Anti-tumor effects of suberoylanilide hydroxamic acid on Epstein–Barr virus-associated T cell and natural killer cell lymphoma

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The ubiquitous Epstein–Barr virus (EBV) infects not only B cells but also T cells and natural killer (NK) cells and is associated with various lymphoid malignancies. Recent studies have reported that histone deacetylase (HDAC) inhibitors exert anticancer effects against various tumor cells. In the present study, we have evaluated both the *in vitro* and *in vivo* effects of suberoylanilide hydroxamic acid (SAHA), an HDAC inhibitor, on EBV-positive and EBV-negative T and NK lymphoma cells. Several EBV-positive and EBV-negative T and NK cell lines were treated with various concentrations of SAHA. SAHA suppressed the proliferation of T and NK cell lines, although no significant difference was observed between EBV-positive and EBV-negative cell lines. SAHA induced apoptosis and/or cell cycle arrest in several T and NK cell lines. In addition, SAHA increased the expression of EBV-lytic genes and decreased the expression of EBV-latent genes. Next, EBV-positive NK cell lymphoma cells were subcutaneously inoculated into severely immunodeficient NOD/Shi-scid/IL-2Rγnull mice, and then SAHA was administered intraperitoneally. SAHA inhibited tumor progression and metastasis in the murine xenograft model. SAHA displayed a marked suppressive effect against EBV-associated T and NK cell lymphomas through either induction of apoptosis or cell cycle arrest, and may represent an alternative treatment option.

More than 90% of the world population is infected by the Epstein–Barr virus (EBV), which is an oncogenic γ-herpesvirus. Not only B cells but also T cells and natural killer (NK) cells can be infected by EBV, a condition that is associated with various lymphoid malignancies, including Burkitt lymphoma, Hodgkin lymphoma, post-transplant lymphoproliferative disorders, extranodal NK/T cell lymphoma, hydroa vacciniforme-like lymphoma, aggressive NK cell leukemia and chronic active EBV infection.[1,2] The ability of EBV to establish latent infection and induction of the proliferation of infected cells make it the significant causative agent in the pathogenesis of many of these malignancies. Some of these EBV-associated T and NK cell malignancies are refractory to conventional chemotherapies and have poor prognoses.[3] For the treatment and prophylaxis of B cell lymphoma and lymphoproliferative disorders, rituximab, a humanized monoclonal antibody (Ab) against CD20, targets B cell-specific surface antigens and has been used with marked success.[4,5] However, novel approaches to molecular targeted therapy are required to effectively treat T and NK cell malignancies.

Histone deacetylase (HDAC) inhibitors induce acetylation of histones, thus affecting transcription, and selectively induce tumor-suppressive genes. In various cancer cell types, HDAC inhibitors induce differentiation, apoptosis and cell cycle arrest.[6,7] Moreover, with notable tumor specificity, HDAC inhibitors have potent anticancer activities, and some exhibit therapeutic potential through their targeting of epigenetic regulation. Previously, we showed that an HDAC inhibitor, valproic acid, induced apoptosis and cell cycle arrest in EBV-positive T and NK lymphoma cells.[8] However, the suppressive effect of valproic acid in cell lines was modest and was not affected by the presence of EBV.

Suberoylanilide hydroxamic acid (SAHA) is an FDA-approved HDAC inhibitor, and its efficacy has been confirmed by clinical trials for malignant diseases such as non-Hodgkin lymphoma, acute myeloid leukemia, breast cancer and cutaneous T cell lymphoma.[9–12] Micromolar concentrations of SAHA have anticancer effects and a well-established safety profile.[9] Furthermore, recent studies have confirmed that SAHA can induce EBV lytic infection and mediate enhanced cell death in EBV-positive gastric carcinoma and nasopharyngeal carcinoma cells.[13,14] Very recently, a gene expression profile study identified SAHA as an effective drug candidate for NK cell neoplasms, including EBV-positive NK lymphoma.[15] However, no *in vivo* study has evaluated the efficacy of SAHA in EBV-positive T and NK lymphoma cells.

In the present study, we evaluate the antitumor effects of SAHA on EBV-positive and EBV-negative T and NK cell lines

and analyze induction of apoptosis, cell cycle arrest and expression of EBV-encoded genes. To further evaluate the effect of SAHA, an *in vivo* model is necessary. A suitable host for xenotransplantation of human lymphoid cells is the NOD/Shi-*scid*/IL-2Rγ^{null} (NOG) mouse, which is completely immunodeficient and lacks T, B, NK and dendritic cells, as well as macrophages.[16–19] Recently, the proliferation of EBV-positive T and NK cells has been confirmed by the xenotransplantation of human peripheral blood mononuclear cells (PBMC) to the NOG mouse.[20] Instead of human PBMC, we applied the xenograft model to evaluate SAHA using an EBV-positive NK cell line, which is more suitable for the evaluation of drugs.

Materials and Methods

Cell lines. Of the cell lines used, SNT13 and SNT16 are EBV-positive T cell lines,[21] Jurkat is an EBV-negative T cell line,[22] KAI3 and SNK6 are EBV-positive NK cell lines,[21,23] and KHYG1 is an EBV-negative NK cell line.[24] EBV-positive MT2/rEBV/9-7 and MT2/rEBV/9-9 cell lines were established by infection of MT2 cells with the hygromycin-resistant B95-8 strain.[25,26] EBV-negative MT2/hyg/CL2 and MT2/hyg/CL3 cell lines were transfected with a hygromycin-resistant gene. These four cell lines were used to verify the presence/absence of EBV in the T cell lines. Similarly, the EBV-negative NKL cell line was derived from a patient with NK cell leukemia, and the EBV-positive TL1 cell line was established from NKL cells infected with an Akata-transfected recombinant EBV strain containing a neomycin-resistant gene.[27,28] TL1 and NKL were used to verify the presence/absence of EBV in the NK cell lines. The characteristics of each cell line are summarized in Table 1.

