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LBH589 Enhances T Cell Activation In Vivo and Accelerates Graft-versus-Host Disease in Mice

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Histone deacetylase inhibitors (HDACis) are a new class of compounds that induce acetylation of histone lysine tails in chromatin and modify gene expression. The Food & Drug Administration approved HDACi, Vorinostat, or suberoylanilide hydroxamic acid (SAHA), has been shown to inhibit tumor cell growth and the production of proinflammatory cytokines. In preclinical allogeneic transplant models, SAHA induces graft-versus-host disease (GVHD) amelioration in treated mice without impairing graft-versus-leukemia. LBH589 (Panobinostat), a structurally novel cinnamic hydroxamic acid class, is an HDACi more potent than SAHA. In the current work, we tested the hypothesis that LBH589 would be highly effective in the prevention of GVHD. Using mouse model of allogeneic bone marrow transplant (BMT), we unexpectedly found that treatment with LBH589 accelerated GVHD, in contrast to the treatment with SAHA that alleviated GVHD. Accelerated GVHD in the recipients treated with LBH589 was associated with elevated Th1 cytokines in recipient serum, enhanced CXCR3 expression on donor T cells, and T cell infiltration in the liver. The current study highlights the distinct effects of pan HDACi on allogeneic BMT and alerts that LBH589 (Panobinostat) could have an adverse effect on GVHD, and possibly on other inflammatory diseases.

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INTRODUCTION

Histone deacetylase (HDAC) are enzymes that modulate chromatin structure and gene expression by removing acetyl groups on histone and other proteins. According to their structure, HDACs are classified into 4 groups: class I (HDAC1, HDAC2, HDAC3, and HDAC8), class IIa (HDAC4, HDAC5, HDAC7, and HDAC9), class IIb (HDAC6 and HDAC10), class III (SIRT1-7), and class IV (HDAC11) [1,2]. Inhibiting HDAC activity by pan-HDAC inhibitors (HDACis) has been shown to cause growth arrest and apoptosis of tumor cells. Therefore, initially, pan-HDACis were applied for cancer therapy. Recent findings showed that pan-HDACi could also prevent or alleviate inflammation in mouse models for various diseases as colitis, lupus, arthritis, and neural stroke [3-6]. Li et al. [5], Reddy et al. [7,8], and Leng et al. [9], showed that SAHA, a pan-HDACi approved for the therapy of cutaneous T cell lymphoma, could alleviate graft-versus-host disease (GVHD) after allogeneic bone marrow transplant (BMT) in mice in an indoleamine 2,3-dioxygenase-dependent manner. LBH589 is a hydroxamic acid-based HDACi with a similar structure with suberoylanilide hydroxamic acid (SAHA). Compared to SAHA, LBH589 has much higher potency in inhibiting each of HDAC family members [10]. In the current study, we evaluated the effect of LBH589 on the prevention of GVHD after allogeneic BMT, and unexpectedly we found that LBH589 worsened GVHD. The accelerated GVHD was related to higher levels of proinflammatory cytokines in serum, and increased CXCR3 expression on donor T cells, and T cell infiltration in the liver.

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MATERIALS AND METHODS

Mice

C57BL/6 (B6, H-2^b), BALB/c (B/c, H-2^d), were purchased from National Cancer Institute/National

Institutes of Health. All animals were housed in an American Association for Laboratory Animal Care-accredited Animal Resource Center at Moffitt Cancer Center. Experiments were carried out under protocols approved by the Institutional Animal Care and Use Committee.

Chemicals and Reagents

LBH589 powder provided by Novartis AG was dissolved in 5% dextrose (Sigma-Aldrich, St. Louis, MO) and sonicated in PBS before use. SAHA was purchased from ChemieTek (Indianapolis, IN). SAHA was first dissolved in DMSO and further diluted in PBS before use. LBH589 and SAHA were administrated via i.p. injection. Carboxyfluorescein diacetate, succinimidyl ester (CFSE)-5 and 6 was purchased from Invitrogen (Grand Island, NY).

Isolation of Donor T and BM Cells

Donor mice T cells were purified by negative selection from pooled spleen and lymph node cells to remove the non-T cells using biotinylated Abs and the MACS system (anti-biotin microbeads and LS column, Miltenyi Biotech, Auburn, CA) according to the manufacture's instruction. The Abs for T cell purification (anti-mouse TER-119, anti-mouse CD49b, anti-mouse CD11b, and anti-human/mouse CD45R) were purchased from eBioscience (San Diego, CA). The purity of T cells was usually over 95%. Bone marrow (BM) was harvested from tibia and femurs, and T cells were depleted by incubation with anti-Thy1.2 Ab (clone 30H12, BioXCell, West Lebanon, NH), and rabbit complement (GTI Diagnostics, Waukesha, WI).

BMT

Female B/c mice at 7 to 8 weeks old were lethally irradiated (800 cGy, single dose) using a Shepherd Mark I Cesium Irradiator (J.L. Shepherd and Associates, San Fernando, CA) 1 day before BMT. On the day of transplantation (Day 0), 5×10^6 T cell depleted (TCD) B6 BM cells were transferred to recipients via tail vein with or without 1×10^6 B6 T cells. Mice were housed in sterilized micro isolator cages and received normal chow and autoclaved hyperchlorinated water for the first 3 weeks after BMT and autoclaved water thereafter. The clinical signs of GVHD (weight loss, ruffled fur, hunched back, and skin lesions) were monitored twice a week.

