main causes of death were multiple organ failure (n = 10), hepatic failure (n = 6), heart failure (n = 5), pulmonary failure (n = 5), sepsis (n = 5), intracranial hemorrhage (n = 5), intestinal hemorrhage or perforation (n = 3), hemophagocytic syndrome (n = 2), and other (n = 6). Of the 47 patients who died, 20 (42%) died after transplantation. Of the 61 surviving patients, 41 were in CR and 4 were in PR without any symptoms, whereas 16 remained in stable disease at the last follow-up.

Clonality analysis

At the time of diagnosis, viral clonality was analyzed by Southern blot analysis using EBV terminal repeat. Of 76 patients with available DNA, EBV-infected cells were monoclonal in 64 (84%) and oligoclonal in 8 (11%). Polyclonal EBV-infected cells were detected in only 4 patients (5%). TCR rearrangement was analyzed in 90 patients at the time of diagnosis, 42 of whom had monoclonal rearrangements. Six patients with NK-cell infection demonstrated TCR rearrangement. Because this analysis uses a PCR-based method, erroneous detection of a seemingly clonal cell population (pseudoclonality) or reduced TCR diversity caused by the prevalence of a few Ag-selected subclones, which are often seen in EBV infection, may occur.[42] Chromosomal aberrations were detected in the peripheral blood or lymph nodes at diagnosis in 6 patients, whereas an additional 6 patients later developed chromosomal aberrations in their clinical course of 1-9 years (median, 5 years). Patterns of chromosomal aberrations in each patient are shown in supplemental Table 2. These results provided additional support to the assertion that patients with EBV$^+$ T/NK-LPDs had clonality at early stages and subsequently developed overt lymphoma or leukemia with an increase of chromosomal aberrations in their clinical course.

Pathologic categories based on the 4th WHO classification

At the time of diagnosis, based on the 4th WHO classification, 53 and 13 patients were classified into systemic EBV$^+$ T-LPD of childhood and hydroa vacciniforme–like lymphoma, respectively. The proportion of these pathologic categories in each clinical group is shown in Figure 1C. Four patients clinically categorized to hydroa vacciniforme without any cellular atypia or systemic symptoms were classified into hydroa vacciniforme–like lymphoma based on the monoclonality of cells with TCR rearrangements. In systemic EBV$^+$ T-cell LPD, T-cell subsets of EBV-infected cells were variable (Figure 1D). In hydroa vacciniforme–like lymphoma, 6 of 13 patients had γδ T-cell infection. Conversely, 42 patients were not classified into either of these pathologic categories because they failed to correspond to criteria in the current WHO classification. Classification of each patient is shown in supplemental Table 1.

At the last follow-up or death, there were 29 patients who were unclassifiable, most of whom had CAEBV of the NK-cell type and severe mosquito bite allergy with NK-cell infection (Figure 1D). In the clinical course, ENKL developed in 6 patients (patients 2, 5, 20, 34, 60, and 81 in supplemental Table 1) after 9 months to 12 years of follow-up after onset (median, 1.5 years), whereas ANKL developed in 4 patients (patients 8, 43, 66, and 80) after 2-17 years of follow-up (median, 12 years); most of these patients had NK-cell infection. EBV$^+$ PTCL developed in 3 patients after 1 year (patient 83), 5 years (patient 93), and 20 years (patient 53) of follow-up. The EBV$^+$ PTCL patients in this study were characterized by their expression of cytotoxic molecules, nodal manifestation, lack of CD56 expression, and TCR gene rearrangement. These features

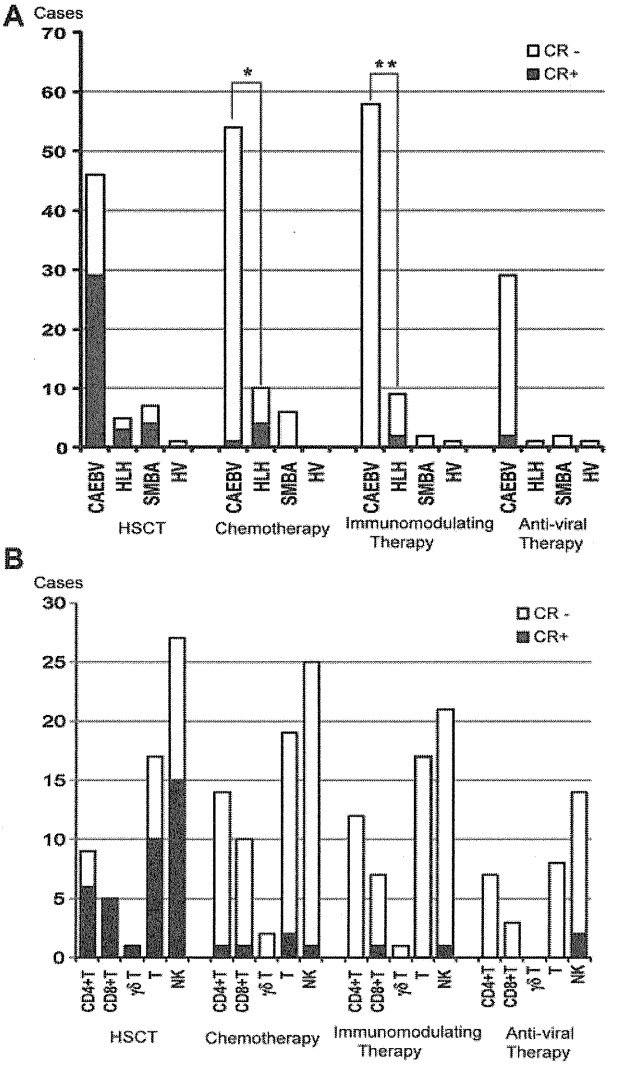

Figure 4. Efficacy of therapeutic interventions. (A) Number of patients treated with each therapy and patients who maintained CR are shown among categories of clinical groups. SMBA indictes severe mosquito bite allergy; and HV, hydroa vacciniforme. *P = .002; **P = .02. (B) Numbers of patients who received each therapy and those who maintained sustained CR are shown among categories of EBV-infected cells.

suggest a pathologic distinction between these EBV$^+$ PTCL and extranasal ENKL.

Representative results of histological examinations are shown in Figure 2. Histological findings and the number of EBER$^+$ cells varied among patients. EBER$^+$ lymphocytes were detected at various frequencies. Infiltrating cells (presumably EBV-infected) expressed cytotoxic molecules such as TIA-1, perforin, and granzyme B. BM aspirations showed various findings, but most patients had normocellular BM without any abnormal findings. Patients with EBV-associated HLH showed normoplastic or hyperplastic BM with mild or moderate hemophagocytosis. In all patients, however, BM findings showed an absence of hematologic malignant disorders at the time of diagnosis.

Differences between patients with T-cell and NK-cell infection

We compared clinical and virological differences between T- and NK-cell infections (Table 2). T-cell infection was characterized by higher rates of primary EBV infection and TCR rearrangement,

Table 3. Univariate and multivariate analyses of factors associated with mortality in 108 patients with EBV$^+$ T/NK-LD

	Univariate analysis		Multivariate analysis†	
	OR (95% CI)	P*	OR (95% CI)	P‡
Female sex	**1.40 (0.98-1.97)**	**.048**	1.26 (0.48-3.31)	.64
Age at disease onset (≥ 8 years)§	**1.63 (1.17-2.28)**	**.003**	**4.43 (1.61-12.2)**	**.004**
Past history of infectious mononucleosis	0.62 (0.35-1.11)	.093	0.36 (0.12-1.02)	.054
Primary infection at diagnosis	0.47 (0.18-1.20)	.079	0.32 (0.08-1.25)	.10
Clinical entity at diagnosis				
CAEBV	1.12 (0.90-1.39)	NS		
HLH	0.65 (0.24-1.77)	NS		
Severe mosquito bite allergy	1.04 (0.30-3.65)	NS		
Hydroa vacciniforme	0.43 (0.05-4.03)	NS		
T-cell infection	1.13 (0.69-1.71)	NS		
NK-cell infection	0.95 (0.69-1.30)	NS		
EBV DNA in mononuclear cells (≥ $10^{4.5}$ copies/μg DNA)	1.16 (0.79-1.71)	NS		
EBV DNA in plasma (≥ $10^{3.5}$ copies/mL)	1.23 (0.84-1.72)	NS		
EBV monoclonality	1.08 (0.89-1.31)	NS		
TCR rearrangement	1.13 (0.73-1.76)	NS		
Chromosomal aberration	1.92 (0.34-10.9)	NS		
Symptoms and signs at diagnosis				
Fever	1.10 (0.98-1.24)	NS		
Liver dysfunction	**1.33 (1.09-1.63)**	**.006**	**4.25 (1.23-14.7)**	**.022**
Splenomegaly	**1.38 (1.01-1.88)**	**.033**	¶	
Anemia	**1.84 (1.18-2.88)**	**.005**	1.36 (0.31-6.01)	.68
Thrombocytopenia	**1.75 (1.13-2.71)**	**.009**	1.80 (0.44-7.33)	.41
Lymphadenopathy	1.24 (0.77-2.00)	NS		
Hemophagocytic syndrome	1.30 (0.72-2.32)	NS		
Hypersensitivity to mosquito bites	0.89 (0.69-1.15)	NS		
Hydroa vacciniforme-like eruption	0.86 (0.34-1.97)	NS		
Chemotherapy	0.84 (0.53-1.34)	NS		
HSCT	**0.67 (0.045-0.98)**	**.022**	**0.34 (0.12-0.96)**	**.041**
T-cell infection group	**0.54 (0.30-0.97)**	**.021**		
NK-cell infection group	0.83 (0.51-1.34)	NS		

NS indicates not significant.

*$P < .10$ are shown; $P < .05$ (shown in bold) are statistically significant.

†For multivariate analysis, factors with $P < .10$ were included.

‡$P < .05$ (shown in bold) are statistically significant.

§Stratified onset ages were analyzed in advance, and ≥ 8 years was chosen as the age factor.

¶Splenomegaly was excluded from multivariate analysis, because this factor was closely associated with anemia, thrombocytopenia, and liver dysfunction.

whereas a significant number (43%) of patients with NK-cell infection had hypersensitivity to mosquito bites (Table 2). Interestingly, 5 patients had both hypersensitivity to mosquito bites and hydroa vacciniforme–like eruptions; these patients all had NK-cell infection (Table 2). Conversely, 8 of 10 patients with hydroa vacciniforme–like eruptions but without hypersensitivity to mosquito bites had T-cell infections (Table 2).

A comparison of viral load in the peripheral blood between patients with T- and NK-cell infections detected similar levels of EBV-DNA in both PBMCs and plasma (Table 2). Correlation of viral loads between PBMCs and plasma was estimated (Figure 3A). The quantity of EBV-DNA in PBMCs was significantly correlated with that in plasma in both T-cell and NK-cell infections, although EBV-DNA was not detected in the plasma from 15 patients. We also compared viral load among clinical groups (Figure 3B-C). Interestingly, the quantity of EBV-DNA in PBMCs was significantly higher in patients with severe mosquito bite allergy and hydroa vacciniforme, but these patients did not have any systemic symptoms.

