Keywords: epidemiological study, HIV, leukemia, lymphoma, non-AIDS-defining hematological malignancy

Introduction

By the end of 2010, a total of 12 648 cases of HIV infection and 5799 cases of AIDS were reported in Japan [1]. The prevalence of HIV infection is estimated to be less than 0.1% [1]. However, the number of newly reported HIV-infected individuals has gradually increased, and the estimated number now ranges from 6300 to 10 000[1]. Most of the HIV-infected population in Japan is men [2]. The morbidity and incidence of infections has decreased as the introduction of highly active antiretroviral therapy (ART). The incidence of AIDS-defining malignancies has also decreased [3,4].

Although the incidence of AIDS-related non-Hodgkin's lymphoma (NHL) is decreasing, other hematological malignancies such as acute myeloid leukemia (AML), Hodgkin's lymphoma, and chronic myeloid leukemia (CML) are often reported. We conducted a nationwide epidemiological study to evaluate the incidence and clinical outcomes of non-AIDS-defining hematological malignancies (NADHMs), excluding NHL, in HIV-infected patients.

Methods

Most HIV-infected patients are followed in regional AIDS centers, and educational hospitals for hematologists cover most patients with hematological malignancies in Japan.

Questionnaires were sent to 429 regional AIDS centers, namely specialty clinical institutes for AIDS in each region certified by the Ministry of Health, Labor, and Welfare, and 497 educational hospitals certified by the Japanese Society of Hematology, namely training institutes for hematologists (207 of these institutions overlapped); in total, both the types of institutes will cover almost all the patients with HIV-related hematological malignancies in Japan. These institutes were requested to report all cases of hematological malignancies between 1991 and 2010, excluding NHL, in HIV-infected patients. We conducted a two-step inquiry. In the first step, all the institutes were required to answer the experience of NADHMs; if yes, the questionnaire was sent. The questionnaire included the date of diagnosis, subtype classification, chromosomal analysis, age, sex, CD4 count at the time of diagnosis, overall survival, treatment, response to treatment, date of relapse, and duration of ART.

Epidemiological data for HIV were acquired from the Joint United Nations program on HIV/AIDS (UNAIDS) [1]. The estimated size of the HIV-infected population in Japan was also based on the data from UNAIDS [1]. Data on AIDS and HIV estimates in Japan were available from 1990 to 2009. We calculated the crude incidence of NADHM from 1991 to 2009. Patient background and clinical data were analyzed using SPSS version 18.0 (IBM Japan, Inc., Tokyo, Japan).

Results

Responses were obtained from 511 institutes (response rate, 71.1%).

Patient characteristics

From 1991 to 2010, 47 patients with NADHMs were reported by 21 institutes, including 19 patients with Hodgkin's lymphoma, 13 with AML, seven with acute lymphoblastic leukemia (ALL), four with CML [three chronic phase (CML-CP) and one accelerated phase (CML-AP)], two with multiple myeloma, one with chronic lymphoid leukemia (CLL), and one with myelodysplastic syndrome–refractory anemia with excess blast one. The median age of the patients was 42.0 years (range, 21–70 years), with 93.6% being male. The median CD4-positive T-cell count was 255/μl (range, 1–1371/μl), and the median HIV viral load was 55 copies/ml. The median duration from the diagnosis of HIV infection to development of hematological malignancy was 28.0 months (range, 0–204 months) (Table 1). Prior to diagnosis, 68.1% patients were treated with ART (mean duration, 27.2 months) and 51.1% had AIDS. The median observation period after the diagnosis of NADHMs was 20.0 months (range, 0–140 months).

Subtypes of leukemia/lymphoma and cytogenetic abnormalities

Varying numbers of subtypes of AML were identified as follows: FAB-M1, M2, M3, M4, and M5. Four of the 13 patients with AML exhibited a normal karyotype; three exhibited recurrent cytogenetic abnormalities such as t(8;21), t(15;17), and inv(16);and three exhibited complex karyotypic abnormalities. Four of the seven patients with AML possessed Burkitt leukemia/lymphoma-type cytogenetic abnormalities such as t(8;14) or t(8;22). Three of the four patients with CML and 1patient with ALL possessed the Philadelphia (Ph^1) chromosome (Ph^1-ALL).

In 19 patients with Hodgkin's lymphoma, mixed-cellularity classical Hodgkin's lymphoma was the most common subtype (68%). Immunostaining revealed that 89% of the patients were positive for Epstein–Barr virus.

Table 1. Characteristics of patients with NADHMs.

		n (%)
Age	49.3 ± 12.9 (21–70) years	
Sex	Male	44 (93.6%)
	Female	3 (6.4)
Disease	Hodgkin's disease	19 (40.4)
	ALL	7 (14.9)
	AML	13 (27.7)
	MDS prior to AML	3 (6.4)
	MDS–RAEB	1 (2.1)
	CML	4 (8.5)
	CLL	1 (2.1)
	Myeloma	2 (4.3)
CD4/μl	Median 255 (1–1371)	
Time since HIV diagnosis	Median 28.0 (0–204) months	
AIDS prior to NADHMs		24 (51.1)
Prior ART		32 (68.1)
Duration of ART	Median 11.5 (0–108) months	

ALL, acute lymphoblastic lymphoma; AML, acute myeloid leukemia; ART, antiretroviral therapy; CLL, chronic lymphoid leukemia; CML, chronic myeloid leukemia; MDS, myelodysplastic syndrome; NADHMs, non-AIDS-defining hematological malignancies; RAEB, refractory anemia with excess blast.

Treatments and outcomes

Most patients with AML and ALL received standard combination chemotherapy (such as cytarabine + daunorubicin and L-asparaginase + adriamycin, vincristine, prednisolone, cyclophosphamide), and the response rates for AML and ALL were 70.0 and 85.7%, respectively. However, the relapse rates for AML and ALL were 62.5 and 50%, respectively. The median overall survival (OS) period was 13 and 16 months for AML and ALL, respectively. The symptoms of three of the four patients with CML-CP were well controlled with imatinib. Four patients (2 AML, 1 Ph^1-ALL, and 1 CML-AP) were treated with allogeneic stem cell transplantation. One patient died because of acute graft-versus-host disease; however, three survived for more than 4 years. One patient with multiple myeloma was treated with autologous stem-cell transplantation without serious toxicity. Sixteen of the 19 patients with Hodgkin's lymphoma were treated with a combination of adriamycin, bleomycin, vinblastine, and dacarbazine or with radiation therapy. Eighty percent achieved complete remission. Details of treatments and outcomes of Hodgkin's lymphoma was described in another report [5].