Abs and Flow Cytometry

The following antibodies were used for cell surface staining: anti-CD4 FITC, or APC (L3T4), anti-CD8α FITC, APC, APC-Cy7 or Alexa Fluor 700 (Ly-2), anti-H-2K^b FITC, PE, or biotin (AF6), anti-mouse CD11b PE-Cy7, anti-mouse CD11c PE, anti-mouse Ly6G (Gr-1) biotin, and anti-mouse CXCR3 biotin were purchased from eBioscience; anti-mouse CCR6 APC were purchased from BioLegend (San Diego, CA); anti-CD4 Pacific blue (RM4-5) and anti-mouse α4β7 PE was purchased from BD Biosciences (San Jose, CA). Detection of biotinylated Abs was performed using APC-Cy7 or APC conjugated to streptavidin (BD Biosciences). Flow data were acquired on FACSCalibur, or LSR II (BD Biosciences) and analyzed using FlowJo (TreeStar Inc, Ashland, OR).

Measurement of T and B Cell Function

Splenic cells were cultured in RPMI 1640 media (Invitrogen) with 10% FBS (Atlanta Biologicals, Atlanta, GA), penicillin (100 units/mL; Invitrogen) and streptomycin (100 μg/mL; Invitrogen) in 96-well round bottom cell culture plates with or without 1 μg/mL anti-CD3 antibody (BioXCell), or 5 μg/mL lipopolysaccharide (LPS, Sigma-Aldrich) for 3 days. Six hours before the end of cell culture, 1 μCi [^{3}H]-thymidine was added to each well. The proliferation of T cell or B cell was measured by a scintillation counter.

Histological Analysis

Representative samples of liver, colon, and small intestines were obtained from patients who underwent transplantation and fixed in 10% neutral-buffered formalin. Samples were then embedded in paraffin, cut into 5-μm thick sections, and stained with H&E. A semiquantitative scoring system was used to account for histologic changes consistent with the GVHD signs in the colon and liver as previously described [11]. Data was presented as individual GVHD target organ scores as well as a composite score from all the tissues. All slides for GVHD analysis were coded and read in a blinded fashion. Images were visualized with an Olympus BX45 microscope. Image acquisition was performed with an Olympus DP70 digital camera (×400) and software package.

Mouse Serum Cytokine Analysis and Intracellular Cytokine Staining

Mouse serum cytokine was analyzed following the protocol of BD Cytometric Bead Array Mouse Th1/Th2/Th17 Cytokine Kit (BD Biosciences).

Statistics

The recipient survival among groups in the GVHD experiments was compared by log-rank test. The *t* test was used for all the other experiments.

RESULTS

Daily Treatment with LBH589 Accelerated GVHD after Allogeneic BMT

Reduction of GVHD after treatment with pan-HDACi SAHA indicates that HDAC can alleviate

the pathogenesis of GVHD. LBH589 is also a pan-HDACi but with much higher potency [10]. Therefore, we originally hypothesized that LBH589 would have a higher efficacy in the prevention of GVHD when compared with SAHA.

Before evaluating the efficacy of LBH589 in GVHD, we first tested its toxicity in recipients after myeloablative conditioning and BM reconstitution to determine the maximum tolerated dose. B/c mice were lethally irradiated 1 day before adoptive transfer of TCD-BM from B6 donors. Recipients were treated every day with LBH589 from day 0 to 4 at 2.5, 5, 10, or 20 mg/kg body weight (BW). By monitoring recipient survival, we found that treatment with 10 mg/kg LBH589 or higher dose caused significant mortality when compared with vehicle control (Figure 1A). Treatment with 5 mg/kg LBH589 or lower dose does not show significant toxicity, although some mice died. To determine whether LBH589 affects donor BM reconstitution, we compared donor B cell, T cell, dendritic cell (DC), and neutrophil reconstitution in recipient spleen at day 28 after BMT. As shown in Figure 1B, daily treatment with 2.5 mg/kg BW LBH589 did not compromise donor lymphocyte reconstitution. In addition, the proliferative function of splenic B cell and T cell was intact (Figure 1C).

Because LBH589 was toxic to allogeneic BMT recipients when given a dose of 10 mg/kg or higher, we decided to evaluate the efficacy of LBH589 in the prevention of GVHD using 1.25 or 2.5 mg/kg doses every day from day 0 to 4. Recipient survival and BW changes were monitored beyond 100 days after BMT. Daily treatment with LBH589 at 2.5 mg/kg did not cause mortality or additional weight loss as compared with vehicle control on recipients who underwent transplantation with TCD-BM alone (Figure 2). Unexpectedly, when T cells were added, the treatment with LBH589 at either dose significantly worsened GVHD, because treated recipients had accelerated death and dramatic weight loss when compared with GVHD controls (BM + T cell + PBS group) (Figure 2A and B).

Treatment with LBH589 Every Other Day had Lower Toxicity but Still Accelerated GVHD

Accelerated GVHD associated with every day treatment could have resulted from LBH589 toxicity or enhanced T cell activation. To lower the potential toxicity of LBH589, we decided to administer it every other day for 2 weeks. No mortality was observed on this treatment schedule when LBH589 was given at 2.5 to 7.5 mg/kg (Figure 3A). Although treatment at 7.5 mg/kg causes noticeably more weight loss than that with vehicle control, all recipients treated with LBH589 shortly recovered their BW to the levels of those treated with vehicle control (Figure 3B). We conclude that the every other day treatment causes lower toxicity compared to every day treatment.

To assess the effect of LBH589 in the prevention of GVHD, we treated the recipients with LBH589 every other day at 2.5 mg/kg for 2 weeks, and found that the treatment with such dose and schedule had no appreciable toxicity in the recipients with TCD-BM alone nor on GVHD in the recipients with TCD-BM plus allogeneic T cells (Figure 4A and B). Further, we evaluated the LBH589 efficacy in the every other day schedule by increasing the dose to 5 mg/kg. Although the every other day treatment with LBH589 at 5 mg/kg for 2 weeks did not cause noticeable toxicity on the TCD-BM group, such treatment significantly accelerated GVHD in the recipients of T cells (Figure 4C and D).