Efficacy of therapeutic interventions

Each patient received a variety of therapies. HSCT was administered to 59 patients, which induced sustained CR in 63% of patients with CAEBV, 60% of HLH patients, and 57% of severe mosquito bite allergy patients (Figure 4A). Seventy patients received chemotherapy such as etoposide/cyclosporine A/dexamethasone, cyclophosphamide/doxorubicin/vincristine/prednisolone (CHOP), CHOP plus etoposide, and high-dose cytosine arabinoside therapy. Chemotherapy was effective in some patients, but the effect was usually transient and failed to induce sustained CR in most cases. Chemotherapy induced sustained CR in only 5 patients, 4 of whom had HLH (Figure 4A). Immunomodulating therapies such as prednisolone, cyclosporine A, high-dose IV immunoglobulin, and methyl prednisolone pulse therapy were administered to 58 patients. The immunomodulating therapies induced sustained CR in 2 patients with HLH (Figure 4A). In patients with HLH, both chemotherapy and immunomodulating therapy induced sustained CR more frequently compared with those with CAEBV ($P = .002$ and $P = .02$, respectively). Antiviral therapies such as acyclovir, adenine arabinoside, and ganciclovir were administered to 32 patients. In 2 patients (patients 11 and 45 in supplemental Tale 1), sustained CR was achieved during oral acyclovir therapy and weekly IV administration of adenine arabinoside (Figure 4A). However, because antiviral therapies had been administered for a long time, it was not clear whether CR was induced by them or if it was spontaneously achieved.

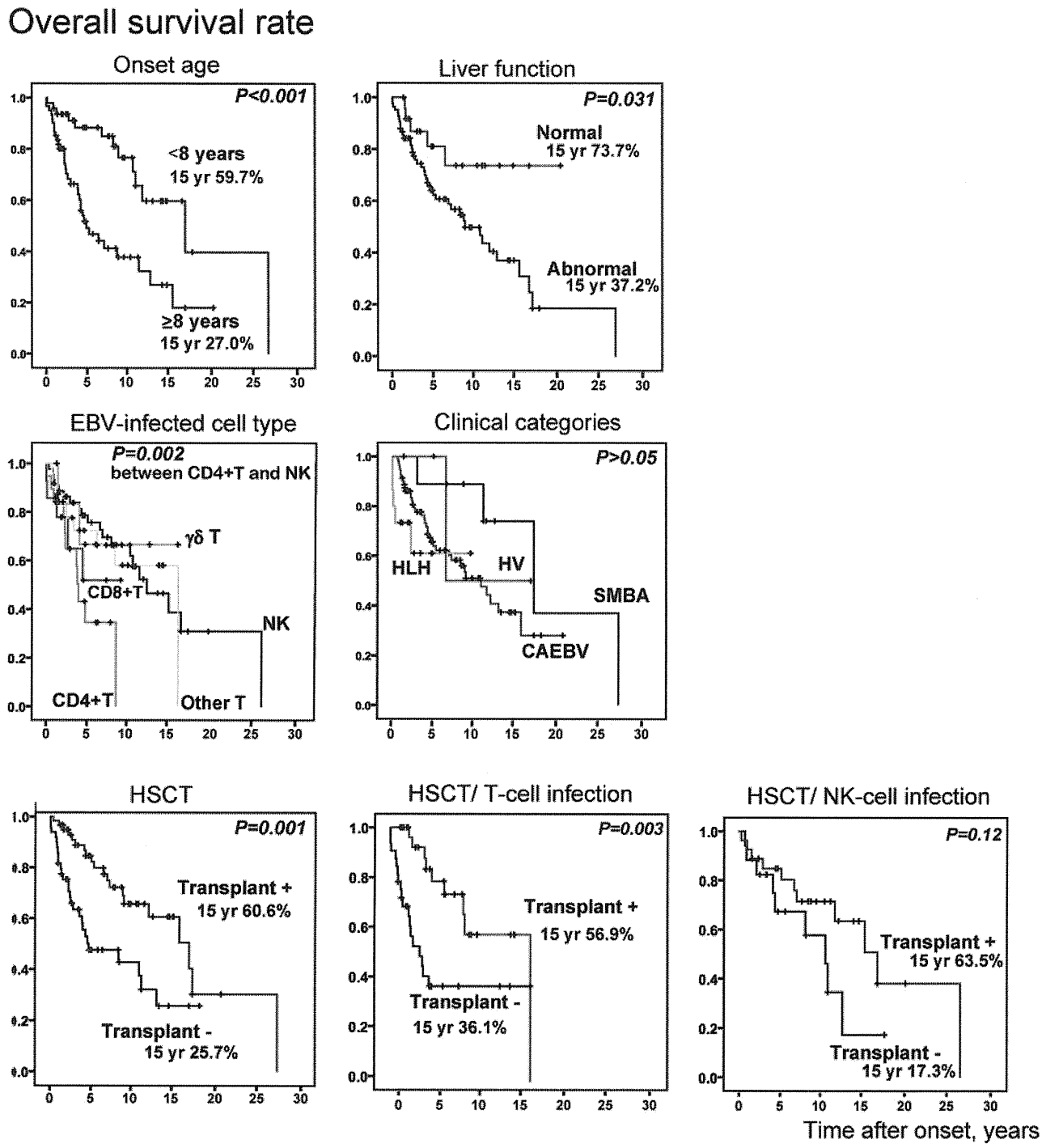

Figure 5. Probability of survival rates from time of disease onset. Overall survival rates from onset (n = 108) were calculated from Kaplan-Meier estimates between each subgroup (onset age ≥ 8 years or < 8 years, with or without liver dysfunction, EBV-infected cell types, clinical categories, and with or without HSCT). HSCT patients were divided into groups based on T-cell infection (n = 64) and NK-cell infection (n = 44) and independently analyzed. SMBA indicates severe mosquito bite allergy; and HV, hydroa vacciniforme.

The effects of each therapy among cell types are shown in Figure 4B. There was no statistical difference in the CR rate of each therapy among cell types.

Factors associated with mortality

The factors associated with mortality were analyzed (Table 3), and univariate analysis showed that sex (female), onset age (≥ 8 years), liver dysfunction, splenomegaly, anemia, and thrombocytopenia were significantly associated with mortality. Conversely, HSCT was inversely correlated with mortality rate (odds ratio, 0.67), and this was statistically significant only in patients with T-cell infection. Multivariate analysis using factors for which $P < .10$ revealed that onset age and liver dysfunction were independently significant factors that increased mortality (Table 3); again, HSCT was an independent factor that decreased mortality rate.

Table 4. Comparison of characteristics based on outcome in 59 patients after transplantation

	Total (n = 59)	Alive (n = 39)	Dead (n = 20)	P*
Sex, male/female	29/30	22/17	7/13	NS
Age at disease onset, y	11.8 ± 9.2	11.0 ± 9.0	13.6 ± 9.5	NS
Clinical category at diagnosis, n				
CAEBV	46	32	14	NS
HLH	5	3	2	NS
Severe mosquito bite allergy	7	4	3	NS
Hydroa vacciniforme	1	0	1	NS
EBV DNA quantity in peripheral blood at diagnosis				
Mononuclear cells, log copies/μg DNA	4.5 ± 0.8	4.4 ± 0.9	4.5 ± 0.89	NS
Plasma, log copies/mL	3.3 ± 1.6	3.3 ± 1.3	3.3 ± 2.0	NS
T-cell infection, n	32	23	9	NS
NK-cell infection, n	27	16	11	NS
Age at HSCT, y	17.5 ± 9.23	**15.6 ± 9.1**	**21.2 ± 8.3**	**.034**
Time from onset to HSCT, mo	65.0 ± 68.2	52.2 ± 54.7	90.0 ± 84.8	.059
Disease status at transplantation, active/inactive	25/34	**13/26**	**12/8**	**.046**
Preceded chemotherapy, n (%)	42 (71)	27 (69)	15 (75)	NS
Stem cell source, BM/peripheral blood/cord blood	35/11/13	22/8/9	13/3/4	NS
Donor, MRD/MUD/MMRD/MMUD	18/11/4/26	10/9/3/17	8/2/1/9	NS
No of mismatched HLA	0.76 ± 0.9	0.76 ± 0.9	0.75 ± 0.9	NS
Preconditioning regimen, myeloablative/reduced	21/38	11/28	10/10	.086

NS indicates not significant; MRD, matched related donor; MUD, matched unrelated donor; MMRD, mismatched related donor; and MMUD, mismatched unrelated donor.
*$P < .10$ are shown; $P < .05$ (shown in bold) are statistically significant.

We compared overall survival rates between each subgroup to confirm association of the above factors with mortality (Figure 5). Overall survival rate in patients whose onset was more than 8 years was significantly low ($P < .001$). Patients with liver dysfunction at the time of diagnosis had lower survival rate ($P = .031$). When patients were divided into 5 groups based on EBV-infected cells, patients with $CD4^+$ T-cell infection had a significantly lower survival rate compared with those with NK-cell infection ($P = .002$). However, there was no statistical difference in survival rate among clinical groups, although the numbers in some groups were small. Patients who received HSCT survived longer ($P = .001$) and, again, this was statistically significant only in patients with T-cell infection ($P = .003$).

Characteristics of patients after HSCT

Of 59 patients who underwent HSCT, 39 patients (66%) survived 1-144 months after transplantation (median, 35.5 months). Conversely, 20 patients (34%) died 1 day to 48 months after transplantation (median, 1.8 months). Detailed characteristics of each patient are shown in supplemental Table 3. Main causes of death were multiple organ failure (n = 5), intracranial hemorrhage (n = 5), sepsis (n = 2), and other (n = 8). Of the 20 deaths, 15 were considered to be treatment related. We compared various factors between patients who lived and those who died after HSCT (Table 4). Univariate analysis showed that age at HSCT was higher and patients with active disease status at the time of HSCT died more frequently after HSCT (Table 4). Time from disease onset to HSCT and intensity of the conditioning regimen (either myeloablative or reduced) were marginally associated with death ($P = .059$ and $P = .086$, respectively). To determine independent risk factors, we performed multivariate analysis using factors for which $P < .10$, and found that none was an independent risk factor for death (data not shown).

We compared overall survival rates (Figure 6A) and event-free survival rates (Figure 6B) of transplanted patients between each subgroup. Although disease status at HSCT was not an independent risk factor by multivariate analysis, overall survival rate was significantly higher in patients with inactive disease at the time of HSCT ($P = .014$); however, its significance diminished for the event-free survival rate. Patients who received HSCT at an age less than 15 years had significantly higher overall ($P = .013$) and event-free survival rates ($P = .015$). Patients whose time from onset to HSCT was less than 30 months also had significantly higher overall ($P = .036$) and event-free survival rates ($P = .033$). Interestingly, these were statistically significant only in patients with T-cell infection.

Discussion

Determining the phenotype of EBV-infected cells is mandatory for our further understanding of the pathogenesis of EBV^+ T/NK-LPDs and related biologic behaviors. In the present study, we used unfixed peripheral blood to determine the phenotypes of EBV-infected cells. One caveat of this study is that we may have missed EBV-associated T/NK-LPDs if EBV-infected cells failed to migrate into the peripheral blood.[33] Furthermore, EBV-infected cells in the peripheral blood might be different from those existing in tissues, although there was no discordant result between tissue biopsy and peripheral blood.

