Histone deacetylase inhibition modulates indoleamine 2,3-dioxygenase–dependent DC functions and regulates experimental graft-versus-host disease in mice

Pavan Reddy, … , Charles A. Dinarello, James L.M. Ferrara


Histone deacetylase (HDAC) inhibitors are antitumor agents that also have antiinflammatory properties. However, the mechanisms of their immunomodulatory functions are not known. We investigated the mechanisms of action of 2 HDAC inhibitors, suberoylanilide hydroxamic acid (SAHA) and ITF 2357, on mouse DC responses. Pretreatment of DCs with HDAC inhibitors significantly reduced TLR-induced secretion of proinflammatory cytokines, suppressed the expression of CD40 and CD80, and reduced the in vitro and in vivo allostimulatory responses induced by the DCs. In addition, injection of DCs treated ex vivo with HDAC inhibitors reduced experimental graft-versus-host disease (GVHD) in a murine allogeneic BM transplantation model. Exposure of DCs to HDAC inhibitors increased expression of indoleamine 2,3-dioxygenase (IDO), a suppressor of DC function. Blockade of IDO in WT DCs with siRNA and with DCs from IDO-deficient animals caused substantial reversal of HDAC inhibition–induced in vitro suppression of DC-stimulated responses. Direct injection of HDAC inhibitors early after allogeneic BM transplantation to chimeric animals whose BM-derived cells lacked IDO failed to protect from GVHD, demonstrating an in vivo functional role for IDO. Together, these data show that HDAC inhibitors regulate multiple DC functions through the induction of IDO and suggest that they may represent a novel class of agents to treat immune-mediated diseases.
Histone deacetylase inhibition modulates indoleamine 2,3-dioxygenase–dependent DC functions and regulates experimental graft-versus-host disease in mice

Pavan Reddy, Yaping Sun, Tomomi Toubai, Raimon Duran-Struuck, Shawn G. Clouthier, Elizabeth Weisiger, Yoshinobu Maeda, Isao Tawara, Oleg Krijanovski, Erin Gatza, Chen Liu, Chelsea Malter, Paolo Mascagni, Charles A. Dinarello, and James L.M. Ferrara

Introduction
Pharmacological suppression of the immune system has revolutionized the treatment of autoimmune diseases and transplant recipients. Traditionally, lymphocytes have been the primary targets of immunosuppressive drugs (1), but accumulating evidence suggests that DCs are master regulators of immune responses (2). DCs are the sentinel of innate immunity that also function as the most potent APCs. DCs initiate innate immune responses primarily through TLRs, and they shape adaptive immunity through modulation of T cell responses (3). After transplantation, DCs present alloantigens to T cells and initiate alloimmune reactions (4). Agents that target both innate and allostimulatory functions of DCs might therefore have therapeutic potential in many immune-mediated disease processes.

Chromatin remodeling by the acetylation and deacetylation of histones helps to regulate gene expression. The acetylation of histones is regulated by 2 classes of enzymes: histone acetyltransferases and histone deacetylases (HDACs) (5, 6). HDAC inhibitors modulate the expression of multiple genes and are currently being evaluated as antitumor agents (6, 7). Several recent experimental studies have demonstrated that HDAC inhibitors can modulate immune responses at concentrations much lower than those needed for antitumor effects (8–12). Suberoylanilide hydroxamic acid (SAHA) and ITF 2357 are 2 such agents; both reversibly hyperacetylate histones by inhibiting HDAC enzymes (7, 13, 14). Phase I clinical trials have demonstrated that they are well tolerated and have antitumor activity even in patients with advanced solid and hematological tumors whose prior treatments were extensive (7, 15, 16).

We and others have demonstrated that administration of SAHA at the time of allogeneic BM transplantation (BMT) suppresses proinflammatory cytokine production and reduces systemic acute graft-versus-host disease (GVHD), the major toxicity of allogeneic BMT (10, 17). HDAC inhibitors have also been shown to regulate several other inflammatory and immune-mediated diseases (8, 9, 11, 13, 18). However, the cellular and molecular mechanisms for the immunomodulatory effects of HDAC inhibitors are not yet known. Here we investigated the effects of hydroxamic acid–containing HDAC inhibitors SAHA and ITF 2357 and showed that they regulated the in vitro and in vivo responses of DCs. Direct injection of HDAC inhibitors early after allogeneic BM transplantation to chimeric animals whose BM-derived cells lacked IDO failed to protect from GVHD, demonstrating an in vivo functional role for IDO. Together, these data show that HDAC inhibitors regulate multiple DC functions through the induction of IDO and suggest that they may represent a novel class of agents to treat immune-mediated diseases.

Results
HDAC inhibitors regulate innate immune responses of DCs. We tested the effects of SAHA on the innate immune responses of DCs that are triggered through TLRs, cell surface molecules that recognize...
We next investigated whether HDAC inhibitors modulated allogeneic T cell–stimulatory capacity of DCs. We next determined the effects of HDAC inhibition on in vitro alloresponses by incubating BALB/c T cells with allogeneic B6BMDCs in a standard mixed leukocyte reaction (MLR) assay. As shown in Figure 2A, addition of 1 μM SAHA or more to the cultures significantly reduced T cell proliferation at 72 h by greater than 70%. IL-2 secretion in culture supernatants collected at 48 h was similarly reduced (Figure 2B).

