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Valproic Acid Ameliorates Graft-versus-Host Disease by Downregulating Th1 and Th17 Cells

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Graft-versus-host disease (GVHD) is the major complication after allogeneic bone marrow transplantation. Valproic acid (VPA) was described as a histone deacetylase inhibitor that had anti-inflammatory effects and reduced the production of proinflammatory cytokines in experimental autoimmune disease models. Using well-characterized mouse models of MHC-mismatched transplantation, we studied the effects of VPA on GVHD severity and graft-versus-leukemia (GVL) activity. Administration of VPA significantly attenuated the clinical severity of GVHD, the histopathology of GVHD-involved organs, and the overall mortality from GVHD. VPA downregulated Th1 and Th17 cell responses and cytokine production in vitro and in vivo, whereas its effect on GVHD was regulatory T cell independent. The effect of VPA was related to its ability to directly reduce the activity of Akt, an important regulator of T cell immune responses. Importantly, when mice received lethal doses of host-type acute leukemia cells, administration of VPA did not impair GVL activity and resulted in significantly improved leukemia-free survival. These findings reveal a unique role for VPA as a histone deacetylase inhibitor in reducing the donor CD4+ T cells that contribute to GVHD, which may provide a strategy to reduce GVHD while preserving the GVL effect. The Journal of Immunology, 2015, 195: 1849–1857.

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Abbreviations used in this article: BMT, bone marrow transplantation; F, forward; GVHD, graft-versus-host disease; GVL, graft-versus-leukemia; HDAC, histone deacetylase; MHC, major histocompatibility complex; mTOR, mammalian target of rapamycin; Th1, T helper 1; Th2, T helper 2; Th17, T helper 17; Tregs, regulatory T cells; TCD-BM, T cell–depleted bone marrow; VPA, valproic acid.

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pathways, such as the β-catenin–Wnt, Notch, MAPK, and NF-κB pathways, which are critical in immune function (30). In this study, we demonstrate that VPA treatment ameliorated GVHD in a mouse BMT model by downregulating Th1 and Th17 cells and reducing inflammatory cytokine levels via inhibition of the phosphorylation level of Akt in CD4+ T cells. Importantly, VPA therapy preserved the GVL effect, resulting in significantly improved survival of mice after BMT and leukemia challenge. Our findings suggest that VPA can be used to prevent and treat GVHD.

Materials and Methods

Mice
Female C57BL/6 (B6; H-2b), BALB/c (H-2d), and FVB (H-2v) mice were obtained from the Shanghai Laboratory Animal Center (Chinese Academy of Sciences, Shanghai, China). The mice were housed in specific pathogen–free conditions at the Research Center for Experimental Medicine of Rui-Jin Hospital. Mice were between 8 and 12 wk of age at the start of the experiments. All experiments were carried out according to the National Institutes of Health’s guidelines for the Care and Use of Laboratory Animals.

GVHD and GVL model
BALB/c or FVB mice received 8 Gy total body irradiation from a 137Cs source as a split dose with a 3-h interval on day −1. On day 0, recipient mice were transplanted with 5 × 10^6 T cell–depleted bone marrow (TCD-BM) cells and 1 × 10^6 spleen T cells from B6 donors. T cell depletion and purification were performed with anti-CD90.2 MicroBeads and a CD25 purification kit (Miltenyi Biotec), according to the manufacturer’s protocols. Donor cells were injected through the tail vein. In GVL experiments, acute myeloid leukemia cells that were developed in our institute were administered on the day of transplantation (31). The development of leukemia was monitored by counting the percentage of peripheral blood GFP+ cells.

Results

GVHD and GVL model
BALB/c or FVB mice were transplanted with 5 × 10^6 T cell–depleted bone marrow (TCD-BM) cells and 1 × 10^6 spleen T cells from B6 donors. T cell depletion and purification were performed with anti-CD90.2 MicroBeads and a CD25 purification kit (Miltenyi Biotec), according to the manufacturer’s protocols. Donor cells were injected through the tail vein. In GVL experiments, acute myeloid leukemia cells that were developed in our institute were administered on the day of transplantation (31). The development of leukemia was monitored by counting the percentage of peripheral blood GFP+ cells through flow cytometry. Death caused by leukemia was defined by enlargement of the spleen and liver on autopsy, whereas death caused by GVHD was defined as the absence of tumor and the presence of GVHD.

