Molecular Therapy
Oncolytics
Original Article

HDAC Inhibitor LBH589 Suppresses the Proliferation but Enhances the Antileukemic Effect of Human γδT Cells

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γδT cells have potent effects on hematological malignancies, and their functions can be regulated by anti-tumor agents. Histone deacetylase inhibitors (HDACis) not only have antileukemic activity on leukemia but also affect immune cells during therapeutic application. In this *in vitro* study, we showed that LBH589, a pan-HDACi, impaired the proliferation of human γδT cells, as well as their proportions in peripheral blood mononuclear cells (PBMCs). At the specific concentration, LBH589 induced significant antileukemic activity of γδT cells against the HL-60 cells and Kasumi cells in a dose-dependent manner. However, the expression levels of activating receptor and molecules, as well as interferon-γ (IFN-γ) expression on γδT cells, were not affected by LBH589. After treatment with LBH589 for indicated times, extracellular-regulated protein kinase (ERK), Akt, and c-Jun N-terminal kinase (JNK) signaling pathways in γδT cells were not activated. In contrast, a stronger expression of Notch was observed and sustained for 72 h. Inhibition of Notch signaling by FLI-06, the γ-secretase inhibitor, significantly reversed the enhanced antileukemic ability of γδT cells induced by LBH589. For the first time, our investigations demonstrate that LBH589 can inhibit proliferation of γδT cells but facilitate their antileukemic effects via activation of Notch signaling.

INTRODUCTION

Human γδT cells have the potential as modulators of innate and adaptive immune responses and are recognized for their anti-tumor properties,[1] and previous studies have demonstrated that γδ T cells could kill primary leukemic cells.[2,3] Therefore, γδT cells have become ideal candidates for antileukemic immunotherapy. Although some patients with refractory malignancies have achieved promising outcomes by induction or adoptive infusion of γδT cells, most of the patients are not responsive or relapse shortly after remission. The therapeutic application of γδ T cells in clinical trials for cancer therapy shows complicated results, indicating a long way in improving the anti-tumor responses of γδ T cells.[4] There is clinical evidence showing that in patients with tumors, the functions and frequencies of γδT cells may correlate with clinical outcome and the anti-tumor efficacy in chemotherapy,[5,6] indicating that it is feasible to promote the antileukemic effectiveness via modifying the function of γδT cells.

Recently, several researchers have found that anti-tumor agents can regulate the functions of γδT cells. Tokuyama et al.[7] suggest that the monoclonal antibody (mAb) drugs, trastuzumab and rituximab, promote the cytotoxicity of Vγ9Vδ2 T cells against B cell lymphoma cells, chronic lymphocytic leukemia (CLL) cells, and breast cancer cells. Similarly, rapamycin is capable of enhancing anti-tumor effects of human γδT cells *in vitro*.[8] We also reported that the tyrosine kinase inhibitor (TKI) dasatinib could promote proliferation and increase cytolytic responses of long-term induced γδT cells.[9] According to these observations, we supposed that certain anti-tumor drugs may regulate functions and proliferation of γδT cells. However, given that the agents described in above studies are applicable for specific diseases, it is reasonable to select the broad-spectrum anti-tumor drugs as the optimal candidates for studying their potential regulation on γδT cells.

Histone deacetylase (HDAC) acts as regulator of gene transcription and contributes to numerous human diseases. Therefore, the HDAC inhibitor (HDACi) has been developed for anti-cancer therapy, including hematological malignancies.[10] Recently, studies have found that HDACi not only exerts direct anti-tumor effects on malignant cells but also exhibits impacts on the immune system,[11] as various HDAC family members have been identified to mediate diverse functions in T cell development and its biological

Received 18 November 2019; accepted 6 August 2020;
https://doi.org/10.1016/j.omto.2020.08.003.

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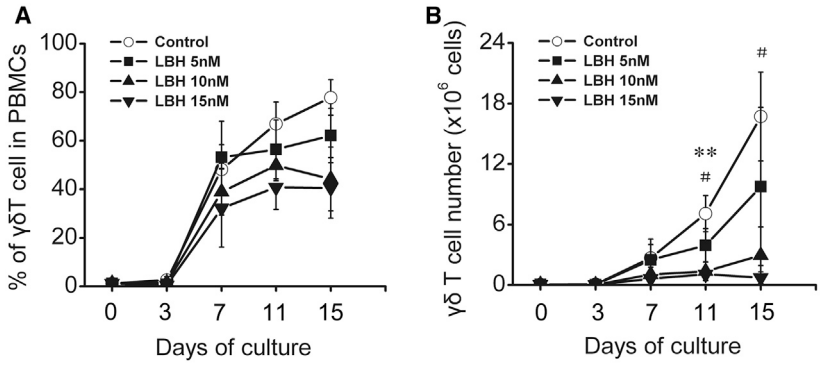

Figure 1. LBH589 Inhibits Human γδT Cell Proliferation in a Long-Term *Ex Vivo* Culture