However, consistent with previous observations (9), SAHA pretreatment did not inhibit T cell responses after stimulation with anti-CD3 and anti-CD28 (Supplemental Figure 1A). Thus, we reasoned that HDAC inhibition in an MLR might primarily affect DC function. To test this directly, we pretreated BMDCs with different concentrations of SAHA for 16–20 h and then washed them thoroughly prior to use as stimulators in an MLR. As shown in Figure 2C and Figure 3, A and B, pretreatment of DCs at SAHA concentrations of 500 nM or greater significantly reduced allogeneic T cell proliferation, even with increasing the responder/stimulator ratios (Figure 2C), and also reduced IL-2 secretion by nearly 50% (Figure 3B). Similar effects were seen upon treatment with ITF 2357 (data not shown). Because cells derived from BM cultures are heterogeneous (21, 22), we purified CD11c+ DCs by fluorescence-activated cell sorting (FACS) from both C57BL/6 and BALB/c BM cultures (>98% CD11c+ DC purity) and found that SAHA had similar regulatory effects on purified DCs (62,198 ± 7,418 cpm versus 29,935 ± 1,1656 cpm, P < 0.02). In contrast, levels of TGF-β, IL-10 and IL-12 in MLR supernatants were unchanged (Supplemental Figure 1, B and C), and their neutralization with anti–TGF-β or anti–IL-10 mAb did not restore T cell proliferation in MLRs with SAHA-treated DCs (Figure 3, C and D). Expression of T cell activation markers, such as CD25 and CD69, was also reduced, with no increase in CD152 expression (data not shown).

We next determined whether coculture of naive T cells with HDAC inhibitor–treated DCs altered their phenotype and/or function into suppressor T cells or Tregs. As shown in Figure 3E, BALB/c pathogen-associated molecular patterns (19). BM-derived DCs (BMDCs) from C57BL/6 mice (B6BMDCs) were incubated with 100 and 500 nM SAHA for 16–20 h prior to stimulation with 100 ng/ml LPS, a potent stimulus of innate immune response via TLR4. SAHA-treated DCs secreted significantly reduced amounts of TNF-α, IL-12, and IL-6 in a dose-dependent manner (Figure 1A). Thus, we reasoned that SAHA and ITF 2357 regulate DC and macrophage responses that are mediated through TLR stimulation.

HDAC inhibitors modulate allogeneic T cell–stimulatory capacity of DCs. We next determined the effects of HDAC inhibition on in vitro alloresponses by incubating BALB/c T cells with allogeneic B6BMDCs in a standard mixed leukocyte reaction (MLR) assay. As shown in Figure 2A, addition of 1 μM SAHA or more to the cultures significantly reduced T cell proliferation at 72 h by greater than 70%. IL-2 secretion in culture supernatants collected at 48 h was similarly reduced (Figure 2B).

However, consistent with previous observations (9), SAHA pretreatment did not inhibit T cell responses after stimulation with anti-CD3 and anti-CD28 (Supplemental Figure 1A). Thus, we reasoned that HDAC inhibition in an MLR might primarily effect DC function. To test this directly, we pretreated BMDCs with different concentrations of SAHA for 16–20 h and then washed them thoroughly prior to use as stimulators in an MLR. As shown in Figure 2C and Figure 3, A and B, pretreatment of DCs at SAHA concentrations of 500 nM or greater significantly reduced allogeneic T cell proliferation, even with increasing the responder/stimulator ratios (Figure 2C), and also reduced IL-2 secretion by nearly 50% (Figure 3B). Similar effects were seen upon treatment with ITF 2357 (data not shown). Because cells derived from BM cultures are heterogeneous (21, 22), we purified CD11c+ DCs by fluorescence-activated cell sorting (FACS) from both C57BL/6 and BALB/c BM cultures (>98% CD11c+ DC purity) and found that SAHA had similar regulatory effects on purified DCs (62,198 ± 7,418 cpm versus 29,935 ± 1,1656 cpm, P < 0.02). In contrast, levels of TGF-β, IL-10 and IL-12 in MLR supernatants were unchanged (Supplemental Figure 1, B and C), and their neutralization with anti–TGF-β or anti–IL-10 mAb did not restore T cell proliferation in MLRs with SAHA-treated DCs (Figure 3, C and D). Expression of T cell activation markers, such as CD25 and CD69, was also reduced, with no increase in CD152 expression (data not shown).

We next determined whether coculture of naive T cells with HDAC inhibitor–treated DCs altered their phenotype and/or function into suppressor T cells or Tregs. As shown in Figure 3E, BALB/c T cells were incubated with diluent (sterile water), SAHA, or ITF 2357 for 16 h and then stimulated overnight with LPS. Pretreatment with SAHA significantly reduced LPS-induced secretion of TNF-α by the peritoneal macrophages (Figure 1E).

We next determined whether SAHA modulated DC responses induced by other TLR ligands. Using IL-6 production as an end point, we found that SAHA attenuated DC function after stimulation with lipoteichoic acid (LTA), a TLR4 ligand, peptidoglycan (PGN), a TLR2 ligand, the dsRNA poly(IC), a TLR3 ligand, and CpG DNA, a TLR9 ligand (Table 1) (19, 20). Similar reductions were also seen in the secretion of TNF-α by both SAHA and ITF 2357 (data not shown).

Taken together, these data demonstrate that SAHA and ITF 2357 regulate DC and macrophage responses that are mediated through TLR stimulation.

**Figure 1**
Effect of HDAC inhibitors on LPS-induced secretion of proinflammatory cytokines by BMDCs. DCs were obtained from C57BL/6 animals as described in Methods. BMDCs were preincubated for 16–20 h with indicated concentrations of SAHA or diluent and were then stimulated overnight with LPS (100 ng/ml). Proinflammatory cytokines levels in the supernatants were measured by ELISA. (A) TNF-α levels. (B) IL-12 levels. (C) IL-6 levels. *P < 0.02 versus stimulated control DCs. (D) DCs were pretreated with the indicated concentrations of SAHA and ITF 2357 or diluent as described in A. TNF-α levels were measured in the supernatants after overnight stimulation with LPS. **P < 0.01 versus stimulated control DCs. (E) Macrophages (F4/80+) were obtained from C57BL/6 mice after peritoneal lavage and pretreated with SAHA or diluent for 14–16 h. They were then stimulated overnight with LPS, and TNF-α from the culture supernatant was measured. *P < 0.02 versus control macrophages. Data (mean ± SEM) are from 1 of 2–3 experiments with similar results. Unlabeled bar(s) at left represent unstimulated controls.
naive T cells that were incubated in primary cultures with SAHA-treated B6BMDCs proliferated normally in a secondary MLR to the same alloantigen [C57BL/6 (H2b)] and also to a third-party alloantigen, C3H/HeJ (H2k). In addition, these cells did not suppress the proliferation of freshly isolated naive BALB/c T cell responses to C57BL/6 stimulation at a 1:1 ratio (Figure 3E), and no increase in the percent or level of expression of Foxp3 in allogeneic T cells was observed after coculture with SAHA-treated DCs compared with control DCs (data not shown). To determine whether the reduction in allogeneic T cell expansion by SAHA-pretreated DCs was caused by greater apoptosis or by reduced proliferation of the allogeneic T cells, we performed serial annexin V staining and CFSE analysis. We cultured CFSE-labeled BALB/c T cells with either HDAC inhibitor–pretreated DCs or control DCs and analyzed the T cells for their proliferative responses or apoptosis by annexin V staining. As shown in Tables 2 and 3, pretreatment of DCs with SAHA reduced allogeneic T cell proliferation as determined by CFSE staining without causing significant changes in apoptosis.