Adoption of VPA
Recipient mice were administered VPA (300 mg/kg body weight; Sigma-Aldrich) or vehicle solution (PBS) by i.p. injection daily from day 0. This dose was chosen based on previous in vivo studies (28, 29, 32), as well as the following dose-conversion formula, as provided by the U.S. Food and Drug Administration in Guidance for Industry: Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers: animal mg/kg dose × animal kow = human mg/kg dose × human kow (kow = body weight [kg]/body surface area [m^2]).

Evaluation of GVHD and histopathological analysis
After BMT, animals were weighed every 2 d, and the degree of systemic GVHD was assessed by a clinical scoring system including five clinical parameters (weight loss, posture, activity, fur ruffling, and skin integrity), as published previously (33). For histopathological analysis, left lung, liver, small intestine, colon, and tongue (used as a surrogate for skin) specimens of recipients were fixed with 10% neutralized formalin, embedded in paraffin, sectioned, mounted on slides, and stained with H&E. Two slides/organ were evaluated and scored by a pathologist blinded to experimental groups using a scoring system described previously (33, 34).

T cell isolation and in vitro polarization
Single-cell suspension was harvested from spleen and lymph node of normal female B6 mice (8–10 wk). CD4+ naive T cells were purified using the CD4+ naive T cell isolation kit (STEMCELL Technologies), according to the manufacturer’s instruction. Purified CD4+ naive T cells (CD4+CD25 CD2Q2LHCD44v) were activated with plate-bound anti-CD3 (3 μg/ml) and soluble anti-CD28 (1 μg/ml) for 5 d under the following polarization conditions: IL-12 (10 ng/ml) and anti-IL-4 (10 μg/ml) for Th1 polarization; and TGF-β (3 μg/ml), IL-6 (30 ng/ml), IL-23 (20 ng/ml), IL-21 (10 ng/ml), IL-1β (10 ng/ml), anti-IL-4 (10 μg/ml), and anti-IFN-γ (10 μg/ml) for Th17 polarization. All cytokines were purchased from R&D Systems.

Flow cytometry assay
Single-cell suspensions were obtained from spleen, mesenteric lymph node (MLN), and organs of mice and were stimulated in vitro with 50 ng/ml PMA and 500 ng/ml ionomycin (both from Sigma-Aldrich) for 5–6 h in the presence of GolgiStop or GolgiPlug (BD Biosciences). Cells were assayed for cytokine production by intracellular flow cytometry staining. The following Abs were used for staining: anti-CD3–allophycocyanin–Cy7 (BD Biosciences), anti-CD4–FITC, anti-CD4–PE-Cy5 (eBioscience), anti-H–2d–allophycocyanin (BD Biosciences), anti-IFN-γ–Alexa Fluor 488, anti-IL-17A–PE, and anti-Foxp3–PE (eBioscience). Intracellular cytokine staining was carried out using a BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences), and the intracellular staining of Foxp3 was carried out with a Foxp3/Transcription Factor Staining Buffer Set (eBioscience). Data were acquired using a FACS LSR II (BD Biosciences) and analyzed with FlowJo software (TreeStar).

Cytokine measurement
Mouse serum was collected on days 7, 14, and 28 after BMT, and the levels of cytokines were measured using ProcartaPlex Multiplex Immunoassays (eBioscience). For cell culture supernatants, levels of IFN-γ and IL-17A were measured by ELISA, according to the manufacturer’s instructions (R&D Systems).

Real-time PCR, Western blot, and immunoprecipitation analysis
Total RNA was extracted with TRIzol reagent (Invitrogen), according to the manufacturer’s instructions. Transcription levels of T-bet, Rorc, Foxp3, Il17a, Tgfb, and Il2 genes were analyzed by real-time quantitative PCR. The primers used were: T-bet (forward) 5′-AGCAACGGAGGCCGAATG-3′ and reverse (R) 5′-GTGGATATAGCTGCTTTTGC-3′; Foxp3, 5′-ACCATTGTGTTTACCTCGATGT-3′ and R 5′-TCCACTGACAAAGCGATT-3′; Tgfb, 5′-TGGTGACACTCGATGA-3′; Il17a, 5′-TTATACATCCTTT GGCCAAAA-3′; and Il2, 5′-CTTCCCTTCCGCTGAC-3′, Tgfb, 5′-CTTCAATACGTGCAATTCCGG-3′ and R 5′-GAACGGA GAATGTTGTC-3′. For Western blot assay and immunoprecipitation analysis, CD4+ T cells were isolated from mice spleen on day 21 after transplantation using a Mouse CD4+ T Cell Isolation Kit (Miltenyi Biotec), and the procedures were performed as described previously (35).