γδT cells were cultured with various doses of LBH589 for up to 15 days. (A and B) The proportions in total PBMCs (A) and absolute number of γδT cells (B) were calculated using flow cytometry at the indicated time (n = 4). Results are presented as mean ± SEM. Significance is expressed as $p < 0.05$. At the indicated culture time, the differences of γδT cell proliferation between LBH589 treatment groups and control groups were analyzed. **$p < 0.01$, LBH589 10 nM-treated groups versus control groups; #$p < 0.05$, LBH589 15 nM-treated groups versus control groups.

conditions.[12] Recently, HDACi has been found to activate T cells in bone marrow transplantation (BMT) mice,[13] facilitating the effector responses and cytokine release of $CD8^+$ T cells.[14,15] HDACi panobinostat (LBH589) can repress the expression of tumor necrosis factor receptor 2 (TNFR2)-positive regulatory T cells (Tregs) in acute myeloid leukemia (AML) patients, while associated with raised release of cytokines from effector T cells.[16]

Accordingly, we design to investigate whether HDACi LBH589 could have impacts on the proliferation and function of human γδT cells as it does on Tregs and attempt further to search for the potential mechanisms behind this effect. In this study, we analyzed the influences of LBH589 treatment on γδT cells *in vitro* and found the following: that (1) expansions of γδT cells were inhibited, (2) effector functions were preserved following long-term exposure to LBH589, and (3) LBH589 enhanced cytolytic activity of γδT cells against AML cells via the Notch signaling pathway.

RESULTS

LBH589 Suppresses the Proliferation of γδT Cells

HDACi has been demonstrated to inhibit the proliferation of tumor cells and induce cell-cycle arrest. We first assessed the anti-proliferative effect of LBH589 on various AML cell lines using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay[17] and established the half-maximal inhibitory concentration (IC_{50}) of LBH589 (Figure S1). To investigate the antileukemic relevant concentrations of LBH589 on γδT cell amplification, we induced a long-term *in vitro* culture added with escalated doses of LBH589, including the concentration of the IC_{50} value. At the indicated time, absolute cell counts and percentages of γδT cells in the peripheral blood mononuclear cells (PBMCs) were analyzed. As the concentrations of LBH589 increased, the proportions of γδT cells reduced, albeit not significantly within groups (Figure 1A). However, a remarkable decrease in absolute number was observed in a dose-dependent manner, especially on day 11; the total number of γδT cells was $1.35 \pm 0.91 \times 10^6$ in 10 nM LBH589-treated groups and $1.07 \pm 0.60 \times 10^6$ in 15 nM LBH589-treated groups, respectively (compared with control groups, $p < 0.05$; Figure 1B).

Subsequently, the fold changes of the γδT cell number were analyzed among groups at the indicated culture periods. Compared with the absolute cell counts on day 0, γδT cells increased in all groups on day 15, resulting in a significant expansion in control groups, as well as in the presence of 5 nM LBH589, but lower fold changes were detected in 10 nM and 15 nM LBH589-treated groups than that in control groups ($p < 0.05$; Figure S2). These results show that LBH589, a pan-HDACi, negatively regulates the proliferation of γδT cells at higher concentration, especially at the cytotoxic concentration against AML cell lines.

LBH589 Facilitates the Antileukemic Activity of γδT Cells

To evaluate the effects of LBH589 on the antileukemic activity of γδT cells, we used the carboxyfluorescein diacetate succinimidyl ester (CFSE)-based assay, as described previously.[18] After being incubated with different doses of LBH589 for 24 h, γδT cells were washed and collected for cytotoxicity assay. The toxicity of LBH589 on γδT cells was detected using flow cytometry with an Annexin V-Fluorescein Isothiocyanate (FITC)/Propidium Iodide (PI) Apoptosis Detection Kit (Figure S3). The HL-60 cells and Kasumi cells were labeled with CFSE separately and cocultured with γδT cells at various ratios of effector-to-target (E/T ratios) cells for 8 h. Before analysis, the mixture of both cells was stained with PI. We gated the AML cells according to the fluorescence intensities of CFSE, and the dead or killed AML cells were $CFSE^+PI^+$ double-positive cells (Figure 2A). Cytotoxicity was measured using γδT cells from 5 individual donors. Interestingly, we found that LBH589 significantly enhanced the cytolytic effects of γδT cells against both HL-60 cells and Kasumi cells in a dose-dependent manner (Figures 2B and 2C). In comparison with control groups, at the E/T ratio of 20:1, the cytotoxic efficiency of γδT cells against HL-60 cells was 44.1% ± 7.3% in 10 nM LBH589-treated groups ($p < 0.05$) and 46.9% ± 9.8% in 15 nM LBH589-treated groups ($p < 0.01$), respectively. At the E/T ratio of 5:1, LBH589-treated γδT cells also efficiently killed HL-60 cells, although there is no significant difference within groups. For Kasumi cells, LBH589-treated γδT cells showed significantly higher cytotoxicity compared to the control group when the E/T ratio was only 5:1. This observation identifies that compared to HL-60 cells, Kasumi cells are more