Jurkat cells were cultured in RPMI 1640 medium supplemented with 10% FBS, penicillin, streptomycin and glutamine (complete medium). SNT13, SNT16, KAI3, SNK6, KHYG1, TL1 and NKL cells were grown in complete medium supplemented with 100 U/mL human interleukin-2 (IL-2). MT2/rEBV/9-7, MT2/rEBV/9-9, MT2/hyg/CL2 and MT2/hyg/CL3 cells were grown in complete medium supplemented with 0.2 mg/mL hygromycin. For xenotransplantation, the SNK6 cell line was grown in complete medium supplemented with human serum and 700 U/mL of human IL-2. All cultures were maintained at 37°C in 5% CO_2.

Cell viability. Suberoylanilide hydroxamic acid (Cayman Chemicals, Ann Arbor, MI, USA) was dissolved in DMSO. Each

cell line (2×10^5 cells per mL) was cultured in 24-well plates. Human PBMC were isolated from healthy volunteers using Ficoll–Paque (GE Heathcare AB BioSciences, Uppsala, Sweden) gradient centrifugation, and 5×10^5 PBMC per mL were cultured in 24-well plates. Cells were treated with various concentrations of SAHA for 96 h. The cell number and viability were quantified by trypan blue exclusion. Viability was calculated as the percentage of viable SAHA-treated cells versus DMSO-treated cells. These experiments were performed in triplicate, and the results were expressed as mean values with SEM.

Apoptosis assay by flow cytometry. Apoptosis was measured by flow cytometry using an annexin V-PE/7-AAD apoptosis assay kit (BD Pharmingen Biosciences, San Diego, CA, USA) according to the manufacturer's protocol.[29] Briefly, 2×10^5 cells were treated with SAHA for 24 h, incubated with annexin V-PE and 7-AAD for 15 min, and then analyzed by flow cytometry. Stained cells were analyzed using the FACSCantoII flow cytometer and the FlowJo software (Tree Star, Ashland, OR, USA).

Immunoblotting. After 24 and 48 h of treatment with various concentrations of SAHA, cell pellets were lysed directly in SDS sample buffer (50 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 6% 2-mercaptoethanol and 0.0025% bromophenol blue). Cell lysates were separated on 10% acrylamide gels by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with Abs. Abs were used against acetyl-histone H3 (Cell Signaling, Boston, MA, USA), poly (ADP-ribose) polymerase (PARP; Sigma, St. Louis, MO, USA), latent membrane protein (LMP) 1 (S12; BD Biosciences, San Jose, CA, USA),[30] EBV nuclear antigen (EBNA) 1[31] and β-actin (Sigma).

Cell cycle assay. Cells were treated with various concentrations of SAHA for 48 h and fixed with 70% ethanol. Fixed cells were treated with DNase-free RNase, stained with propidium iodide (Sigma) for 15 min, and analyzed by flow cytometry. Stained cells were analyzed using a FACSCalibur (Becton Dickinson, San Jose, CA, USA) flow cytometer and the ModFit LT software (Verity Software House, Topsham, ME, USA).

RT-PCR assay. RNA was extracted using the QIAmp RNeasy Mini Kit (Qiagen, Hilden, Germany), and contaminating DNA was removed by on-column DNase digestion using the RNase-free DNase Set (Qiagen). Viral mRNA expression was quantified by one-step multiplex real-time RT-PCR using the Mx3000P real-time PCR system (Stratagene, La Jolla, CA, USA) as described previously.[32,33] β2-Microglobulin was used as an endogenous control and reference gene for relative quantification.[34] Each experiment was performed in triplicate and was shown as the mean of three samples with the SEM.

Xenograft model using the NOG mouse. Female 6-week-old or 7-week-old NOG mice were obtained from the Central Institute of Experimental Animals, Kawasaki, Japan, and maintained under specific pathogen-free conditions by the approval and guidelines of the Nagoya University Experimentation Animal Committee. On day 0, 1×10^6 SNK6 cells were inoculated subcutaneously as described previously.[35] Each day from days 4 to 28, the mice were treated i.p. with 100 mg/kg SAHA or DMSO (control). Tumor volume was quantified using calipers twice per week and calculated using the following formula: $\pi \times$ short axis $\times$ long axis $\times$ height/6. On day 30, mice were killed, and the tumor and organs were excised. RNA was extracted from the tumor and subjected to real-time RT-PCR to quantify viral gene expression. Peripheral blood was collected, and plasma was separated. DNA was extracted from the plasma and quantified by quantitative real-time PCR.[36]

Table 1. Characteristics of the cell lines

Name	Cell type	EBV	Cell origin
SNT13	T	+	Chronic active EBV infection
SNT16	T	+	Chronic active EBV infection
Jurkat	T	−	Acute T lymphoblastic leukemia
KAI3	NK	+	Chronic active EBV infection
SNK6	NK	+	Extranodal NK/T cell lymphoma
KHYG1	NK	−	Aggressive NK cell leukemia
MT2/rEBV/9-7	T	+	MT2 cell line
MT2/rEBV/9-9	T	+	MT2 cell line
MT2/hyg/CL2	T	−	MT2 cell line
MT2/hyg/CL3	T	−	MT2 cell line
TL1	NK	+	NKL cell line
NKL	NK	−	NK-cell leukemia

EBV, Epstein–Barr virus; NK, natural killer.