LBH589 and SAHA had an Opposite Effect on GVHD

LBH589 is a pan-HDACi with much higher potency than SAHA. Given that independent groups showed that SAHA alleviates GVHD after allogeneic BMT [7,9], it was highly unexpected to observe the accelerated GVHD with LBH589 treatment (Figures 2 and 4). To exclude the possibility that the experimental systems we chose might contribute to our unexpected results, we directly compared the therapeutic effects of SAHA and LBH589 on GVHD prevention. We used the same dose and schedule of SAHA treatment shown to be effective in previously published reports by others [7,8], and confirmed that SAHA significantly alleviated GVHD in sharp contrast with LBH589, which significantly accelerated GVHD (Figure 5A and B). All comparisons were made between PBS vehicle, SAHA and LBH589 treatment groups. The distinct outcomes on GVHD resulted from the treatment with LBH589 or SAHA were further supported by pathologic evidence. Figure 5C shows significantly increased damage ($P < .01$) in the liver from the LBH589 treated recipients when compared with the ones that received vehicle alone. In contrast, the treatment with SAHA produced lower liver damage than observed in the control group. We also examined pathologic changes in the colon and small intestine, and found that the pathologic scores were higher in the recipients treated with LBH589 than those with vehicle control, although the differences were not statistically significant (data not shown). In conclusion, LBH589 and SAHA had an opposite effect on GVHD development.

In Vivo T Cell Activation was Decreased by SAHA, but Increased by LBH589

To understand the underlying mechanisms by which LBH589 and SAHA had opposite effects on GVHD development, we evaluated the T cell activation