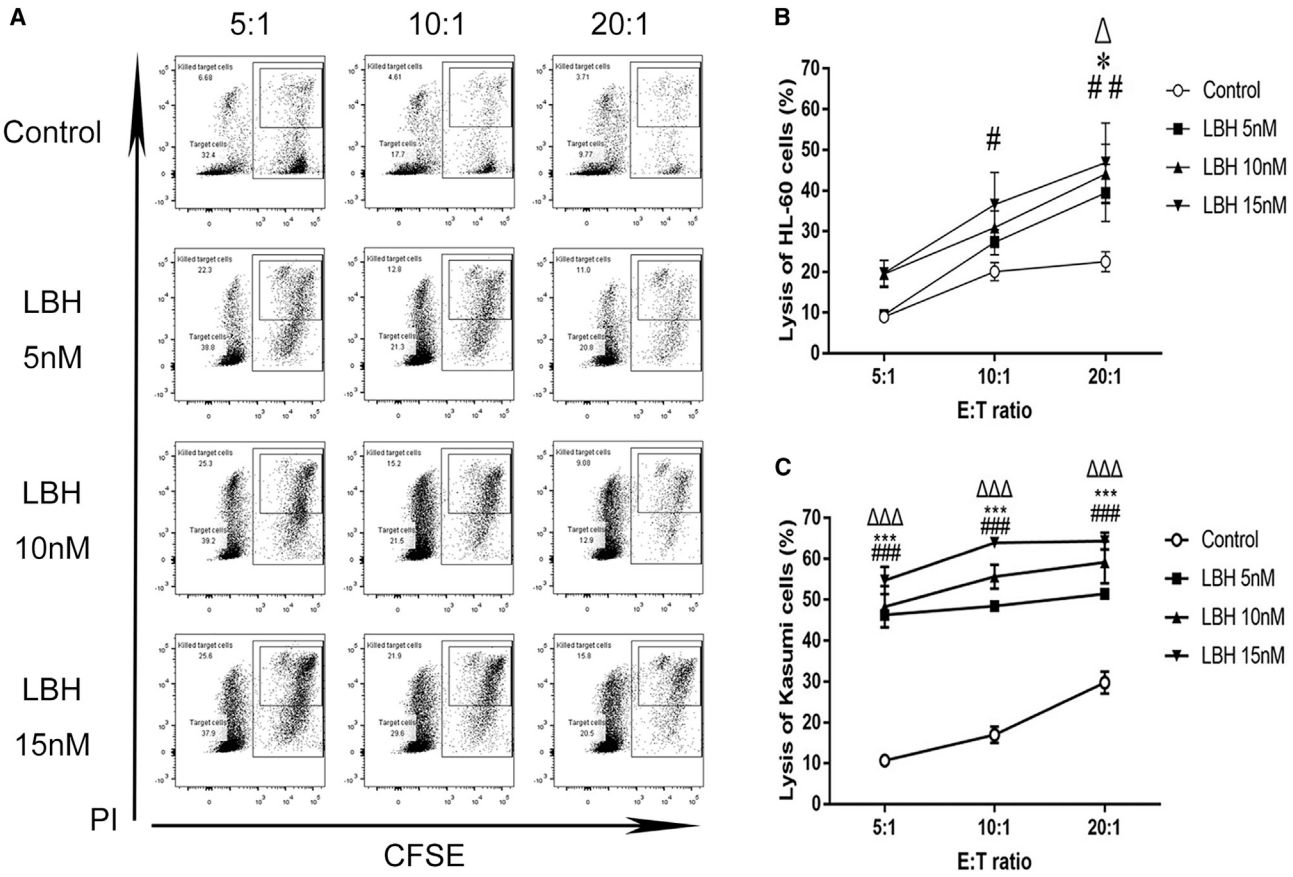

Figure 2. LBH589 Enhances the Antileukemic Activity of γδT Cells

After 24 h of different concentrations of LBH589 treatment, γδT cells were washed and harvested for cytotoxicity assay. The antileukemic effects of γδT cells were measured by a CFSE-based assay using flow cytometry. AML cell lines (target cells) were labeled with CFSE and incubated with or without LBH589-treated γδT cells (effector cells) at various E/T ratios for 8 h, thereafter stained with PI to distinguish dead cells. (A) The CFSE-positive cells were gated as target cells, whereas PI-positive cells represented dead cells, and CFSE/PI double-positive cells were calculated as killed target cells. (B and C) Lysis of HL-60 cells (B) or Kasumi cells (C) by γδT cells, which were treated by LBH589 or left untreated, was analyzed by flow cytometry. The efficacy of cytotoxicity was calculated as follows: % lysis = ([absolute number $CFSE^+$ PI^+ target cells]/[absolute number $CFSE^+$ target cells] − [absolute number $CFSE^+$ PI^+ target cells without effector cells]/[absolute number $CFSE^+$ target cells without effector cells]) × 100. Data are shown as mean ± SEM of five independent experiments performed. Δp < 0.05, ΔΔΔp < 0.001, 5 nM-treated groups versus control groups; *p < 0.05, ***p < 0.001, LBH589 10 nM-treated groups versus control groups; #p < 0.05, ##p < 0.01, ###p < 0.001, LBH589 15 nM-treated groups versus control groups (n = 5).

susceptible to γδT cells. Moreover, the cytotoxic effects can be enhanced by LBH589 in a concentration-dependent manner.