The Mann–Whitney *U*-test was used to compare tumor volumes, viral mRNA expression and quantity of EBV-DNA. *P*-values <0.05 were deemed to indicate statistical significance.

Epstein–Barr virus-encoded small RNA *in situ* hybridization. Formalin (20%)-fixed and sucrose (0.1%)-fixed tissues were sectioned into 10-μm slices and treated with 1:10 diluted proteinase K. The tissues were incubated at room temperature for 30 min, and were then washed with pure water and ethanol (96%). The tissues were stained for Epstein–Barr virus-encoded small RNA (EBER) by *in situ* hybridization (ISH). EBER-ISH was performed using the EBER PNA Probe (Y5200; Dako) and the PNA ISH detection kit (Dako, Glostrup Denmark) according to the manufacturer's protocol.[33]

Results

Effect of suberoylanilide hydroxamic acid on the viability of T and natural killer cell lines. Epstein–Barr virus-positive and EBV-negative T and NK cell lines were cultured with various concentrations of SAHA. SAHA increased acetylated histone H3 levels, confirming that SAHA worked as an HDAC inhibitor (Fig. 1a). SAHA reduced the viability of all treated cell lines in a dose-dependent manner (Fig. 1b). Next, the same six cell lines were treated with 5 μM SAHA and assessed at different time points. The viability of all six cell lines was reduced by treatment with SAHA for 96 h (Fig. 1c). The effects of SAHA did not differ between EBV-positive and EBV-negative cell lines. In addition, to compare its effects on EBV-positive and EBV-negative cell lines, we treated MT2/rEBV/9-7 and MT2/rEBV/9-9 cells (EBV-positive T cell lines), MT2/hyg/CL2

and MT2/hyg/CL3 cells (EBV-negative T cell lines), TL1 cells (EBV-positive NK cell line) and NKL cells (EBV-negative parental NK cell line) with SAHA. SAHA had similar effects on the EBV-positive and EBV-negative cell lines (Fig. 2a). Moreover, human PBMC were treated with SAHA to evaluate the adverse effects. Viability remained >69% at 96 h, indicating the absence of adverse effects (Fig. 2b).

Effects of suberoylanilide hydroxamic acid on apoptosis and the cell cycle of T and natural killer cell lines. To determine whether apoptosis was induced by SAHA in the tested cell lines, early apoptotic cells were quantified by annexin V and 7-AAD staining. SAHA increased early apoptotic cells in the Jurkat, KAI3 and KHYG1 cell lines (Fig. 3a). In other cell lines, the proportions of early apoptotic cells were not increased. Next, the cleavage of PARP was analyzed by immunoblotting. With the exception of the SNT16 cell line, SAHA induced the cleavage of PARP in the five cell lines (Fig. 3b). Next, effects on the cell cycle were investigated. In the SNT16 and KAI3 cell lines, the population of cells in G1 phase was increased, whereas that in G2 phase was increased in the SNK6 cell line (Fig. 4). In Jurkat and KHYG1 cells, the cell cycle assay was indeterminate because of the massive cell death caused by SAHA.

Effects of suberoylanilide hydroxamic acid on Epstein–Barr virus-encoded genes of Epstein–Barr virus-positive T and natural killer cell lines. The expression of eight EBV-related genes, including lytic genes (BZLF1 and gp350/220) and latent genes (EBNA1, EBNA2, LMP1, LMP2, EBER1 and Bam HI-A rightward transcripts [BART]) were analyzed using real-time RT-PCR. In the SNT13, KAI3 and SNK6 cell lines, the
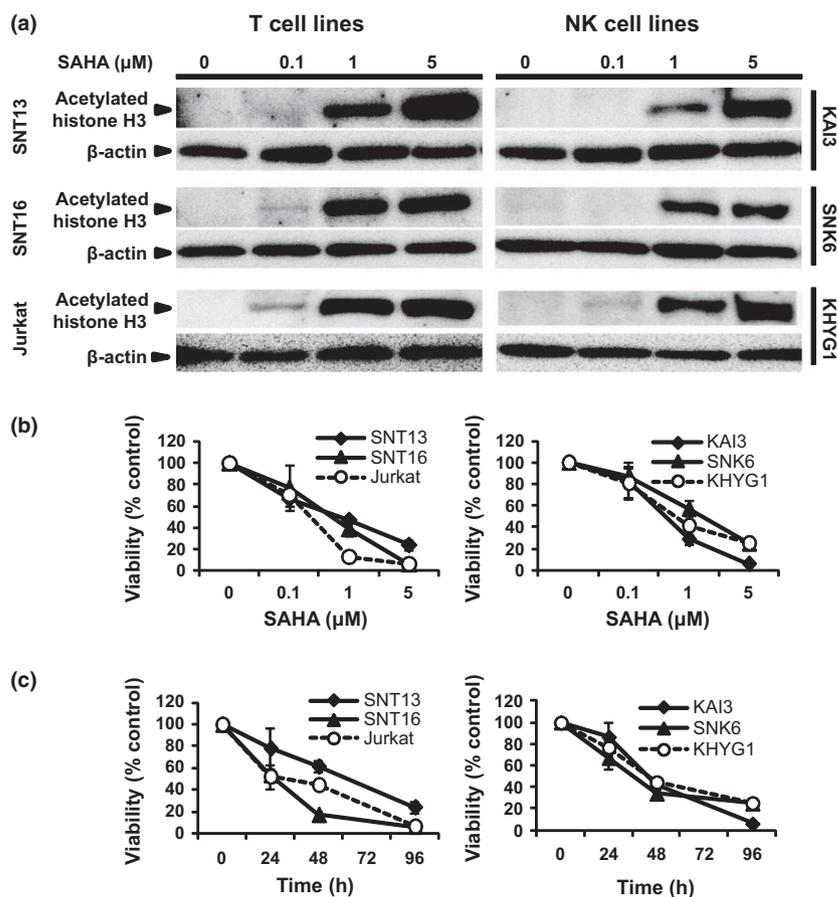

