the role of HDACs in the complex regulation of GVHD and GVL, and discuss several other studies offering potential molecular mechanisms of ac-

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Figure 1. Acute GVHD of the skin. Photograph courtesy U of Michigan, BMT program.

tion for HDAC inhibition and prevention of alloresponses. Finally, we describe an ongoing Phase II clinical trial that attempts to translate the preclinical studies on HDAC inhibition and GVHD into a proof-of-concept clinical trial.

Clinical Features of GVHD

GVHD occurs when donor T cells respond to histoincompatible antigens on the host tissues and clinically presents in an acute or chronic form. Historically, acute and chronic forms were defined arbitrarily on the basis of the time frame after transplant. Classically, acute GVHD develops within the first 100 days of transplant or can occur beyond 100 days after transplant with persistent, recurrent or late-onset symptoms. The principle target organs include the skin, liver and GI tract. The signs and symptoms can be characterized by diffuse maculopapular rash (Figure 1), anorexia, profuse diarrhea, nausea, vomiting, ileus and cholestatic hepatitis (Table 1). Despite HLA identity between a patient and donor and the current immunoprophylaxis, about 40% of patients with acute GVHD require treatment with high-dose steroids (1). The incidence of acute GVHD is even higher in patients who re-

ceived mismatched donor grafts. Chronic GVHD is a complex, multisystem disorder with myriad manifestations that can involve essentially any organ and, typically, is characterized by fibrosis (Table 2) (19). Chronic GVHD may emerge from acute disease (progressive type), develop following a period of resolution from acute disease (quiescent or interrupted type), or occur *de novo*. Some patients may experience overlap syndrome in which clinical features of acute and chronic GVHD appear together (20). The incidence of chronic GVHD is 60% to 70%, depending on the type of donor (19). Specific signs and symptoms, including erythematous rash, nausea, vomiting, diarrhea and liver dysfunction are shared between the two (Table 2).

Pathophysiology of GVHD

The pathophysiology of GVHD is complex and can be considered as a normal immune response that has gone awry. GVHD also can be considered as a complex immune response that has gone awry and can be understood as a pathway that consists of triggers, sensors, mediators and effectors of GVHD.

Triggers for induction of GVHD

As with all immune responses, certain triggers are critical for induction of acute GVHD.

(a) Disparities between Histocompatibility Antigens. Antigen disparity can be at the level of major histocompatibility complex (MHC), that is, MHC mismatched or at the level of minor histocompatibility antigens (miHA) that is, MHC matched but miHA mismatched.

Table 1. Acute GVHD symptoms.

Skin	Maculopapular skin rash
Upper GI tract	Nausea and/or anorexia plus positive histology
Lower GI tract	Watery diarrhea ≥ 500 mL ± severe abdominal pain ± bloody diarrhea or ileus (after exclusion of infectious etiology)
Liver	Cholestatic hyperbilirubinemia

In humans, the *MHC* gene is on chromosome 6 and encodes the human leukocyte antigens (HLA) (21). The severity of acute GVHD is directly related to the degree of MHC mismatch (22). In bone marrow transplants (BMT) that are MHC matched but miHA disparate, donor T cells still recognize MHC peptide derived from the products of recipient polymorphic genes, the *miHAs* (23–25). The expression of *miHAs* is wide and variable. Some *miHAs* such as *HA-1*, *HA-2*, *HB-1* and *BCL2A1* are found primarily on hematopoietic cells, whereas some others such as the H-Y antigens, *HA-3*, *HA-8*, and *UGT2B17* are ubiquitous (26).

(b) Damage induced by conditioning regimens and underlying diseases. Under most circumstances, the initiation of an adaptive immune response is triggered by the innate immune response. The innate immune system is triggered by certain exogenous and endogenous

Table 2. Chronic GVHD symptoms.

Skin	Dyspigmentation, new-onset alopecia, poikiloderma, lichen planuslike eruptions or scleroticfeatures
Nails	Nail dystrophy or loss
Mouth	Xerostomia, ulcers, lichen-type features, restrictions of mouth opening from sclerosis
Eyes	Dry eyes, sicca syndrome, cicatricial conjunctivitis
Muscles, fascia, joints	Fasciitis, myositis, or joint stiffness from contractures
Female genitalia	Vaginal sclerosis, ulcerations
GI tract	Anorexia, weight loss, esophageal web or strictures
Liver	Jaundice, transaminitis
Lungs	Restrictive or obstructive defects on pulmonary function tests, bronchiolitis obliterans, pleural effusions
Kidneys	Nephrotic syndrome (rare)
Heart	Pericarditis
Marrow	Thrombocytopenia, anemia, neutropenia

molecules. This is likely the case in the induction of acute GVHD. Pattern recognition receptors such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain containing 2 (NOD2) (27) play an essential role in innate immunity by recognizing conserved damage or pathogen-associated molecular patterns (PAMPs) and initiating the cellular signaling pathways that activate cytokine secretion, such as NF-κB (28,29). The PAMPs such as lipopolysaccharide (LPS), CpG, and MDP2 which are recognized by TLR-4, TLR-9 and NOD2 respectively, are released during the chemotherapeutic and radiotherapeutic conditioning regimens performed before the infusion of BMT donor cells (30–34). In this way, the conditioning regimens amplify the secretion of proinflammatory cytokines such as IL-1, TNFα (31,35,36), IL-6 (37) and other interferon family members in a process described as a "cytokine storm." In addition to the exogenous microbial associated molecules, endogenous triggers as a consequence of damage, called damage-associated molecular patterns (DAMPs) might also play a critical role in GVHD (29). In fact the proinflammatory cytokines themselves might serve as DAMPs.