Epidemiological analysis

There is an upward trend in the number of patients with NADHMs and the estimated number of HIV-infected patients [1] (Fig. 1). The estimated incidence of total NADHMs, Hodgkin's lymphoma, AML, ALL, and chronic leukemia (CLL, CML) was 32.6 (minimum–maximum, 27.2–41.3), 12.7(10.6–16.1), 8.0(6.6–10.1), 5.6 (4.6–7.1), and 4.0(3.3–5.0)/100 000 persons per year, respectively, between 1991 and 2009. The estimated crude incidence of total NADHMs increased 4.5-fold (4.3–5.4) from 1991–2000 to 2001–2009.

Discussion

The present study aimed to clarify the epidemiological status of NADHMs in Japan. Our results showed an estimated crude incidence rate of leukemia (CLL, CML, ALL and AML) of 17.6 (14.5–22.2)/100 000 persons per year in estimated HIV-infected individuals in Japan, which is 2.2-fold higher than that of leukemia (ICD10, C91–95) in the general population [6]. In addition, the estimated incidence of NADHMs has increased 4.5-fold (4.3–5.4) over the past decade.

The introduction of ART has improved the immunological status of HIV-infected individuals and reduced the incidence of AIDS-defining malignancies; however, the incidence of non-AIDS-defining malignancies is increasing. Several studies on non-AIDS-defining solid tumors suggest that aging; concomitant viral infection, such as that with Epstein–Barr virus, human papilloma virus, hepatitis C virus, and hepatitis B virus; low CD4 T-cell count with long-term immune suppression; and smoking are the possible causes of cancer [7–10]. However, the reason for the increased incidence of NADHMs remains unknown.

Recent developments in ART and supportive therapy for HIV-infected patients help facilitate long-term survival. Generally, aging is a key factor in carcinogenesis. In this study, we found that the mean age of patients with NADHMs was 49.3 years and that more than 30% were more than 60 years old, which is consistent with overall cancer trend.

Immune suppression is thought to be a risk factor for non-AIDS-defining malignancies. Krishnan *et al.* [9] analyzed prospective data of 3158 ART-naïve HIV-infected individuals and found that a recent low CD4 T-cell count is associated with non-AIDS-defining malignancies. In the present study, more than half of the patients with NADHMs had prior AIDS and their median CD4 T-cell count was less than 200/μl, suggesting that a low CD4 T-cell count is common in patients with NADHMs and may be one of the risk factors.

Uncontrolled HIV viral load is considered to be a risk factor for both AIDS-defining and non-AIDS-defining malignancies [11,12], and HIV itself may play a role in the onset of cancer. The regulatory proteins Tat and Vpr, which are encoded by the HIV genome, may contribute to oncogenesis [13,14]. However, a study by the AIDS Therapy Evaluation in the Netherlands (ATHENA) cohort showed no significant effect of viremiaon non-AIDS-defining malignancies [15]. In the present study, the HIV viral load was well controlled in more than 50% patients. Careful discussion is required to assess the association between carcinogenesis and HIV infection.

Before being diagnosed with NADHMs, the majority of patients were treated with ART, with the median

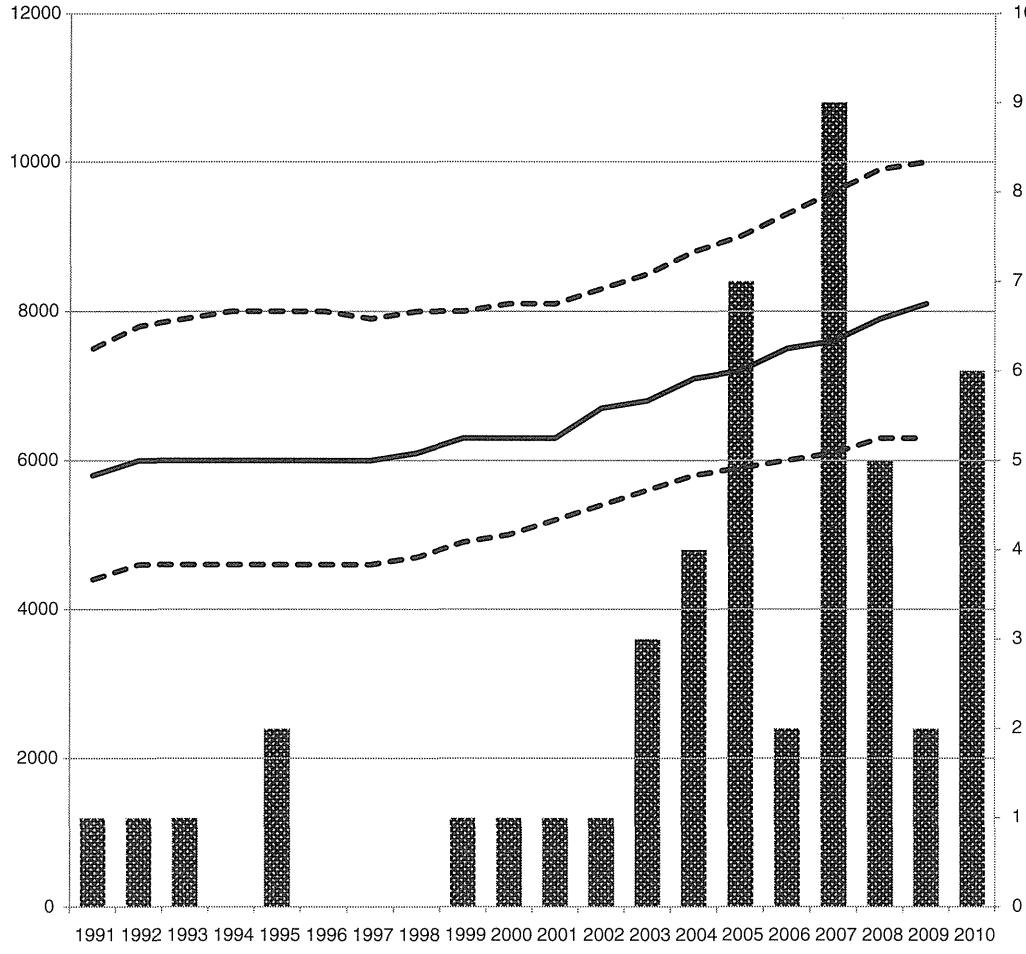

Fig. 1. The solid line shows the trend of the number of people in Japan estimated to be infected with HIV [1], and the dashed lines show the lower and upper ranges of the estimation. The solid bars indicate the number of patients with non-AIDS-defining hematological malignancies in each year.

duration of treatment being less than 1 year. Antiretroviral drugs can affect hematopoiesis, and zidovudine-containing regimens induce myelosuppression and possibly carcinogenesis [16]. Even when the duration of exposure to ART is short, its contribution to the development of NADHMs cannot be excluded.