In the present study, EBV-infected cells in EBV^+ T/NK-LPDs were immunophenotypically divided into $CD4^+$ T cells, $CD8^+$ T cells, γδ T cells, and NK cells, the variable proportions of which were observed in each of the clinical categories. Kasahara et al reported that CAEBV and EBV-associated HLH were largely caused by $CD4^+$ T or NK cells and $CD8^+$ T cells, respectively.[22] We demonstrated that CAEBV was caused by not only $CD4^+$ T and NK cells but also by $CD8^+$ T and γδ T cells. We also demonstrated that EBV-infected cells in nearly half of hydroa vacciniforme or hydroa vacciniforme–like lymphoma patients were γδ T cells, which is in agreements with our previous observations.[36] Interestingly, all of these cells express molecules characteristic of cytotoxic cells. In fact, $EBER^+$ lymphocytes in EBV^+ T/NK-LPDs usually express cytotoxic molecules including perforin, granzyme B, and TIA-1, as shown in this study and in previous studies.[7,44]

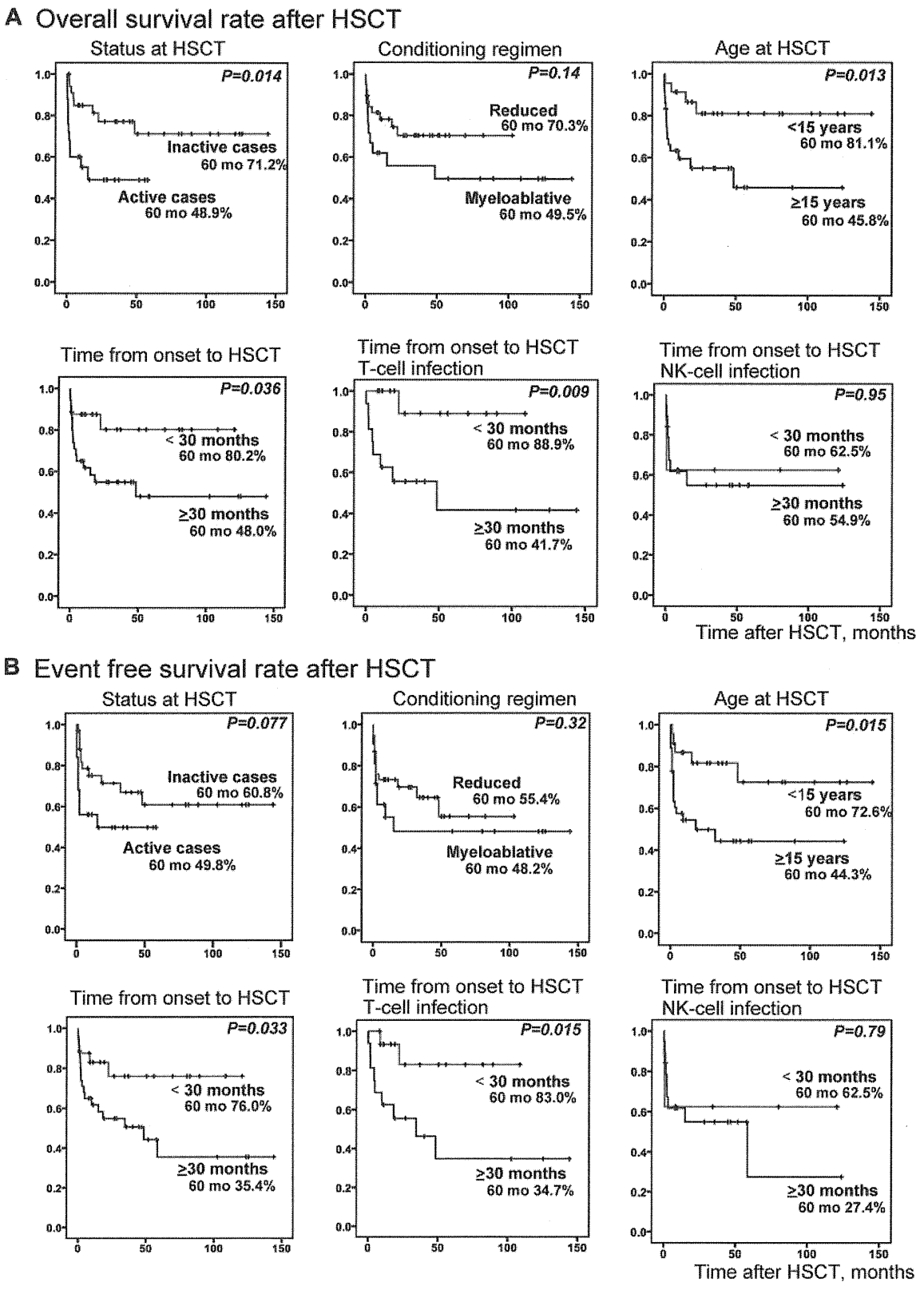

Figure 6. Probability of survival rates after HSCT. Survival rates after HSCT were calculated from Kaplan-Meier estimates between each subgroup (inactive or active cases at HSCT, reduced or myeloablative conditioning, age ≥ 15 years or < 15 years at HSCT, and time from onset to HSCT ≥ 30 months or < 30 months). Stratified ages were analyzed in advance, and ≥ 15 years was chosen as the age factor. Similarly stratified times from onset to HSCT were analyzed in advance, and ≥ 30 months was chosen as the time factor. (A) Overall survival rate after HSCT (n = 59). (B) Event-free survival rate after HSCT (n = 59). For time from onset to HSCT, patients were divided into T-cell infection (n = 32) and NK-cell infection (n = 27) groups and independently analyzed.

The mechanism underlying EBV infection of T and NK cells, which do not express CD21, remains unresolved. It has been shown that NK cells activated by EBV-infected B cells acquire CD21 by synaptic transfer, and these ectopic receptors allow EBV binding to

NK-cell hosts.[45] It is plausible that killer cells in close contact with EBV-infected B cells may acquire EBV infection directly and then proliferate with clonality.

In the present study, we evaluated prognostic factors among patients with EBV$^+$ T/NK-LPDs. Multivariate analysis showed that age at onset of disease ($\geq$ 8 years) and liver dysfunction were independent risk factors for mortality, and that patients receiving transplantations had a better prognosis. We found previously that older onset age ($\geq$ 8 years) was associated with mortality in patients with CAEBV.[29] Furthermore, a recent report demonstrated that adult patients with CAEBV had progressive and more aggressive courses than those of childhood onset cases.[46] Interestingly, patients with CD4$^+$ T-cell infection had shorter survival rates than those with NK infection, whereas clinical categories were not correlated with survival rates. Onset age of patients with CD4$^+$ T-cell infection was high (median, 14.5 years). These results suggest that adult patients with CD4$^+$ T-cell infection may have more aggressive features and are likely to develop multiple organ failure. Although the reason is unclear, we should be cautious about rapid progression in patients with CD4$^+$ T-cell infection.

We surveyed administered therapies based on physician questionnaire responses. A potential limitation of this study design was the use of retrospective questionnaires; therefore, we should be cautious about the evaluation of treatment efficacy. Nevertheless, it seems that only HSCT induced CR in patients with EBV-associated T/NK-LPDs except for HLH. Some EBV-associated HLH patients responded well to chemotherapy and immunomodulating therapies,[47] but patients with CAEBV were generally refractory to chemotherapy. Similar findings were reported in patients with CAEBV in the United States.[20] Furthermore, Kaplan-Meier estimates indicated that shorter time from onset to HSCT ($<$ 30 months) and inactive disease at HSCT resulted in long survival times, suggesting that earlier HSCT in patients in good condition is preferred. Patients with CAEBV have a higher risk of transplantation-related complications.[41,48] Recently, Kawa et al reported excellent outcome of HSCT with reduced-intensity conditioning.[40] Although the superiority of reduced-intensity conditioning over myeloablative conditioning did not reach statistical significance in that study, it appears that a reduced-intensity regimen is sufficient to prevent transplantation-related deaths.[40,49]

The concept of EBV$^+$ T/NK-LPD was initially proposed by Kawa et al, and then examined by other researchers.[27,44] This umbrella term encompasses specific clinical diseases of the CAEBV T/NK–cell type, EBV-associated HLH, severe mosquito bite allergy, and hydroa vacciniforme, the distinction of which are differentiated based on clinical manifestations. However, if the clinical data are absent regarding the prodromal phase of expansion of EBV$^+$ T/NK–cells with variable clonality, we cannot discriminate systemic diseases such as ANKL and extranasal ENKL from EBV$^+$ NK-LPDs, because EBV$^+$ proliferating cells are indistinguishable in morphology and phenotype. Recently, this issue was highlighted by Takahashi et al.[50] Interestingly, 4 patients of the present series developed ANKL in their clinical course, 2 of whom had only skin symptoms categorized as severe mosquito bite allergy at the time of the diagnosis. In addition, 6 patients who were clinically categorized as CAEBV NK-cell type (4 cases) and T-cell type (2 cases) developed ENKL; the major clinical difference from de novo ENKL was its early onset (median age, 8.5 years). Three patients had hypersensitivity to mosquito bites. There were no differences in pathologic features between these patients and de novo ENKL patients.[50] Furthermore, new development of chromosomal aberrations was seen in 6 patients during follow-up. In this study, most of the patients with EBV$^+$ T/NK-LPDs had clonality of EBV-infected cells. These results indicate that patients with clonally expanding EBV-infected T or NK cells in EBV$^+$ T/NK-LPD eventually develop overt leukemia and lymphoma, the clinicopathologic findings of which are in keeping with those well documented in extranasal ENKL, ANKL, and PTCL, with additional mutations in cancer genes or tumor-suppressor genes.

In 2008, an international meeting was organized at the National Institute of Health to better define the pathogenesis, classification, and treatment of EBV-associated LPDs in nonimmunocompromised hosts.[39] At that meeting, acute and chronic EBV syndromes of T cells and NK cells were clarified to have a broad spectrum, in which hydroa vacciniforme, hydroa vacciniforme–like lymphoma, severe mosquito bite allergy, and systemic EBV$^+$ T-LPD of childhood were listed as EBV$^+$ T/NK-LPDs under an umbrella term of CAEBV of T/NK–cell type.[39] In the present study, EBV$^+$ T/NK-LPD is characterized by the systemic distribution of EBV$^+$ clones beyond the clinical categorization currently proposed as CAEBV, HLH, severe mosquito bite allergy, and hydroa vacciniforme. Furthermore, we also shed light on the clinicopathologic distinctiveness of patients with NK-cell infection, which has not been well addressed in the past even though these patients comprise approximately 40% of EBV$^+$ T/NK-LPD cases. This phenotype was more closely associated with hypersensitivity to mosquito bite and a relatively indolent clinical course, the biologic significance of which should be clarified in the future.