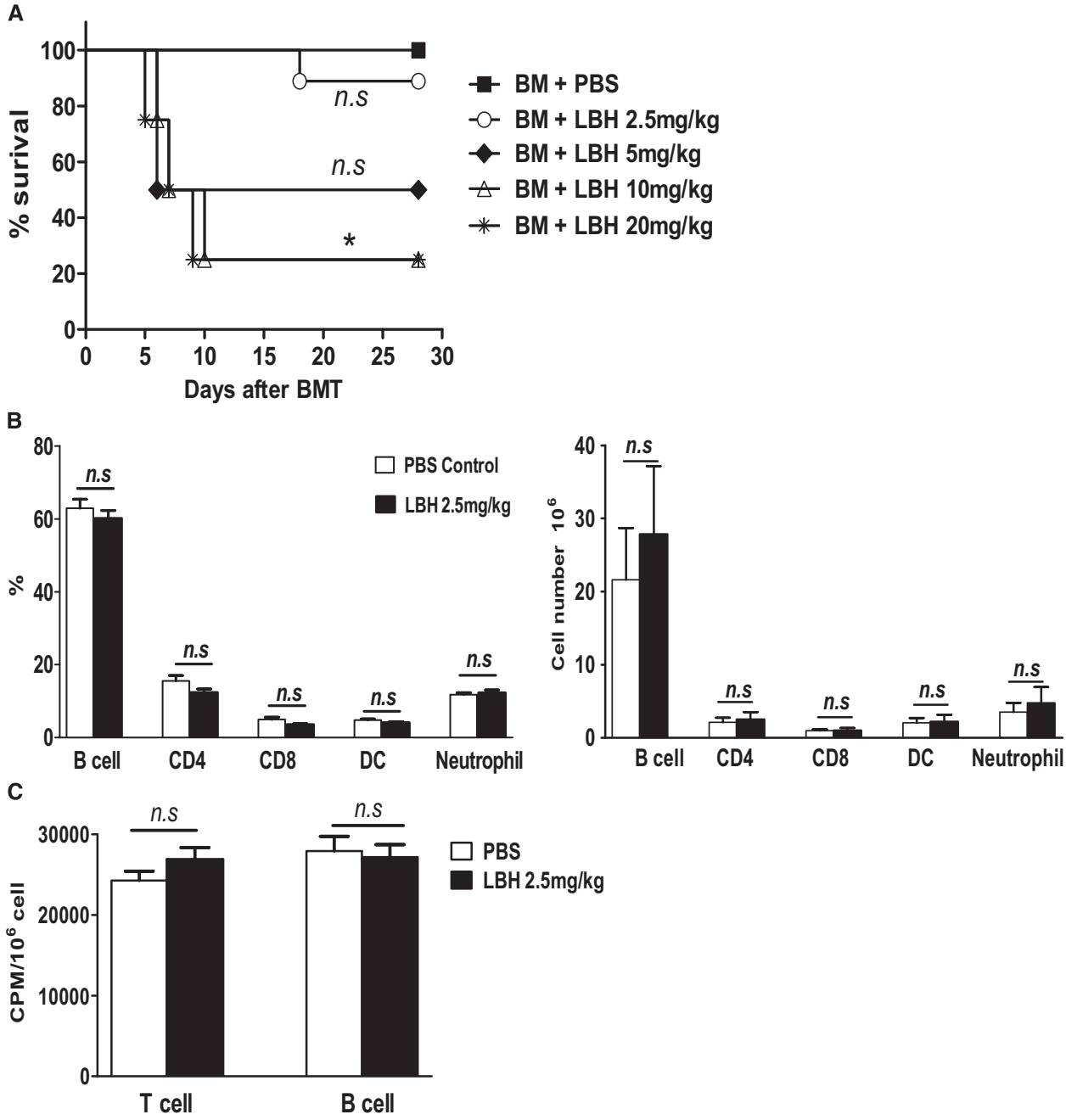

Figure 1. Toxicity of LBH589 with every day treatment. B/c mice (n = 4-5) were lethally irradiated 1 day before bone marrow transplant (BMT). On the day of transplantation, 5×10^6 T cell depleted (TCD) B6 bone marrow (BM) cells were transferred to B/c recipients via tail vein. Recipients were treated with PBS vehicle or different dose of LBH589 daily from day 0 to 4 via i.p. injection. (A) Mice survival. (B) On day 28, recipients (n = 5) treated with vehicle control or LBH589 at 2.5 mg/kg BW were killed, and the percentages of donor B cells (B220), T cells (CD4/CD8), dendritic cell (DC; CD11c), and neutrophils (CD11b/Gr-1) in the spleen were analyzed by flow cytometry. (C) The 4×10^5 splenic cells from recipient mice (n = 5) were stimulated with 1 μg/mL anti-CD3 or 5 μg/mL lipopolysaccharide (LPS) for 3 days. The proliferation of T cells or B cells was compared by [^{3}H]-thymidine incorporation. This experiment was repeated 3 times and similar phenomenon was observed. The representative data from one experiment was shown. CPM, count per minute; n.s., no significant difference. *$P < .05$.

in response to alloantigens in vivo by measuring TNF-α and IFN-γ in recipient serum because either inflammatory cytokine plays a critical role in GVHD development. As shown in Figure 6A and B, the levels of TNF-α and IFN-γ were significantly lower in the recipients treated with SAHA than those treated with vehicle control, consistent with published reports by others that SAHA reduces the inflammatory cytokines [9]. In contrast, the levels of IFN-γ and TNF-α were significantly higher in the recipients treated with LBH589 than those treated with vehicle control, suggesting that LBH589 enhanced T cell activation in vivo. We also compared other Th1/Th2/Th17 cytokines like IL-2, IL-4, IL-6, IL-10, and IL-17, and

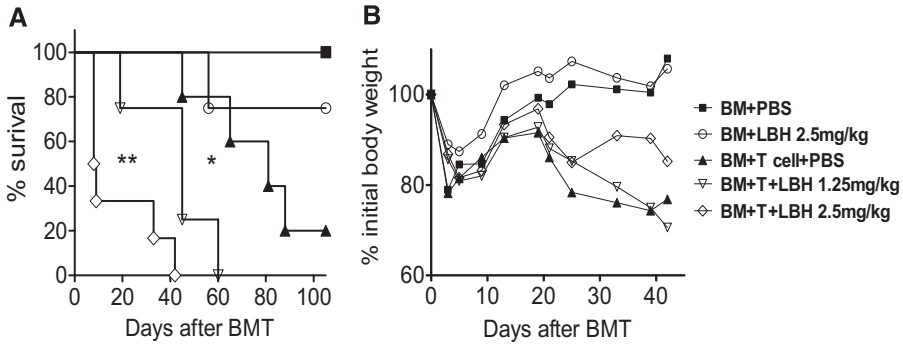

Figure 2. Effect of LBH589 with every day treatment on graft-versus-host disease (GVHD). B/c mice (n = 5-6) were lethally irradiated and the next day underwent transplantation with 5×10^6 T cell depleted (TCD) B6 bone marrow (BM) cells with or without 1×10^6 T cells via tail vein. Recipients were treated with PBS vehicle or different dose of LBH589 daily from day 0 to 4 via i.p. injection. Mice survival (A) and body weight (BW) (B) are shown. This experiment was repeated 3 times and similar results were obtained. One representative experiment was shown here. BMT, bone marrow transplant. $^*P < .05$; $^{**}P < .01$.

observed similar levels among all experimental groups (data not shown).

Donor T cell expansion and subsequent migration into target organs are essential for the development of GVHD. We thus measured donor T cells in recipients' spleens on day 14 after BMT, and found that treatment with LBH589 reduced the number of donor T cells in the spleen, whereas SAHA had no effect (Figure 7A). Because migration of activated T cells into GVHD target organs primarily relies on chemokine receptors expression, we compared the expression of chemokine receptors on donor T cells in the recipients treated with LBH589, SAHA, or vehicle control. Among the receptors (CXCR3, $\alpha 4\beta 7$, and CCR6) tested, we found that donor $CD4^+$ and $CD8^+$ T cells expressed significantly higher levels of CXCR3 after LBH589 treatment (Figure 7B). Given that CXCR3 is preferentially expressed on Th1 cells, these data are consistent with elevated Th1 cytokines (IFN-γ and TNF-α) in the recipients treated with LBH589 (Figure 5). As a consequence, significantly higher numbers of donor $CD4^+$ and $CD8^+$ T cells were found in the liver of the recipients treated with LBH589 compared to those with vehicle control (Figure 7C). Taken together, treatment with LBH589 increased T cell activation and migration to GVHD target organ liver in particular, which likely resulted in accelerated GVHD after allogeneic BMT. To exclude the possibility that LBH589 treatment led to BM failure, we compared the percentage of donor BM-derived cells in the spleen at day 13. As seen in Figure 7D and E, the percentage of H-2kb+ cell population (donor BM-derived) were very similar among the 3 experimental groups.