Cytotoxicity assays
Cytotoxicity assays were performed as described (36).

Statistics
Data analysis was performed using Prism software. Survival comparisons were performed using the log-rank test. Other differences between experimental groups were analyzed using a two-tailed unpaired Student t test. A p value < 0.05 was deemed to be significant in all experiments.

Results

VPA treatment significantly reduces the severity of GVHD
To determine whether VPA treatment had a salutary effect on the severity of acute GVHD, we used a classical GVHD model (B6 into BALB/c). lethally irradiated BALB/c mice were transplanted with 5 × 10^6 B6 TCD-BM cells with or without 1 × 10^6 B6 spleen T cells. T cell recipients were injected i.p. with either VPA (300 mg/kg/d) or vehicle daily from day 0 of BMT. As expected, BALB/c mice receiving B6 TCD-BM alone developed no sign of GVHD, whereas all control BALB/c mice receiving B6 donor TCD-BM + spleen T cells died of GVHD (Fig. 1A). In contrast, VPA treatment inhibited GVHD in T cell recipients, with ~40% of them surviving without severe GVHD (Fig. 1A). This was accompanied by less weight loss (Fig. 1B), and the clinical score also was lower in VPA recipients (Fig. 1C). We performed histological examination of GVHD target organs (liver, lung, small intestine, and colon) and the tongue, which served as a surrogate for skin damage (37), 4 wk after transplantation. There was a significant reduction in pathological damage in all sites of VPA-treated mice compared with vehicle-control mice (Fig. 1D, 1E). Administration of VPA to mice reconstituted with TCD-BM only also was lower in VPA recipients (Fig. 1C). We performed histological examination of GVHD target organs (liver, lung, small intestine, and colon) and the tongue, which served as a surrogate for skin damage (37), 4 wk after transplantation. There was a significant reduction in pathological damage in all sites of VPA-treated mice compared with vehicle-control mice (Fig. 1D, 1E). Administration of VPA to mice reconstituted with TCD-BM only also was lower in VPA recipients (Fig. 1C).
VPA administration reduces Th1 and Th17 cell responses

To understand the mechanism by which VPA treatment reduced GVHD, we first monitored the kinetics of T cell responses during GVHD development. We found that, in the allogeneic group, IFN-γ-, IL-17A-, and IFN-γ/IL-17A double-positive CD4+ T cells derived from spleen (Fig. 2A, 2B) and MLN (Supplemental Fig. 1A, 1B) increased since the early stage after transplantation compared with the syngeneic group, whereas the IFN-γ- and/or IL-17A-producing CD8+ T cells increased only at the late stage (Supplemental Fig. 1C, 1D). These data conform to the notion that the B6 into BALB/c GVHD model is primarily driven by CD4+ T cells. The numbers of IFN-γ- and/or IL-17A-expressing CD4+ T cells in the liver (Supplemental Fig. 1E) and lung (Supplemental Fig. 1F) of allogeneic recipients were significantly greater compared with syngeneic recipients. The characteristic cytokines produced by Th1 and Th17 cells also were elevated in serum from allogeneic recipients (Supplemental Fig. 1G). We performed similar experiments in a second BMT model (B6 into FVB), and confirmed the upregulated Th1 and Th17 cell responses in this model (Supplemental Fig. 2). These data suggest that Th1 and Th17 cells play an important role in the development of acute GVHD.

We then treated BMT recipients with VPA or vehicle and recovered donor T cells from spleen and MLN of recipients on the indicated days after transplantation. Flow cytometry analysis showed that, compared with control, VPA treatment markedly reduced the frequencies of Th1 and Th17 cells in the spleen (Fig. 2C, 2D) and MLN (Supplemental Fig. 3A, 3B). The absolute number of Th1 and Th17 cells also decreased significantly on days 7 and 14 (Supplemental Fig. 3C, 3D); because control mice became severely wasted and developed GVHD-induced lymphopenia on day 28, the cell numbers on that day were not counted. The numbers of Th1 and Th17 cells in the lung and liver of VPA recipients were significantly less than those of the control group (Fig. 3A, 3B). We also detected substantially lower serum levels of Th1- and Th17-related cytokines (e.g., TNF-α, IFN-γ, IL-17A, IL-21, and IL-23) in VPA recipients (Fig. 2G). Taken together, these data suggest that the decrease in Th1 and Th17 cells may be the primary cause of VPA-mediated GVHD inhibition.