Sensors of GVHD

The triggers that initiate an immune response have to be sensed and presented. Antigen presenting cells (APCs) might be considered the sensors for acute GVHD. The APCs sense the DAMPs (i.e., present the MHC-disparate or miHA-disparate protein and provide the critical secondary (costimulatory) and tertiary (cytokine) signals for activation of the alloreactive T cells), the mediators of acute GVHD. APCs sense allodisparity through MHC and peptide complexes. Dendritic cells (DCs) are the most potent APCs and the primary sensors of allodisparity (38). Recipient DCs that have been primed by the conditioning regimen will process and present MHC and peptide complexes to donor T cells at the time of transplant (39). At later time points, donor DCs may take

over this role (40,41). In the case of hematopoietic cell transplants (HCT), recipient DCs present the endogenous and the exogenous antigens to donor CD8+ and CD4+ T cells, respectively. There is no predilection for allopeptides to be recognized by either CD4+ or CD8+ mediated presentation. As noted earlier, DCs are important initiators of GVHD. The role of DC subsets in GVHD is just beginning to be understood (42–44). However, the kinetics of the switch from recipient to donor APCs, the contributions of different APC subsets, the importance of direct alloantigen presentation, and the magnitude of indirect alloantigen presentation in GVHD remain to be determined.

APCs provide the critical costimulation signals for turning on the acute GVHD process. The interaction between the MHC/allopeptide complex on APCs and the TCR of donor T cells along with the signal via T-cell costimulatory molecules and their ligands on APCs is required to achieve T-cell activation, proliferation, differentiation, and survival (45,46) and the *in vivo* blockade of positive costimulatory molecules (such as CD28, ICOS, CD40, CD30 and so on) (47–52), or inhibitory signals (such as PD-1 and CTLA-4) mitigate or exacerbate acute GVHD respectively (53).

As mentioned above, the inflammatory cytokines and DAMP ligands released during pretransplant conditioning regimens act as a third signal to enhance recipient APC and donor T-cell interactions. In addition, various modulations of APCs can influence GVHD development. Recent data show that exposure to granulocyte colony-stimulating factor (G-CSF) shortly after HCT, in combination with a TBI-conditioning regimen, significantly worsened GVHD in mice (54). Histone deacetylase inhibitors such as suberonylanilide hydroxamic acid (SAHA) and ITF 2357 have been shown to reduce development of GVHD in murine models by modulating host DC functions (55–57) (discussed below).

Mediators of GVHD. These primarily include the donor T-cell subsets and the

donor NK cells. Evidence suggests that alloreactive donor T cells consist of several subsets with different stimuli responsiveness, activation thresholds, and effector functions. The alloantigen composition of the host determines which donor T-cell subsets differentiate and proliferate. As mentioned previously, in the majority of HLA-matched HCT, acute GVHD may be induced by either or both CD4+ and CD8+ subset responses to MiHAs (58). The repertoire and immunodominance of the GVHD-associated peptides presented by MHC class I and class II molecules has not been defined (59). Donor naïve CD62L+ T cells are the primary alloreactive T cells that drive the GVHD reaction, while the donor effector memory CD62L− T cells do not (60,61). Interestingly, donor Tregs expressing CD62L are also critical to the regulation of GVHD (62,63). We now know that it is possible to modulate the alloreactivity of naïve T cells by inducing anergy with costimulation blockade, deletion via cytokine modulation or mixed chimerism. Donor effector memory T cells that are nonalloreactive do not induce GVHD, yet are able to transfer functional memory (60) and mediate GVL (64). In addition, lymphopenia-induced proliferation gives rise to cells that are like memory T cells and enhance the graft-versus-tumor effect after donor leukocyte injection (DLI) (65). In contrast, memory T cells that are alloreactive can cause severe GVHD (66–68).

GVHD is regulated negatively by regulatory T cells (Tregs). Distinct subsets of Tregs exist: the naturally occurring CD4+ CD25+ Tregs that express the Forkhead Box Protein P3 (FOXP3), CD4+ CD25− IL10+ Tr cells, γδ T cells, double negative (DN) T cells, and NKT cells (69–74). In mouse BMT models, naturally occurring donor-derived Tregs suppress the proliferation of conventional T cells, prevent GVHD and preserve GVL effects depending upon the ratio of effector T cells to Tregs (75–80). Furthermore, viral immunity is preserved in the presence of Tregs after allogeneic HCT (81). Mechanisms that enhance Treg numbers and

function might therefore be very effective in enhancing alloBMT. HDACi have been shown to have such salutary effects of natural Tregs (discussed further below). In addition, based on the dominant cytokines that are produced upon activation, T cells can be distinguished into various subsets such as Th1, Th2 and Th17 cells. The Th1 cytokines (IFN-γ, IL-2 and TNF-α) have been implicated in the pathophysiology of acute GVHD (82–84). IL-2 production by donor T cells remains the main target of many current clinical therapeutic and prophylactic approaches, such as cyclosporine, tacrolimus and monoclonal antibodies (mAbs) against the IL-2 and its receptor to control acute GVHD (85,86). But emerging data indicate an important role for IL-2 in the generation and maintenance of $CD4^+CD25^+$ $Foxp3^+$ Tregs, suggesting that prolonged interference with IL-2 may have an unintended consequence in the prevention of the development of long-term tolerance after allogeneic HCT (87–90). Furthermore the role of Th1/Th2 and Th17 cytokines is complex and might be model dependent (91–103). Moreover these cells are required for the GVL effects.