DISCUSSION

A pan-HDACi, SAHA, was shown to alleviate GVHD after allogeneic BMT in various mouse models [5,7-9]. Here we tested another highly potent pan-HDACi, LBH589, in the prevention of GVHD after allogeneic BMT with the expectation that LBH589 would diminish GVHD with a high efficacy. Contrary to our expectation, LBH589 accelerated rather than alleviated GVHD. The accelerated GVHD was associated with increased secretion of Th1 inflammatory cytokines. Pathological T cell infiltration in the liver was also increased by LBH589,

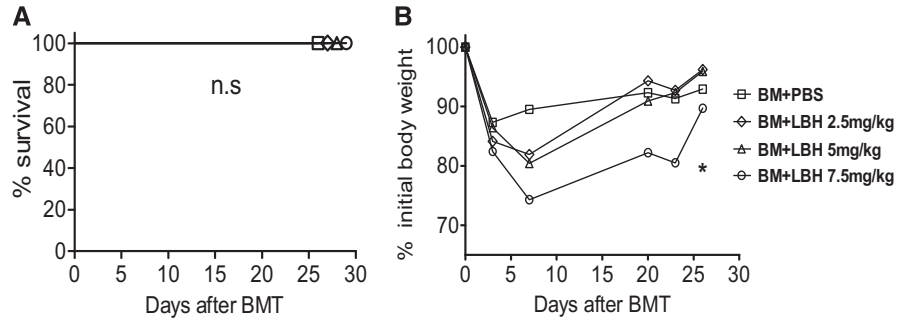

Figure 3. Toxicity of LBH589 with every other day treatment. B/c mice (n = 4) were lethally irradiated and underwent transplantation with 5×10^6 T cell depleted (TCD) B6 bone marrow (BM) cells via tail vein. Recipients were treated with PBS vehicle or different doses of LBH589 every other day from day 0 to 13 via i.p. injection. Mice survival (A) and body weight (BW) (B) are shown. The experiment was repeated 3 times and similar results were obtained. One representative experiment was shown. BMT, bone marrow transplant; n.s., no significant difference. $^*P < .05$.

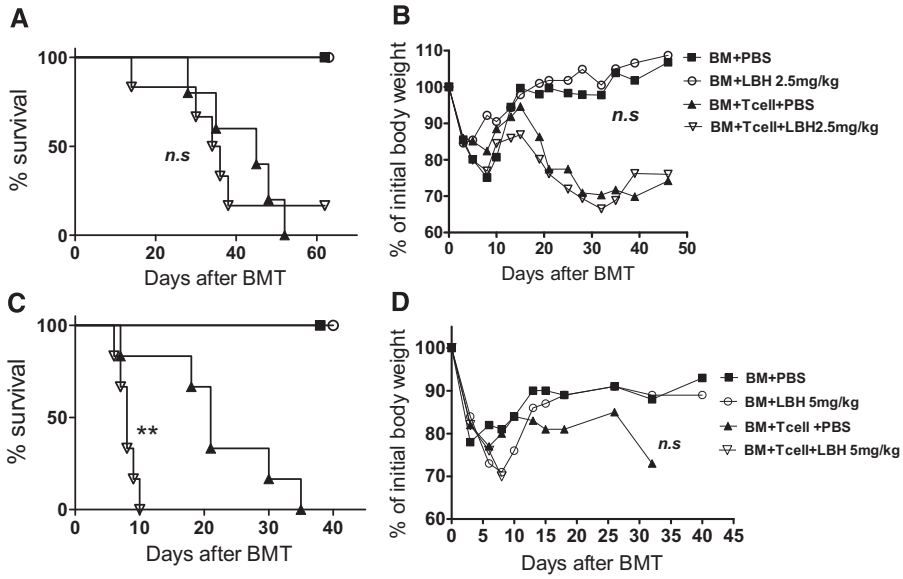

Figure 4. Effect of LBH589 with every other day treatment on graft-versus-host disease (GVHD). B/c mice (n = 5-6) were lethally irradiated and underwent transplantation with 5×10^6 T cell depleted (TCD) B6 bone marrow (BM) cells with either 0.25×10^6 or 1×10^6 B6 CD25 T cells. Recipients were treated for 12 days every other day via i.p. injection with PBS vehicle or LBH589 (2.5 mg/kg body weight [BW] for 0.25×10^6 T cell recipients and 5 mg/kg BW for 1×10^6 T cell recipients). Mice survival (A, C) and BW (B, D) are shown here. The experiment was repeated 3 times and similar results were observed. One representative experiment was shown. BMT, bone marrow transplant; n.s., no significant difference; $^{**}P < .01$.

likely through upregulation of CXCR3 expression on donor T cells.

It is known that Th1 cytokines, including IFN-γ and TNF-α, are the critical effectors for acute GVHD, although recent reports showed that IFN-γ has dual effects in the immune regulation [12-14]. IFN-γ is secreted by activated donor T cells, and increased serum levels of IFN-γ were related to acute GVHD [15,16]. TNF-α was normally secreted by host antigen presenting cells (APC) and donor T cells [17]. Higher TNF-α level indicated higher host APC activity, which was correlated with increased severity of GVHD. Here, we show that LBH589 treatment significantly increased IFN-γ and TNF-α level in serum from recipients treated with LBH589. These elevated inflammatory cytokines likely contributed to accelerated GVHD. In addition, our in vitro experiments showed that at certain doses, LBH589 significantly increased IFN-γ secretion, proliferation of T cells, and the antigen presentation ability of DC (Supplementary Figures 1 and 2), consistent with our observation in vivo (Figure 6). Therefore, the LBH589 doses used in vivo may fall in the concentration range that promoted T cell activation. Because LBH589 was toxic at high doses in vivo, we could not demonstrate the protective effect of LBH589 in GVHD. In accordance with our observation regarding the effect of LBH589 on cytokine production, Wang et al. [18] recently reported that LAQ824, another pan-HDACi, augmented inflammatory responses in