The effect of VPA on GVHD is Treg independent

A number of previous studies showed that HDAC inhibitors could increase the generation of Tregs by promoting the expression of Foxp3 protein in CD4+ T cells (23) or enhance the immune-regulatory function of Tregs by modulating the acetylation and stability of Foxp3 protein (38, 39). In addition, VPA was shown to confer the molecular profile, including microRNA signature and FOXP3 expression, of natural Tregs to human cord blood CD4+ effector T cells (40). As mentioned above, Tregs can reduce the severity of GVHD (7–10). Therefore, we wondered whether the effect of VPA on GVHD resides in its effect of promoting Tregs and quantified the frequencies and absolute numbers of Foxp3-expressing CD4+ T cells in spleen on the indicated days after transplantation. Recipients administered VPA showed no difference in the frequencies of Foxp3+ cells (Fig. 3A, 3B). The numbers of Foxp3+ cells in the control group were even slightly greater than in VPA recipients on day 14 (Fig. 3C). Foxp3 mRNA expression in spleen also was decreased in VPA recipients (Fig. 3D). To further confirm that the effect of VPA on GVHD was not associated with Tregs, mice were injected with whole T cells or CD25-depleted T cells from B6 donors. As shown in Fig. 3E and 3F, although depletion of CD25+ cells intensified GVHD (Fig. 3D), depletion only slightly increased the frequency of Foxp3+ cells (Fig. 3E). Therefore, these data suggest that the effect of VPA treatment is not associated with Tregs.

VPA inhibits the differentiation and immune function of Th1 and Th17 cells in vitro and in vivo

Because the salutary effect of VPA on GVHD is not dependent on Tregs, we reasoned that VPA may inhibit Th1 and Th17 cells directly. As GVHD was thought to be a result of naive T cell response (2), we first examined the effect of VPA on Th1 and Th17 cell differentiation. Purified naive CD4+ T cells from normal B6 mice were cultured under Th1-polarizing conditions with or without VPA. As shown in Fig. 4A, VPA inhibited the in vitro differentiation of Th1 cells. We detected a similar effect of VPA on Th17 differentiation (Fig. 4B). Moreover, annexin V/propidium iodide staining showed that VPA only marginally decreased the viability of in vitro–differentiated Th1 and Th17 cells at a relatively high concentration (1 mM) (Supplemental Fig. 4A, 4B), whereas VPA readily inhibited the differentiation of Th1 and Th17 cells at a low concentration (0.2–0.5 mM), indicating that apoptosis induction was not the primary mechanism for Th1 and Th17 cell differentiation inhibition (28, 29). We then examined whether
VPA regulated immune homeostasis and ameliorated GVHD through inducing apoptosis of CD4+ T cells in vivo. Spleen CD4+ T cells were harvested from VPA recipients and control mice on day 21 after transplantation. Annexin V/propidium iodide staining showed no difference in apoptosis rate between the two groups (Supplemental Fig. 4C). Thus, VPA did not reduce GVHD by inducing apoptosis of CD4+ effector T cells.

Next, we tried to clarify whether VPA directly inhibits the immune function of in vivo–differentiated Th1 and Th17 cells. Spleen CD4+ T cells were isolated from mice 21 d after transplantation and cultured or not with VPA for 24 h to determine cytokine production. VPA inhibited IFN-γ (Fig. 4C) and IL-17A (Fig. 4D) production in a dose-dependent manner. In parallel, the mRNA expression of key transcriptional factors for Th1 cells (T-bet), Th17 cells (Rorc), and Tregs (Foxp3), as well as Ifng and Il17a, also were decreased in a dose-dependent manner (Supplemental Fig. 4D). Next, BMT recipients were administered VPA or vehicle from day 0 of transplantation, and cytokine production was assessed in spleen CD4+ T cells harvested on day 21. Data showed that spleen CD4+ T cells from VPA-treated recipients produced significantly less Th1- and Th17-related cytokines after stimulation (Fig. 4E). Altogether, these data suggest that VPA directly downregulates Th1 and Th17 cells in vitro and in vivo.