Donor natural killer (NK) cells which are inhibited by recognition of class I alleles on target cells via their killer cell immunoglobulin-like receptors (KIR) are emerging as key effectors in the GVH process. They have been shown to specifically downregulate host APC-mediated activation of alloreactive T cells perhaps by directly killing APCs without losing the beneficial effects (104–106).

Effectors and Amplifiers of GVHD. The effector phase that leads to GVHD target organ damage is a complex cascade that involves cytolytic cellular effectors such as CD8 CTLs, CD4 T cells, NK cells and inflammatory molecules such as IL-1b, TNFα, IFNγ and reactive oxygen species. The cellular effectors require cell-to-cell contact to kill the cells of the target tissues via activation of perforin-granzyme, Fas–FasL (CD95-CD95L), or TNFR-TRAIL pathways. Other CTL-killing mechanisms such as TWEAK, and LTβ/LIGHT pathways also have been
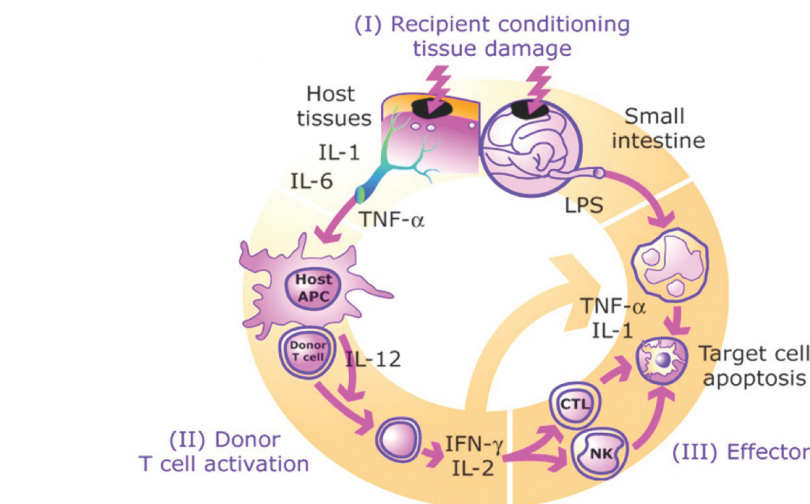
Figure 2. Pathophysiology of GVHD. Three phases of GVHD pathophysiology. From: Reddy P, Ferrara JLM. (2009 Feb 28) Mouse models of graft-versus-host disease. In: StemBook (Internet). Cambridge (MA): Harvard Stem Cell Institute; 2008–. Available at: http://www.stembook.org/node/548

implicated in GVHD (107–114). It is important to note that CTL pathways are essential for GVL effects as well. Inflammatory pathways, by contrast, based on animal models, do not require cell-to-cell contact to kill target cells and are not particularly critical of GVL. GVHD damage by the cellular effectors is amplified by these inflammatory mediators including IFNγ produced by T cells, TNFα (115) and IL-1 (116) produced by T cells and monocytes/macrophages, and nitric oxide (NO) produced by monocytes/macrophages (117,118).

All of the above aspects of the biology of acute GVHD may be summarized in a cyclical three-step model: (step 1) conditioning regimen-related damage and the release of DAMPs such as LPS, (step 2) donor T-cell proliferation and (step 3) target organ damage by effectors (Figure 2). While this allows for accessing the biology of GVHD, it is important to note that GVHD is a complicated systemic process with many unknowns and is not a simplified, linear or cyclical process. Nonetheless, based on our current understanding, agents that reduce inflammatory cytokines such as TNF and IL-1, but spare T-cell CTL functions and enhance donor Tregs and NK cell functions

may be ideal for reducing GVHD without compromising GVL significantly. Experimental data suggests that HDACi may be able to provide such an effect (see Figure 3 and discussion).

Histone Deacetylase Inhibitors (HDACi)

Histones are major structural proteins that package DNA into chromatin and play an important role in gene regulation. DNA wraps around a histone octamer composed of histones H2A, H2B, H3 and H4 to form a nucleosome and the histone H1 links the octameric core into chromatin. Covalent modification on the amino terminal of the core histones through methylation, ADP-ribosylation, phosphorylation, ubiquitylation and acetylation (10,119,120) affect nuclear replication, chromatin assembly and transcription (121–123), and thus provide insight into the epigenetic regulation of gene expression (10,124).