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Authorship

Contribution: H.K. designed the study, followed the patients, analyzed the data, and wrote the manuscript; Y.I. contributed to the study design, followed the patients, and helped to edit the manuscript; S. Kawabe, K.G., and S.E. performed the experiments; Y.T., S. Kojima, and T.N. followed the patients, collected the clinical data, and helped to edit the manuscript; A.K., A.S., and K.K. followed the patients and collected the clinical data; K.O. performed the experiments and helped to edit the manuscript; and S.N. contributed to the study design, performed the experiments, and wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Bortezomib induces apoptosis in T lymphoma cells and natural killer lymphoma cells independent of Epstein-Barr virus infection

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Epstein-Barr virus (EBV), which infects not only B cells, but also T cells and natural killer (NK) cells, is associated with multiple lymphoid malignancies. Recently, the proteasome inhibitor bortezomib was reported to induce apoptosis of EBV-transformed B cells. We evaluated the killing effect of this proteasome inhibitor on EBV-associated T lymphoma cells and NK lymphoma cells. First, we found that bortezomib treatment decreased the viability of multiple T and NK cell lines. No significant difference was observed between EBV-positive and EBV-negative cell lines. The decreased viability in response to bortezomib treatment was abrogated by a pan-caspase inhibitor. The induction of apoptosis was confirmed by flow cytometric assessment of annexin V staining. Additionally, cleavage of caspases and polyadenosine diphosphate-ribose polymerase, increased expression of phosphorylated IκB, and decreased expression of inhibitor of apoptotic proteins were detected by immunoblotting in bortezomib-treated cell lines. We found that bortezomib induced lytic infection in EBV-positive T cell lines, although the existence of EBV did not modulate the killing effect of bortezomib. Finally, we administered bortezomib to peripheral blood mononuclear cells from five patients with EBV-associated lymphoproliferative diseases. Bortezomib had a greater killing effect on EBV-infected cells. These results indicate that bortezomib killed T or NK lymphoma cells by inducing apoptosis, regardless of the presence or absence of EBV.

Key words: Epstein-Barr virus, proteasome inhibitor, apoptosis, lytic infection, NF-κB

Abbreviations: 7-AAD: 7-aminoactinomycin D; BARTs: *Bam*HI-A rightward transcripts; cIAP: cellular IAP; DMSO: dimethyl sulfoxide; EBV: Epstein-Barr virus; EBER: EBV-encoded small RNA; EBNA: EBV nuclear antigen; FISH: Flow cytometric in situ hybridization; IAPs: inhibitors of apoptotic proteins; LMP: latent membrane protein; LCL: lymphoblastoid cell line; MNCs: mononuclear cells; NK: natural killer; NF-κB: nuclear factor-κB; PBS: phosphate-buffered saline; PE: phycoerythrin; PARP: polyadenosine diphosphate-ribose polymerase; RT: reverse transcription; SDS: sodium dodecyl sulfate; XIAP: X-linked IAP; β2m: β2-microglobulin

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The ubiquitous Epstein-Barr virus (EBV) infects most individuals by early adulthood and typically remains latent throughout life. EBV not only infects B cells but also T cells and natural killer (NK) cells and has been associated with multiple lymphoid malignancies such as Burkitt lymphoma, diffuse large B cell lymphoma, Hodgkin lymphoma, post-transplant lymphoproliferative disorders, nasal NK/T lymphoma, hydroa vacciniforme-like lymphoma, aggressive NK cell leukemia, and chronic active EBV infection.[1–4] EBV plays an important role in the pathogenesis of many of these malignancies through its ability to establish latent infection and induce the proliferation of infected cells.[5] Some of these EBV-associated lymphoid malignancies are refractory and resistant to conventional chemotherapies. Rituximab, a humanized monoclonal antibody against CD20, targets B cell-specific surface antigens present on EBV-transformed malignant cells. Currently, Rituximab is used in the treatment and prophylaxis of B cell lymphoma and lymphoproliferative disorders.[6,7] However, a continuing need exists for effective treatments of T and NK cell lymphoid malignancies, and novel approaches of molecular target therapy are desirable.

Recently, bortezomib was reported to induce apoptosis in EBV-transformed B cells and prolong survival of mice inoculated with EBV-transformed B cells.[8] Bortezomib is an inhibitor of the 26S proteasome.[9] Proteasomes are multi-protein

complexes that degrade ubiquitinated proteins, including those involved in cell cycle regulation, oncogenesis, and apoptosis. Inhibition of the proteasome can result in apoptosis in malignant cells.[10,11] Bortezomib is approved for the treatment of multiple myeloma and is in clinical trials for non-Hodgkin lymphoma, prostate cancer, and lung cancer.[12,13] A key factor in the ability of bortezomib to kill myeloma cells is that it blocks the activation of nuclear factor-κB (NF-κB).[14] In normal cells, NF-κB is bound to the inhibitory protein IκB, which maintains it in an inactive form in the cytoplasm. Once activated, NF-κB can then enter the nucleus and initiate many actions in the tumor cell that help the cell to survive and proliferate. As a result of inhibiting the proteasome and thus the activation of NF-κB, bortezomib may induce apoptosis by reducing the expression of inflammatory molecules and cell adhesion molecules.[14] Additionally, a study has reported that bortezomib can lead to EBV lytic infection.[15] In EBV-infected lymphocytes, only a few viral genes are expressed to maintain latency and to avoid host immune mechanisms.[1,5] Bortezomib may alter the pattern of viral gene expression thus converting a latent infection to a lytic infection.

In our study, we evaluated the killing effect of bortezomib on EBV-associated T/NK lymphoma cells. To investigate the mechanism of killing, we administered bortezomib to multiple EBV-positive and -negative T and NK cell lines. We further administered bortezomib *ex vivo* to lymphoma cells from patients with EBV-associated lymphoid malignancies.

Material and Methods

Cell lines and reagents

Raji, a latency type III cell line, is an EBV-positive B cell line derived from Burkitt lymphoma.[16] Lymphoblastoid cell line (LCL)-1 and LCL-2, latency type III cell lines, are EBV-positive B cell lines transformed with B95-8 EBV from peripheral blood B lymphocytes. BJAB is an EBV-negative B cell line. SNT-13 and SNT-16[17] are EBV-positive T cell lines. SNK-6[17] and KAI-3[18] are EBV-positive NK cell lines. SNT-13, -16, SNK-6, and KAI-3 were derived from patients with chronic active EBV infection or nasal NK/T cell lymphomas. Jurkat[19] and KHYG-1[20] are EBV-negative T and NK cell lines, respectively. Jurkat was derived from a patient with acute T lymphoblastic leukemia. KHYG-1 was derived from a patient with aggressive NK cell leukemia.

MT-2 cell line was established from cord mononuclear cells by co-culture with adult T cell leukemia cells, and harbors human T cell-leukemia virus type I.[21] MT-2/rEBV/9-7 cell line was established by infection of MT-2 cells with the hygromycin-resistant B95-8 strain.[22] MT-2/hyg cell line, transfected with a hygromycin-resistant gene, and MT-2/rEBV/9-7 were used to verify the difference of EBV presence in the T cell lines. Similarly, NKL cell line[23] was derived from a patient with NK cell leukemia, and TL1 cell line[24] was established from NKL cells infected with Akata-transfected recombinant EBV strain containing a neomycin-resistant gene. TL1 and NKL were used to verify the difference of EBV presence in the NK cell lines.

Raji, LCL-1, LCL-2, BJAB, and Jurkat cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, penicillin, and streptomycin (complete media). The medium for SNT-13, -16, KAI3, SNK-6, KHYG1, TL1, and NKL was complete media supplemented with 100 U/ml human interleukin-2. The medium for MT-2/hyg and MT-2/rEBV/9-7 was complete media supplemented with 0.2 mg/ml hygromycin. TL1 underwent periodic selection with G418.

Bortezomib, a gift from Millennium Pharmaceuticals (Cambridge, MA), was dissolved in phosphate-buffered saline (PBS). The pan-caspase inhibitor Q-VD-OPH (Calbiochem, La Jolla, CA) and the proteasome inhibitor MG132 (Biomol International, Plymouth Meeting, PA) were dissolved in dimethyl sulfoxide (DMSO).

Cell viability and apoptosis assays

Cells (2×10^5/ml) were cultured in 24-well plates and cell viability was quantified by trypan blue exclusion. The pan-caspase inhibitor Q-VD-OPH (50 μM) was added at 1 hr prior to the addition of bortezomib. These experiments were performed in duplicate and the results are shown as the mean of two wells.

Apoptosis was measured by flow cytometry using an annexin V-phycoerythrin (PE)/7-aminoactinomycin D (7-AAD) apoptosis assay kit (BD Pharmingen Biosciences, San Diego, CA) according to the manufacturer's protocol. Briefly, 2×10^5 cells were treated with various concentrations of bortezomib for 6 hr, washed with ice-cold PBS, resuspended in binding buffer, incubated with annexin V-PE and 7-AAD for 15 min, and then analyzed by flow cytometry. Viable cells were defined as those negative for annexin V-PE and 7-AAD staining, and early apoptotic cells were defined as those positive for annexin V-PE and negative for 7-AAD staining. Stained cells were analyzed using a FACSCalibur and CellQuest software (Becton Dickinson, Franklin Lakes, NJ).

Immunoblots

Whole-cell extracts were lysed directly with sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 6% 2-mercaptoethanol, 0.0025% bromophenol blue). Cell lysates were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Immobilon-P membrane; Millipore), and immunoblotted with antibodies. Antibodies were directed against caspase-3, cleaved caspase-3, caspase-9, phosphorylated IκBα, and polyadenosine diphosphate-ribose polymerase (PARP) (Cell Signaling Technology, Beverly, MA); β-actin (Sigma, St. Louis, MO); XIAP and cIAP-2 (R&D Systems, Minneapolis, MN); and p53 (BD Pharmingen Biosciences). To compare the amounts of each protein, densitometoric analysis was performed using ImageJ software ver. 1.43 (NIH, Bethesda, MD).