Fig. 1. Suberoylanilide hydroxamic acid (SAHA) inhibits the deacetylation of histone H3 protein and decreases the viability of T and natural killer (NK) cell lines. (a) SNT13, SNT16 (Epstein–Barr virus [EBV]-positive T cell line), Jurkat (EBV-negative T cell line), KAI3, SNK6 (EBV-positive NK cell line) and KHYG1 (EBV-negative NK cell line) cells were treated with the indicated SAHA concentrations for 24 h, and acetylated histone H3 was detected by immunoblotting. β-Actin was used as a loading control. (b) Each cell line was treated with the indicated concentrations of SAHA for 96 h or (c) with 5 μM SAHA for the indicated times. Data are expressed as means ± SEM.
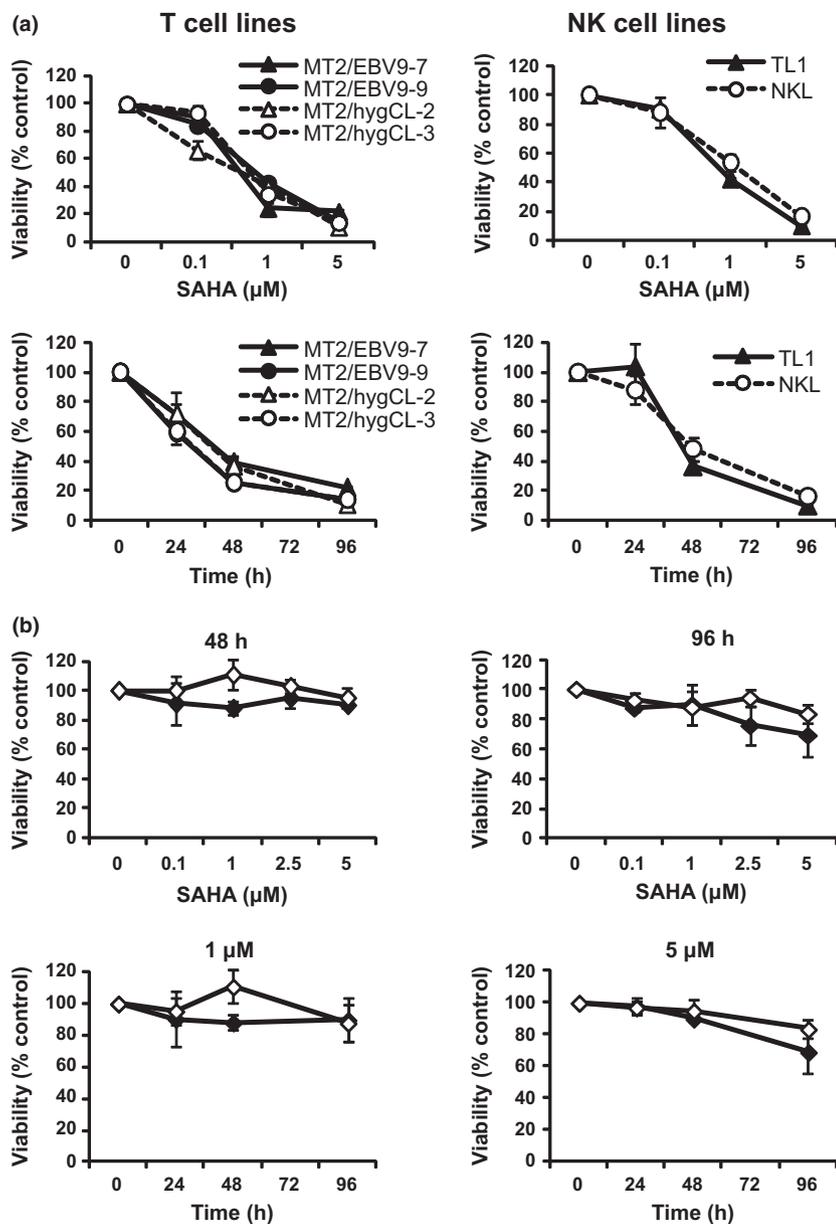
Fig. 2. The effects of suberoylanilide hydroxamic acid (SAHA) do not differ between Epstein–Barr virus (EBV)-positive and EBV-negative cell lines, and SAHA exerts no adverse effects on human peripheral blood mononuclear cells (PBMC). (a) MT2/rEBV/9-7, MT2/rEBV/9-9 (EBV-positive T cell lines), MT2/hyg/CL2, MT2/hyg/CL3 (parental cell lines), TL1 (EBV-positive natural killer [NK] cell line) and NKL (parental cell line) cells were treated with the indicated concentrations of SAHA for 96 h or with 5 μM SAHA for the indicated times. (b) Human PBMC were isolated from two volunteers and treated with the indicated concentrations of SAHA for 48 and 96 h or with 1 and 5 μM SAHA for the indicated times. Data are expressed as means ± SEM.