We next analyzed whether the proliferative responses were restored in the primary MLR cultures with HDAC inhibitor–treated DCs by the addition of exogenous IL-2. As shown in Supplemental Figure 3A, addition of exogenous IL-2 significantly rescued T cell proliferation, suggesting that HDAC inhibitor–treated DCs induced T cell anergy (23). Thus, pretreatment of DCs with HDAC inhibitor did not induce apoptosis of DCs, nor did it trigger regulatory function in T cells cocultured with HDAC inhibitor–treated DCs. We next examined whether SAHA-treated DCs affect the CTL functions of allogeneic T cells against allo-specific targets. BALB/c T cells were stimulated in a bulk MLR with either SAHA-treated or control DCs for 5 d. T cells were harvested and tested for their CTL functions against allogeneic C57BL/6 concanavalin A blasts in a 6-h chromium release killing assay. BALB/c T cells caused equivalent lyses of host-type B6BMDCs treated with either diluent or 500 nM SAHA (Figure 3F). We used a similar experimental approach to evaluate the in vivo responses of CD8+ T cells to SAHA-treated DCs in vivo. MHC class I-deficient (Hla-/-) recipient mice were conditioned and transplanted as described above with 3 × 106 allogeneic CD8+ T cells from bm1 donors (see Methods), which differ from the recipient animals by a single MHC class I antigen. SAHA pretreatment of DCs also significantly inhibited donor CD8+ T cell proliferation 21 d after BMT in this model (Figure 3G). Together, these data demonstrate that pretreatment of DCs with SAHA regulates the in vivo alloproliferation of both CD4+ and CD8+ T cells.

### Table 1

<table>
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<tr>
<th>Treatment</th>
<th>Diluent</th>
<th>SAHA</th>
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<tr>
<td>Control DCs</td>
<td>ND</td>
<td>973 ± 86</td>
</tr>
<tr>
<td>SAHA DCs</td>
<td>ND</td>
<td>278 ± 97</td>
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</tbody>
</table>

DCs were harvested from C57BL/6 BM, treated with either 500 nM SAHA or diluent control for 16–18 h, and stimulated overnight as indicated. IL-6 was measured in the culture supernatants at the end of culture with TLR ligands. ND, not detected. Data (mean ± SEM of triplicate wells) are from 1 of 2 independent experiments with similar results. *TLR4 ligand. **TLR2 ligand. ***TLR3 ligand. ****TLR9 ligand.

P < 0.02 versus control DCs. P < 0.001 versus control DCs.


T cells from either syngeneic C57BL/6 or allogeneic bm12 donors (see Methods), which differ from the recipient animals by a single MHC class II antigen. Analysis of donor T cells in the spleen at 7 d — chosen because most recipients were dead by 10 d — revealed fewer CD4+ T cells in animals that received DCs pretreated with SAHA (Figure 3F). We used a similar experimental approach to evaluate the in vivo responses of CD8+ T cells to SAHA-treated DCs in vivo. MHC class I-deficient (Hla-/-) recipient mice were conditioned and transplanted as described above with 3 × 106 allogeneic CD8+ T cells from bm1 donors (see Methods), which differ from the recipient by a single MHC class I antigen. SAHA pretreatment of DCs also significantly inhibited donor CD8+ T cell proliferation 21 d after BMT in this model (Figure 3G). Together, these data demonstrate that pretreatment of DCs with SAHA regulated the in vivo alloproliferation of both CD4+ and CD8+ T cells.

**Ex vivo treatment of DCs with HDAC inhibitors regulates experimental GVHD.** In light of these results, we evaluated whether the regulatory effects of HDAC inhibitor–treated DCs modulated clinically relevant alloreactions by using a well-established murine allogeneic BMT model [BALB/c (H2b) C57BL/6 (H2b)], in which activation of donor T cells by host APCs is critical for induction of acute GVHD (24). C57BL/6 Ly5.2 BMT recipients were injected on days –1, 0, and 2 with 4 × 106 to 5 × 106 host-type B6BMDCs treated with either diluent or 500 nM SAHA for 16–20 h. This schedule was chosen to modulate the initial donor T cell interaction with host APCs during the first 72 h after BMT (25–27). As shown in Figure 4A, injection of SAHA-treated DCs to allogeneic animals significantly improved survival compared with injection of control DCs (50% versus 10%; P = 0.01). We also observed significantly lower GVHD clinical disease scores in mice injected with SAHA-treated DCs compared with controls early after BMT (7 d, 3.7 ± 0.2 versus 5.2 ± 0.6, P = 0.037; 14 d, 2.3 ± 0.4 versus 4.4 ± 0.9, P = 0.014; 21 d, 3.1 ± 0.4 versus 4.9 ± 0.8, P < 0.05; 28 d, 4.3 ± 0.7 versus 5.1 ± 1; P = 0.21). Consistent with the reduction in clinical GVHD, injection of SAHA-treated DCs also reduced serum levels of TNF-α (Figure 4B) and attenuated the expansion of donor CD3+ lymphocytes 7 d after BMT (Figure 4C) (25). We next stained for Foxp3 on the H2d’CD4+ double-positive cells and analyzed for donor CD4+Foxp3+ Treg expansion 7 and 10 d after allogeneic BMT. There was no significant increase in the percent positivity of donor Tregs in the splenocytes of the recipient animals after infusion of SAHA-treated DCs compared with control DC infusion on days 7 and 10 (7 d, 12.6% ± 3.2% versus 8.8% ± 3.6%, P = 0.19; 10 d, 13.1% ± 2.2% versus 10.4% ± 2.9%, P = 0.23), suggesting lack of early donor Treg enhancement.