LBH589 Has No Effect on the Costimulatory Molecules and Cytokine Expression of γδT Cells

To investigate the mechanisms of LBH589 on effector functions of γδT cells, the activating molecules and receptor were evaluated by flow cytometry analysis. After 12–15 days' culture, we analyzed CD25, CD95, CD69, NKp44, and NKG2D expression levels on γδT cells. Results showed that there was no significant difference in the median fluorescent intensity (MFI) levels of CD25 or CD95 among groups, and it also had little or no effect on NKG2D expression and CD69-positive cell proportions by LBH589 treatment on γδT cells (Figure 3A). In addition, we detected no NKp44 expression on γδT cells in any group (data not shown).

As the phenotype reflects the diverse functional status of T cells, we detect the percentage of CD45RA and CD27 expression on γδT cells. Almost all γδT cells within each group displayed as the $CD45RA^-CD27^-$ double-negative type (effector memory cells; Figure 3B).

Activated human γδT cells can produce cytokines, such as interferon-γ (IFN-γ), to regulate functions other than immune cells. After 12–15 days' culture with different concentrations of LBH589, γδT cells were harvested and assayed by intracellular staining. The frequency of IFN-γ-producing γδT cells increased slightly as the dose of LBH589 escalated (Figure 3C). Overall, the above results suggest that LBH589 might not interfere with the activating molecules and receptors, as well as their phenotypes of γδT cells.

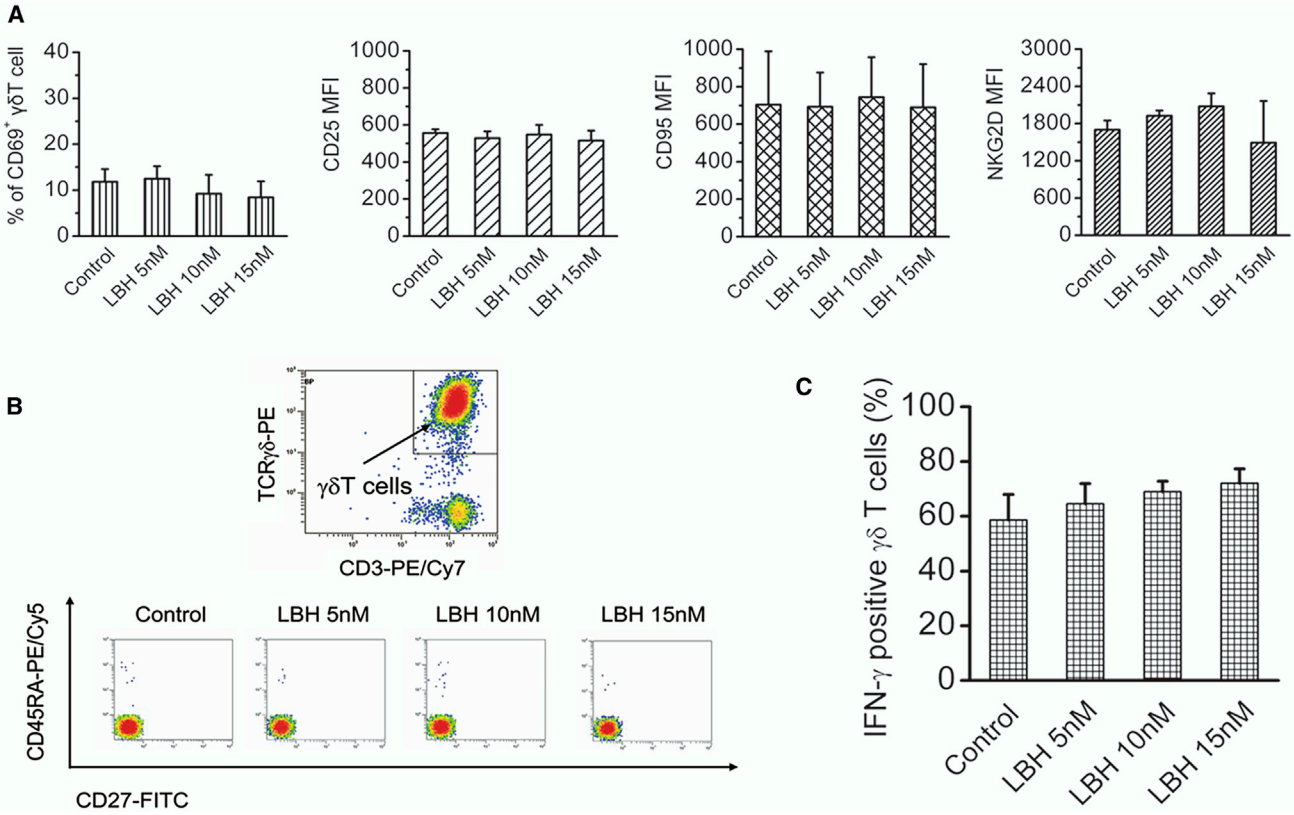

Figure 3. LBH589 Has No Effects on the Activation Modalities and Cytokine Expression of γδT Cells after 12–15 Days' Culture