expression of BZLF1, which is an immediate-early gene in the lytic infection cycle, was increased by SAHA (Fig. 5). However, the expression of the late lytic gene gp350/220 was increased only in the SAHA-treated SNT13 cell line. These results indicated that SAHA induced lytic infection in some EBV-positive T and NK cell lines, although it was abortive. The expression of BZLF1 was decreased in the SAHA-treated SNT16 as time went by, while that in mock-treated SNT16 was also decreased. Of the EBV latent genes tested, the expression of EBNA1, LMP1 and BART was decreased in most of the cell lines, whereas that of LMP2 was increased by SAHA (Fig. 5). Next, the EBNA1 and LMP1 protein levels were determined by immunoblotting. SAHA decreased the EBNA1 protein level in all cell lines, and that of LMP1 in the SNT16, KAI3 and SNK6 cell lines (Fig. 6).

***In vivo* effects of suberoylanilide hydroxamic acid using the mouse xenograft model.** After confirmation of the *in vitro* effect of SAHA, we extended our work to an *in vivo* xenograft

model. Initially, we inoculated six T and NK cell lines into immunodeficient NOG mice via various routes. Of the EBV-positive T or NK cell lines used, only the SNK6 cell line was engrafted after subcutaneous or intravenous inoculation (Suppl. Table S1). The Jurkat cell line, which is EBV-negative and IL-2 independent, could also be engrafted, raising the possibility that IL-2 dependency may be associated with the engraftment. We cultured six cell lines with the different concentration of IL-2, and found that SNK6 was less dependent of IL-2 compared with other T/NK cell lines (Suppl. Fig. S1). We considered that the independency of IL-2 can explain the success of engraftment, at least partially. Because evaluation of the former was easier, the subcutaneous model was used in subsequent experiments.

We subcutaneously inoculated 1×10^6 SNK6 cells into NOG mice. All of the mice developed tumors at the site of inoculation. Four days after the inoculation, mice were treated with SAHA daily up to day 28. The treated mice normally
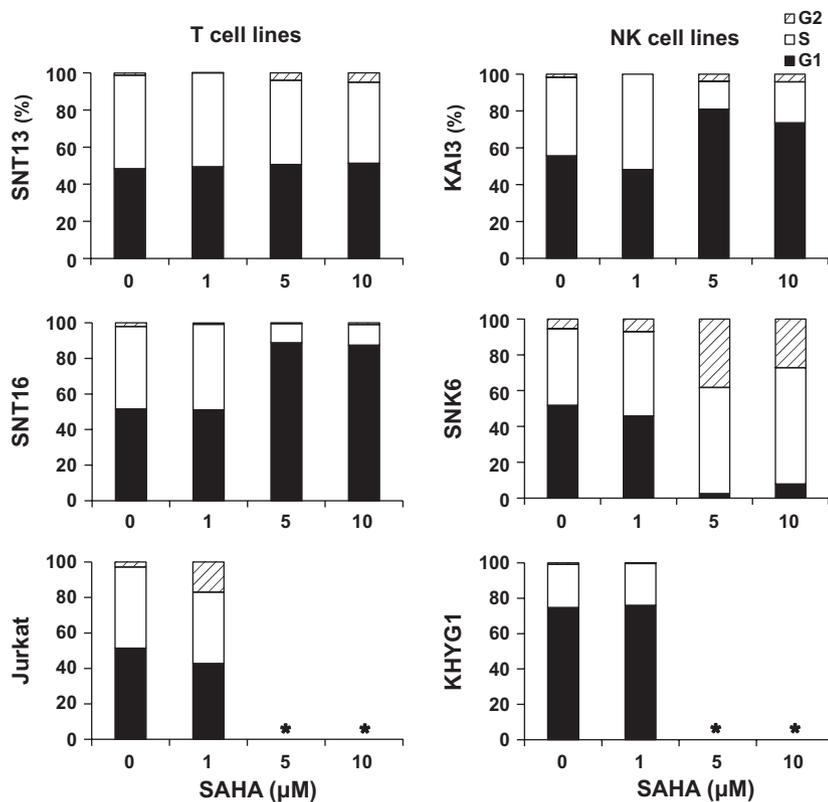
Fig. 3. Suberoylanilide hydroxamic acid (SAHA) induces apoptosis in several T and natural killer (NK) cell lines. (a) Epstein–Barr virus (EBV)-positive and EBV-negative T and NK cell lines were treated with 5 μM SAHA for 24 h. Viable cells were defined as those negative for both annexin V-PE and 7-AAD staining, and early apoptotic cells were defined as those positive for annexin V-PE but negative for 7-AAD staining. (b) T and NK cell lines were treated with the indicated concentrations of SAHA for 24 or 48 h. The cleavage of poly (ADP-ribose) polymerase (PARP) was detected by immunoblotting. β-Actin was used as a loading control.