We found that HIV-infected patients suffered from a variety of hematological malignancies, with a spectrum of NADHMs similar to that of HIV-negative patients. Cytogenetic analysis revealed typical chromosomal abnormalities of both acute and chronic leukemia. Moreover, adverse karyotypic abnormalities such as complex karyotype and/or monosomy, seven in AML and Burkitt-type karyotypes in ALL were often present. In three patients with AML with adverse karyotype, the duration of ART was more than 24 months. Long-term exposure to ART may cause therapy-related cytogenetic abnormalities.

In the present study, most patients with acute leukemia were treated with standard combination chemotherapy, and no deaths due to therapy were observed. Therapy appeared to be well tolerated, and the remission rate was similar to that of HIV-negative patients. In retrospective analysis, Sutton *et al.* [17] reported no deaths related to treatment of HIV-related AML in France, with a remission rate of 73.3%. Long-lasting remission of leukemia is rare in HIV-infected patients [18]. In the present study, more than 50% of the patients relapsed or represented primary refractory cases. However, two patients with AML and two with ALL continued in complete remission for more than 5 years. In the study by Sutton *et al.* [17], the estimated 5-year OS of 18 patients with AML was 19.9%, with a median survival period of 11 months [18]. There are several case reports of HIV-infected patients who have undergone stem-cell transplantation [19–21]. Five patients were found in our survey; however; only one death related to this therapy was observed. We conclude that stem-cell transplantation appears to be a feasible treatment for selected patients.

To summarize, a nationwide epidemiological study in Japan revealed that HIV-infected patients are at high risk for hematological malignancies, and the incidence of

these malignancies has increased in the past decade. The prognosis of HIV-infected patients was similar to that of HIV-negative patients. Standard chemotherapy may be a feasible treatment option for HIV-infected patients with hematological malignancies. Further study focusing on the mechanism of carcinogenesis in HIV-infected individuals is required.

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Conflicts of interest

The authors have no conflicts of interest.

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ORIGINAL ARTICLE

Clinical characteristics of human immunodeficiency virus-associated Hodgkin lymphoma patients in Japan

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Abstract The incidence of Hodgkin lymphoma (HL) is paradoxically increasing in the combination anti-retroviral therapy (cART) era. However, there has been no nation-wide survey of human immunodeficiency virus (HIV)-associated HL (HIV-HL) in Japan. We retrospectively examined the clinical characteristics and outcomes of 19 newly diagnosed HIV-HL patients at 11 HIV/AIDS and hematology regional hospitals in Japan between 1991 and 2010. At the time of HL diagnosis, 79 % of patients were receiving cART. All the patients, but one received HL diagnoses in the cART era. The median CD4+ cell count at HIV-HL diagnosis was 169/μl. Mixed-cellularity classical Hodgkin lymphoma was the most common subtype occurring in 68 % of the patients; 89 % of the patients were positive for Epstein–Barr virus. Of these 19 patients, 84 % were in advanced stages, with bone marrow

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involvement observed in 47 % of the patients; 58 % had extranodal sites. All the treated patients were given cART concurrent with HL therapy. The complete remission rate of the treated patients was 87 %. The median OS of the entire cohort was 17 months. These results suggest that the characteristics of HIV-HL in Japan are more aggressive than those of non-HIV-associated HL in Japan, but standard chemotherapy is effective and feasible.

Keywords Hodgkin lymphoma · HIV infection · ABVD · Antiretroviral therapy · EBV

Introduction

The introduction of combination antiretroviral therapy (cART) has led to improvements in immune status among human immunodeficiency virus (HIV)-infected individuals, reducing acquired immune deficiency syndrome (AIDS)-related morbidity and prolonging survival. The incidence of non-Hodgkin lymphoma (NHL) and other AIDS-defining malignancies has declined substantially over the past 10 years. In contrast, the incidence of non-AIDS-defining malignancies including Hodgkin lymphoma (HL) does not appear to have decreased, and some studies have shown that its incidence may have increased [1–6].

The behavior of HIV-associated HL (HIV-HL) is known to be more aggressive than that of non-HIV-HL, with a higher frequency of poor prognostic features, such as an advanced stage, extranodal involvement, bone marrow involvement, B symptoms, with Epstein–Barr virus (EBV) positivity [7–9]. Several studies reported that the standard combination chemotherapy, such as doxorubicin, bleomycin, vinblastine, and dacarbazine (ABVD) and the Stanford V regimen in conjunction with cART were tolerable and effective in patients with HIV [9–11].

The number of HIV-1 infected individuals is continuously increasing in Japan, with increased prevalence of AIDS-related NHL [12]. Since the incidence of non-HIV-associated HL in Japan is approximately one-third of that in Western countries [13, 14], the incidence and features of HIV-HL in Japan need to be clarified. In this study, we performed a retrospective analysis to elucidate the specific features of HIV-HL in Japan.

Methods

We surveyed all 429 regional AIDS centers and all 497 educational hospitals certified by the Japanese Society of Hematology (207 of these institutions overlapped) and obtained data from 511 institutions (71.1 %). In this retrospective cohort study, we examined the clinical characteristics and outcomes of patients with diagnoses of HIV-HL who visited 11 regional hospitals for HIV/AIDS and/or hematological diseases in Japan between 1991 and 2010. This study was approved by the responsible Ethics Committee.

Patients

The patients included in this study had received new diagnoses of HIV-HL during the study period. The pathological diagnosis of each institution was accepted. All patients who satisfied the above-mentioned criteria were consecutively examined. Data from all examined patients were then statistically analyzed.

Clinical characteristics of the patients

Data regarding age, prior AIDS diagnosis, prior administration of antiretroviral therapy, CD4+ cell count at diagnosis, HIV viral load at diagnosis, and the performance status (PS) according to the criteria of the Eastern Cooperative Oncology Group (ECOG) at diagnosis were analyzed. Complete remission (CR) was defined as the disappearance of all clinical evidence of disease at the completion of the first induction therapy. The presence of residual disease, but with $\geq$50 % decrease in the sum of the product of the greatest diameter was defined as partial response (PR). Overall survival (OS) was defined as the interval from diagnosis to death from any cause. An International Prognostic Score (IPS) is defined as the number of adverse prognostic factors present at diagnosis, and predicts the rate of freedom from progression of disease [15].

Statistical analysis

Kaplan–Meier survival curves were used to evaluate OS. Data were statistically analyzed using Statcel2 for Excel 2007 (The Publisher OMS, Saitama, Japan).