(A) The levels of activating molecules and receptor expression were determined on γδT cells (n = 3). (B) Representative dot graph showed the phenotypes of γδT cells within LBH589 treatment groups. (C) Proportions of IFN-γ-producing γδT cells after 4 h PMA/ionomycin stimulation were analyzed by flow cytometry (n = 3). Data represent as mean ± SEM.

Activated Notch Signaling Correlates with Enhanced Antileukemic Activity of γδT Cells by LBH589

As described above, the cell-surface molecules, which expressed on the activated γδT cells, were unaffected by LBH589. Therefore, we speculated that the intracellular signaling pathway or molecules might play an important role in γδT cells' activation by LBH589. It was previously observed that some signaling pathways, such as Janus kinase/signal transducer and activator of transcription (JAK-STAT), mitogen-activated protein kinase/extracellular-regulated protein kinase (MEK/ERK), phosphatidylinositol 3-kinase/Akt (PI3K/Akt), and Notch signals, were involved in the activation of γδT cells.[19–21] Accordingly, we evaluated whether these signaling pathways might correlate with the antileukemic responses of γδT cells induced by LBH589. As shown in Figure 4A, after being incubated with 15 nM LBH589 for various times, the upstream of ERK and Raf-1 expression increased at a relatively high level, but the phosphorylation of ERK1/2 decreased significantly in a time-dependent manner. Additionally, the phosphorylated Akt or c-Jun N-terminal kinase (JNK)1/2 was hardly detected in γδT cells associated with reduction of JNK1/2 total protein levels. In contrast, a stronger expression of Notch2 was induced by LBH589 in γδT cells and sustained up to 72 h, as seen in Figure 4B. However, the recombination signal binding protein Jκ (RBP Jκ), a transcription factor downstream of Notch, had no significantly detectable change on expression level.

To confirm the relation between Notch signaling and an enhanced antileukemic effect of γδT cells by LBH589, we evaluated the cytotoxic capability of γδT cells against HL-60 cells in the presence or absence of the γ-secretase inhibitor, FLI-06 (Selleck Chemicals).[22] The purified γδT cells were untreated or pretreated with FLI-06 at a concentration of 10 μM for 30 min, and then indicated concentrations of LBH589 were added together for 24 h incubation. The CFSE-based cytotoxicity assay demonstrated that at an E/T ratio of 20:1, FLI-06 pretreatment significantly suppressed the antileukemic ability of γδT cells compared with those treated with LBH589 alone (Figure S4; $p < 0.05$). We next checked the gene expression of downstream targets of the Notch pathway using quantitative real-time PCR. After LBH589 treatment, the expression of Hes1, DLL3, and DLL4 in γδT cells significantly increased (Figure 4C). Therefore, these findings confirm that Notch signaling induced by LBH589 enhances the antileukemic activity of γδT cells, which may be through the upregulation of Hes1, DLL3, and DLL4.

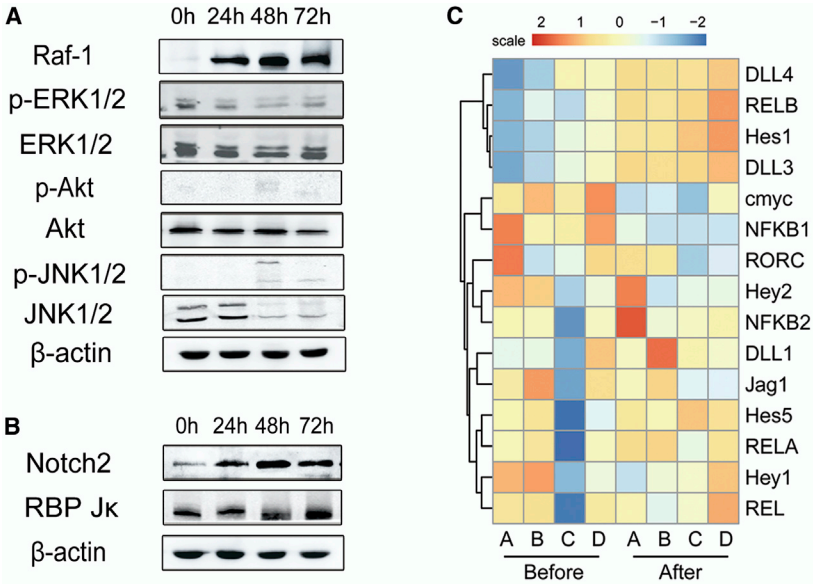

Figure 4. LBH589 Regulates the Cytotoxicity Activity of γδT Cells via Notch Signaling