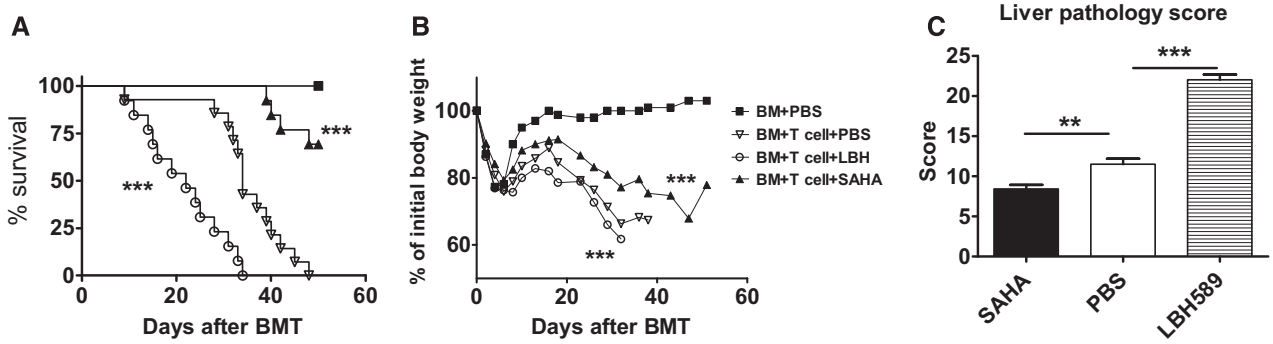

Figure 5. Effect of LBH589 and suberoylanilide hydroxamic acid (SAHA) on graft-versus-host disease (GVHD). B/c mice were lethally irradiated and underwent transplantation with 5×10^6 T cell depleted (TCD) B6 bone marrow (BM) cells with or without 1×10^6 T cells. Recipients were treated with PBS vehicle, SAHA (25 mg/kg body weight [BW], daily treatment from day 0 to 7) or LBH589 (1.25 mg/kg BW, every other day, from day 0 to 12) via i.p. injection. (A) Mice survival was the combined data from 3 independent experiments (n = 12-13). (B) Mice BW (n = 4-5). Similar results were found in the repeated experiments. (C) GVHD pathological score of mice livers from the combination of 2 independent experiment (n = 8). BMT, bone marrow transplant. $^{**}P < .01$; $^{***}P < .001$.

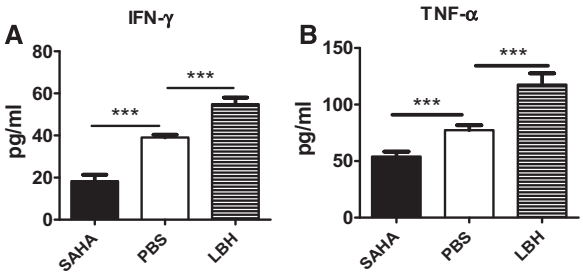

Figure 6. Effect of LBH589 and suberoylanilide hydroxamic acid (SAHA) on cytokine production. B/c mice were lethally irradiated and underwent transplantation with 5×10^6 T cell depleted (TCD) B6 bone marrow (BM) cells with or without 1×10^6 T cells. Recipients were treated with PBS vehicle, SAHA (25 mg/kg body weight [BW], daily treatment from day 0 to 7), or LBH589 (1.25 mg/kg BW, every other day from day 0 to 12) via i.p. injection. Mice were killed on day 13, and serum IFN-γ (A) and TNF-α (B) were analyzed by flow cytometric bead array. The data shown here are pooled from 2 replicate experiments (n = 8) with the same setting. BMT, bone marrow transplant. ***$P < .001$.

macrophages through transcriptional regulation of IL-10. SAHA does not always downregulate the inflammatory response; it was reported to suppress the LPS-induced mRNA expression of the proinflammatory mediators Edn1, CCL7/MCP-3, and Il-12p40, but amplify the expression of the proatherogenic factors Cox-2 and Pai-1/serpine1 in primary mouse BM-derived macrophages [19]. Thus, the effect of pan-HDACi on inflammation may be drug and disease-specific.

When comparing T cell expansion in spleen and T cell infiltration in GVHD target organs at day 14, we found that there were much less donor T cells in the recipient spleen after the LBH589 treatment (Figure 7), but more donor T cells infiltrated the liver. Consistent with this finding, pathological analysis showed higher GVHD scores in the livers from the LBH589 treated group (Figure 5C). Less splenic T cells might reflect less T cell expansion, or more extensive T cell migration from the spleen to the GVHD target organs upon LBH589 treatment. In fact, we found that after the LBH589 treatment, significantly more donor T cells expressed CXCR3, a chemokine receptor that mediates T cell migration to the liver and the intestine [20]. We did not observe more severe GVHD in the colon and small intestine in LBH589 treated recipients. It was likely due to the difference in the spatiotemporal expression of cytokine and chemokine gradients. After total body irradiation, the increase of proinflammatory cytokines and the forming of chemokine gradients (CXCL9-11) may occur earlier in the liver than in the gut. In addition, the expression of T cell gut homing-receptor, α4β7, was not altered by LBH589 treatment (data not shown). Therefore, we surmised that LBH589 aggravated GVHD by increasing T cell activation, Th1 cytokine production, and T cell migration to the recipient liver through upregulating CXCR3 expression.

Previous studies by others have shown that pan-HDACis have therapeutic effect on many inflammatory disease models. Glauben et al. [3] showed that oral administration of either valproic acid (another HDACi) or SAHA reduced the disease severity in dextran sulfate sodium-induced colitis in mice. The reduction of disease severity was associated with a marked suppression of colonic proinflammatory cytokines and apoptosis of lamina propria lymphocytes. Three other groups showed that SAHA alleviated GVHD after allogeneic BMT [5,7-9]. It appears that our data are contradictory to those published; however, we believe that the distinct outcomes with SAHA and LBH589 can be readily reconciled.