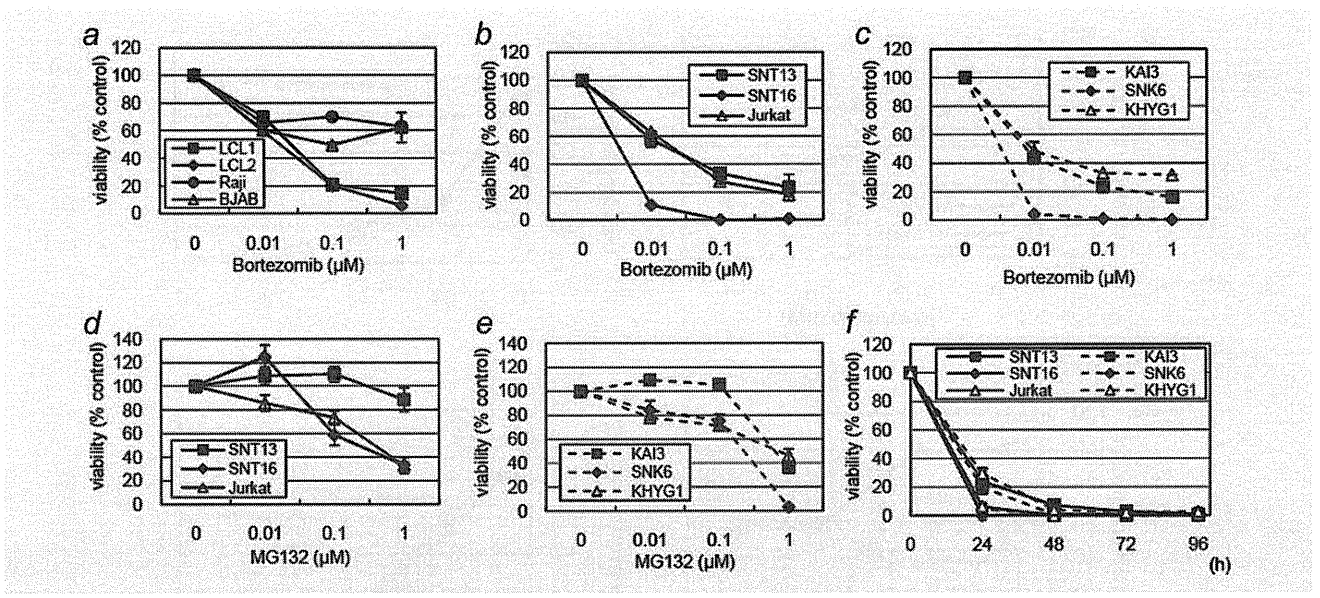

Figure 1. Bortezomib decreases the viability of B, T and NK cell lines. (*a*) B cell lines (Raji, LCL-1, LCL-2, BJAB), (*b*) T cell lines (SNT-13, SNT-16, Jurkat), and (*c*) NK cell lines (KAI-3, SNK-6, KHYG-1) were treated with bortezomib at the indicated concentrations for 24 hr. (*d*) T cell lines or (*e*) NK cell lines were treated with MG132 at various concentrations for 24 hr. (*f*) T or NK cell lines were treated with 1 μM bortezomib for 4 days and viability was determined every 24 hr. The filled markers represent EBV-positive cell lines and the open markers represent EBV-negative cell lines. Viability was calculated as the percentage of viable cells in bortezomib-treated cells *versus* PBS-treated cells. Bars indicate standard errors.

Real-time RT-PCR assay

RNA was extracted from 1×10^6 cells using the QIAmp RNeasy Mini Kit (Qiagen, Hilden, Germany). Contaminating DNA was removed by on-column deoxyribonuclease digestion using the RNase-Free DNase Set (Qiagen). Viral mRNA expression was quantified by a one-step multiplex real-time reverse transcription (RT)-PCR using a Mx3000P real-time PCR system (Stratagene, La Jolla, CA), as described previously.[25,26] The stably expressed housekeeping gene β2-microglobulin (β2m) was used as an endogenous control and reference gene for relative quantification.[27] Each experiment was conducted in triplicate and results are shown as the mean of three samples with standard errors. The Mann-Whitney *U*-test was used to compare the expression levels. p values <0.05 were deemed to be statistically significant.

Patients

Peripheral blood mononuclear cells (MNCs) were collected from five patients with EBV-associated diseases. None of these patients had received any immunosuppressive treatment such as steroid therapy or chemotherapy. Patients T-1 (7-year-old boy), T-2 (6-year-old girl), and T-3 (12-year-old boy) had hydroa vacciniforme-like lymphoma, a newly classified EBV-associated T cell lymphoma.[2] In these patients, 10∼20% of the MNCs were infected with EBV, and the EBV-infected cells were primarily γδT cells.[28] The other two patients, Patients NK-1 (14-year-old boy) and NK-2 (9-year-old boy), had chronic active EBV infection, NK cell type.[29–31] In these patients, ∼40% of MNCs were infected with EBV, and the EBV-infected cells were NK cells. MNCs from three healthy donors were used as controls. Informed consent was obtained from all participants or their guardians. Our study was approved by the institutional review board of Nagoya University Hospital.

MNCs were isolated using Ficoll-Paque (Amersham Pharmacia Biotech AB, Uppsala, Sweden) gradient centrifugation. Cells (2×10^5/ml) were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum. For the cell viability study, each experiment was performed in duplicate, and the results are shown as the mean of two wells.

Magnetic cell sorting

The primarily infected cell fractions were separated by magnetic sorting with a TCRγ/δ$^+$ T Cell Isolation Kit or CD56 Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, cells were magnetically labeled with MicroBeads and separated on a column placed in the MACS separator. The flow-through was collected as a negative fraction depleted of the labeled cells. The magnetically retained cells were flushed out as the positive fraction. The respective purity and recovery rates were 98.3% and 80.0% with the TCRγ/δ $^+$ T Cell Isolation Kit, and 96.4% and 80.9% with CD56 Microbeads.

Flow cytometric in situ hybridization (FISH) assay

To verify that the sorted fraction contained EBV-infected cells, a FISH assay was used.[28] Briefly, cells were fixed with 1% acetic acid/4% paraformaldehyde, permeabilized with 0.5% Tween 20/PBS, and hybridized with a fluorescein-labeled

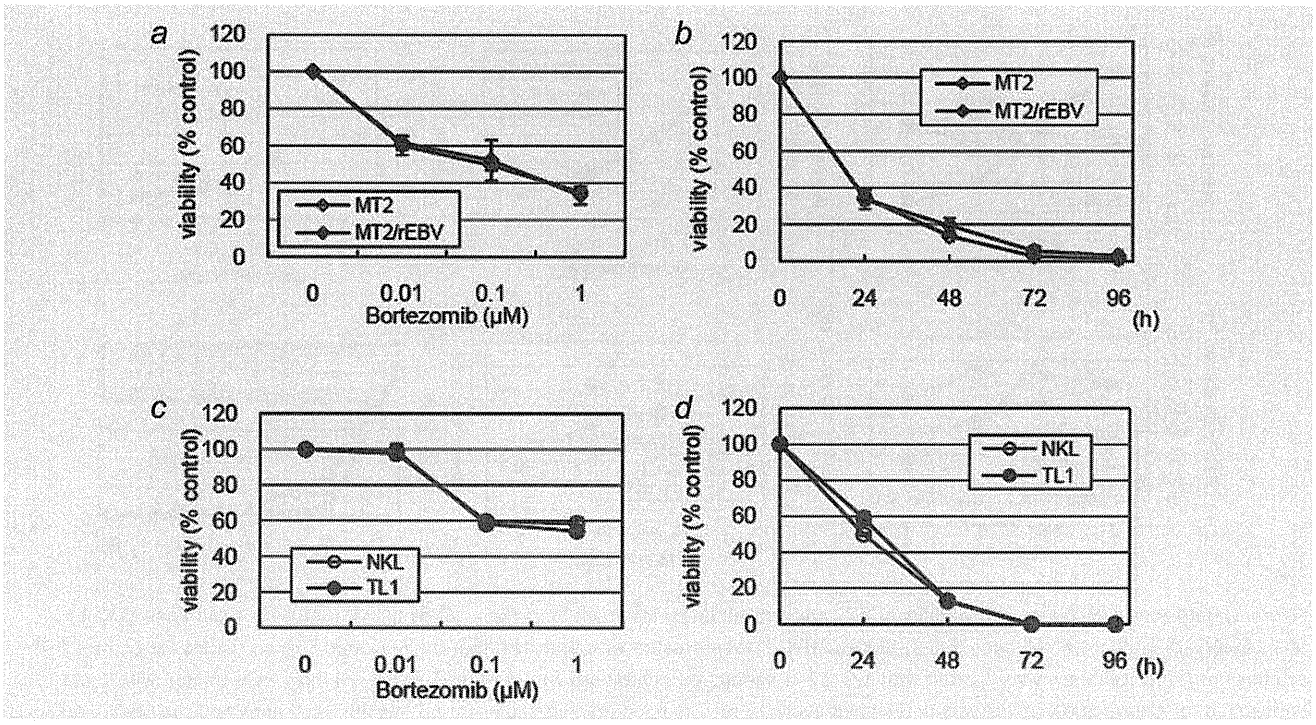

Figure 2. Bortezomib induces similar effects in EBV-positive and -negative cell lines. An EBV-positive T cell line (MT-2/rEBV) and a control cell line (MT/hyg) were treated with various concentrations of bortezomib for 24 hr (*a*) and 1 μM bortezomib for 4 days (*b*). EBV-positive NK cell line (TL1) and its parental line (NKL) were treated with various concentrations of bortezomib for 24 hr (*c*) and 1 μM bortezomib for 4 days (*d*). Bars indicate standard errors.

EBV-encoded small RNA (EBER)-specific peptide nucleic acid probe (Y5200; Dako, Glostrup, Denmark). The fluorescence intensity was enhanced using the AlexaFluor 488 Signal Amplification Kit (Molecular Probes, Eugene, OR), and stained cells were analyzed using a FACSCalibur and CellQuest software (Becton Dickinson).

Results

Bortezomib decreased the viability of B, T and NK cell lines

First, we administered bortezomib to B, T and NK cell lines for 24 hr and counted viable cells. Bortezomib decreased the viability of all four B cell lines: three EBV-positive cell lines (Raji, LCL-1, and LCL-2) and one EBV-negative cell line (BJAB) (Fig. 1*a*). LCL-1 and LCL-2 were more sensitive to bortezomib than Raji or BJAB, consistent with the results of a previous study reporting that bortezomib killed both EBV-positive and -negative B cell lines, but more efficiently in LCLs.[8] Next, we administered bortezomib to T cell lines (SNT-13, SNT-16, Jurkat) and NK cell lines (KAI-3, SNK-6, KHYG-1). Bortezomib decreased the viability of all six target cell lines in a dose-dependent manner (Figs. 1*b* and 1*c*). SNT-16 and SNK-6 seemed to be more sensitive to bortezomib than other cell lines. MG132, another proteasome inhibitor, had less effect on these cell lines (Figs. 1*d* and 1*e*). We administered 1 μM bortezomib to these cell lines for 4 days and determined their viability every 24 hr. We found that bortezomib decreased the viability of these six cell lines by less than 10% at 48 hr or later (Fig. 1*f*). There were no obvious differences on the effect of bortezomib between the EBV-positive and -negative cell lines. Furthermore, to directly compare the effect of bortezomib between EBV-positive and -negative cell lines, we administered bortezomib to MT-2/hyg and MT-2/rEBV/9-7 (Figs. 2*a* and 2*b*), and NKL and TL1 (Figs. 2*c* and 2*d*). We found that bortezomib had almost equal effects on the two cell lines.

Bortezomib induces apoptosis in T and NK cell lines

The induction of apoptosis was confirmed by flow cytometry with annexin V and 7-AAD staining. Bortezomib decreased viable cells, defined as those negative for both annexin V-PE and 7-AAD staining, and increased early apoptotic cells, defined as those positive for annexin V-PE and negative for 7-AAD staining in the four cell lines (Fig. 3*a*): EBV-positive (SNT-16) and EBV-negative T cell lines (Jurkat); and EBV-positive (KAI-3) and EBV-negative NK cell lines (KHYG-1). This result showed that bortezomib induced apoptosis in both T and NK cell lines.