Histone acetylation is tightly regulated by the balance of histone acetyltransferases (HATs) and histone deacetylases (HDACs). HAT enzymes, which now include more than 20 members (125), act by acetylating specific lysine residues of the histone components of chromatin
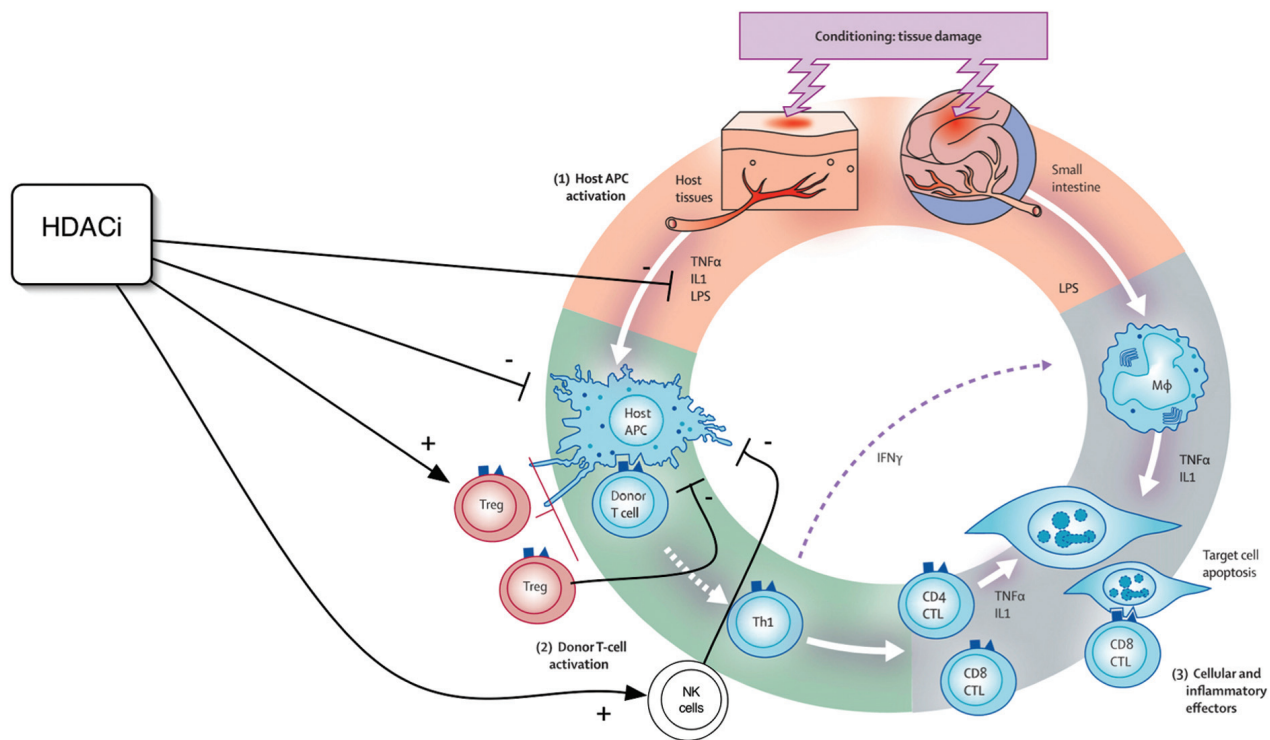
Figure 3. The regulation of immune cells by histone deacetylase inhibitors (HDACi). HDACi have direct and indirect effects on various immune cellular subsets: HDACi play an important role in the negative regulation of APCs, reduce the secretion of inflammatory cytokines, increase the numbers and function of naturally occurring regulatory T cells (Tregs), and activate natural killer (NK) cell–mediated activity.

while HDACs deacetylate the lysine residues. HDACs comprise a family of 18 genes subdivided into four distinct classes: Class I (HDAC1, 2, 3, and 8), class II (HDAC5, 6, 7, 9, and 10), and class IV (HDAC 11) share sequence similarity and require Zn^+ dependent enzymatic activity (126–128). Class III is a structurally unrelated NAD^+ dependent subfamily and belongs to the Sirtuin family (127). Much research on these enzymes has focused on their ability to modulate acetylation of histones and the regulation of chromatin (129,130). Emerging data demonstrate that HDACs can also target nonhistone cellular proteins (127). It is now becoming increasingly known that acetylation of several nonhistone proteins by the HATs and HDACs is an important posttranslational modification that regulates their function, stability, protein-protein/protein-DNA interactions, signaling and functions (131) and that disruption in the balance of acetylation and deacetylation

affects a broad range of human disorders, including oncogenesis and immune dysfunction (132). However, not all HDACs are expressed in all cells; even in those that express them, their location, target proteins and functions might thus vary. Thus, the specificity of different HDACs and their nonhistone proteins, and more importantly, the consequences of targeting specific HDACs in modulating cellular growth, differentiation and immune responses are poorly understood.

However, HDAC inhibitors have emerged as an important class of anti-cancer agents (133–135). HDACi are diverse and can be divided into six classes based on their chemical structure, which include hydroxamic acid derivatives, carboxylates, benzamides, electrophilic ketones, cyclic peptides and miscellaneous compounds (136,137). These agents inhibit the enzymatic activity of primarily class I and II HDACs with varying efficiency (126,138,139), thereby causing increased histone acetylation and gene tran-

scription. Two of them, SAHA and ITF 2357, are hydroxamic-containing agents, and the former was approved by the FDA for treatment of cutaneous T-cell lymphoma (135,140,141). The HDACi, including SAHA and ITF 2357, have differential effects on various zinc-dependent HDAC enzymes, that is, class I and II HDACs (18,142). Thus, the specific HDACs that are critical for the various biological and clinical effects observed upon treatment with HDACi are not known (126). While a large range of different HDACi have been studied and developed for cancer therapy, we and others have demonstrated that HDACi at lower and noncytotoxic concentrations possess a novel and potent antiinflammatory and immunoregulatory effect (17,18). Emerging data from multiple laboratories demonstrate that HDACi can suppress several inflammatory and immune-mediated diseases such as lupus, sepsis, inflammatory bowel disease, rheumatoid arthritis, autoimmune diabetes, allograft tolerance and GVHD in

preclinical models (17,18,55,56,143–148). Several of these are discussed in accompanying articles. Here we will focus on GVHD and discuss potential mechanisms of regulation by HDACi (Figure 3).