Results

Baseline characteristics of the patients

Table 1 shows the baseline characteristics of the 19 patients with HIV-HL. The median age was 48 years (range 31–66 years), and 89 % of the patients were men. A total of 16 patients (84 %) were Japanese. Of the 19 patients, 10 had received a diagnosis of AIDS before the development of HIV-HL, and 15 (79 %) were receiving cART at diagnosis. All the patients except 1 received diagnoses of HL in the cART era (Fig. 1).

Table 1 Baseline characteristics at the time of Hodgkin lymphoma (HL) diagnosis

Men	17 (89 %)
Median Age, years (range)	48 (31–66)
Prior AIDS diagnosis	10 (53 %)
Absolute CD4 cell counts, cells × 10^9/l (range)	169 (1–567)
Viral load <500 copies/ml	11/17 (65 %)
ECOG PS, *n* (%)	
0–1	12 (63 %)
2	4 (21 %)
3–4	3 (16 %)
Histologic subtype *n* (%)	
Classical Hodgkin lymphoma	18 (95 %)
MCCHL	13 (68 %)
NSCHL	3 (16 %)
LDCHL	2 (11 %)
Non-specific	1 (5 %)
EBV positivity	16/18 (89 %)
Ann Arbor stage *n* (%)	
I	2 (11 %)
II	1 (5 %)
III	7 (37 %)
IV	9 (47 %)
B symptoms	11 (58 %)
Extranodal sites	11 (58 %)
Bone marrow involvement	9 (47 %)
IPS	
0–2	7 (37 %)
>3	12 (63 %)

ECOG Eastern Cooperative Oncology Group, *PS* performance status, *MCCHL* mixed-cellularity classical Hodgkin lymphoma, *NSCHL* nodular sclerosis classical Hodgkin lymphoma, *LDCHL* lymphocyte-depleted classical Hodgkin lymphoma, EBV Epstein–Barr virus, *IPS* International Prognostic Score

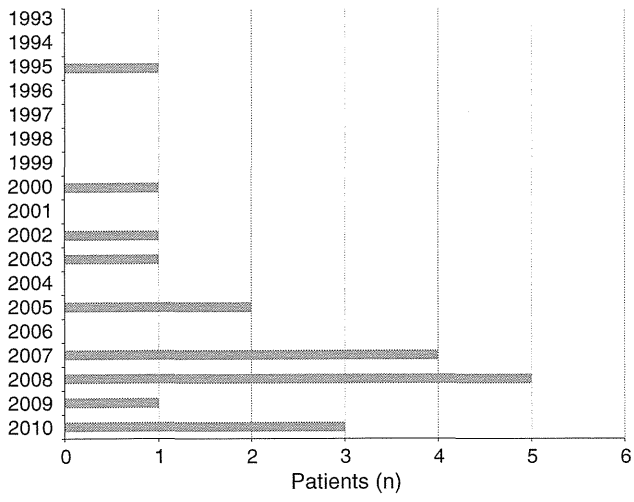

Fig. 1 Annual incidence of HIV-associated HL in Japan

The median CD4+ cell count at HIV-HL diagnosis was 169/μl (range 1–567/μl) and 1 patient with a CD4+ cell count of 1/μl had hemophagocytic syndrome at diagnosis. A total of 65 % of the patients had achieved an HIV viral load of <500 copies/ml at the diagnosis of HL, and 37 % of the patients had an ECOG PS of ≥2 at diagnosis. Mixed-cellularity classical Hodgkin lymphoma (MCCHL) was the most common subtype occurring in 13 (68 %) of the 19 HL patients, followed by nodular sclerosis classical Hodgkin lymphoma (NSCHL) occurring in 3 (16 %). A high frequency (89 %) of EBV association (EBER and/or LMP-1) was observed in HL tissues. A total of 84 % of the patients showed advanced stages (III, IV), with bone marrow involvement in 47 % of the patients. A total of 11 patients (58 %) had B symptoms and 11 patients (58 %) had extranodal sites. The number of patients with an International Prognostic Score (IPS) of 0–2, and the number of patients with an IPS of 3 or greater than 3 were 7 (37 %) and 12 (63 %), respectively.

Treatment and initial response

Of 3 patients who showed a localized stage, 2 were treated with ABVD therapy and radiotherapy and 1 was treated with radiotherapy alone. Among the patients who showed an advanced stage, 7 were treated with ABVD therapy. Of these 7 patients, 1 was not given bleomycin because of pre-existing interstitial pneumonia. Furthermore, 1 was given a reduced dose (/m^2 → /body) because of poor PS and pre-existing bone marrow suppression due to HIV infection without bone marrow infiltration of HL. A total of 6 patients were treated with ABVD therapy with a lower dose of dacarbazine (AVBd, 250 mg/m^2) and 3 patients could not be treated (2 patients received diagnoses at autopsy, and 1 with poor PS received a diagnosis during the pre-cART era). All the treated patients ($n = 16$) were given cART therapy concurrently with HL therapy.

Figure 2 shows the OS probabilities of the 19 patients. The median OS of the entire cohort was 17 months. The CR rate of the treated patients ($n = 15$) was 87 %: 1 patient achieved PR and 1 patient developed progressive disease (PD); 2 relapsed after achieving CR. The median progression-free survival (PFS) ($n = 19$) was also 17 months (Fig. 3).

Table 2 shows the status, CD4 cell counts and IPS of the patients. The 5-year survival rates of the patients with IPS of 0–2 and ≥3 were 86 % and 35, respectively ($n = 19$; $p = 0.095$ by logrank test) (Fig. 4). The CR rate of the advanced-stage patients who were treated with ABVD/ABVd therapy ($n = 12$) was 83 % (2 relapsed afterwards) and their survival rate ($n = 13$) was 56 % (Fig. 5). The survival rates of the advanced-stage patients treated with ABVD/ABVd ($n = 13$) with IPS of 0–2 and ≥3 were 75