Next, to analyze the mechanism of bortezomib-induced apoptosis, cleavages of caspase and PARP were investigated by immunoblotting. Bortezomib induced cleavage of caspase-3, caspase-9, and PARP in all four cell lines (Fig. 3*b*). The decrease in viability caused by bortezomib was inhibited by pretreatment with Q-VD-OPH, a pan-caspase inhibitor (Fig. 3*c*).

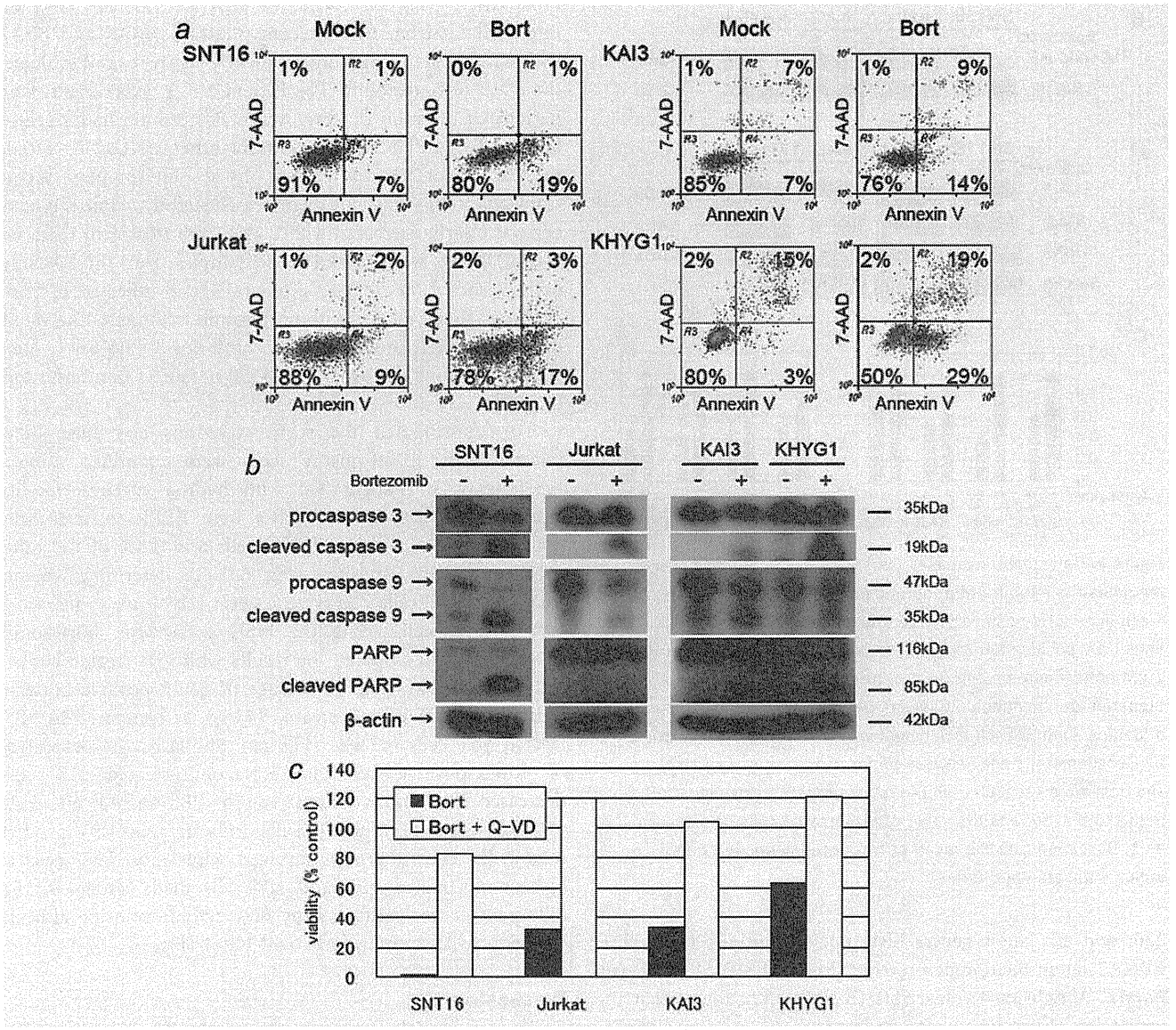

Figure 3. Bortezomib induces apoptosis in T/NK cell lines. (*a*) T cell lines (EBV-positive SNT-16 and -negative Jurkat) and NK cell lines (EBV-positive KAI-3 and -negative KHYG-1) were treated with 1 μM bortezomib for 6 hr. Viable cells were defined as those negative for annexin V-PE and 7-AAD staining, and early apoptotic cells were defined as those positive for annexin V-PE and negative for 7-AAD staining. (*b*) Bortezomib induces cleavage of caspase-3, caspase-9, and PARP. T/NK cell lines were treated with 1 μM bortezomib for 6 hr. Protein lysates were prepared and immunoblotting was performed. β-actin was used as a loading control. Each experiment was performed at least twice. (*c*) Q-VD-OPH inhibits the decrease in viability induced by bortezomib. The pan-caspase inhibitor Q-VD-OPH (50 μM) was added 1 hr prior to the addition of 1 μM bortezomib for 24 hr. Viability was calculated as the percentage of viable cells to PBS-treated cells as assessed by trypan blue exclusion.

Bortezomib blocks activation of NF-κB by inhibiting the proteasome, reducing antiapoptotic factors

In myeloma cells, bortezomib blocks the activation of NF-κB through increasing phosphorylation of IκB.[14] Thus, we confirmed the increase in phosphorylated IκB, which should have been degraded in proteasomes, in bortezomib-treated cell lines by immunoblotting (Fig. 4*a*). Furthermore, we evaluated the effect of bortezomib on two inhibitors of apoptotic proteins (IAPs). Bortezomib down-regulated cellular IAP (cIAP)-2 and X-linked IAP (XIAP) in bortezomib-treated T/NK cell lines (Fig. 4*b*). Densitometoric analysis was performed to confirm that cIAP-2 and XIAP were decreased in all bortezomib-treated cells (Fig. 4*c*). Additionally, bortezomib up-regulated p53, facilitating apoptosis, in some cell lines (Fig. 4*b*).

Bortezomib induces lytic infection of EBV in T cell lines

Next, we analyzed the expression of eight viral genes using a real-time RT-PCR assay: two lytic genes, BZLF1 and gp350/

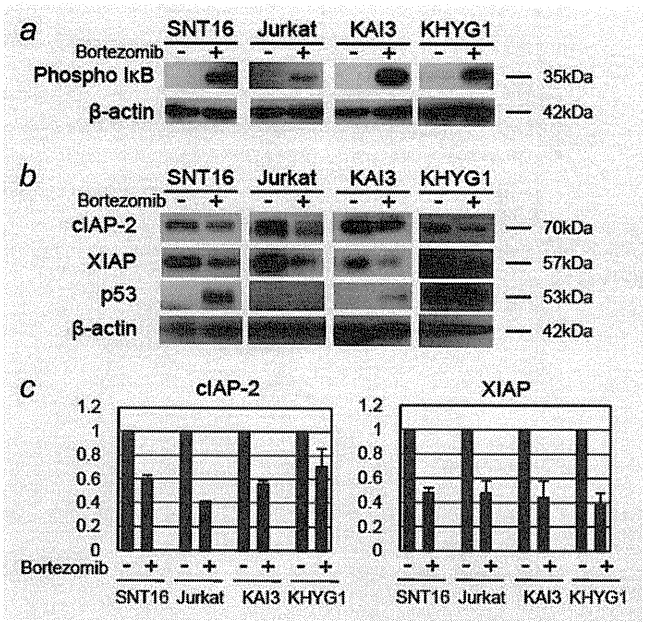

Figure 4. Bortezomib increases phosphorylated IκB and down-regulates the inhibitor of apoptotic proteins in T/NK cell lines. T cell lines (EBV-positive SNT-16 and -negative Jurkat) and NK cell lines (EBV-positive KAI-3 and -negative KHYG-1) were treated with 1 μM bortezomib for 2 hr (*a*) or 8 hr (*b*). Protein lysates were prepared and immunoblotting was performed. β-actin was used as a loading control. Each experiment was performed at least twice. (*c*) Densitometoric analysis was performed using ImageJ software. The data were calculated as the ratios of cIAP-2 (left) and XIAP (right) to β-actin, and the value of PBS-treated cells was assigned as 1. Boxes indicate the mean of two experiments and bars indicate the standard errors.

220; and six latent genes, EBV nuclear antigen (EBNA) 1, EBNA2, latent membrane protein (LMP) 1, LMP2, EBER1, and *Bam*HI-A rightward transcripts (BARTs). We found that the expression of two lytic genes, an immediate early gene (BZLF1) and a late gene (gp350/220), were increased in bortezomib-treated T cell lines (SNT-13 and SNT-16 in Fig. 5). In NK cell lines, however, no such effect was observed. Regarding latent genes, expression of LMP2 was increased in T cell lines. The expressions of other latent genes (EBNA1, EBNA2, LMP1, EBER1, and BARTs) were not obviously different between bortezomib-treated cells and controls (Fig. 5).

Bortezomib decreases the viability of EBV-infected cells from patients with EBV-associated lymphoma

Finally, we investigated the *ex vivo* effect of bortezomib in MNCs from five patients with EBV-associated malignancies. We separated γδT cells and other MNCs from three patients (Patients T-1, T-2, and T-3) with hydroa vacciniforme-like lymphoma by magnetic sorting. Bortezomib (0.5 μM) was administered to each sample of cells, and the viable cells were counted for 3 days. Bortezomib had a greater killing effect on γδT cells that were primarily infected with EBV than on the other MNCs (Fig. 6*a*). Next, we separated the NK cells and other MNCs from two patients (Patients NK-1, and NK-2) with chronic active EBV infection, NK cell-type, and evaluated the effect of bortezomib. For Patient NK-1, experiments were performed twice on different visits. Bortezomib had a greater killing effect on NK cells than on the other MNCs (Fig. 6*b*). In the γδT cell or NK-cell fraction, the absolute number of control viable cells was stable or increased slightly, while the number was clearly decreased with bortezomib treatment (data not shown). Next, we collected blood samples from three healthy donors, sorted the γδT cells, NK cells, and other MNCs, and evaluated their viability with bortezomib treatment. The viability of the cells treated with bortezomib for 3 days was around 100%, indicating that bortezomib did not affect nontumor cells (Fig. 6*c*).

To confirm that the sorted fractions contained EBV-infected cells, EBER-positive cells were quantified using a FISH assay. In Patient T-1 with hydroa vacciniforme-like lymphoma, 19.8% of the MNCs were EBER-positive. After magnetic sorting, 54.7% of γδT cells and 3.8% of the other MNCs were EBER-positive (Fig. 6*d*). To determine whether EBV-infected cells survive selectively, we quantified EBV-positive cells using the FISH assay after bortezomib treatment, and compared the results with PBS-treated control cells. After 3 days, the percentage of EBER-positive bortezomib-treated γδT cells decreased (4.0%), as compared to PBS-treated γδT cells (47.8%) (Fig. 6*e*). Similarly, the percentage of EBER-positive cells in bortezomib-treated NK cells decreased (0.5%), as compared to PBS-treated NK cells (34.1%) (Fig. 6*e*). These results indicate that EBV-positive cells in the control groups survived, while most EBV-positive bortezomib-treated γδT and NK cells died. Moreover, this killing effect was confirmed by flow cytometry using annexin V and 7-AAD staining in Patient NK-1 (Fig. 6*f*).