VPA inhibits Th1 and Th17 cells by reducing the phosphorylation level of Akt

Because Akt is an important factor in the differentiation and immune function of T cells, and previous studies showed that VPA and other HDAC inhibitors could inhibit Akt activity in cancer cells (41, 42), we examined whether Akt activity was modulated by VPA treatment. Spleen CD4+ T cells were harvested from mice 21 d after transplantation and cultured or not with VPA for 24 h. Western blot assay showed that VPA attenuated the phosphorylation level of Akt in a dose-dependent manner (Fig. 4F). The phosphorylation levels of downstream target proteins of Akt also were downregulated in parallel (Fig. 4F). Western blot analysis showed that phosphoinositide-dependent protein kinase 1, the upstream kinase that phosphorylates Akt, and several phosphatases (phosphatase and tensin homolog deleted on chromosome 10, protein phosphatase 1, and protein phosphatase 2A), known as negative regulators of the PI3K/Akt signaling pathway, were unchanged upon VPA treatment (Supplemental Fig. 4E), indicating that VPA might act on Akt directly. A study reported that acetylation of Akt on several lysine sites could reduce Akt activity (35). Thus, we examined whether VPA treatment could increase the acetylation level of Akt and, in turn, decrease the phosphorylation level of Akt.
VPA treatment does not increase CD4+ Tregs in vivo. (A) Donor-derived spleen T cells from VPA recipients and control mice on days 7, 14, and 28 were stained for Foxp3 expression. (B) Representative percentage of donor-derived spleen T cells expressing Foxp3 on days 7, 14, and 28. (C) Absolute numbers of CD4+Foxp3+ Tregs in spleens on days 7 and 14 from VPA recipients and control mice. (D) Foxp3 mRNA expression in spleen on day 21. (E and F) Lethally irradiated BALB/c mice were transplanted with 5 × 10^6 TCD-BM plus 1 × 10^6 total spleen T cells or CD25-depleted T cells from B6 mice. VPA was administered i.p. to these recipients from day 0, with vehicle treatment as control (n = 20/group). The survival of BMT recipients was monitored over time (E), and the clinical score was assessed every week (F). (G) mRNA expression of TGF-β and IL-2 in spleen on day 21. Results are representative of two or three independent experiments. Data are mean ± SEM. *p < 0.05, **p < 0.01. ns, p > 0.05.

VPA preserves GVL activity after BMT

To evaluate the impact of VPA treatment on the preservation of GVL activity, we challenged BALB/c recipients with host-type GFP+ acute myeloid leukemia cells (H-2d) to mimic residual leukemia in patients receiving allogeneic BMT. All mice transplanted with TCD-BM and challenged with leukemia cells died from leukemia (Fig. 5A). In vivo administration of VPA slightly prolonged the survival of mice transplanted with TCD-BM and challenged with leukemia cells, which might be explained by the anti-tumor activity of HDAC inhibitors (Fig. 5A, 5C, 5D). However, VPA treatment failed to eliminate leukemia, and these mice eventually died from leukemia. Autopsy showed spleen and liver enlargement (Fig. 5E). No leukemia growth was observed in mice receiving TCD-BM + spleen T cells, indicating a potent GVL effect, but these mice developed severe GVHD after transplantation (Fig. 5B–D). In contrast, VPA administration from the day of transplantation had no effect on the control of leukemia, and ~40% of VPA recipients survived free of leukemia (Fig. 5B–D). Autopsy also showed no sign of leukemia infiltration in BMT recipients, regardless of VPA treatment, confirming the GVL effect (Fig. 5E). In addition, we observed that donor T cells recovered from VPA-treated BMT recipients and controls on day 14 showed comparable cytolytic activity against host-type leukemia cells (Fig. 5F). Therefore, VPA treatment had no adverse effect on GVL activity.