tolerated SAHA without showing any obvious toxicity. During this period, no significant difference in the body weights of SAHA-treated and control mice was noted (data not shown). Until the end of the experiment, the size of tumors in SAHA-treated mice increased gradually, but the tumor volume was significantly less than the control group (Fig. 7a). EBER ISH showed the extent of the tumor in each mouse (Fig. 7b). In the SAHA-treated mouse, the tumor was regressed with degenera-

tion. Additionally, SAHA-treated mice showed a significantly lower plasma EBV-DNA level (Fig. 7c). Furthermore, SAHA showed significant inhibitory effects on most EBV-encoded genes in tumor tissues (Fig. 7d). Finally, we collected samples from organs at 30 days after inoculation and performed EBER ISH. EBER-positive cells were detected in the organs of control mice, but not SAHA-treated mice (Fig. 7e). In the spleen, liver and lung, EBER-positive cells were sporadically

Fig. 4. Suberoylanilide hydroxamic acid (SAHA) arrests the cell cycle in T and natural killer (NK) cell lines. After treatment with the indicated concentrations of SAHA for 48 h, T and NK cell lines were fixed and stained with propidium iodide. Cell cycle profiles were assessed by flow cytometry. *Because of cell death, the cell cycle assay was indeterminable.

observed in focal lesions, indicating hematogenous dissemination of tumor cells. Conversely, the expansion of EBER-positive cells from the renal capsule to parenchyma was observed in the kidney, indicating direct invasion. These results indicated that SAHA inhibited metastasis and invasion of lymphoma cells.

Discussion

Histone deacetylase inhibitors affect tumor cell growth and survival through the induction of cell death by their characteristics of apoptosis.[6] By the upregulation of CDKNIA, HDAC inhibitors induce cell cycle arrest at the G1/S-phase. Moreover, through elongation of G2-phase, HDAC inhibitors can mediate G2/M-phase arrest, but this event occurs less frequently than G1 arrest. HDAC inhibitors can also reduce the expression of proangiogenic factors, resulting in the suppression of angiogenesis. Furthermore, HDAC inhibitors show immunomodulatory effects, which enhance tumor cell antigenicity and alter the expression of key cytokines, such as tumor necrosis factor-α, interleukin-1 and interferon-γ.[6] In the present study, SAHA markedly suppressed the proliferation of T and NK lymphoma cell lines, irrespective of the presence of EBV. The suppressive effect of SAHA was greater than that of valproic acid as demonstrated in our previous study.[8] In several T and NK cell lines, SAHA-induced apoptosis was confirmed by the increase in annexin V-positive cells and cleavage of PARP. SAHA also induced cell cycle arrest in several T and NK cell lines. The mechanism of killing appeared to differ among the cell lines. A recent study by Karube et al.[15] shows that suppression of the JAK-STAT pathway contributes to the suppressive effect of SAHA against NK cell lymphoma cells. Given the pleiotropic biological effects of HDAC inhibitors, it is unlikely that a single molecular pathway leading to tumor cell death will be identified in all cell types.[6]

Suberoylanilide hydroxamic acid has been reported to induce EBV lytic infection in EBV-positive gastric and nasopharyngeal carcinoma cells.[13,14] For the treatment of EBV-associated malignant diseases, induction of lytic infection is advantageous because it causes lysis of EBV-infected tumor cells. Furthermore, lytic infection should produce viral proteins with antigenicity that could induce host cellular responses. BZLF1 is an immediate-early gene and a hallmark to switch from latent gene to lytic infection.[37] In the present study, SAHA increased the expression of BZLF1 in most EBV-positive T and NK cell lines, although the late lytic gene gp350/220 was increased in only one cell line. The lytic infection induced by SAHA may play a role in its effects on EBV-infected T and NK cells. Interestingly, BZLF1, which was not expressed in the SNK-6 cell line in vitro, was expressed in the SNK6-derived tumor from both control and SAHA-treated mice. We speculate that the expression of BZLF1 was induced in in vivo culture conditions presumably by nutrients or cytokines, although there is no direct poof of this.