Discussion

Bortezomib, which is used in the treatment of myeloma, has also been assessed for a variety of other malignancies. In recent studies, this proteasome inhibitor was reported to induce apoptosis in NK lymphoma/leukemia cells,[32,33] and has been tested in cutaneous T cell lymphomas and aggressive T/NK cell lymphomas, including EBV-associated ones.[34,35] Although promising data have accumulated, these trials were small and must be considered preliminary. Furthermore, experience with EBV-associated cases is limited. To our knowledge, there have been no *in vitro* studies to compare the efficacy of bortezomib between EBV-positive and EBV-negative T/ NK lymphoma cells.

In our study, we treated T and NK cell lines with bortezomib to investigate this proteasome inhibitor's ability to induce apoptosis in T and NK lymphoma cells. Bortezomib markedly decreased the viability of T and NK cell lines by inducing apoptosis. Consistent with previous reports, the cleavage of caspases and PARP, increased phosphorylated IκB, and the decreased inhibitor apoptotic proteins indicated

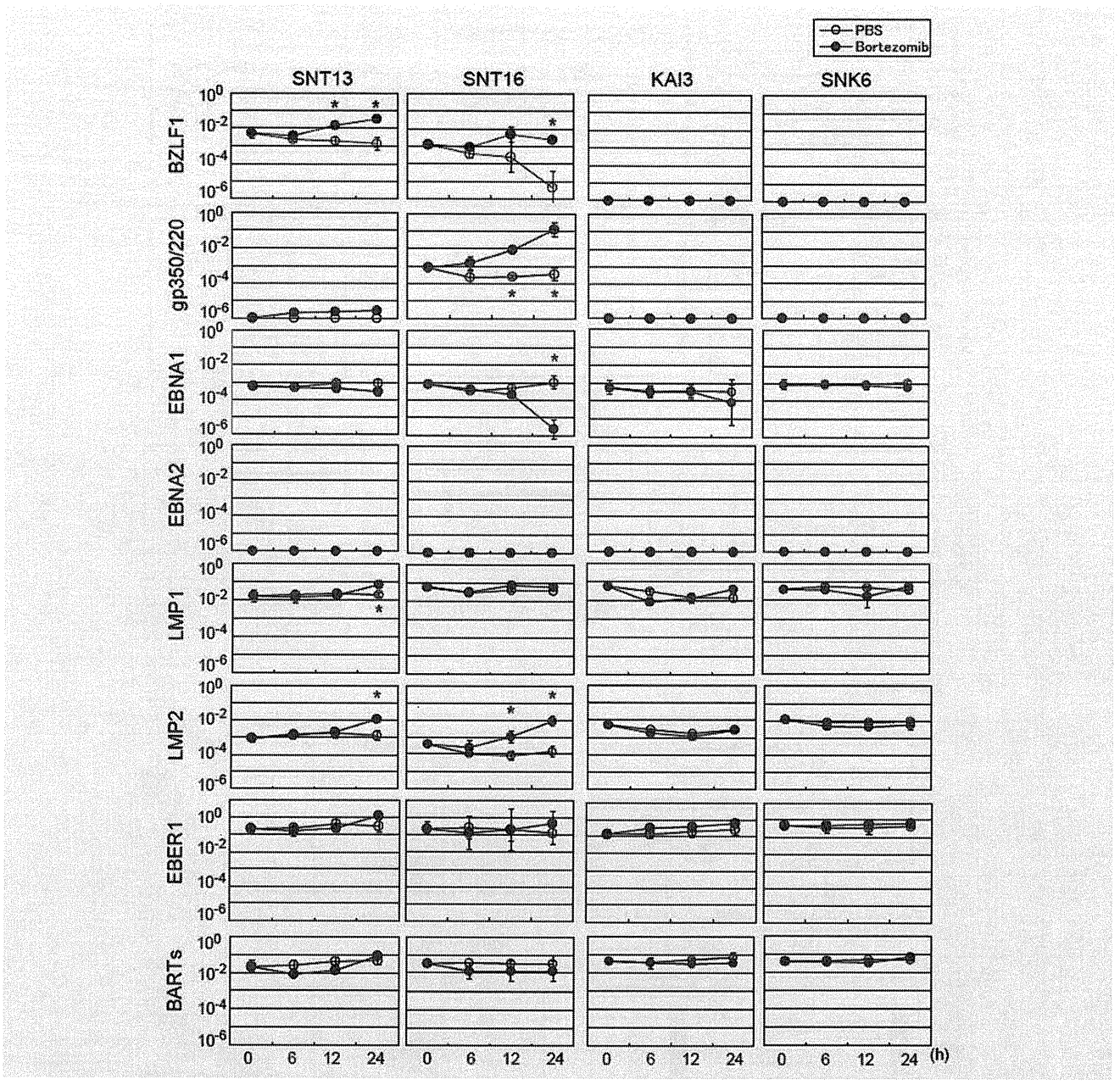

Figure 5. Bortezomib induces lytic infection of EBV in T cell lines. EBV-positive T cell lines (SNT-13 and SNT-16) and EBV-positive NK cell lines (KAI-3 and SNK-6) were treated with 1 μM bortezomib and collected at 0, 6, 12, and 24 hr to evaluate the expression of EBV-encoded genes by real-time RT-PCR. BZLF1 is an immediate early gene and gp350/220 is a late gene. EBNA1, EBNA2, LMP1, LMP2, EBER1, and BARTs are latent genes. β2-microglobulin was used as an endogenous control and reference gene for relative quantification and assigned an arbitrary value of 1 (10^0). Closed circles indicate bortezomib-treated cells, while open circles denote PBS-treated cells. Bars indicate standard errors. *$p < 0.05$ by Mann-Whitney *U*-test.

that this apoptosis was due to bortezomib inhibiting the degradation of phosphorylated IκB.[8,14,36] The inhibition of phosphorylated IκB degradation restrains the activation of NF-κB, which is associated with chemoresistance and poor survival in T and NK cell lymphomas.[37,38] We found, however, no significant difference between EBV-positive and -negative cell lines. The findings suggest that the mechanism of bortezomib in eliminating malignant cells involves the inhibition of NF-κB activation inducing apoptosis and preventing the immortalization and proliferation of cells. Other mechanisms by which bortezomib acts against malignancies, such as the stabilization of p53, disruption of the cell cycle, induction of endoplasmic reticulum stress, and increased intracellular reactive oxygen species, have been reported.[39,40]

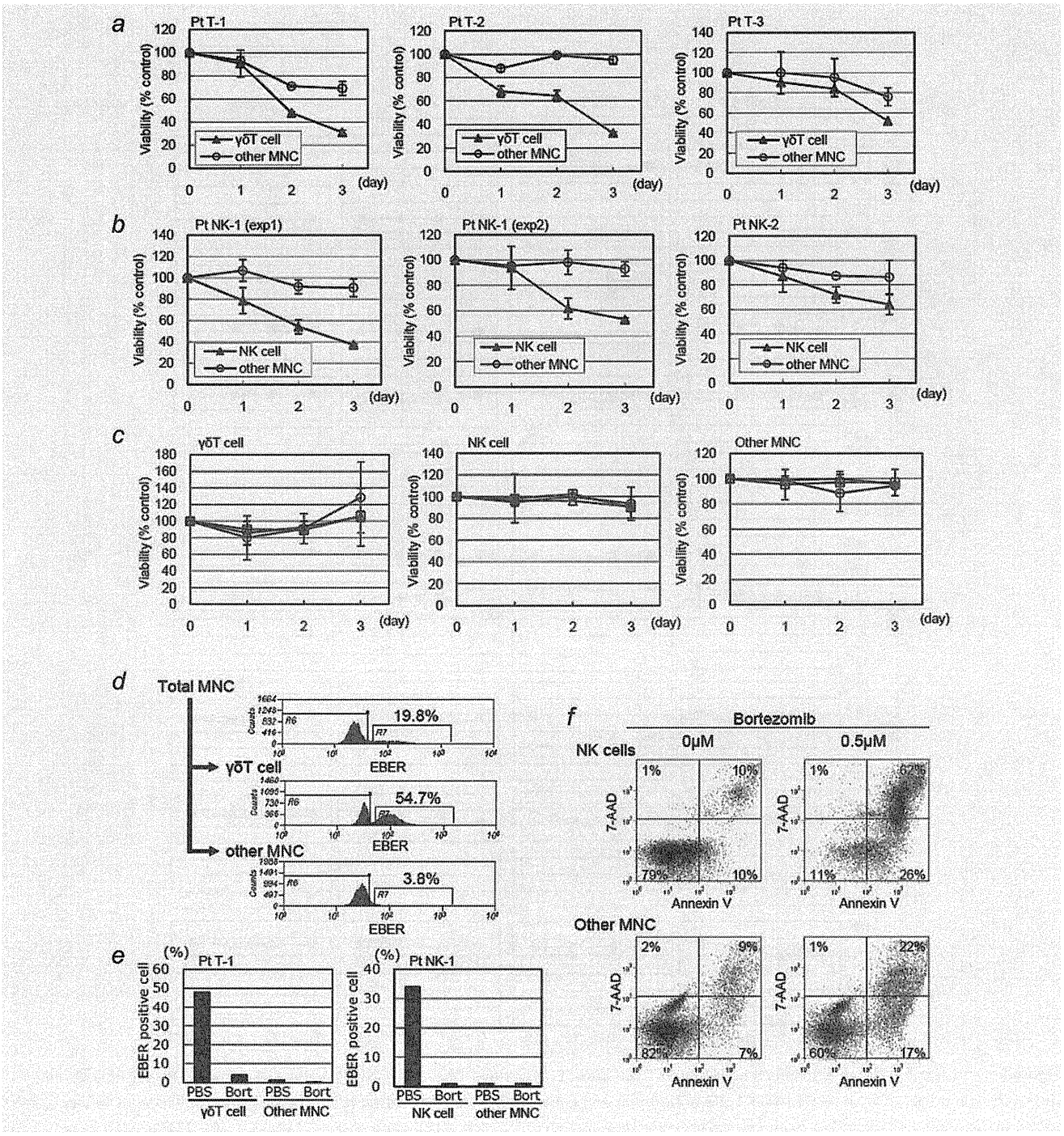

Figure 6. Bortezomib decreases the viability of EBV-infected cells from patients with EBV-associated diseases. (*a, b, c*) Bortezomib (0.5 μM) was administered to each sample of cells, and the viable cells were counted for 3 days. (*a*) γδT cells and other MNCs from three patients (Patients T-1, T-2, and T-3) with hydroa vacciniforme-like lymphoma, (*b*) NK cells and other MNCs from two patients (Patients NK-1, and NK-2) with NK cell-type chronic active EBV infection, and (*c*) γδT cell, NK cell, and non-γδT, non-NK cell (other MNC) from three healthy donors were separated by magnetic sorting. For Patient NK-1, experiments were performed twice on different visits (exp.1 and exp.2). Bars indicate standard errors. (*d*) EBER-positive cells of Patient T-1 with hydroa vacciniforme-like lymphoma were quantified using a FISH assay on unsorted MNCs (total MNCs), the γδT cell fraction (γδT cell), and the non-γδT cell fraction (other MNCs). (*e*) The EBER-positive rate of each sorted cells from Patient T-1 (left) and Patient NK-1 (right) were quantified using a FISH assay after 3 days treatment with bortezomib or PBS. (*f*) NK cells and other MNCs of Patient NK-1, with NK cell-type chronic active EBV infection were separated by magnetic sorting. Cells were treated with 0.5 μM bortezomib for 24 hr and analyzed by flow cytometry. Viable cells were defined as those negative for annexin V-PE and 7-AAD staining, and early apoptotic cells were defined as those positive for annexin V-PE and negative for 7-AAD staining.