Impact of HDAC Inhibition on Experimental GVHD

Insights into the cellular and molecular pathogenesis of GVHD implicate proinflammatory cytokines and host APCs, such as DCs, as important targets for reducing GVHD (3,149). SAHA or ITF 2357 are such agents that are currently in clinical trials for treatment of cancers (150). Micromolar concentrations of SAHA are required for antitumor effects, whereas nanomolar concentrations of SAHA reduce the secretion of inflammatory cytokines such as TNF-α, IFN-γ, IL-1β and IL-12 (17,143). Given the antiinflammatory properties of these agents, and based on the central role of proinflammatory cytokines in the pathogenesis of acute GVHD, we investigated the role of SAHA in a well-characterized murine model of allogeneic HCT. SAHA or ITF 2357 were administered during the amplification of the proinflammatory cascade early in the time course of transplant without interrupting the initial donor T-cell interaction with host APCs (35,151,152). SAHA significantly reduced serum levels of TNF-α, IL-1, and IFN-γ after alloBMT (55). Furthermore, this reduction in the proinflammatory cytokines was associated with a reduction in the GVHD mortality and GVHD-specific target organ damage in multiple murine models (55). SAHA administration following allogeneic HCT did not affect donor T-cell responses to host antigens as determined by their proliferative and CTL responses (55). In addition, the inhibition of cytokines by SAHA temporally correlated with enhanced acetylation of histone H3 and was associated with the downregulation of TNF-α and IFN-γ mRNA after allogeneic HCT (55). Thus the reduction in GVHD likely was primarily due to the inhibition of the inflammatory cytokines and not due to a direct effect on donor T-cell responses in these models.

It is often challenging to separate the toxicity from GVHD with the beneficial GVL effects, a well-recognized and potent form of immunotherapy for malignancies (1). While inflammatory cytokines contribute to the toxicity of GVHD, they have a more limited role in the eradication of residual leukemia, which is primarily mediated by donor CTLs and NK cells (76,95,153–155). Consistent with the preservation of donor T-cell functions, we have found that SAHA administration led to the disruption of inflammatory cytokine cascades, but maintained CTLs, thereby attenuating GVHD mortality and preserving GVL effects and improving leukemia-free survival (55). By contrast, the syngeneic animals that received SAHA did not eliminate the tumor completely, demonstrating the requirement of GVL for tumor eradication. These observations were confirmed in additional tumor and alloBMT murine models, thus ruling out any tumor- or model-specific artifacts. Thus, the maintenance of donor T-cell responses to host antigens after SAHA treatment also preserved the beneficial GVL effect in multiple mouse models of allogeneic HCT. Similar observations on GVHD reduction were made by other groups in other different models (148).

HDAC inhibition modulates the function of APCs. DCs serve as the sentinels of the immune response and function as the most potent APCs (156). They initiate innate immune responses primarily through PRRs, and shape adaptive immunity through the modulation of T-cell responses (157). Because host APCs are critical for the induction of allo-responses and are the major sources of proinflammatory cytokines, following our initial observation that SAHA administration suppressed proinflammatory cytokines and reduced GVHD (55), we investigated the effect of SAHA and ITF 2357 on the function of DCs (56). Bone marrow–derived DCs treated with SAHA or ITF 2357 and then stimulated with TLR agonists such as LPS or other TLR ligands (lipoteichoic acid, peptidoglycan, dsRNA poly[IC] and CpG DNA) (158,159), secreted significantly reduced amounts of proinflammatory cytokines such as IL-1b, TNF-α, IL-12, and IL-6 in a dose-dependent manner (56). DCs treated with SAHA and ITF 2357 also demonstrated reduced *in vitro* allostimulatory responses. This was due to decreased proliferation of the allogeneic T cells and not a consequence of enhanced apoptosis. More importantly, despite the reduction in proliferation of the T cells, their CTL functions were preserved against the allotargets. Consistent with the decreased *in vitro* alloproliferative responses and reduced amounts of cytokines, when the host type DCs were treated with SAHA and infused in the alloBMT recipients early after BMT, they reduced both CD4 and CD8 driven GVHD (56). The reduction in GVHD was once again associated with reduced levels of proinflammatory cytokines (56).

HDACi induce IDO and regulate DCs. Indoleamine 2,3-dioxygenase (IDO) is an intracellular enzyme that degrades tryptophan, an amino acid that is essential for T-cell activation (160). Treatment of DCs with SAHA increased IDO expression at the protein and mRNA levels (56). The increase in IDO expression was associated with histone (H4) acetylation in the IDO promoter region (56). Utilizing three complementary approaches, siRNA, pharmacologic inhibition by 1-MT, and genetically deficient $IDO^{-/-}$ mice, the importance of IDO induction in the DCs by HDACi was investigated. IDO-specific siRNA silenced the mRNA expression of IDO in the SAHA-treated DCs and significantly reversed the suppression of the proinflammatory cytokine, TNF-α, upon LPS stimulation. Likewise, LPS-stimulated DCs from $IDO^{-/-}$ animals or those treated with 1-MT also demonstrated the loss of suppression of proinflammatory cytokine secretion by HDACi. Consistent with our data from murine BM DCs, HDACi also reduced the innate and allostimulatory responses of DCs derived from

healthy human volunteers (56). Furthermore, when bone marrow chimeras generated by utilizing IDO-deficient animals ($IDO^{-/-} \rightarrow$ B6) (such that only host hematopoietic-derived APCs were incapable of generating IDO), were used as recipients in alloBMT, these animals were not protected by administration of SAHA or ITF 2357, demonstrating a loss of HDACi-induced reduction in GVHD. These data indicate a key role for IDO induction by host APCs in the HDACi induced GVHD protection.