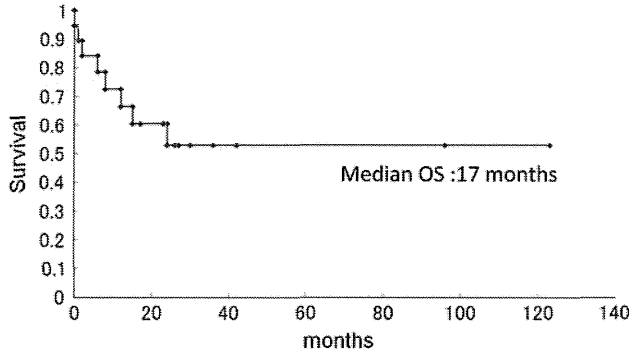

Fig. 2 Overall survival of the 19 patients

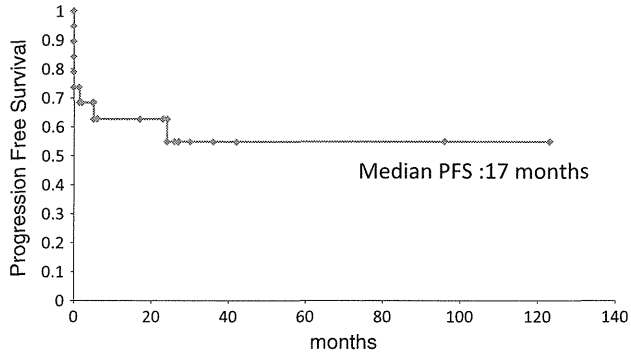

Fig. 3 Progression-free survival of the 19 patients

Table 2 Outcome, CD4 cell count and IPS of all patients

Patient No.	CD4 cell count	IPS	Survival (months)	Outcome
1	379	1	6	Alive (CR)
2	130	4	24	Dead (CR)
3	384	2	36	Alive (CR)
4	341	1	30	Alive (CR)
5	75	4	8	Dead (no treatment)
6	535	1	17	Alive (CR)
7	124	3	96	Alive (CR)
8	74	4	23	Alive (CR)
9	567	2	27	Alive (CR)
10	23	2	2	Dead (PD)
11	24	4	0	Dead (no treatment)
12	409	2	123	Alive (CR)
13	179	3	6	Dead (PR)
14	293	4	15	Dead (relapse)
15	452	3	26	Alive (CR)
16	1	4	12	Dead (relapse)
17	169	3	2	Alive (receiving treatment)
18	146	4	42	Alive (CR)
19	24	5	1	Dead (no treatment)

IPS International Prognostic Score, *CR* complete response, *PR* partial response

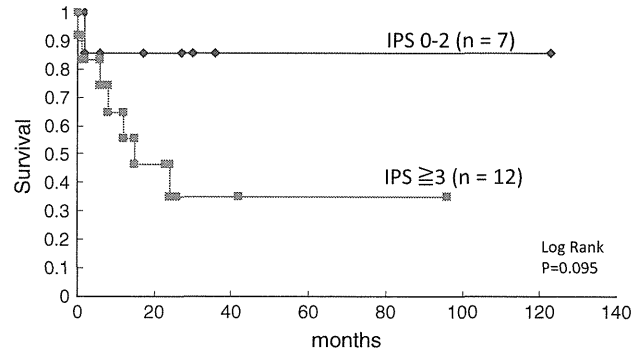

Fig. 4 Overall survival of the 19 patients according to IPS

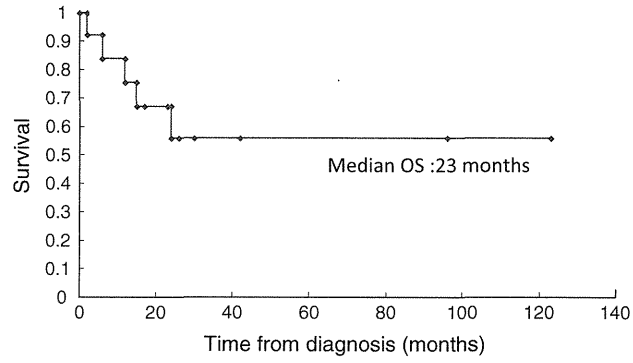

Fig. 5 Overall survival of the advanced-stage patients treated with ABVD/ABVd ($n = 13$)

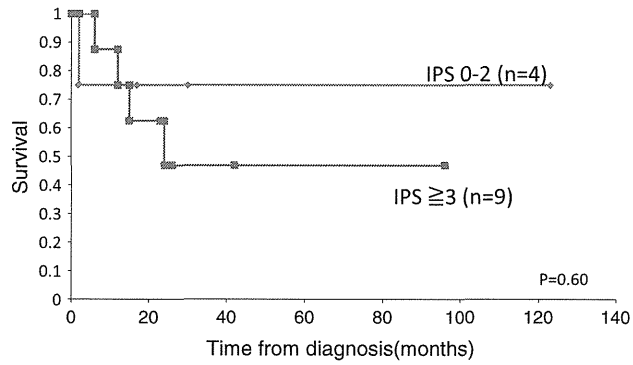

Fig. 6 Overall survival of the advanced-stage patients treated with ABVD/ABVd ($n = 13$) according to IPS

and 47 %, respectively (Fig. 6; $p = 0.60$ by logrank test). Of the 8 patients who died, the cause of death in 7 was HL (no treatment, $n = 3$; PR, $n = 1$; PD, $n = 1$; relapse, $n = 2$) and 1 patient died of infection and gastrointestinal perforation after the completion of therapy while in CR. The duration of grade 4 neutropenia was 0–12 days (median 7 days). With regard to bone marrow suppression, ABVD/ABVd was tolerable and no treatment-related deaths occurred during therapy. We were not able to assess the incidence of opportunistic infections.

Discussion

Although HL is not an AIDS-defining malignancy, its incidence is approximately 10 times higher in HIV-positive populations than in HIV-negative populations [16]. While the incidence of many opportunistic infections and some malignancies such as Kaposi sarcoma and NHL has been decreasing since the development of cART [1, 17], the incidence of HIV-HL has been increasing even in the cART era [2–5]. Recent data from the United States show that the estimated numbers of AIDS-defining cancers decreased by greater than threefold from 1991 to 1995 and from 2001 to 2005, whereas those of non-AIDS-defining cancers increased approximately threefold during the same periods and that of HL increased twofold [6]. The incidence of HIV-HL in Japan has also been increasing in the cART era after the year of 1996, based on the findings of the present study in which HL developed in all cases except 1 (Fig. 1).

In the present study, cART had been initiated in 79 % of the patients before the development of HL. A total of 65 % of the patients had achieved a viral load of <500 copies/ml at HL diagnosis. The suppression of RNA load and the presence of a higher CD4+ cell count did not appear to be useful to prevent the development of HL in the current series. Biggar et al. [3] showed that the incidence of HL after AIDS onset was higher in the cART era and in patients with a higher CD4 cell count. They suggested that the development of HL requires an inflammatory background which is absent in those with severe immunosuppression. Lanoy et al. [18] recently reported that HL risk was particularly elevated in months 1–3 of cART therapy, which suggests that immune reconstitution may play a role in some cases. HL developed in only 1 patient in months 1–3 in the current series of patients. A detailed physical examination of superficial lymph nodes even in patients with a good virological response condition is essential for an early diagnosis of HIV-HL.