**Mechanisms of HDAC inhibitor–mediated regulation of DCs.** We explored the cellular mechanisms critical for regulation of DCs mediated by HDAC inhibitors. Although the immunomodulatory effects were observed at low concentrations, because HDAC inhibitors can induce cellular apoptosis (28), we sought to determine whether the regulation of DC function was secondary to loss of DC viability. We found no significant increase in annexin V–positive CD11c+ DCs when pretreated with 100 and 500 nM SAHA (the dose used for most in vitro studies), but observed a significant increase at 2- and 5-μM concentrations, thus ruling out apopto-
We then evaluated the effect of HDAC inhibition on the expression of costimulatory molecules CD80, CD86, and CD40. Control B6BMDCs demonstrated high expression of CD80, CD86, and CD40, as reported previously (29, 30), and stimulated proliferation of allogeneic T cells. HDAC inhibitor treatment markedly reduced the surface expression of all these molecules (Figure 5). These data suggest that treatment of DCs with HDAC inhibitors causes active downregulation of costimulatory molecule expression.

To test the potential clinical applicability of our findings, we evaluated the effect of HDAC inhibitor pretreatment on human PBMC–derived DCs. PBMC-derived DCs from normal healthy volunteers were treated with 500 nM SAHA for 18 h and then washed prior to using them as stimulators in an allogeneic MLR. SAHA treatment of DCs reduced proliferation and IFN-γ secretion by allogeneic responder PBMCs (Supplemental Figure 2, A and B) and, consistent with a recent report (31), significantly reduced the expression of CD40 and CD80 on human PBMC–derived CD11c+ DCs (Supplemental Figure 2, C and D). Pretreatment with both SAHA and 100 nM ITF 2357 also reduced LPS-induced TNF-α expression from these cells (data not shown).

HDAC inhibitors cause immunodominant regulation of DCs. We explored whether HDAC inhibition would dominantly regulate the function of DCs. SAHA-treated DCs were cocultured at increasing ratios with control DCs, whose numbers were kept constant. As shown in Figure 6A, SAHA-treated DCs significantly inhibited the proliferation of allogeneic responder T cells in a contact-dependent manner. We then evaluated whether regulation at this high ratio was contact dependent. As shown in Figure 6B, when SAHA-treated DCs were separated from control B6BMDCs and BALB/c allogeneic T cells by transwell, allogeneic T cells proliferated briskly. These data show that HDAC inhibition dominantly regulates the allostimulation of control DCs in a contact-dependent manner.

Next, to address whether the contact-dependent regulation of HDAC inhibitor–treated DCs requires contact with T cells or control DCs, we devised a 3-cell experiment in which SAHA-treated DCs lacked the capacity to present antigens to T cells. We reasoned that if regulation depended on contact with control DCs alone, rather than T cells, it would be preserved even if the SAHA-treated DCs were deficient in MHC molecules and thus incapable of direct interaction with responder T cells. C4d74−/− (H2b) mouse B6BMDCs were treated with SAHA as described above and then cocultured with WT (H2b) B6BMDCs together with purified BALB/c (H2d) responder CD4+ T cells in an MLR. Contrary to our hypothesis, allogeneic CD4+ T cells proliferated briskly in this 3-cell experiment (Figure 6C), demonstrating that the critical points of contact in the regulation of T cell proliferation lie between HDAC inhibi-
tor–treated DCs and T cells. These data also provide an explanation for the reduction of GVHD by ex vivo–treated DCs we observed and thus collectively show that HDAC inhibition can dominantly regulate the function of DCs both in vitro and in vivo.

**HDAC inhibitors induce the expression of IDO.** We next sought to determine the potential molecular mechanisms underpinning the regulation of DCs by HDAC inhibitors. IDO is an enzyme that degrades tryptophan, which suppresses DC function and induces T cell anergy (32, 33). Therefore, to elucidate the molecular mechanism of the effect of HDAC inhibition on DC responses, we tested the hypothesis that treatment of DCs with HDAC inhibitors enhances the expression of IDO. B6BMDCs were harvested and treated overnight with increasing concentration of SAHA or ITF 2357; the controls were treated with diluent alone. Cells were harvested and analyzed for the induction of IDO mRNA by RT-PCR, as described in Methods. As shown in Figure 7A, both SAHA and ITF 2357 increased the expression of IDO mRNA, which suggests that HDAC inhibition increases the transcription of IDO. We confirmed the presence of IDO protein by Western blot analysis using these same cellular preparations (data not shown), which suggests that the regulatory effects of HDAC inhibitors on DCs correlates with increased expression of the immunoregulatory enzyme IDO. We next evaluated whether HDAC inhibitors regulate the transcription of IDO by performing a chromatin immunoprecipitation assay (see Methods). As shown in Supplemental Figure 4A, acetylated H4 was bound to the IDO promoter, demonstrating a direct role for acetylation of histones in promoting transcription of IDO by SAHA.

**IDO is critical for HDAC inhibitor–mediated regulation of DCs.** To address whether IDO is critical for HDAC inhibitor–mediated regulation of DCs, we used 3 distinct but complementary approaches with siRNA, with pharmacologic inhibition by 1-MT, and with DCs from *Indo*–/– mice (34, 35). B6BMDCs were treated with 500 nM SAHA, 10 ng/ml IFN-γ, or diluent and were transfected with either IDO-specific or control siRNA as described in Methods. At 48 h, IDO-specific siRNA efficiently silenced the mRNA expression of IDO both in the SAHA group and in IFN-γ–treated controls (Fig 7B). The lack of effect of siRNA on the increased expression of SOD2 in IFN-γ–treated cells further confirmed the specificity of the silencing of *Indo* gene expression.