(A) γδT cells were treated with 15 nM LBH589 for 24 h, 48 h, and 72 h, and then cells were subjected to western blot for assessment of various activation-associated signaling pathways. (B) Western blot analysis detected the Notch/RBP Jκ signaling in 15 nM LBH589-treated γδT cells for various times. The representative results shown in this figure were obtained from 3 independent experiments. (C) Heatmap showing the expression of downstream targets of the Notch pathway from untreated γδT cells and LBH589-treated γδT cells (n = 4). Heatmap scale is the *Z* score.

DISCUSSION

HDACis have been shown to regulate the functions and immune responses of various immune cells in previous observations.[23,24] To enhance anti-tumor immunity, it is crucial to identify the possible influences of therapeutic drugs on the activity of γδT cells in cancer therapy. In this study, we aim to assess the potential effects of LBH589, a pan-HDACi, on the proliferation and antileukemic capability of γδT cells. The results have demonstrated that LBH589 reduces the expansion of γδT cells and on the contrary, enhances their antileukemic effects through the activation of Notch signaling.

The anti-proliferative activity of LBH589 on tumor cells is definite, but whether it had similar inhibitory effects on the proliferation of immune cells is still not clear. Recently, it was reported that relatively low concentrations of LBH589 (10 nM and 15 nM) could reduce the frequencies of $TNFR2^+$ Tregs after 3 days' culture.[16] Similarly, our results indicate that LBH589 inhibited the expansion of human γδT cells at the cytotoxic concentrations for AML cells in *ex vivo* long-term culture. Moreover, the inhibitory effect was specific for reduction of the absolute number and fold expansion, except for the proportion of γδT cells in PBMCs. However, other researchers revealed no effects of high doses of LBH589 (>50 nM) on the survival of $CD8^+$ or $CD4^+$ PBMCs within 21 h.[25] The peak plasma concentration of LBH589 is around 60 nM *in vivo*, and Rasmussen et al.[26] suggested that treatment of 62.5 nM LBH589 for 18 h could not induce significant death of PBMCs isolated from healthy donors. These results are controversial, and the possible explanations of this discrepancy might be the treatment time and concentrations. It is previously demonstrated that HDACis blocked the actions of HDACs and induced cell-cycle arrest and cell apoptosis by dose and time dependently.[27–29] Although the time-dose relationships were not completely analyzed in this study, the above findings suggest a negative role of LBH589 in γδT cell frequencies. Thus, further investigations are needed to determine the roles of LBH589 on γδT cell proliferation at clinically relevant concentrations.

In addition to inhibition of cell proliferation, HDACis also modify the functions of γδT cells. In the present study, we show that LBH589 enhanced antileukemic responses of γδT cells against AML cell lines in a dose-dependent manner. Different from the inhibitory effects on cell expansion, LBH589, at higher concentrations, facilitated the cytotoxicity of γδT cells. However, the expression of activating molecules and the receptor was not affected, and the proportions of IFN-γ-producing γδT cells remained unchanged after LBH589 treatment. Furthermore, most of the γδT cells express as $CD45RA^-CD27^-$ effector memory cells. The $CD45RA^-CD27^-$ γδT cells were identified as lack of proliferation efficacy but capable of producing IFN-γ and having the cytotoxicity functions.[30] This suggests that LBH589 could enhance the antileukemic effects of γδT cells without being interfered with their activation modalities. So far, LBH589 has been proven to promote the TNF-α and IFN-γ production in BMT mice, suggesting that LBH589 could boost T cell activation *in vivo*. It was demonstrated that 20 nM LBH589 could downregulate the NKG2D and NKp46 expression on NK cells, whereas pretreatment of both target cells and PBMCs with 10 nM LBH589 induced significant lysis of target cells (Hodgkin lymphoma cell line), as well as increased TNF-α secretion at 50:1 E/T ratio.[31] These findings, together with our observations, indicate that LBH589 may improve the cytotoxicity of γδT cells independent of the activating molecules or receptor expression levels. Although the mechanisms of enhanced cytolytic activity are still unknown, based on the current results, we supposed that the intracellular signaling pathway may contribute to the enhanced cytotoxicity of γδT cells under LBH589 exposure.