STAT-3 is necessary for induction of IDO by HDACi. The critical pathways responsible for the induction of IDO following treatment with HDACi was further dissected. Signaling via Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathways positively and negatively regulate all cell types involved in immune responses (161,162). There are seven STAT transcription factors (161) and while each of the STAT proteins have distinct and overlapping functions, STAT-3 is critical for negative regulation of proinflammatory cytokine secretion by monocyte/DCs but for enhancing T-cell function (163). Data from lethal STAT-3 knock-out mice (164), tumor immunotherapy models (165–167), and, more importantly, humans with loss of function mutations of STAT-3 (Hyper IgE syndrome patients) show enhanced inflammatory phenotype (168–170), demonstrating an essential role for STAT-3 in suppressing immune responses. These findings indicate that STAT-3 plays a critical role in negative regulation of DCs (171). Posttranslational modification of STAT-3 either by phosphorylation and/or acetylation activates its functions (172–178). We therefore, reasoned that HDACi might activate STAT-3 by acetylation and that may be critical for induction of IDO and regulation of DCs (174). STAT-3 was acetylated following SAHA- or ITF 2357-treatment of DCs. Although HDACi acetylated STAT-3 it did not alter its phosphorylation status (174). Furthermore, when the effects of SAHA and ITF 2357 were tested on the induction of IDO in cell lines expressing pcDNA3 empty
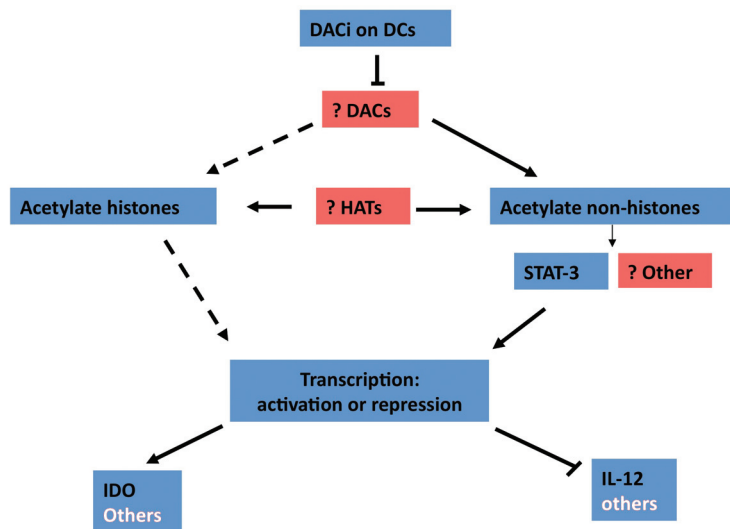
Figure 4. The regulation of DCs by histone deacetylase inhibitors (HDACi).

vector (STAT-3 null), wild type STAT-3, and STAT-3 mutant^{K685R} (that contains Lys685-to-Arg substitution and therefore cannot be acetylated-K685R), HDAC inhibition enhanced IDO expression in the WT STAT-3 transfected cells but not in the null control or the acetylation resistant STAT-3 mutant^{K685R}. These data suggest a critical role for STAT-3 acetylation in the induction of IDO. The relevance of STAT-3 in altering DC function by HDACi was evaluated further by utilizing a drug that disrupts STAT-3 DNA complex formation, JSI-124 specifically (179). Murine DCs, when treated with JSI-124 and then conditioned with SAHA and ITF 2357, did not show suppression of LPS-induced secretion of proinflammatory cytokines such as TNF-α secretion or a reduction in allogeneic T-cell proliferation when compared with diluents treated DCs. Thus, the disruption of STAT-3 activity with JSI-124 mitigated the suppressive effects of HDAC inhibition on DCs. Other studies have demonstrated that STAT3 acetylation by the HAT CBP has been correlated with increased DNA-binding and transactivation activity (174,180,181,182). Conversely, deacetylation of STAT3 by the HDAC Sirtuin 1 correlates with decreased STAT3 tyrosine phosphorylation and activity (183). Nonetheless, collec-

tively, these data demonstrate that acetylation of STAT-3 is necessary for its activation and for the regulation of DCs. However, whether acetylation alone is sufficient in the absence of phosphorylation remains to be investigated. These and other potential effects of HDACi on DCs are summarized in Figure 4.