We all used pan-HDACi without considering the functional difference of individual HDAC members. Actually, HDAC members have similar or opposite effects on the regulation of immune response. Conditional deletion of HDAC1 in T cells leads to enhanced airway inflammation and increased Th2 cytokine production [21]. HDAC2 activity is indispensible to control chronic obstructive pulmonary disease by corticosteroid [22,23]. A recent report showed that HDAC6-deficiency increases the suppressive potency of regulatory T cells [24,25], whereas our unpublished data indicated that HDAC11-deficiency resulted in T cell hyperactivation leading to increased severity of GVHD in mice. When a pan-HDACi was tested, what we observed is a compound effect on all 11 HDAC members. Considering the wide range of potency of individual HDACis on different HDAC members, and that each HDAC member plays a distinct role, it is plausible that 2 inhibitors would likely have distinct effects on a certain disease. Specifically, SAHA and LBH589 have very different potency in inhibiting each member of the HDAC family (eg, LBH589 inhibits HDAC11 40-fold more potently than SAHA) [10]. Given that HDAC11 may negatively regulate T cell function as supported by our unpublished data, effective blockade of HDAC11 by LBH589, but not by SAHA, could contribute to the different GVHD outcome after treatment with LBH589 vs SAHA.

In addition, the substrate of HDACs are not limited to histones [26]. There are more than 1750 nonhistone substrates that make the effect of HDACi even more complex [27]. Given the paucity of knowledge on the functions of nonhistone substrates, it is currently impossible to precisely predict the effect of HDACis at the cellular level until the role of individual HDAC is uncovered, and isoform-specific HDACis are developed. Finally, LAQ824, another pan-HDACi derived from hydroxamic acid, enhanced antitumor activity of tumor antigen-specific lymphocytes both in vitro and in vivo [28]. This is consistent with our finding that LBH589 enhanced allogeneic T cell activation in vivo. The LBH589 structure is