Figure 3
HDAC inhibitors regulate in vitro and in vivo functions of DCs. B6BMDCs were pretreated with diluent or SAHA and used as stimulators in MLR cultures as described in Methods. (A) BALB/c T cell proliferation after 72 h of culture. (B) IL-2 levels in supernatants at 48 h of culture. *P = NS, control versus 0.1 μM SAHA. **P < 0.01 versus control. (C and D) Addition of (C) anti–TGF-β or (D) anti–IL-10 to SAHA-treated DC cultures did not reverse SAHA-treated DC–mediated suppression. *P = NS, SAHA-treated versus control DCs. **P < 0.05 versus control. (E) Naive BALB/c T cells, or those obtained after 48 h of culture with C57BL/6 (B6) DCs pretreated with 0.5 μM SAHA, were reconstituted in secondary cultures with control C57BL/6 DCs either separately or together at a 1:1 ratio. T cells cultured from primary SAHA-treated DCs were restimulated with third-party C3H/HeJ BMDCs. *P = NS for all between-group comparisons. Data are mean ± SEM of triplicate cultures. (F and G) *Cd74*–/– and HLA-G–/– animals were irradiated and transplanted as described in Methods. Syngeneic (*n* = 3–4) and some allogeneic animals (*n* = 4–5) received 4 × 10^6 to 5 × 10^6 control B6BMDCs, while some allogeneic recipients (*n* = 5) received similar numbers of SAHA-treated B6BMDCs, on days −1, 0, and 2 relative to BMT. (F) Donor CD4+ cell number was evaluated in recipients’ spleens on day 7. **P < 0.001 versus allogeneic. Data (mean ± SEM) are from 1 of 3 similar experiments. (G) Donor CD8+ cell number was evaluated in recipients’ spleens on day 21. *P < 0.05 versus allogeneic. Data (mean ± SEM) are from 1 of 2 similar experiments.
We directly examined the functional role of IDO in mediating the effects of SAHA by measuring the response of DC production of TNF-α to LPS stimulation. B6BMDCs were treated with SAHA or diluent, transfected with either scrambled siRNA control or IDO-specific siRNA, and then stimulated with LPS for the last 12 h of a 48-h culture (see Methods). TNF-α levels were measured in the supernatants to determine DC function as described above. As we observed in Figure 1A, SAHA treatment suppressed TNF-α production by more than 70% (Figure 7C). Blockade of IDO by siRNA significantly, but not completely, reversed this suppression of TNF-α production by SAHA-treated DCs (Figure 7C). Marked reversal of suppression of the allogeneic T cell proliferation was also observed when IDO was knocked down (data not shown).

We further confirmed the critical role of IDO in HDAC inhibitor-mediated regulation by stimulating BMDCs from Indo–/– mice and B6BMDCs with LPS. We observed significantly less suppression of TNF-α secretion by SAHA-treated Indo–/– B6BMDCs (Figure 7D), confirming a substantial role for IDO in HDAC inhibitor-mediated suppression of DCs. The role for functional IDO expression by HDAC inhibitor treatment of DCs in the suppression of allogeneic T cell proliferation was further confirmed by cotreatment with 1-MT (data not shown). Thus, IDO directly mediates, at least in part, the regulation of DCs by HDAC inhibitors.

We next determined whether the effects of HDAC inhibitor-treated DCs modulated in vivo GVHD responses in an IDO-dependent manner. We once again used the same allogeneic BMT model (BALB/c into C57BL/6), in which activation of donor T cells by host APCs are critical for induction of acute GVHD. C57BL/6 Ly5.2 recipients were injected on days –1, 0, and 2 relative to BMT with either diluent or 5 mg/kg ITF 2357 or the clinical severity of GVHD compared with control-treated mice. As expected, all allogeneic BALB/c –→ C57BL/6 mice treated with either diluent or 500 nM SAHA for 12–16 h at a 4:1 ratio. T cells were harvested and stained with FITC-conjugated annexin V and allophycocyanin-conjugated CD3 for FACS analysis. Differences were not significant between groups on all days: 3 d, P = 0.88; 5 d, P = 0.11; 6 d, P = 0.51. Data (mean ± SEM) are from 1 of 2 similar experiments.

<table>
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<th>Treatment</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 6</th>
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<tbody>
<tr>
<td>Control</td>
<td>10.95 ± 0.3175</td>
<td>11.43 ± 5.895</td>
<td>9.500 ± 1.365</td>
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<tr>
<td>SAHA</td>
<td>10.30 ± 0.2887</td>
<td>13.33 ± 1.411</td>
<td>15.83 ± 8.587</td>
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CD90+ T cells from BALB/c mice were cultured with B6BMDCs pretreated with either diluent or 500 nm SAHA for 12–16 h at a 4:1 ratio. T cells were harvested and stained with FITC-conjugated annexin V and allophycocyanin-conjugated CD3 for FACS analysis. Differences were not significant between groups on all days: 3 d, P = 0.88; 5 d, P = 0.11; 6 d, P = 0.51. Data (mean ± SEM) are from 1 of 2 similar experiments.

We then generated C57BL/6 BM chimeras using TCD BM from Indo–/– C57BL/6 mice transplanted into recipient C57BL/6 mice (Indo–/– C57BL/6 –→ C57BL/6) so that BM-derived APCs would be incapable of IDO expression. Chimeras were reirradiated on day –1 relative to BMT, then injected on day 0 with TCD BM and 3 × 10^6 purified CD3+ T cells from either allogeneic BALB/c or syngeneic C57BL/6 donors. The allogeneic recipients were injected with either diluent or 5 mg/kg ITF 2357 on days –1, 0, 1, and 2 relative to BMT in order to modulate the host hematopoietic-derived APCs that initiate graft-versus-host reaction (27). As expected, all allogeneic BALB/c –→ C57BL/6 mice treated with diluent died of GVHD (Figure 8C), while 50% of allogeneic animals that received ITF 2357 died, and survivors demonstrated mild clinical GVHD (Figure 8, C and D). However, no significant survival difference was noted between the control-treated [C57BL/6 –→ C57BL/6] and [Indo–/– C57BL/6 –→ C57BL/6] animals. GVHD was also confirmed by target organ histopathology (i.e., gastrointestinal tract and liver; data not shown). These data collectively demonstrate that expression of IDO by host APCs alone was not critical for the modulation of GVHD in control-treated animals but was critical for HDAC inhibitor-mediated regulation of GVHD.