The existence of EBV may have little effect on cell death induced by bortezomib. In a previous study, the killing effect of bortezomib was not different between EBV-positive and -negative Burkitt lymphoma cell lines,[8,41] although Zou *et al.*[8] reported that bortezomib had a greater effect in B cell lines with latency type III than those with Type I. The effect of bortezomib may be different in cells with different latency types due to distinct patterns of viral gene expression. The existence of EBV may have some effect on bortezomib because LMP1 is known to activate NF-κB.[42] In the study by Zou *et al.*,[8] however, transfection of LMP1 into an EBV-negative B cell line did not change the sensitivity to bortezomib. The EBV-positive T and NK cell lines used in our study are classified as latency type II[25] and express LMP1. Bortezomib seemed to have little impact on LMP1-mediated NF-κB activation because the presence of EBV did not influence its effects in our study. Comparing cell lines that are naturally EBV positive with derivative cell lines that have lost EBV is necessary to prove that EBV does not affect the outcome of bortezomib treatment. However, establishing such EBV-depleted cell lines is very difficult and, to our knowledge, there are no existing EBV-depleted T- or NK-cell lines. Alternatively, we compared EBV-negative cell lines and those that have been infected with EBV *in vitro*. We admit that this is an artificial system that may have limited relevance.

Bortezomib induced lytic infection only in T cell lines. Inhibition of the NF-κB pathway has been shown to induce the EBV lytic cycle.[43] Although the reason for the difference in lytic induction between T and NK cell lines is unclear, T cell lines seem to express lytic infection genes more often than NK cell lines, consistent with our previous report.[25] Histone deacetylase inhibitors, such as butyric acid and valproic acid, and phorbol 12-myristate 13-acetate are reported to induce an EBV lytic cycle in B cell and epithelial cell lines.[5,26] To our knowledge, however, no agent induces a lytic cycle in EBV-positive NK cell lines. We administered valproic acid, a class I histone deacetylase inhibitor, to NK-cell lymphoma cell lines. Lytic induction was not induced in the NK cell lines (data not shown).

Such lytic induction could contribute to the decreased viability of T cell lines. However, this lytic induction is not a major mechanism of bortezomib-induced cell death because no significant difference was observed in its effect between T and NK cell lines, or between EBV-positive and -negative cell lines. In SNT-16 cells, bortezomib induced a marked decrease in viability, and the recovery in viability following administration of a pan-caspase inhibitor was incomplete (Fig. 3*c*). Thus, the induction of lytic infection by bortezomib could play a partial role in this cell line. Additionally, this result suggests the potential utility of bortezomib as a novel strategy against EBV-positive T cell lymphoma. In EBV-infected T cells, a few latent genes with low antigenicity are expressed.[25] Moreover, the increased expression of lytic proteins, which are generally antigenic, can be recognized in virus-specific cytotoxic T lymphocytes, resulting in lysis of EBV-infected T cells.

Bortezomib killed EBV-infected γδT cells of hydroa vacciniforme-like lymphoma and EBV-infected NK cells of chronic active EBV infection. Taken together with the flow cytometry results, bortezomib induced apoptosis in EBV-infected immortalized cells. These results indicate that bortezomib may be an effective therapy against EBV-associated T and NK lymphoma/lymphoproliferative diseases. We administered 0.5 μM bortezomib to peripheral blood cells in an *ex vivo* study. This concentration is approximately equal to the plasma concentration after administering 1.3 mg/m^2 bortezomib in a recent Phase I and II study of multiple myeloma.[44]

Although recent studies have shown that the effect of bortezomib could be enhanced synergistically in combination with a histone deacetylase inhibitor,[45–47] further studies with appropriate *in vivo* models are essential to confirm this possibility. EBV infects only humans and no good animal models exist, although recently, humanized mouse models with reconstituted human lymphocytes and EBV infection have been reported.[48–50] Despite the complexity of this mouse model, it could be useful for evaluating new therapies, including molecular targeted therapy. The *ex vivo* administration model, coupled with magnetic sorting, is a convenient method to evaluate molecular targeted therapy against EBV-associated lymphoma.

In conclusion, bortezomib killed T/NK lymphoma cells by inducing apoptosis. No significant difference in killing was observed between EBV-positive and -negative cell lines, although bortezomib induced lytic infection in EBV-infected T cells. Following *ex vivo* administration, bortezomib had a greater killing effect on EBV-positive cells than other MNCs. These results give rationale for the use of bortezomib on T/NK lymphomas, although existence of EBV may have little effect on cell death induced by bortezomib.

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Antitumor activities of valproic acid on Epstein–Barr virus-associated T and natural killer lymphoma cells

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Epstein–Barr virus (EBV), which infects B cells, T cells, and natural killer (NK) cells, is associated with multiple lymphoid malignancies. Recently, histone deacetylase (HDAC) inhibitors have been reported to have anticancer effects against various tumor cells. In the present study, we evaluated the killing effect of valproic acid (VPA), which acts as an HDAC inhibitor, on EBV-positive and -negative T and NK lymphoma cells. Treatment of multiple T and NK cell lines (SNT13, SNT16, Jurkat, SNK6, KAI3 and KHYG1) with 0.1-5 mM of VPA inhibited HDAC, increased acetylated histone levels and reduced cell viability. No significant differences were seen between EBV-positive and -negative cell lines. Although VPA induced apoptosis in some T and NK cell lines (SNT16, Jurkat and KHYG1) and cell cycle arrest, it did not induce lytic infection in EBV-positive T or NK cell lines. Because the killing effect of VPA was modest (1 mM VPA reduced cell viability by between 22% and 56%), we tested the effects of the combination of 1 mM of VPA and 0.01 μM of the proteasome inhibitor bortezomib. The combined treated of cells with VPA and bortezomib had an additive killing effect. Finally, we administered VPA to peripheral blood mononuclear cells from three patients with EBV-associated T or NK lymphoproliferative diseases. In these studies, VPA had a greater killing effect against EBV-infected cells than uninfected cells, and the effect was increased when VPA was combined with bortezomib. These results indicate that VPA has antitumor effects on T and NK lymphoma cells and that VPA and bortezomib may have synergistic effects, irrespective of the presence of EBV. (*Cancer Sci* 2012; 103: 375–381)

The ubiquitous Epstein–Barr virus (EBV) infects most individuals by early adulthood and typically remains latent throughout life. Not only does EBV infect B cells, T cells, and natural killer (NK) cells, but it is also associated with multiple lymphoid malignancies, including Burkitt lymphoma, diffuse large B cell lymphoma, Hodgkin lymphoma, post-transplant lymphoproliferative disorders, nasal NK/T-cell lymphoma, hydroa vacciniforme-like lymphoma, aggressive NK cell leukemia, and chronic active EBV disease.[1–4] Epstein–Barr virus plays an important role in the pathogenesis of many of these malignancies via its ability to establish latent infection and induce the proliferation of infected cells.[5] Some of these EBV-associated lymphoid malignancies are refractory and resistant to conventional chemotherapies. Rituximab, a humanized monoclonal antibody against CD20, targets B cell-specific surface antigens present on EBV-transformed malignant cells. Currently, rituximab is used for the treatment and prophylaxis of B cell lymphoma and lymphoproliferative disorders.[6,7] However, the need remains for effective treatments for T and NK cell lymphoid malignancies and novel approaches to molecular targeting are desirable.

Sodium valproate (VPA) is a short chain fatty acid that is widely used to treat epilepsy. It is easily accessible and has a well-established safety profile. Recently, VPA was reported to be a potent histone deacetylase (HDAC) inhibitor and inducer of DNA demethylation.[8] It has been found that HDAC inhibitors have potent anticancer activities, with remarkable tumor specificity, and some have even demonstrated therapeutic potential.[9] The HDAC inhibitors can affect tumor cell growth and survival through multiple biological effects. For example, they induce tumor cell death with all of the biochemical and morphological characteristics of apoptosis. Several HDAC inhibitors have been used in the treatment of leukemias and lymphomas, such as cutaneous T cell lymphoma, myelodysplastic syndrome, and diffuse B cell lymphoma.[9] They have been used alone or in combination with DNA demethylating agents or other anticancer chemotherapies. Valproate has been reported to induce cell death in human leukemia cell lines[10] and endometrial tumor cells,[11] and to enhance the efficacy of chemotherapy in EBV-positive tumors.[12] Furthermore, VPA was shown to activate lytic viral gene expression in cells infected with EBV.[12,13]

Previously, we reported that the proteasome inhibitor bortezomib induced apoptosis in T and NK lymphoma cells.[14] Bortezomib produced a stronger killing effect in EBV-infected tumor cells compared with uninfected cells from patients with EBV-associated lymphoproliferative diseases, although the killing effect of bortezomib in cell lines was not affected by the presence of EBV. In the present study, we administered VPA to EBV-positive and -negative T cell lines and NK cell lines, and evaluated its antitumor effects by analyzing cell viability, the induction of apoptosis, cell cycle arrest, and expression of EBV-encoded genes. Finally, we evaluated the antitumor effect of the combination of VPA and bortezomib using both *in vitro* cell lines and *ex vivo* primary cultures of EBV-infected T and NK lymphoma cells.

Materials and Methods

Cell lines and reagents. Of the cell lines used in the present study, SNT13 and SNT16 are EBV-positive T cell lines,[15] SNK6[15] and KAI3[16] are EBV-positive NK cell lines, and Jurkat[17] and KHYG1[18] are EBV-negative T and NK cell lines, respectively. The SNT13, SNT16, SNK6, and KAI3 cells were derived from patients with chronic active EBV disease or nasal NK/T-cell lymphoma. The MT2/rEBV/9-7 cell line[19] was established through infection of MT2 cells with the hygromycin-resistant B95-8 strain.[20] The MT2/hyg cell line was transfected with a hygromycin resistance gene. Similarly, the NKL cell line[21] was derived from a patient with NK cell leukemia, and the TL1 cell line[22] was established from NKL cells infected with an Akata-transfected recombinant EBV strain carrying a neomycin resistance gene.

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