Discussion

A variety of mice models has been developed to clarify the mechanism of GVHD (43, 44), and T cells are thought to be the primary mediator of this disease (2). Because of their ability to differentiate into various effector T subsets, Th1, Th2, Th17, and Tregs, CD4+ T cells are particularly important in the initiation of GVHD in mice and have been considered potential targets for the treatment and prevention of GVHD in the clinic. In humans, GVHD is reduced in recipients of CD4+ T cell–depleted grafts (45). Many studies reported that Th1 and Th17 cells had a causative role in the development of acute GVHD. Yu et al. (46) used nude mice deficient for both T-bet and RORγt as T cell donors and showed that blockade of both Th1 and Th17 differentiation is required to prevent acute GVHD. In the current study, we observed that Th1 and Th17 cells were increased during the early stage of GVHD. The infiltration of Th1 and Th17 cells into GVHD target organs (liver and lung), as well as the serum level of related cytokines, also were increased in allogeneic BMT recipients.

Because HDAC inhibitors showed potent anti-inflammatory or immunomodulatory activities in many studies (47), we tried to investigate whether VPA might be beneficial for GVHD amelioration. In a phase 1/2 clinical trial, the maximum tolerated dose of VPA was 50 mg/kg/d for a 7-d cycle (32). In two other studies that assessed the therapeutic effect of VPA on experimental colitis or experimental autoimmune encephalomyelitis, the investigators showed that VPA was effective and safe up to 400 mg/kg/d (28, 29). Therefore, based on these in vivo studies, as well as the formula provided by the U.S. Food and Drug Administration for converting between human doses and animal doses, we chose 300 mg/kg/d VPA for this study. We demonstrated that administration of VPA ameliorated GVHD, and this was accompanied by a marked reduction in the frequency and number of Th1 and Th17 cells. Moreover, the infiltration of Th1 and Th17 cells into GVHD...
target organs, as well as the serum level of proinflammatory cytokines, were significantly decreased. Our results suggest that combined blocking of Th1 and Th17 by VPA may represent a promising strategy to prevent or treat GVHD.

The mechanism by which VPA controls GVHD may differ from that of other HDAC inhibitors. For example, SAHA prevented the development of GVHD by modulating dendritic cell functions (25). However, due to the various effectiveness in inhibiting different members of the HDAC family, especially HDAC11, LBH589 administration worsened GVHD through enhancing T cell activation (27). Other organ-transplantation studies showed that administration of HDAC inhibitors increased the generation of Tregs or enhanced the stability of Tregs in an inflammatory microenvironment (23, 38). In contrast, we found that VPA caused a slight decrease in the number of CD4+ Tregs in allogeneic BMT recipients. The mRNA level of Foxp3 gene also was decreased in the spleen of VPA recipients. In addition, the depletion of Tregs from donor T cells did not have an adverse influence on the effect of VPA. Thus, modulation of Th1 and Th17 cell differentiation and immune function, in contrast to the promotion of Tregs, may represent a different mechanism by which VPA controls GVHD. Previous studies observed similar activity in other disease models. For example, the combination of HDAC inhibitors trichostatin A and butyrate was shown to reduce IFN-γ production by T cells (48). More recently, another HDAC inhibitor, ITF2357, was shown to modulate IL-6-dependent CD4+ T cell polarization into Th17 cells through targeting the IL-6/STAT3/IL-17 pathway, and it reduced the severity of experimental colitis (49). In our study, we observed that VPA treatment inhibited the differentiation of Th1 and Th17 cells.

Because GVHD is primarily a result of a naive T cell response (50), treating GVHD with VPA may offer a great advantage. We also observed that the in vitro and in vivo functions of Th1 and Th17 cells were suppressed upon VPA treatment. Therefore, our results suggest a direct suppressive effect of VPA on the differentiation and immune function of Th1 and Th17 cells.

The Akt/mTOR signaling pathway has a critical role in CD4+ T cell differentiation and function. The Akt/mTORC1 axis was shown to promote the generation of Th1 and Th17 cells through down-regulating suppressor of cytokine signaling protein 3 (15). It was shown that chronically stimulated T cells proliferating in response to