**Discussion**

HDAC inhibitors possess potent antimetastatic properties at high concentrations (36); however, at lower concentrations they modulate many immune responses (8, 9, 11, 12, 37–39). Histone acetylation is critical in regulating gene expression for many immune processes (40), but the exact cellular effects and the molecular mechanisms that are critical for immunosuppression caused by these agents are not well understood. Because DCs are master regulators of immunity, they are attractive potential targets for modulating immune reactions. In this study we showed that pretreatment of DCs with SAHA and ITF 2357 modulated murine BMDC functions in vitro and in vivo and also regulated in vitro functions of human PBMC–derived DCs. HDAC inhibitors increased the mRNA expression of immunoregulatory enzyme IDO (32), which peaked by 6 h and returned to baseline by 36 h (data not shown) and coincided with the transient, but immunodominant, regulation of DCs by HDAC inhibitors. Chromatin immunoprecipitation assay demonstrated that HDAC inhibitors directly enhanced IDO transcription.

<table>
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<th>Treatment</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
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<td>Control</td>
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<td>45.77 ± 5.387</td>
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<td>SAHA</td>
<td>15.75 ± 0.1443a</td>
<td>23.40 ± 1.250</td>
<td>25.97 ± 0.233A</td>
<td>39.00 ± 1.404A</td>
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</table>

CD90+ T cells from BALB/c mice were cultured with B6BMDCs pretreated with either diluent or 500 nm SAHA for 12–16 h at a 4:1 ratio. T cells were harvested and stained with FITC-conjugated annexin V and allophycocyanin-conjugated CD3 for FACS analysis. Differences were not significant between groups on all days: 3 d, P = 0.025; 4 d, P = 0.043; 5 d, P = 0.003; 6 d, P = 0.001.
through acetylation of histone H4. Gene silencing with siRNA, pharmacologic inhibition with 1-MT, and use of Indo–/– mice confirmed functional relevance for IDO. We thus confirm and extend recent observations on the regulation of DC function by HDAC inhibition and additionally demonstrate what we believe to be a novel molecular mechanism and in vivo relevance for HDAC inhibitor–mediated modulation of DCs (31, 39, 41). Given the impact of acetylation on gene transcription and protein functions, it is nonetheless possible that HDAC inhibitors will also affect other targets in addition to IDO in the DCs. Furthermore, although IDO is constitutively expressed by B220+CD19+CD11c-low plasmacytoid DCs, it can also be induced in a variety of other cell types (42). Because BM-derived DCs contained less than 1% B220+CD19+CD11c+ cells (data not shown), our findings suggest that HDAC inhibitors induce expression of IDO primarily in the nonplasmacytoid DC fraction of BMDCs. Nonetheless, the effect of HDAC inhibition on different DC subsets, the additional molecular targets, and the critical pathways that modulate IDO-dependent mechanisms will warrant further studies.

Host DCs, proinflammatory cytokines, donor T cell responses, and TLR (LPS) signaling play critical roles in the pathogenesis of acute GVHD (43), the most serious complication of allogeneic BMT (44). Injection of host-type DCs treated ex vivo with the HDAC inhibitor SAHA early after BMT significantly controlled acute GVHD mortality. Direct administration of a second HDAC inhibitor, ITF 2357, also reduced acute GVHD that was dependent, at least in part, on the expression of IDO by host-type APCs in response to HDAC inhibition. These data thus confirm a critical role for host expression of IDO and additionally provide what we believe to be a novel molecular mechanism for our and others’ previous observations regarding the ability of these agents to suppress GVHD (10, 45). Furthermore, our data were suggestive, but not conclusive, that HDAC inhibitors might also have direct salutary effects on epithelial cells during both alloimmune and autoimmune responses (17, 18). A recent study suggested an association between histone acetylation and regulation of STAT-1 expression in GVHD in some target organs (18); however, a direct cause and effect relationship of STAT-1 regulation by HDAC inhibition for GVHD protection remains to be demonstrated.

Figure 4
BMDCs treated ex vivo with HDAC inhibitor regulate acute GVHD. C57BL/6 animals were irradiated and transplanted with TCD BM and T cells from either allogeneic BALB/c or syngeneic C57BL/6 Ly5.2 (n = 4) animals as described in Methods. Allogeneic recipients were injected with diluent (n = 10) or with 4 x 10^6 to 5 x 10^6 control (n = 10) or SAHA-treated (n = 10) B6BMDCs on days –1, 0, and 2 relative to BMT. (A) Survival. Open circles, syngeneic; open triangles, diluent-injected allogeneic; filled circles, SAHA-treated DC–injected allogeneic; filled triangles, control DC–injected allogeneic. P < 0.05, SAHA-treated DCs versus control DCs. (B) Serum levels of TNF-α 7 d after BMT. *P < 0.05 versus allogeneic control recipients. (C) Day-7 donor T cell expansion. *P < 0.05 versus allogeneic control recipients. Data (mean ± SEM) were combined from 2 experiments and represent 2 of 3 experiments with similar results.

Figure 5
Effect of SAHA on the phenotype of murine DCs. BM cultures enriched for DCs from C57BL/6 mice were sorted by FACS for CD11c+ DCs after being stained with FITC-conjugated anti-CD11c. Cells were then incubated with 0.5 μM SAHA or diluent for 16–18 h and stained for PE-labeled IgG (black) or anti-CD80, anti-CD86, and anti-CD40 (gray) as described in Methods. Increased expression relative to control IgG is shown by arrows and percentages. Results are representative of 3 replicate experiments.
Importantly, HDAC inhibitors do not suppress the CTL responses to allogeneic tumor targets (10) and increase the immunogenicity of tumors, making them more susceptible to T cell–mediated cytotoxicity (47–50). Furthermore, treatment of P815 and EL-4 tumor cell lines with subcytotoxic doses of SAHA and ITF 2357 did not increase the expression of IDO (data not shown). Given these differential effects, HDAC inhibitors might enhance graft-versus-leukemia by increasing tumor immunogenicity (48–50) and maintaining CTL responses while simultaneously reducing GVHD through regulation of DC functions and proinflammatory cytokine secretion. Nonetheless, IDO can be expressed in a variety of other cell types (32, 51–53), and how HDAC inhibitors affect the expression of IDO in other cells, and the interactions amongst these cells, remains to be explored.