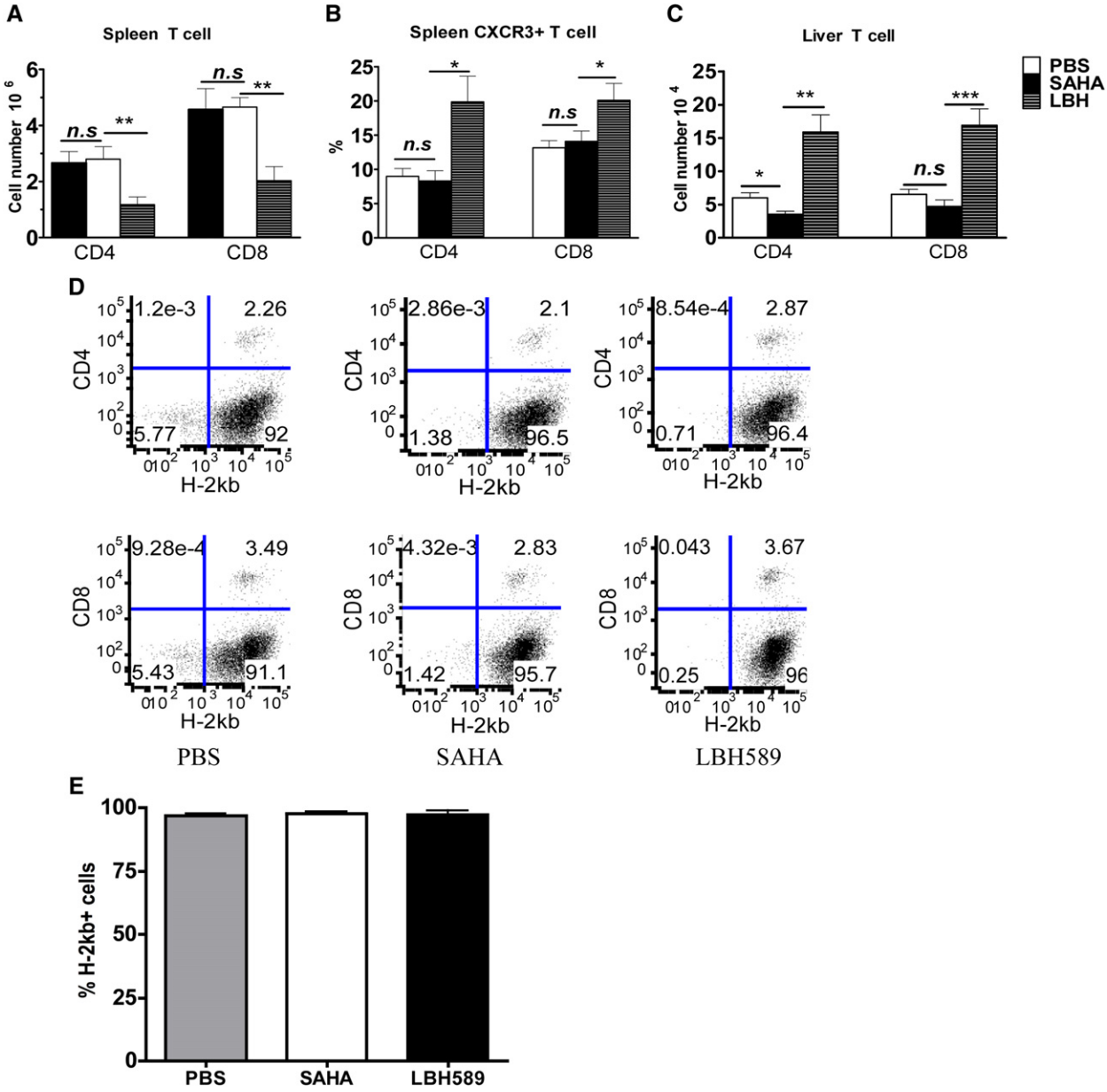

Figure 7. Effect of LBH589 and suberoylanilide hydroxamic acid (SAHA) on donor T cell expansion and migration in vivo. B/c mice (n = 4) were lethally irradiated and underwent transplantation with 5 × 10^6 T cell depleted B6 bone marrow (BM) cells with or without 1 × 10^6 T cells. Recipients were treated with PBS vehicle, SAHA (25 mg/kg body weight [BW], daily treatment from day 0 to 7) or LBH589 (1.25 mg/kg BW, every other day from day 0 to 12) via i.p. injection. Mice were killed on day 13. Splenic T cell number (A), CXCR3 expression on splenic donor T cells (B), and liver T cell infiltration (C) were analyzed by flow cytometry. The data shown are pooled from 2 replicate experiments (n = 8). (D) The flow cytometry data of H-2kb+ CD4-CD8- cells in the spleen of each group are shown. The data of 1 mouse from each group was shown here. Only the cells in live gate were studied. (E) The average percentage of H-2kb+ cells in spleen are shown here. Only the cells in live gate were studied. The data shown was pooled from 2 replicate experiments (n = 8). n.s., no significant difference; *$P < .05$; **$P < .01$; ***$P < .001$.

closer to LAQ824 than SAHA and accordingly, LBH589 IC50 to HDAC members is quite similar to LAQ824. This may be the reason why both drugs improved T cell activation in vivo.

In summary, we evaluated the effect of LBH589 on the prevention of GVHD and found that LBH589 accelerated rather than alleviated GVHD after allogeneic BMT in mice. The accelerated GVHD was associated with increased systemic Th1 cytokines and donor T cell infiltration in the recipient liver. This study highlights the distinct effects of pan-HDACi on allogeneic BMT, and demonstrates that LBH589 (Panobinostat) could have an adverse effect on GVHD or other inflammatory diseases if used in a treatment scheme to replace other HDACis. Thus, caution must be taken in consideration not to assume that pan-HDACis would lead to similar therapeutic effects. Instead, the information learned from this study strongly encourages investigations on the function of individual HDAC members, and the development of

isoform-specific HDACis for higher efficacy and better selectivity.

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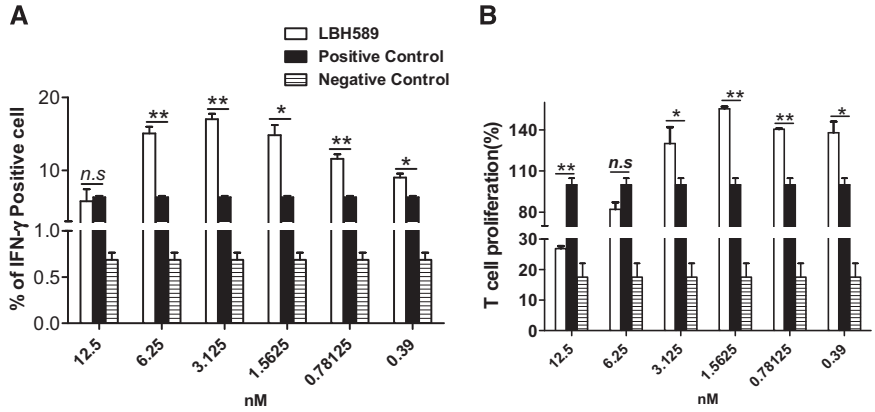
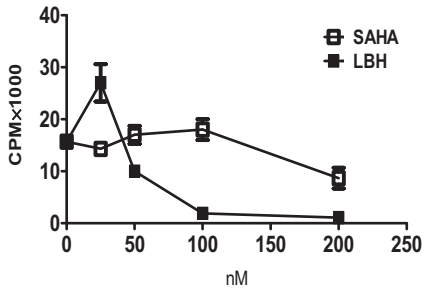


Supplementary Figure 1. Effect of LBH589 on mouse T cell in vitro. Purified T cells from B6 mice were labeled with CFSE and stimulated with plate bound anti-CD3 (10 μg/mL) and soluble anti-CD28 (1 μg/mL) with LBH589 at indicated concentrations for 3 days. Cells were then stimulated with PMA (50 ng/mL) plus ionomycin (500 ng/mL) for 4 hours. Golgi Plug was added to cell culture at 1 μL/well 2 hours after starting cell culture. (A) The cells were harvested and IFN-γ secretion was measured by flow cytometry. (B) T cell proliferation was measured by CFSE dilution and normalized to positive control. Data are shown on gated $CD4^+$ cells, and similar results were seen on $CD8^+$ cells (not shown). This experiment was repeated 2 times and the results were very similar. One representative experiment was shown here. n.s., no significant difference. *$P < .05$; **$P < .01$.



Supplementary Figure 2. LBH589 enhanced the antigen presentation ability of dendritic cell (DC) at certain dose range. B/c bone marrow-derived DCs were generated by adding supernatant of J558L (including granulocyte macrophage-colony stimulating factor) to the cell culture. On day 5, DCs were treated with LBH589 doses (serial dilution from 12.5 nM to 0.39 nM) for 2 hours before adding lipopolysaccharide (LPS). On day 6, DCs was washed and mixed leukocyte reaction was set up using B6 T cells as responder and irradiated DCs as stimulator. T cell proliferation was measured by $[^3H]$-thymidine incorporation. The repeated experiment showed the same trend.