Specific HDAC enzymes in the regulation of DCs. HDACi mediated suppression of DCs demonstrate the overall impact on DCs from global suppression of class I/II HDACs. The role of specific HDAC enzymes in the regulation of DCs is being evaluated currently. To this end, recent studies by Sotomayor and colleagues have evaluated the impact of HDAC11 and HDAC6 on DCs (184,185). HDAC11 is a newly characterized member of the HDAC family (186). Villagra *et al*. (184), showed that HDAC11 regulates the expression of IL-10 negatively in mouse and human APCs (DCs and macrophages), primarily by interacting with the distal segment of the promoter of the gene encoding this cytokine. IL-10 is an antiinflammatory cytokine that is an important mediator in influencing the function of APCs at the site of antigen encounter, and, thus, serves a key role in tolerance induction and regulation of inflammation (187–189). Overexpression of HDAC11 abrogated the expression of

IL-10 mRNA in LPS-treated macrophages. When HDAC11 was "knocked down" by the transduction of primary mouse macrophages with short hairpin RNA, LPS stimulation resulted in higher expression of IL-10 mRNA. These findings were confirmed in additional experiments using two macrophage cell lines (derived from RAW264.7) lacking HDAC11 expression. Furthermore, in RAW264.7 cells transfected with an enzymatically inactive mutant HDAC11 with deletion of its deacetyltransferase domain, demonstrated increased expression of IL-10 mRNA, suggesting that intact deacetylase activity was required for HDAC11-mediated inhibition of IL-10 in APCs. APCs overexpressing or lacking HDAC11 altered CD4$^+$ T-cell proliferation. Specifically, overexpression of HDAC11 in APCs activated naïve antigen-specific CD4$^+$ T cells and restored the responsiveness of tolerant T cells, whereas APCs lacking HDAC11 functionally impaired CD4$^+$ T-cell proliferation and they produced less IL-2 and IFN-γ. More recently, Dubovsky *et al*. (185) report that overexpression of HDAC6 induced transcriptional activation of IL-10 gene expression, the opposite effect seen with HDAC11. These data demonstrate the role of specific HDACs, HDAC11 and HDAC6, and the inflammatory response of APCs. They demonstrate that, in contrast to global inhibition of HDACs, the inhibition of specific HDACs might lead to a distinct and/or opposite effect on DC responses. Nonetheless, they collectively demonstrate that HDACs could serve as potential therapeutic targets for influencing APC/DC-mediated immune responses.

HDAC inhibition and regulatory T-cell (Tregs) function. As noted above, donor Tregs reduce GVHD, but do not diminish GVL significantly after alloBMT. Recently, Tao *et al*. (147) reported that HDACi expands *in vivo* Treg cell population and also increased the activity of these cells. Several mouse models of alloimmunity and autoimmunity were studied for the *in vivo* analysis of HDAC inhibition. Namely, the recombination-ac-

tivating gene-2 (Rag2)-deficient mouse model and two adoptive transfer models. In each model, HDAC inhibition increased the absolute numbers and proportion of Treg cells, primarily in the CD4$^+$ CD25$^+$ Foxp3$^+$ T-cell subset. When the effects of HDACi therapy were evaluated *in vivo*, there was increased expression and acetylation of FOXP3$^+$ and also an increase in the Treg associated genes, such as *CTLA4* and *GITR*, while IL-2 was repressed. Thus, the expression of multiple Treg-associated genes was increased with HDACi. HDAC inhibition promoted acetylation of histones in Treg cells and increased acetylation on several lysines in the forkhead domain of FOXP3$^+$. When these lysines were mutated, FOXP3$^+$ could not repress IL-2 expression and was less able to suppress conventional T-cell activity *in vitro*, demonstrating that the enhanced function of Tregs is in part due to direct targeting (acetylation) of nonhistone protein Foxp3. They also demonstrate that HDAC9, expressed in higher amounts in Treg cells than conventional T cells, was critical for modulating Tregs. They further demonstrated that the beneficial effects of HDACi on allograft rejection and IBD models. The direct impact of HDACi on donor Tregs after alloBMT is under active investigation currently by our group. Nonetheless, data extrapolated from Tao *et al*.'s observation suggest that HDACi might also have Treg enhancing effects after alloBMT and that this may be another potential GVHD protective effect of HDACi.

HDAC inhibition on NK-cell function and tumor immunogenicity. Donor NK cells reduce GVHD by eliminating host APCs while promoting GVL by direct elimination of the host tumors. Acetylation and deacetylation also may play an important role in NK-cell activity (186). HDAC inhibition with SAHA treatment has been reported to increase the functional expression of NK cell–mediated killing through NKG2, member D (NKG2D) ligands including MHC class I-related chain A and B (MICA/B) in Jurkat T-cell leukemia, thereby making them more sensitive to NK cell–mediated

lyses (190). The effect of HDACi on NK-cell activity also was investigated by Armeanu *et al*. (191) who showed that treatment of human hepatocellular carcinoma cells with the HDACi sodium valproate (VPA) mediated the lyses of malignant cells via NKG2D expressed on cytotoxic lymphocytes. VPA induced the transcription of MICA/B in hepatocellular carcinoma cells, which led to increased cell surface expression, followed by lyses of the cancer cells. These data support a role for HDACi in stimulating NK cell–mediated activity, which may contribute to antitumor immune responses while regulating GVHD. This, however, remains to be demonstrated directly in experimental GVHD models.