Immunomodulatory agents, such as IL-10, sirolimus, or vitamin D3, alter the phenotype of DCs by reducing the expression of expression costimulatory molecules, and these agents eventually generate Tregs (54–61). Although HDAC inhibitor treatment of DCs reduced the surface expression of CD40, CD80, and CD86 (31), it did not lead to the generation of the conventional suppressor cells, such as CD4+CD25+Foxp3+ or Tr-1 cells. Moreover, the proliferative responses were restored in the primary MLR cultures by the addition of exogenous IL-2. These data suggest that HDAC inhibitor–treated DCs induced T cell anergy (23) and are consistent with previous observations that IDO-expressing cells induce T cell anergy (23) and are consistent with previous observations (68). Nevertheless, our data suggest that the doses needed for antitumor efficacy, and whether reduced doses alter immune surveillance, infection, and immune modulation, need to be explored.

Methods

Mice. Female C57BL/6 (K\(^{d}\)D\(^{p}\)-I\(^{-}\)A\(^{-}\)E\(^{-}\)), B6.Ly-5a (CD45.1\(^{-}\)), CD45.2\(^{-}\), HLA-G\(^{-}\)/C57BL/6 (H2\(^{d}\); CD45.2\(^{-}\)), C3H/HeJ (H2\(^{b}\); bm12 (K\(^{d}\)D\(^{p}\)-I\(^{-}\)A\(^{-}\)m12/I\(^{-}\)E\(^{-}\); CD45.2\(^{-}\)), Indo\(^{-}\)/C57BL/6, and bm1 (K\(^{d}\)D\(^{p}\)-I\(^{-}\)A\(^{-}\)E\(^{-}\); CD45.2\(^{-}\))
mice were purchased from The Jackson Laboratory. C57BL/6-background Cd74−/− mice (B6.129-AbbN5; CD45.2) were purchased from Taconic Farms. The bm12 and bm1 mice possess mutant MHC class II and MHC class I alleles, respectively, that differ from C57BL/6 mice. The age of mice used for experiments ranged between 8 and 12 wk. Mouse were housed in sterilized microisolator cages and received filtered water and normal chow or autoclaved hyperchlorinated drinking water for the first 3 wk after BMT.

DC isolation. To obtain DCs, BM cells from C57BL/6 mice were cultured with murine recombinant GM-CSF (10 ng/ml; BD Biosciences — Pharmingen) and 10 ng/ml IL-4 (Peprotech) for 5–8 d and harvested as described previously (69). DCs from normal healthy human volunteers were also isolated from fresh heparinized blood following culture with murine recombinant GM-CSF (10 ng/ml; BD Biosciences — Pharmingen) and 10 ng/ml IL-4 (Peprotech) for 5–8 d and harvested as described previously (69). DCs from normal healthy human volunteers were also isolated from fresh heparinized blood following culture with murine recombinant GM-CSF (10 ng/ml; BD Biosciences — Pharmingen) and 10 ng/ml IL-4 (Peprotech) for 5–8 d and harvested as described previously (69).

HDAC inhibitors. SAHA and ITF2357 were obtained from Italfarmaco (Cinisello Balsamo) in the form of dry lyophilized powder and were stored at –80°C. Vials of SAHA and ITF2357 were first dissolved in 50 μl DMSO, diluted in sterile H2O, and heated to boiling for complete dissolution before being added to the cultures (14). DCs were harvested and either sorted or normalized for CD11c+ DCs and cultured for 16–18 h with various concentrations of SAHA and ITF 2357. Sterile H2O was used as the diluent control.

Cell cultures. The TLRs on DCs were stimulated overnight with specific pathogen-associated molecular patterns: 1 μg–100 ng/ml LPS and 10 μg/ml LTA, both for TLR4; 10 μg/ml PGN for TLR2; 100 μg/ml polyIC for TLR3; and 10 μM CpG for TLR9. The supernatant from the cultures was analyzed for proinflammatory cytokines, including TNF-α, IL-12p70, and IL-6. For analysis of proliferative responses, mixed lymphocyte cultures were performed with 2 × 10^5 cells/ml of allogeneic BALB/c CD90+ T cells cultured in flat-bottomed 96-well Falcon plates (BD) in the presence of SAHA- or control-treated CD11c+ DCs (2 × 10^5 cells/ml) from C57BL/6 animals for 72 h, or cultivated with or without 1 μg/ml anti-CD3e and anti-CD28 mAb (BD Biosciences) for 48 h by incorporation of [3H] thymidine (1 μCi; NEN Life Sciences Products) for the last 24 h of incubation (69). Transwell experiments were performed with BALB/c T cells and unsorted control C57BAL/6 DCs (2 × 10^5 cells/ml) from C57BL/6 animals for 72 h, or cultivated with or without 1 μg/ml anti-CD3e and anti-CD28 mAb (BD Biosciences) for 48 h by incorporation of [3H] thymidine (1 μCi; NEN Life Sciences Products) for the last 24 h of incubation (69). Transwell Costar. Proliferative responses were measured after incorporation of [3H] thymidine for the last 24 h of incubation. For proliferative analysis, BALB/c CD90+ T cells were labeled with CFSE and cultured with B6MDMCs. These DCs had been pretreated with diluent or 500 nm SAHA for 16–18 h, washed, and placed in an MLR culture with CFSE-labeled allogeneic T cells as stimulators. T cells were harvested and stained with allopurinol-conjugated CD3 for CFSE fluorescence intensity analysis.