HIV-HL is reported to be more malignant than non-HIV-HL [19]. HIV-HL is characterized by a high incidence of unfavorable histological subtypes such as MCCHL and lymphocyte-depleted classical Hodgkin lymphoma [3]. Our survey also revealed MCCHL to be the most common HL subtype in Japanese HIV-HL patients, whereas NSCHL is the most common subtype in Japanese non-HIV-HL patients according to 2 previous reports [20, 21]. A high frequency of EBV association (80–100 %) has been shown in the HL tissue of HIV-HL patients [3, 19, 22, 23], and LMP-1 is expressed in virtually all HIV-HL cases which support an etiologic role of EBV in the pathogenesis of HIV-HL, in contrast to an EBV-association incidence of only 20–50 % in non-HIV-HL [24, 25]. In the current series, 89 % of the patients with HIV-HL showed EBV positivity, similar to that of previous reports. It has been reported that the prevalence of EBV in Hodgkin and Reed-Sternberg cells varies according to the histological subtype and epidemiologic factors. The highest frequency (75 %) is found in MCCHL and the lowest (10–40 %) in NSCHL [26]. This may account for the finding that EBV positivity was high in HIV-HL patients in the present series. Previously, a decrease in the incidence of EBV-positive AIDS-related lymphoma from 88 % in the pre-cART era to 58 % in the cART era has been reported in Japan [27]. The major histological subtype was diffuse large B-cell lymphoma (DLBCL) and only 3.5 % of these patients had HL. In the current series, HL developed in only 1 patient in the pre-cART era, making a comparison difficult. However, high EBV positivity, even in the cART era, appears to be a marked characteristic of HIV-HL compared with DLBCL. However, it is still unclear why EBV-positive HIV-HL develops in well-controlled HIV-1 patients.

In non-HIV-HL patients, primary extranodal involvement is rare. More than 60 % of patients have localized disease (Stages I and II). Bone marrow involvement has been reported in only 5 % of cases [28]. The frequency of an advanced stage at HL diagnosis is high in patients with HIV, similar to that observed in patients with AIDS-related NHL. In the present study, 84 % of the patients were in an advanced stage. A high frequency of B symptoms (70–100 %), the presence of extranodal sites (30 %) and bone marrow involvement (40–50 %) have been reported in HIV-HL [29, 30]. The incidence rates of B symptoms, extranodal sites and bone marrow involvement were 58, 58, and 47 %, respectively, in the current series which is consistent with previous reports. The characteristics of HIV-HL in Japan were found to differ from those of non-HIV-HL in Japan, but similar to those of HIV-HL in other countries.

The prognosis of HIV-HL was poor in the pre-cART era. The AIDS Clinical Trial Group (ACTG) reported that in a prospective study of 21 HIV-HL patients treated with standard ABVD therapy without antiretroviral therapy (ART), the median survival was only 1.5 years. Despite the routine use of granulocyte colony-stimulating factor, opportunistic infections occurred in 29 % of the patients during and shortly after the study [30]. The ACTG reported the results of a phase II study of 21 patients (Stages III–IV patients, 81 %) treated with ABVD therapy without cART in which the CR rate was 43 % and the median survival was 18 months [31]. Little et al. [32] reported that cART administration during or at the end of systemic chemotherapy for HIV-associated lymphoma could prolong patient survival. A previous study of 62 retrospectively analyzed advanced-stage HIV-HL patients in Spain who were treated with ABVD concomitantly with cART reported a CR rate of 87 % and a 5-year OS of 76 % [4].

Prognosis has thus improved in the cART era, and patients can be treated similarly as immunocompetent patients.

The median OS of the entire cohort in our study ($n = 19$) was 17 months, and no treatment-related deaths were noted. The cause of death ($n = 8$) was HL in 88 % of the patients. Disease control, rather than infection control was more difficult in patients in our study which reflected the aggressive nature of HIV-HL. The contribution of EBV infection to the development of HIV-HL is still unknown. Keegan et al. have reviewed the association of EBV infection in non-HIV-HL patients, and found different types of association (i.e., worse prognosis, no association or better prognosis) [33]. These results suggest that the poorer prognosis of HIV-HL is not due to the presence of EBV. However, more cases are required to further clarify the contribution of EBV infection to the development of HIV+ HL.

ABVd therapy with a low dose of dacarbazine (250 mg/m^2) has been used for Japanese patients with advanced non-HIV-HL to prevent severe adverse effects, and has shown to be effective for Japanese patients with non-HIV-HL [21]. In the current series of patients, the CR rate of the advanced-stage patients who were treated with ABVD/ABVd ($n = 12$: ABVD, $n = 6$; ABVd, $n = 6$) was 83 %, and the 5-year survival rate ($n = 13$) was 56 %, which was inferior to that described in a previous report from Spain [4]. Further prospective studies are needed to evaluate the efficacy of AVBd therapy for Japanese HIV-HL patients.

IPS, defined as the number of adverse prognostic factors present at diagnosis, predicts the rate of freedom from progression of disease in patients with non-HIV-HL [15, 34]. Spina et al. [35] reported that a high IPS is also predictive of a worse outcome for patients with HIV-HL. In the current series of patients, the 5-year OS of patients with an IPS of 0 to 2 and ≥ 3 were 86 and 35 %, respectively ($n = 19$). Although it is not significant, a high IPS tends to result in lower survival rate. A randomized trial of aggressive conventional chemotherapy compared with high-dose chemotherapy with autologous hematopoietic stem-cell transplantation (HSCT) for relapsed chemosensitive non-HIV-HL patients showed significantly better freedom from treatment failure for patients given HSCT [36]. High-dose chemotherapy and autologous stem-cell transplantation (ASCT) in the salvage setting of AIDS-related lymphoma (ARL) including HL have been demonstrated to be feasible [37] and have shown good tolerability and favorable disease-free survival and OS [38].

In conclusion, we conducted a nationwide survey of HIV-HL patients in Japan. We found that the incidence of HIV-HL in Japan is increasing, and that most of the patients with HIV-HL in Japan showed an advanced stage. Because the number of HIV-1-infected individuals receiving cART in Japan is expected to increase, clinicians who specialize in HIV infection need to be careful in the diagnosis of HIV-HL.

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Potent antitumor activity of zoledronic acid-induced Vγ9Vδ2 T cells against primary effusion lymphoma

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ABSTRACT

Primary effusion lymphoma (PEL) is a subtype of aggressive and resistant non-Hodgkin lymphoma that occurs predominantly in patients with advanced AIDS. In this study, we examined the antitumor activity of zoledronic acid (Zol)-induced Vγ9Vδ2 T cells against PEL cells *in vitro* and *in vivo*. Vγ9Vδ2 T cells recognized endogenous mevalonate metabolites and MICA/B of PEL cell lines, inducing cytotoxicity via granule exocytosis and TRAIL-mediated pathway. Vγ9Vδ2 T cells suppressed the development of PEL cells and existed in a PEL xenograft mouse model. These results show that immunotherapy with Zol-induced Vγ9Vδ2 T cells could demonstrate an efficient strategy for PEL.