**Figure 4.** VPA suppresses Th1 and Th17 cell differentiation and immune function in vitro and in vivo through direct inhibition of Akt. (A) Purified CD4+ naive T cells were cultured with different concentrations of VPA under Th1-polarizing conditions. The differentiation of Th1 cells was analyzed by intracellular staining of IFN-γ (left panels). Bar graph shows the representative percentage of in vitro-differentiated Th1 cells (right panel). (B) Differentiation of Th17 cells. Spleen CD4+ T cells isolated from BMT recipients on day 21 were treated with vehicle solution or VPA for 24 h, the supernatants were collected, and ELISA was performed to determine the level of IFN-γ (C) and IL-17A (D). (E) Spleen CD4+ T cells from BMT recipients treated with VPA or vehicle on day 21 were stimulated with PMA and ionomycin. Five hours later, the supernatants were collected to determine cytokine levels by ProcartaPlex Multiplex Immunoassays. Bar graphs represent the levels of cytokines secreted per 1 × 10^6 stimulated CD4+ T cells. (F) Spleen CD4+ T cells isolated from BMT recipients on day 21 were treated with different concentrations of VPA for 24 h and analyzed by Western blot assay for the expression and phosphorylation level of Akt and downstream target proteins. (G) Immunoprecipitation analysis of the acetylation of Akt. (H) Spleen CD4+ T cells harvested from VPA recipients and control mice on day 21 were analyzed by Western blot assay for the expression and phosphorylation level of Akt and downstream target proteins. Results are representative of three independent experiments. Data are mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
alloantigen in the context of GVHD greatly increased both glycolysis and mitochondrial oxidative phosphorylation compared with normal T cells (51). The Akt/mTOR pathway plays a key role in the induction of aerobic glycolysis and anabolic processes in T cell growth. Activation of mTORC1 induces genes involved in glycolysis, the pentose phosphate pathway, and sterol and lipid biosynthesis (52). Inhibition or deficiency in this pathway leads to T cell anergy, despite the presence of costimulation (53). Therefore, the Akt/mTOR pathway is a potential target for T cell immunosuppression. In the current study, VPA dose dependently decreased the phosphorylation level of Akt, as well as the downstream target proteins in CD4⁺ T cells, in vitro and in vivo. Thus, Akt activity was reduced upon VPA treatment. Because Akt is an important oncogene that is frequently deregulated and shows high activity in many hematological and nonhematological malignancies, using VPA to prevent or treat GVHD may reduce the severity of GVHD, as well as have a salutary effect on inhibiting the relapse of hematological malignancies. In summary, our findings demonstrate a role for the HDAC inhibitor VPA in inhibiting Th1 and Th17 cells and related proinflammatory cytokines that contribute to GVHD while preserving the GVL effect (54). In addition, studies showed that the elimination of residual leukemia was primarily mediated by donor CD8⁺ cytotoxic T lymphocytes and NK cells, whereas the inflammatory cytokines that are secreted mainly by CD4⁺ Th cells have a very limited role in leukemia eradication but contribute significantly to the toxicity of GVHD (55). Therefore, inhibiting the function of CD4⁺ Th cells and suppressing the secretion of inflammatory cytokines might be a useful way to control GVHD and preserve the GVL effect. In our study, we demonstrated that VPA administration in tumor models maintained cytolytic activity of donor T cells while inhibiting the proinflammatory function of Th1 and Th17 cells and, thereby, separated GVHD and the GVL effect. Moreover, VPA treatment might add another beneficial layer of anti-leukemia effect, because a recently published study showed that inhibition of Akt signaling promoted the generation of leukemia-reactive T cells (56).

In summary, our findings demonstrate a role for the HDAC inhibitor VPA in inhibiting Th1 and Th17 cells and related proinflammatory cytokines that contribute to GVHD while preserving GVL effects. Because the long-term use of VPA in the clinical setting amassed pharmacokinetics and safety data, it is rational to test VPA in allogeneic BMT patients in combination with standard GVHD prophylaxis or treatment.
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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure 1

A. CD4\(^+\) (MLN) from Day 7, Day 14, and Day 28.

B. CD4\(^+\) (MLN) showing percentage distribution on Day 7, Day 14, and Day 28.

C. CD8\(^+\) (Spleen) from Day 7, Day 14, and Day 28.

D. CD8\(^+\) (MLN) from Day 7, Day 14, and Day 28.

E. Liver cells (10\(^5\)) for CD4\(^+\)IFN-\(\gamma\), CD4\(^+\)IL-17A\(^+\), and CD4\(^+\)IFN-\(\gamma\)\(\&\)IL-17A\(^+\) from Day 7 and Day 14.

F. Lung cells (10\(^5\)) for CD4\(^+\)IFN-\(\gamma\), CD4\(^+\)IL-17A\(^+\), and CD4\(^+\)IFN-\(\gamma\)\(\&\)IL-17A\(^+\) from Day 7 and Day 14.