γδT cells can be activated by transformed or tumor cells and exhibit cytolytic responses. The molecules and activating receptors, as well as the intracellular signaling pathways, are involved in the anti-tumor activities of γδT cells. We performed further analysis to study the mechanisms underlying the enhanced antileukemic activity of γδT cells induced by LBH589. Our observations suggested that only the Notch expression increased markedly in γδT cells after exposure to LBH589. Stockhausen et al.[32] reported that valproate sodium

(VPA) could affect the levels of the intracellular Notch signaling cascade for interference with neuroblastoma differentiation. Recent studies showed that trichostatin A (TSA) inhibited transforming growth factor-β2 (TGF-β2)-induced epithelial-mesenchymal transition (EMT) via suppression of the Notch pathway,[33] with dual roles in regulating the Notch signaling-mediated smooth muscle cell (SMC) differentiation.[34] Nevertheless, we do not know the relationship between activated Notch signaling and increased cytolytic effector functions of γδT cells induced by LBH589. Recently, numerous investigations have demonstrated that the Notch signaling pathway regulates the functions and survival of $CD4^+$ and $CD8^+$ T cells[35–37] and γδT cells.[21] Since Notch is known to play an important role in the generation of effector functions of T cells, it should be realized that the effects of Notch differ in various T cells. Recently, Notch signaling was demonstrated to be responsible for the efficient stimulation of $CD4^+$ T cell activation and increase their antigen sensitivity.[38] Kuijk et al.[39] revealed that activated Notch signaling was needed for IFN-γ expression but not for cytotoxicity of $CD8^+$ T cells. Nevertheless, inhibition of Notch significantly impaired the cytolytic functions of γδT cells against tumor cells.[21] This is consistent with our research that blockade of Notch by FLI-06 could impair the enhanced antileukemic effects of γδT cells following LBH589 treatment. Our data reveal that Notch activation may participate in the improved antileukemic effects of γδT cells by LBH589.

Altogether, our data suggest that LBH589 suppresses the proliferation of γδT cells but preserves their immune functions and activation status *in vitro*. In addition, the antileukemic activity of γδT cells was facilitated by LBH589, and the activated Notch signaling may contribute to this effect. Combined with other observations, it is clear that LBH589 may improve the effector functions of cytotoxic T lymphocytes (CTLs), such as γδT cells, in anti-tumor therapy. However, the adverse effects of higher drug dosage should be under consideration for impairment in the proliferation of immune cells. Our findings may provide more comprehensive understanding of the potential effects of LBH589 treatment in patients with cancer through γδT cells.

MATERIALS AND METHODS

In Vitro Culture of γδT Cells from Healthy Volunteers

Human peripheral blood was collected from healthy adult volunteers after being approved by the Zhejiang University Ethics Committee and obtaining consent from each volunteer. The protocol for γδT cell expansion had been described previously,[9] and PBMCs were cultured at 2.5×10^6 cells/mL in 24-well plates.

Chemicals

LBH589 was purchased from Selleck Chemicals (Houston, TX, USA). Different doses of LBH589 (control wells, no drug added) were added from day 0 and set as a dose-escalation manner, according to the IC_{50} of LBH589 on AML cell lines and the concentrations used in an *in vitro* assay of Govindaraj et al.[16] Since day 1, various doses of LBH589 were added every other 2 days, together with fresh RPMI 1640 medium and recombinant human interleukin-2 (rhIL-2; PeproTech, Rocky Hill, NJ, USA) at a final concentration of 250 IU/mL.

AML Cell Line Cultures

HL-60 cells and Kasumi cells were obtained from the Shanghai Institute for Biological Sciences, Chinese Academy of Science (Shanghai, China), and established for acute myelogenous leukemia. These cells were cultured in complete RPMI 1640 medium with 10% fetal bovine serum (FBS) (Gibco, NY, USA) at 37°C in a fully humidified atmosphere of 5% CO_2 in air as suspension cultures.

Flow Cytometry Analysis

For determination of γδT cell frequency, cells were subjected to flow cytometric assay before (day 0) and after (days 3, 7, 11, and 15) LBH589 treatment. The absolute number or the percentage of γδT cells in total PBMCs was counted and analyzed by trypan blue exclusion on a Cedex XS cell count analyzer (Roche, Basel, Switzerland) and flow cytometry (Beckman Coulter; FC500MCL). The cells were stained with fluorescence-conjugated antibodies to label the surface markers expressed on γδ T cells: FITC-conjugated anti-CD45 (BioLegend), phycoerythrin-Cy7 (PE-Cy7)-conjugated anti-CD3 (BioLegend), FITC, or PE-conjugated anti-T cell receptor (TCR)γδ (BD Pharmingen). Dead cells were excluded with 7-amino-actinomycin (7-AAD; BD Pharmingen).

The activating molecules or receptor were stained with relevant antibodies as follows: anti-CD25-PE, anti-CD69-FITC, anti-CD95-PE, and allophycocyanin (APC)-conjugated anti-NKG2D (BioLegend). Subsets of γδT cells were determined by phenotypic markers CD27 and CD45RA. γδT cells can be divided as $CD27^+CD45RA^+$ naive, $CD27^+CD45RA^-$ central memory, $CD45RA^-CD27^-$ effector memory, and $CD27^-CD45RA^+$ late effector cells.