In the present study, SAHA decreased the expression of the LMP1 gene and protein in some EBV-positive T and NK cell lines. LMP1 is a major oncoprotein that is responsible for the immortalization of primary human B lymphocytes and activation of the NF-κB, PI3K and JNK pathways.[38] Expression of LMP1 induces several pleiotropic effects, including the upregulation of adhesion molecules, anti-apoptotic proteins and cytokines. Recently, we showed that heat shock protein 90 inhibitors repress the LMP1 expression and proliferation of EBV-positive NK cell lymphoma.[35] SAHA also decreased the expression of EBNA1 in all of the cell lines. EBNA1 is essential for the maintenance of the viral episome, as well as for the initiation of latent viral replication.[38] EBNA1 also
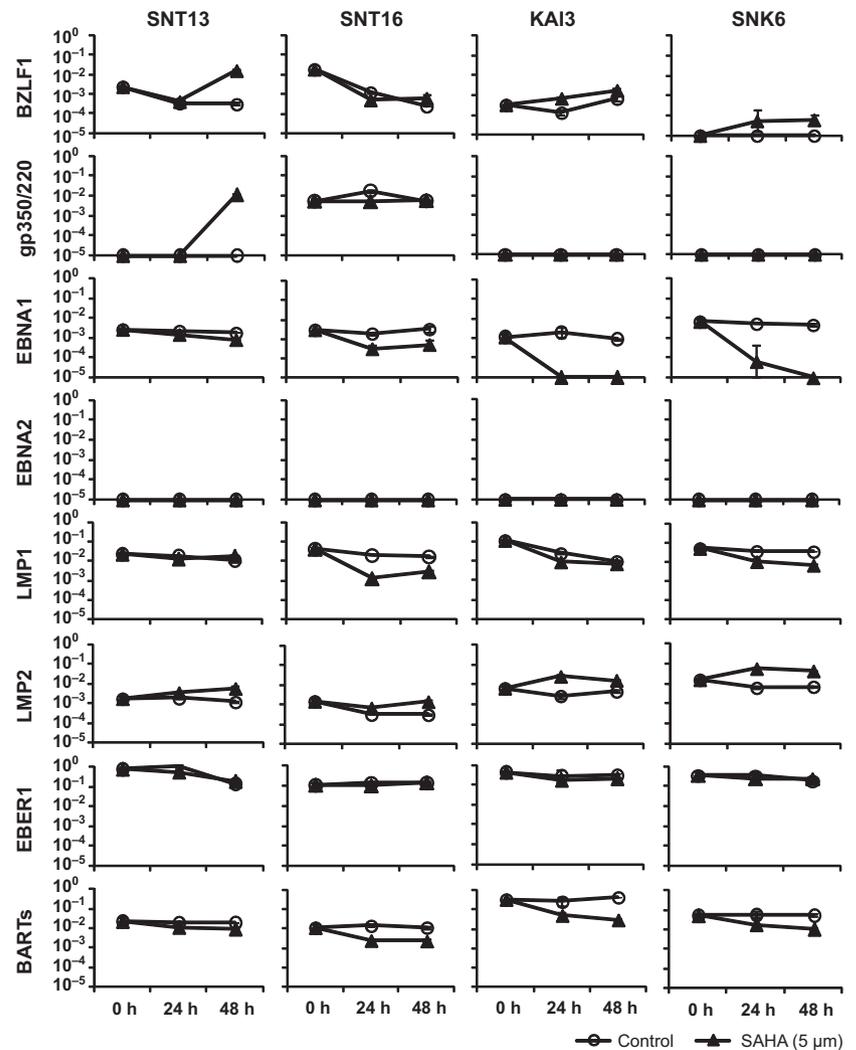
Fig. 5. Suberoylanilide hydroxamic acid (SAHA) increases the expression of Epstein–Barr virus (EBV)-lytic genes and decreases the expression of EBV-latent genes. EBV-positive T cell lines and natural killer (NK) cell lines were treated with 5 μM SAHA (closed triangles) or control (open circles) and cultured for 24 or 48 h. EBV-encoded gene expression was analyzed using real-time RT-PCR. β2-microglobulin was used as a reference gene for relative quantification, and was assigned an arbitrary value of 1 (10^0). Data are expressed as means ± SEM.
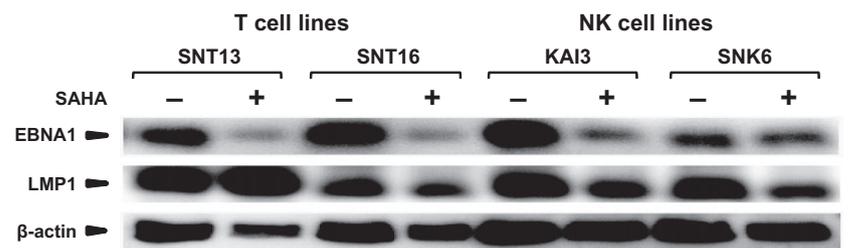
Fig. 6. Suberoylanilide hydroxamic acid (SAHA) decreases the expression of LMP1 and EBNA1 proteins in Epstein–Barr virus (EBV)-positive T and natural killer (NK) cell lines. EBV-positive T and NK cell lines were treated with 5 μM SAHA or DMSO for 48 h. EBNA1 and LMP1 were detected by immunoblotting. β-Actin was used as a loading control.

plays an important role in inhibiting apoptosis.[39] Downregulation of EBNA1 may also be associated with the suppressive effect on the proliferation of EBV-positive T and NK cell lines. Although SAHA downregulated EBV-encoded genes such as EBNA1 and LMP1, the effects of SAHA did not differ between EBV-positive and EBV-negative cell lines. The discrepancy is not clear. The EBV-positive and EBV-negative cell line sets, which were used to verify the presence/absence of EBV in the T and NK cell lines (Fig. 2a), might be inappropriate for the purpose. The EBV-positive cell lines were produced by artificial EBV infections using marker selections.[26,28] The parent cell lines can proliferate vigorously, so they did not need the help of EBV. It is also possible that the change of EBV-associated genes and proteins may be not

the cause, but the result. These questions should be clarified in future studies.