Skov *et al*. (190) demonstrated enhanced NK cell–mediated killing on multiple types of cancer cells following HDACi treatment, which included B-cell acute lymphoblastic leukemia, acute myelogenous leukemia, multiple myeloma, malignant non-Hodgkin lymphoma, T-cell acute lymphoblastic leukemia, mantle cell lymphoma, multiple myeloma, epithelial breast adenocarcinoma, epithelial cervix adenocarcinoma, and epithelial colorectal adenocarcinoma. This was consistent with other studies, which have shown a range of different cancer types characterized by constitutive expression of MICA/B (192–194). Interestingly, Skov *et al*. (190) showed that two other cancer cell lines tested did not respond to HDACi treatment by increased MICA/B expression. Therefore, the molecular basis for the selective expression of MICA/B on different cancer cells by HDACi treatment remains unknown. In any event, another study demonstrated that HDACi enhanced the immune susceptibility of two specific forms of primary human acute myeloid leukemia (195). HDACi also induced apoptosis of leukemic blasts AML expressing the PML-RAR or AML1-ETO oncoproteins, independent of p53, through activation of a specific death receptor pathway (TRAIL and Fas signaling pathways) (196). Collectively, these results suggest that HDACi regulate GVHD

by modulating proinflammatory cytokine secretion, host APC function and perhaps by enhancing Tregs. However, they still may preserve GVL by preservation of donor T-cell CTL functions, increasing NK reactivity and the immunogenicity of the tumor cells.

The impact of HDAC inhibition on other allograft models. The effects of HDACi on other allograft models, including rat and canine transplant models, also have been investigated (197,198). FR276457, a hydroxamic derivative HDAC inhibitor, was shown to prevent allograft rejection in a rat cardiac transplant model. When administered alone as monotherapy, the drug demonstrated strong efficacy and demonstrated dramatic allograft survival when used in combination with tacrolimus. Another HDAC inhibitor, FR235222, also prolonged graft survival in rat cardiac transplant model (199). HDACi therapy allograft survival of rapamycin in murine cardiac and islet cell transplant models (40). The data support the role for HDACi as potential therapeutic agents in mitigating alloresponses after BMT and solid organ allografting and, perhaps, can be used as useful adjuncts to current standard immunoprophylaxis drugs such as the CNI and mTOR inhibitors.

Ongoing Translation of HDAC Inhibition for GVHD Prevention

The literature reviewed herein suggests that HDACi have direct and indirect effects on various immune cellular subsets, depicted in Figure 3. HDACi play an important role in the negative regulation of APCs (56), reduce the secretion of inflammatory cytokines, such as TNF-α, IFN-γ, IL-1β and IL-12 (17,55,143), increase the numbers and function of natural $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs (147, 200), and activate NK cell–mediated activity (190,191). HDAC inhibition reduced GVHD and preserved GVL in murine models (55) and regulated both murine and human APCs (57,147,200,201). The HDACi, SAHA and ITF 2357 thus appear to exhibit the desirable regulatory effects on multiple mediators of GVHD (that is, in-

hibit proinflammatory secretion, host APCs but promote donor Treg and NK-cell responses) (3,202, see Figure 3). In addition, the pharmacokinetics of oral ITF2357 recently has been studied (203) and both oral SAHA (vorinostat) and ITF2357 (givinostat) have good safety profiles in humans.

Therefore, given these preclinical observations and the good therapeutic index of the oral preparations of some of these agents, such as SAHA and ITF2357, a clinical trial has been launched at the University of Michigan and at Washington University to test the concept that deacetylase inhibition, when used as an adjunct with standard prophylaxis with CNI, will reduce the incidence and severity of acute GVHD after reduced intensity conditioning (RIC) in a matched related donor allogeneic HCT. This trial is based on the institutional experience of GVHD with RIC regimens. Prior experience with this approach showed a 42% incidence of grades II–IV GVHD, with a 50% two-year survival rate (204). The trial is now built on this experience to evaluate whether adding HDACi, SAHA (vorinostat) will reduce the incidence of grade II-IV GVHD to 25%. It is an open label, nonrandomized, Phase II clinical trial using the same RIC regimen (fludarabine and busulfan) that has been utilized for approximately 10 years at our institution. The GVHD prophylaxis backbone consists of tacrolimus (day 3 to day 56, followed by a taper over 4 months) and mycophenolate mofeitil (day 0 to day 28). Vorinostat currently is being administered orally from day 10 to day 100 at 100 mg twice daily. So far, 20 patients have been treated on this trial. Because SAHA has not been tested previously in this setting, it was started at 100 mg twice daily based on clinical data demonstrating that this dose of induced acetylation in circulating PBMCs. Because the 100 mg dose appeared safe in the first cohort of 10 patients, the dose was escalated to 200 mg twice daily in an attempt to enhance efficacy. However, even though no dose limiting toxicities were reached on the 200 mg twice daily

dose, there appeared to be a greater incidence of thrombocytopenia on the increased dose. Therefore the study dose has been deescalated back to the 100 mg twice daily after 19 patients, and this dosing will be used for the remainder of the study. Thus far, all 20 patients on the trial have engrafted successfully. There have been no dose-limiting toxicities. No serious adverse events related to drugs, including myelosuppression, liver or kidney toxicities have been observed in this early cohort of patients. Only four patients have developed grade 2 GI GVHD, and in all four of these patients, GVHD resolved with systemic and topical therapy. To our knowledge, this is the first human study of HDAC inhibition in allogeneic BMT patients. If successful, it could lead to the development of an entirely new class of immunomodulatory therapy for GVHD, and perhaps for immune/inflammatory diseases. Alternatively, even if the primary endpoint of the clinical trial is not met, this study will generate data that likely will allow for new lines of laboratory investigation that may foster a better understanding of the biology and role of HDACs in GVHD and immune responses.

DISCLOSURES

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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