G. TNF-\(\alpha\) and IFN-\(\gamma\) levels in pg/ml on Day 7, Day 14, and Day 28.

IL-17A and IL-23 levels in pg/ml on Day 7, Day 14, and Day 28.

IL-21 levels in pg/ml on Day 7, Day 14, and Day 28.
Supplemental Fig. 1 Th1 and Th17 cells are increased during GVHD development. Lethally irradiated BALB/c recipients were transplanted with either $5 \times 10^6$ TCD-BM plus spleen $1 \times 10^6$ T cells (H-2$^b$) from B6 mice (allogeneic group; n=15) or the same dose of TCD-BM and spleen T cells (H-2$^d$) from BALB/c mice (syngeneic group; n=10). (A and B) Flow cytometry analysis of intracellular IFN-γ and IL-17A on donor derived CD4$^+$ T cells from MLN on indicated days for syngeneic and allogeneic mice. (C and D) Intracellular IFN-γ and IL-17A on donor-derived CD8$^+$ T cells from MLN and spleen on indicated days for syngeneic and allogeneic mice. (E and F) Absolute numbers of IFN-γ-, IL-17A-, and IFN-γ/IL-17A-producing CD4$^+$ T cells in recipients’ liver (E) and lung (F) on day 7 and 14. (G) Serum levels of cytokines from syngeneic and allogeneic mice. Results are representative of 2 to 3 independent experiments. Data are expressed as mean ± SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. 
Supplemental Fig. 2. Up-regulated Th1 and Th17 cell responses in B6 into FVB model. Lethally irradiated FVB recipients were transplanted with either TCD-BM plus spleen T cells (H-2^b) from B6 mice (allogeneic group; n=15) or the same dose of TCD-BM and spleen T cells (H-2^a) from FVB mice (syngeneic group; n=10). (A) Flow cytometry analysis of intracellular IFN-γ and IL-17A on donor derived CD4^+ T cells from MLN and spleen on day 21 for syngeneic and allogeneic mice. (B) Representative percentage of donor derived CD4^+ T cells from MLN and spleen expressing IFN-γ and/or IL-17A on day 21. Results are representative of 2 independent experiments. Data are expressed as mean ± SEM. ns, P >0.05; * P < 0.05; ** P < 0.01.
Supplemental Fig. 3. VPA treatment decreases Th1 and Th17 cells. Donor B6 mouse-derived TCD-BM plus spleen T cells (H-2\textsuperscript{b}) were transplanted into lethally irradiated BALB/c mice (H-2\textsuperscript{d}). VPA was administered i. p. to these recipients from day 0, with vehicle treatment as control. (A and B) Flow cytometry analysis of intracellular IFN-\(\gamma\) and IL-17A on donor-derived CD4\(^+\) T cells from MLN on indicated days from control group and VPA recipients. (C and D) Graph shows the numbers of Th1 and Th17 cells from Spleen and MLN. Results are representative of 3 independent experiments. Data are expressed as mean ± SEM. ns, \(P>0.05\); * \(P<0.05\); ** \(P<0.01\).
Supplemental Fig. 4. VPA suppresses Th1 and Th17 cells via Akt inhibition. Purified CD4+ naive T cells were cultured with different concentration of VPA under Th1 or Th17 polarizing conditions. The viability of in vitro differentiated Th1 (A) and Th17 (B) cells was analyzed by Annexin V/PI staining. (C) Spleen CD4+ T cells harvested from VPA recipients and control mice were stained by Annexin V/PI for viability analysis. (D) Spleen CD4+ T cells derived from BMT recipient on day 21 after transplantation were cultured with different concentration of VPA for 24 hours, the mRNA level of T-bet, Rorc, and Foxp3 genes as well as Ifng and Il17a genes were analyzed by real-time PCR. (E) Spleen CD4+ T cells isolated from BMT recipients on day 21 were treated with different concentration of VPA for 24 hours, and were analyzed by Western blot assay for the expression and phosphorylation level of indicated proteins. Results are representative of 2 to 3 independent experiments. Data are expressed as mean ± SEM. ns, \( P > 0.05 \); ** \( P < 0.01 \); *** \( P < 0.001 \); **** \( P < 0.0001 \).