We applied the murine xenograft model to further evaluate the efficacy of SAHA. Using this model, we have shown that SAHA prevented not only tumor growth but also metastasis of EBV-positive NK cell lymphoma. However, the progressive tumor growth was renewed subsequently, suggesting a limitation of single-agent therapy. Synergistic effects of HDAC inhibitors and their combination with mTOR inhibitors have been demonstrated in renal cell and prostate carcinoma cell lines.[40,41] In nasopharyngeal carcinoma, bortezomib and SAHA synergistically induced reactive oxygen species-driven caspase-dependent apoptosis and blocked the replication of EBV.[42] The combination of these agents and SAHA could
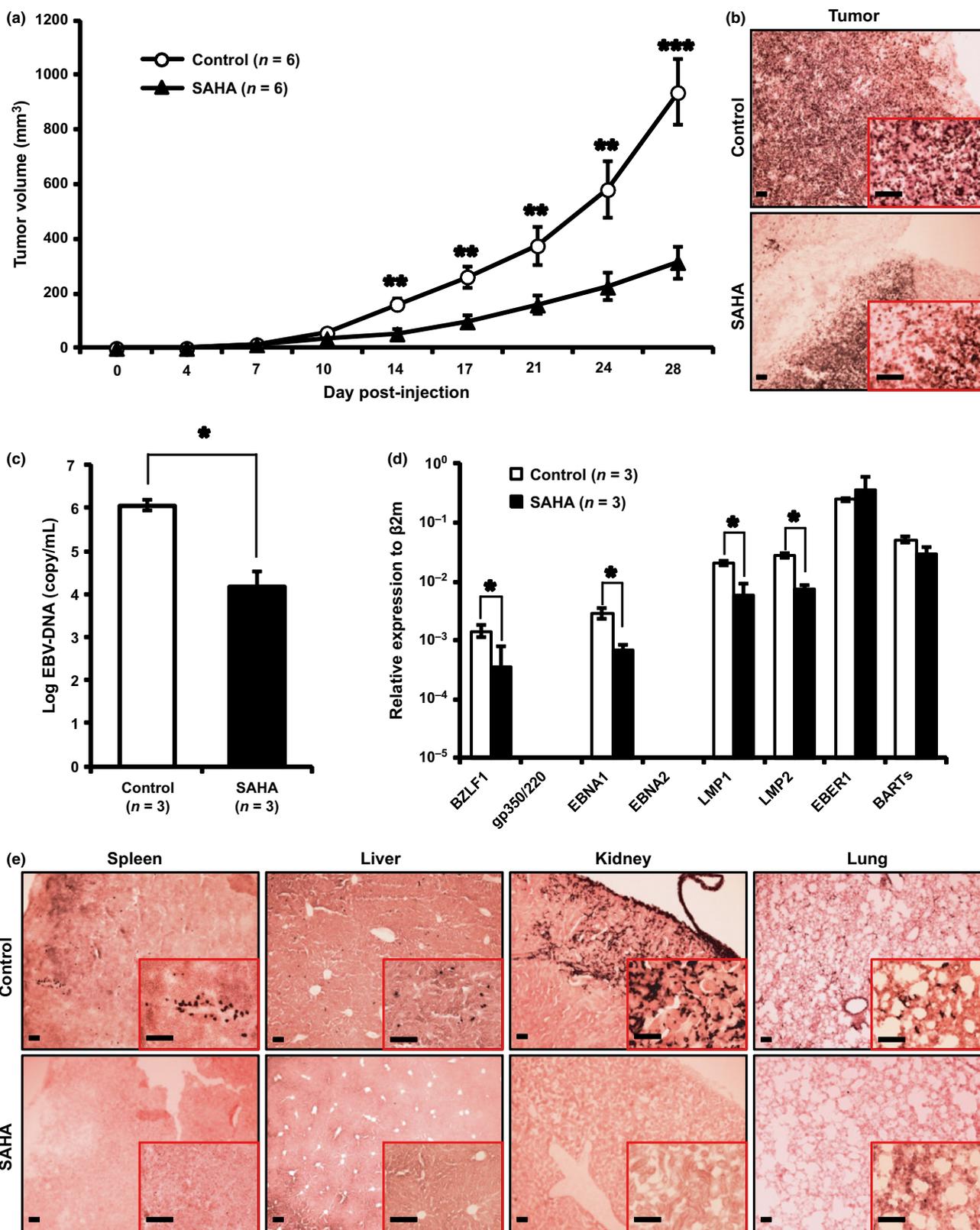
Fig. 7. Suberoylanilide hydroxamic acid (SAHA) inhibits tumor growth and metastasis of Epstein–Barr virus (EBV)-positive natural killer (NK) cell lymphoma. (a) SNK6 cells (1×10^6 per mouse) were inoculated subcutaneously into NOG mice. Mice were treated with 100 mg/kg SAHA or DMSO (control) daily from days 4 to 28. Tumor sizes were measured twice per week. (b) At 30 days after inoculation, mice were killed, and Epstein–Barr virus-encoded small RNA-positive cells were detected by *in situ* hybridization in tumor tissues of SAHA-treated or control mice (scale bars: 200 μm). (c) At 30 days after inoculation, peripheral blood was collected, and plasma was separated. EBV-DNA was quantified by real-time PCR. (d) At 30 days, EBV-related gene expression in tumor tissues was quantified. β2-Microglobulin was used for relative quantification and assigned an arbitrary value of 1 (10^0). (e) EBER-positive cells, which meant EBV-positive lymphoma cells, were detected by *in situ* hybridization in organ tissues of SAHA-treated or control mice (scale bars: 200 μm). *$P < 0.05$, **$P < 0.01$, ***$P < 0.002$ by Mann–Whitney U-test.

improve the therapy of EBV-associated T and NK cell lymphoma.

In conclusion, SAHA suppressed the proliferation of T and NK cell lines, although no significant difference was observed between EBV-positive and EBV-negative cell lines. SAHA induced apoptosis and/or cell cycle arrest in some T and NK cell lines. Furthermore, SAHA inhibited tumor progression and metastasis in a murine xenograft model. Thus, SAHA had a marked suppressive effect against EBV-associated T and NK cell lymphomas, which was mediated by either induction of apoptosis or cell cycle arrest, and could represent an alternative treatment.

Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. SNK6 is less dependent of human interleukin-2 (IL-2) compared with other Epstein–Barr virus (EBV)-positive T and natural killer (NK) cell lines.

Table S1. Engraftment of cell lines into NOG mice.