Cytotoxicity assay. BALB/c T cells from mice were stimulated in bulk primary cultures with control- or SAHA-pretreated B6MDMCs at 37°C in a 5% CO2 atmosphere for 6 d and used as effector cells after normalizing for CD8+ T cell numbers. C57BL/6 or BALB/c spleen cells (2 × 10^5) were cultured with concanavalin A (Sigma-Aldrich) at a concentration of 5 μg/ml.
IDO is critical for regulation of GVHD by HDAC inhibitors. (A and B) WT C57BL/6 mice were irradiated and transplanted with TCD BM and T cells from either allogeneic BALB/c or syngeneic C57BL/6 Ly5.2 (black line; n = 6) animals. Allogeneic recipients were injected with 4 × 10^6 to 5 × 10^6 of either diluent control (gray line; n = 4) or SAHA-treated (dotted line; n = 4) B6BMDCs from Indo→C57BL/6 mice on days −1, 0 and 2. All mice were evaluated for survival (A) and clinical GVHD severity (B). P = NS, SAHA-treated versus control allogeneic recipients. Data (mean ± SEM) are from 1 of 2 similar experiments. (C and D) [C57BL/6→C57BL/6] and [C57BL/6→Indo< 0.05→C57BL/6] chimeras were generated as described in Methods and used as recipients in allogeneic BM. Chimeras were reirradiated and transplanted with TCD BM and T cells from either allogeneic BALB/c or syngeneic C57BL/6 Ly5.2 (gray line; n = 4) animals. Allogeneic [C57BL/6→C57BL/6] recipients were injected with either diluent (thin line; n = 6) or ITF 2357 (thick line; n = 6) as described in Methods. [C57BL/6→Indo< 0.05→C57BL/6] recipients were also injected with diluent (dotted line; n = 5) or ITF 2357 (dashed line; n = 6). All mice were evaluated for (C) survival and (D) clinical severity of GVHD. *P < 0.05 versus ITF 2357. Data (mean ± SEM) are from 1 of 2 similar experiments.
period. Cells were then recovered, washed, and immediately used for analysis of IOD expression by RT-PCR. Prior to using the IOD-specific siRNA, we tested the transfection efficiency of the above conditions. BLOCK-it Fluorescence Oligo (Invitrogen), a fluorescent-labeled dsRNA oligomer designed for RNAi transfection analysis, was used to assess and optimize the cationic liposome–mediated transfection of DCs, a good indication of transfection efficiency with our standard siRNA. After 24 h of transfection, the cells were examined under an inverted reflected-light fluorescence microscope (model 1X70, with filter BAS15 for fluorescence; Olympus) and a 100-W high-power pressure mercury lamp. Digital images for phase-contrast and FITC were captured by a SPOT Xplorer camera (Diagnostic Instruments Inc.) attached to the microscope. The total number of cells and the number of FITC-labeled cells in the same fields were identified and counted in 10 randomly selected areas per high-power field (original magnification, ×100) and revealed that we had a transfection efficiency of 75%–90%.

**Chromatin immunoprecipitation assay.** Chromatin immunoprecipitation assay was performed to evaluate the binding of acetylated histone H4 to the IOD promoter, as described previously (72). Briefly, cells were crosslinked with 1% formaldehyde for 30 min, quenched with 1.25 mm glycine for 5 min, harvested, and washed twice with cold PBS. Cells were resuspended in lysis buffer (50 mm HEPES, pH 8.0; 1 mM EDTA; 0.5 mM EGTA; 140 mM NaCl; 10% glycerol; 0.55 mM NP-40; 0.25% Triton X-100; 1 mM PMSF; and 1× protease inhibitor mixture; Pierce) and sonicated for 5 bursts of 15 s each, followed by a cooling period of 40 s in an ice bath using Dimmerator 550 (Fisher Scientific) and centrifugation at 13,000 g for 10 min. One-tenth of the total lysate was used for total genomic DNA as input control. Supernatant was treated with salmon sperm DNA (Sigma-Aldrich), BSA-coated (Fisher Scientific) and centrifugation at 13,000 × g for 10 min. One-tenth of the input DNA was used for PCR. Primers corresponded to sequences within IOD promoter γ-IFN–activated site regions as follows: forward, 5′-CTCTTTTTATAGGTGTAGTTTCCC-3′; reverse, 5′-GAGACTCCTAGTTTACCCAGC-3′.

**BMT.** BMT was performed according to a standard protocol, as described previously (10). WT C57BL/6, C571/6; or HLA-G−/− C57BL/6 mice were irradiated with 11 Gy total body irradiation (137Cs source) on day –1 relative to BMT and were injected with 4 × 106 to 5 × 106 BM-derived host-type (WT C57BL/6 or Indo−/−) DCs on days –1, 0 and 2 as otherwise specified following total body irradiation. On the day of BMT, mice received 2 × 106 purified splenic CD3+ cells along with 5 × 106 TCD BM cells from either syngeneic or allogeneic BALB/c donors. In experiments with MHC class II– and MHC class I–deficient animals, mice received 2 × 106 CD4+ T cells or 3 × 106 CD8+ T cells (along with 5 × 106 TCD BM cells) from bm12 or bm1 donors, respectively. In preliminary experiments, administration of 1 × 106 or 2 × 106 DCs had no significant effects on GVHD severity. BM chimeras using TCD BM from [C57BL/6−/−C57BL/6] and [Indo−/−C57BL/6−/− C57BL/6] animals were generated following irradiation of 11 Gy. After 3–4 mo, these animals were conditioned with 10 Gy on day –1 and used as recipients in a second BMT with 3 × 106 to 4 × 106 T cells and 5 × 106 BM cells from allogeneic BALB/c donors. The chimeras received 5 mg/kg ITF2357 or diluent on days –1 to 2 relative to BMT.

**Statistics.** Survival curves were plotted using Kaplan–Meier estimates. The Mann–Whitney U-test was used for the statistical analysis of in vitro data and clinical scores, while the Mantel–Cox log-rank test was used to analyze survival data. A P value less than 0.05 was considered statistically significant.

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