1. Introduction

Primary effusion lymphoma (PEL) is an infrequent and distinct entity of aggressive non-Hodgkin B-cell lymphoma that shows serous lymphomatous effusion in body cavities (pleura, peritoneum and pericardium) and is universally associated with Kaposi sarcoma-associated herpes virus/human herpes virus-8 (KSHV/HHV-8) [1,2]. PEL is diagnosed most commonly in patients with HIV/AIDS, accounting for 4% of all non-Hodgkin lymphomas in this population [3]. The lack of optimal therapy combined with the aggressive nature of PEL results in a short median survival of less than 6 months [4]. There is therefore an urgent need for the development of new therapeutics.

Human γδ T cells account for 1–10% $CD3^+$ cells in the peripheral blood of healthy adults [5]. The majority expressed Vγ9Vδ2 T cell receptor (TCR) [6]. Unlike αβ T cells, Vγ9Vδ2 T cells recognize non-peptidic antigens without antigen processing and major histocompatibility complex (MHC) restriction. There is growing evidence of the antitumor activity of Vγ9Vδ2 T cells against a large range of tumor types, including hematopoietic tumor cells [7].

Phosphoantigens, such as isopentenyl pyrophosphate (IPP) and the synthetic analog bromohydrin pyrophosphate (BrHPP), activate Vγ9Vδ2 T cells *in vitro*, inducing their cytotoxic activity against tumor or infected target cells [8]. Nitrogen-containing bisphosphonates (N-BPs), such as zoledronic acid (Zol), indirectly stimulate Vγ9Vδ2 T cells by inhibiting farnesyl diphosphate synthase, a key enzyme of the mevalonate pathway, causing the intracellular accumulation of IPP [9].

We expanded Vγ9Vδ2 T cells from healthy donors with Zol and evaluated the efficacy of Vγ9Vδ2 T cells against PEL cells *in vitro* and *in vivo*. Vγ9Vδ2 T cells recognized endogenous mevalonate metabolites and MHC class I-related chain A and B (MICA/B), of PEL cells. The cytotoxic activity was mediated by granule exocytosis and the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) pathway. In the *in vivo* assessment using a PEL xenograft mouse model, Vγ9Vδ2 T cells inhibited the growth and invasion of PEL cells and were found in the spleen and peripheral blood of the treated mice.

These results show the effect of Vγ9Vδ2 T cell-based immunotherapy against PEL cells and provide insight into the role of Vγ9Vδ2 T cells in tumor immunity for PEL.

2. Materials and methods

2.1. Cell lines and reagents

Human PEL cell lines, BCBL-1 (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH), BC-1 (purchased from ATCC, Rockville, MD), BC-3 (purchased from ATCC) and Burkitt lymphoma cell line, Daudi (obtained from RIKEN Cell Bank, Tsukuba, Japan) were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified incubator at 37 °C and 5% CO_2. Zol was obtained from Novartis Pharma AG (Basel, Switzerland).

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2.2. Human Vγ9Vδ2 T cell preparations and culture

Informed consent was obtained for the collection of peripheral blood from healthy volunteers. Peripheral blood mononuclear cells (PBMCs) were separated individually from blood samples donated by three healthy volunteers using Pancoll (PAN Biotech GmbH, Aidenbach, Germany). PBMCs were stimulated with 1 μM Zol and cultured for 14 days at 37 °C in a humidified atmosphere of 5% CO_2. Medium consisted of RPMI 1640 supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). On day 1, 5, 9, and 13, 100 U/ml interleukin-2 (IL-2) was added to the culture. On day 14, γδ T cells were isolated from expanded γδ T cells by negative selection using γδ T-cell isolation kits (Miltenyi Biotec, Bergisch Gladbach, Germany), as per the manufacturer's instructions. After isolation, the percentage of γδ T cells was more than 95.0%.

2.3. Flow cytometry

Cells were stained with the following monoclonal antibody (mAb): TCR Vγ9-phycoerythrin (PE), TCR Vδ2-fluorescein isothiocyanate (FITC), DR4(TRAIL-R1)-PE, DR5(TRAIL-R2)-PE, CD107a-PE, human CD45-pacific blue (PB), mouse CD45-Alexafluor 700 (AF 700), MICA/B-Alexafluor 647 (AF 647) from BioLegend (San Diego, CA); CD138-FITC from e-Bioscience (San Diego, CA); Fas(CD95)-PE from BD PharMingen (San Diego, CA). To determine perforin expression, cells were fixed with 1% paraformaldehyde and permeabilized with 0.1% saponin. The frequency of degranulating Vγ9Vδ2 T cells was quantified by measuring CD107a expression. Vγ9Vδ2 T cells were co-incubated with PEL cell lines as an effector to target a cell (E:T) ratio of 1:1 for 1 h at 37 °C in 5% CO_2, after which brefeldin A (Sigma–Aldrich, St. Louis, MO) was added at a final concentration of 10 μg/ml and incubated for an additional 4 h at 37 °C in 5% CO_2. Subsequently, the cells were washed and stained with TCR Vδ2-FITC. After staining, cells were washed twice, resuspended in staining medium (PBS with 3% FBS and 0.05% sodium azide), and immediately analyzed on an LSR II flow cytometer (BD Bioscience, San Jose, CA). Data were analyzed with FlowJo software (Tree Star, San Jose, CA).

2.4. Tetrazolium dye methylthiotetrazole (MTT) assay

The antiproliferative activities of Zol against PEL cell lines were measured by the methylthiotetrazole (MTT) method (Sigma–Aldrich). Briefly, 2×10^4 cells were incubated in triplicate in a 96-well microculture plate in the presence of different concentrations of Zol in a final volume of 0.1 ml for 24 h at 37 °C. Subsequently, MTT (0.5 mg/ml final concentration) was added to each well. After 3 h of additional incubation, 100 μl of a solution containing 10% SDS plus 0.01 N HCl was added to dissolve the crystals. Absorption values at 595 nm were determined with an automatic enzyme-linked immunosorbent assay (ELISA) plate reader (Multiskan; Thermo ElectronVantaa, Finland). Values were normalized to untreated (control) samples.