IFN-γ Production Assay

After 12–15 days' culture, the intracellular IFN-γ expression assay was performed using flow cytometry. γδT cells were incubated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA), 250 ng/mL ionomycin, and monensin (6 μg/mL) for 4 h at 37°C in 5% CO_2. Then, γδT cells were collected, and the following antibodies were used for intracellular staining: PE-Cy7-conjugated anti-CD3 (BioLegend), PE-conjugated anti-TCRγδ (BD Pharmingen), and APC-conjugated anti-IFN-γ (BioLegend). We used a Cytofix/Cytoperm Plus Kit (BD Pharmingen) for intracellular staining, according to the manufacturer's protocols.

Cytotoxicity Assay

γδT cells from control wells were purified by BD FACSAria II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) prior to functional assay. LBH589 was added to γδT cells at varying doses for 24 h incubation. Then, γδT cells were washed and used as effector cells. We choose HL-60 and Kasumi cells (AML cell lines) as target cells, because these cell lines are not susceptible to NK cell-mediated killing.[40] The cytotoxicity analysis was performed by a CFSE-based assay to determine the antileukemic functions of γδT cells.[18] As target cells, AML cell lines were resuspended to 2×10^6 cells/mL and labeled with 10 μM CFSE (Molecular Probes) for 10 min at 37°C. The reaction was stopped by adding ice-cold FBS to the cells for 5 min on ice

and washed twice with fresh culture media. The target cell concentration was adjusted to 5×10^5 cells/mL, and 100 μL cell suspension was added into each 5 mL sterile polystyrene tube. γδT cells and HL-60 cells or Kasumi cells were cocultured at different E/T ratios for 8 h in a humidified atmosphere of 5% CO_2 and 37°C. The tubes that had HL-60 cell or Kasumi cell incubation without γδT cell addition were set as self-control for exclusion of dead cells. After 8 h culture, both of the two cells were washed with phosphate-buffered saline (PBS) containing 5% bovine serum albumin (BSA). Then, 1 μg/mL PI (Sigma-Aldrich) was added to stain dead cells and analyzed by flow cytometry. The percentage of cytotoxicity was calculated as follows: % lysis = ([absolute number $CFSE^+$ PI^+ target cells]/[absolute number $CFSE^+$ target cells] − [absolute number $CFSE^+$ PI^+ target cells without effector cells]/[absolute number $CFSE^+$ target cells without effector cells]) × 100.

Western Blot Analysis

After being treated with 15 nM LBH589 for 24 h, 48 h, and 72 h, γδT cells were harvested and lysed in ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer containing 10 μM phenylmethanesulfonyl fluoride (PMSF) and 1 μg/mL protease inhibitor plus phosphatase inhibitors (Roche). After being incubated for 30–60 min, lysed cells were centrifuged, and supernatants were collected and heated at 95°C for 5 min. Proteins were loaded on 10% or 12% SDS-polyacrylamide gel electrophoresis (PAGE), then transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore). Blots were probed by using various primary mAbs in 4°C overnight and then incubated with IRDye-conjugated secondary Abs (Li-Cor Biosciences) as needed. Detection of the blots was performed by Odyssey infrared imaging (LI-COR Biosciences).

Gene Expression Analysis

γδT cells were harvested and reseeded at 2.5×10^6 cells/mL in 24-well plates and then treated with 15 nM LBH589 for 24 h. RNA was respectively isolated from γδT cells before and after LBH589 treatment using the EZ-press RNA Purification Kit (EZBioscience, Roseville, MN, USA) and reverse transcribed into cDNA (Takara, Japan). Quantitative real-time PCR was performed in triplicate using SYBR Premix Ex Taq (Takara), according to the manufacturer's instructions. The expression level of each gene was standardized to the housekeeping gene Gapdh and the cycle threshold (Ct) method was used to calculate fold induction relative to levels found in control samples. The primers used were shown in Table S1.

Statistical Analysis

All of the data were analyzed by SPSS 17.0 software and presented as mean ± standard error of the mean (SEM). Statistical significance among treatment groups (≥3 experimental groups) were determined by one-way ANOVA. Student's t test was used to evaluate the effect of blocking experiments. $p < 0.05$ was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omto.2020.08.003.

AUTHOR CONTRIBUTIONS

H.H. and Y.Z. designed the experiments, interpreted the results, and wrote the manuscript. Y.H. and L.X. performed the research and analyzed the data. J.F. and K.W. participated in some of the experiments.

CONFLICTS OF INTEREST

The authors have no potential conflicts of interest. The work described has not been published previously. It is not under consideration for publication elsewhere. Its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and if accepted, it will not be published elsewhere in the same form, in English, or in any other language, including electronically without the written consent of the copyright holder.

ACKNOWLEDGMENTS

We appreciate Novartis Pharma for providing zoledronic acid for this study. This study was financially supported by grants from the National Natural Science Foundation of China (81670148), National Key Basic Research Program of China (81730008), and Key Research Program of Zhejiang Province (2019C03016).

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