2.5. In vitro cytotoxicity assay and blocking studies

Purified Vγ9Vδ2 T cells were resuspended at final concentrations of 2×10^5, 1×10^6, and 2×10^6 cells/ml, and 100 μl was then added to round-bottomed polystyrene tubes together with target cells (100 μl) to obtain E:T ratios of 1:1, 5:1, and 10:1. Cytotoxicity was measured by flow cytometric analysis using CFSE and propidium iodide (PI) as previously described [10]. Briefly, 50 μl CFSE (Invitrogen, Carlsbad, CA) was added to 1 ml target cell suspension (5×10^5 cells/ml) in PBS to obtain the final concentration of 2.5 μM CFSE. The cells were incubated for 10 min at 37 °C and gently mixed every 5 min. At the end of incubation, 1 ml FBS was added to the cell suspension to stop the staining reaction, and the cells were centrifuged at 400*g* for 5 min at room temperature, washed twice with cold PBS, and resuspended in complete medium. Control tubes containing only labeled target cells and effector cells were also prepared to establish background levels of cell death. Tubes were gently mixed, centrifuged at 300*g* for 2 min, and incubated at 37 °C in 5% CO_2 for 4 h.

At the end of the incubation period, 300 μl complete medium and 1 μl of 1 mg/ml PI were added to each tube for 15 min before acquisition on LSR II cytometer. The measurement of cytotoxic activity was based on the degree of reduction of viable target cells with the ability to retain CFSE and exclude PI ($CFSE^{high}$ PI^-). To inhibit perforin-mediated cytotoxicity, Vγ9Vδ2 T cells were incubated with 300 nM concanamycin A (CMA) (A.G. Scientific, Inc., San Diego, CA) for 30 min at 37 °C prior to coculture, without further washing. To inhibit recognition of endogenous mevalonate metabolites, PEL cells were incubated with 25 μM mevastatin (Mev) (Sigma–Aldrich) for 60 min at 37 °C prior to coculture, without further washing. For blocking mAbs, anti-FasL (clone NOK-1), anti-TRAIL (clone RIK-2), anti-MICA/B (clone 6D4), mouse IgG1 Isotype Ctrl (clone MG1-45), mouse IgG2a Isotype Ctrl (clone MOPC-173) (Biolegend) were used to evaluate the mechanisms of Vγ9Vδ2 T cell-mediated cytotoxicity of PEL cell lines at the final concentration of 10 μg/ml. Vγ9Vδ2 T cells were cultured with PEL cell lines at an E:T ratio of 10:1 in the presence of CMA, Mev or blocking Abs. Pretreatment of Vγ9Vδ2 T cells or PEL cells with CMA or Mev at the concentrations used in this study did not have any cytotoxic effect.

2.6. Xenograft mouse model

NOD Rag-2-deficient (Rag-$2^{-/-}$) mice and NOD Jak3-deficient (Jak$3^{-/-}$) mice were established by crossing Rag-$2^{-/-}$ mice or Jak$3^{-/-}$ mice with the NOD strain for 10 generations, respectively. NOD Rag-2/Jak3 double-deficient (Rag-$2^{-/-}$Jak$3^{-/-}$) mice (NRJ mice) were established by crossing NOD Rag-$2^{-/-}$ mice and NOD Jak$3^{-/-}$ mice, and were housed and monitored in our animal research facility according to institutional guidelines. All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee at Kumamoto University. Eight to twelve week-old NRJ mice were intraperitoneally inoculated with 7×10^6 BCBL-1 cells or BC-3 cells suspended in 200 μl PBS. The mice were then treated with intraperitoneal injections of PBS or *ex vivo* expanded 3×10^7 Vγ9Vδ2 T cells on day 3 after cell inoculation and once a week. The mice of both groups were also treated with 2 μg Zol (on day 3) and 3000 U IL-2 (twice a week) intraperitoneally. Tumor burden was evaluated by measuring the body weight gain, the volume of ascites and the percentage of human CD45 gated $CD138^+$ cells in the spleen.

2.7. Immunohistochemistry

To investigate the expression of KSHV/HHV-8 ORF73 (LANA) protein, tissue samples were fixed with 10% neutral-buffered formalin, embedded in paraffin and cut into 4-μm sections. The sections were deparaffinized by sequential immersion in xylene and ethanol, and rehydrated in distilled water. They were then irradiated for 15 min in a microwave oven for antigen retrieval. Endogenous peroxidase activity was blocked by immersing the sections in methanol/0.6% H_2O_2 for 30 min at room temperature. Affinity-purified PA1-73N antibody [11], diluted 1:3000 in PBS/5% bovine serum albumin (BSA), was then applied, and the sections were incubated overnight at 4 °C. After washing in PBS twice, the second and third reactions and the amplification procedure were performed using kits according to the manufacturer's instructions (catalyzed signal amplification system kit; DAKO, Copenhagen, Denmark). The signal was visualized using 0.2 mg/ml diaminobenzidine and 0.015% H_2O_2 in 0.05 mol/l Tris–HCl, pH 7.6.

2.8. Statistical analysis

Data are expressed as the mean ± SD. The statistical significance of the differences observed between experimental groups was determined using Student's *t*-test, and $P < 0.05$ was considered significant.

3. Results

3.1. Proliferation of Vγ9Vδ2 T cells by Zol

Fig. 1A shows representative data of Vγ9Vδ2 T cell expansion. Consistent with other reports, Vγ9Vδ2 T cells represented a minor subset of peripheral lymphocytes (1–10%) before culture [5]. Following exposure to 1 μM Zol and IL-2 (100 U/ml, on day 1, 5, 9, 13), the percentages of Vγ9Vδ2 T cells reached up to more than 80% on day 14 (Fig. 1A). Determination of the absolute number of Vγ9Vδ2 T cells increased for 14 days and ranged from 88.0 to 134.1-fold expansion after 14 days in three normal volunteers (Fig. 1B), indicating the efficient expansion of Vγ9Vδ2 T cells by our method.

3.2. In vitro cytotoxicity of ex vivo expanded Vγ9Vδ2 T cells

Three PEL cell lines (BCBL-1, BC-1, and BC-3),the Burkitt lymphoma cell line, Daudi, and non-target cells, PBMCs, were cultured with Vγ9Vδ2 T cells at each E/T ratio (0, 1, 5 and 10) for 4 h, and cytotoxicity was measured by flow cytometric analysis using CFSE and PI as described in Materials and Methods. The percentage of specific lysis against PEL cell lines by *ex vivo* expanded Vγ9Vδ2 T cells increased in an E/T ratio-dependent manner, whereas Vγ9Vδ2 T cells had no obvious cytotoxic effect on PBMCs (Fig. 2). Vγ9Vδ2 T cells had the same levels of cytotoxicity against PEL cell lines as the standard target Daudi Burkitt lymphoma cell line, which possessed strong sensitivity to Vγ9Vδ2 T cells [12]. These findings indicated that Vγ9Vδ2 T cells killed PEL cell lines efficiently. Although N-BPs have been reported to have direct antitumor effects on several cancer cell lines via the inactivation of Ras-related proteins [13], the methylthiotetrazole assay showed that Zol did not have a direct antitumor effect against PEL cell lines with the concentrations

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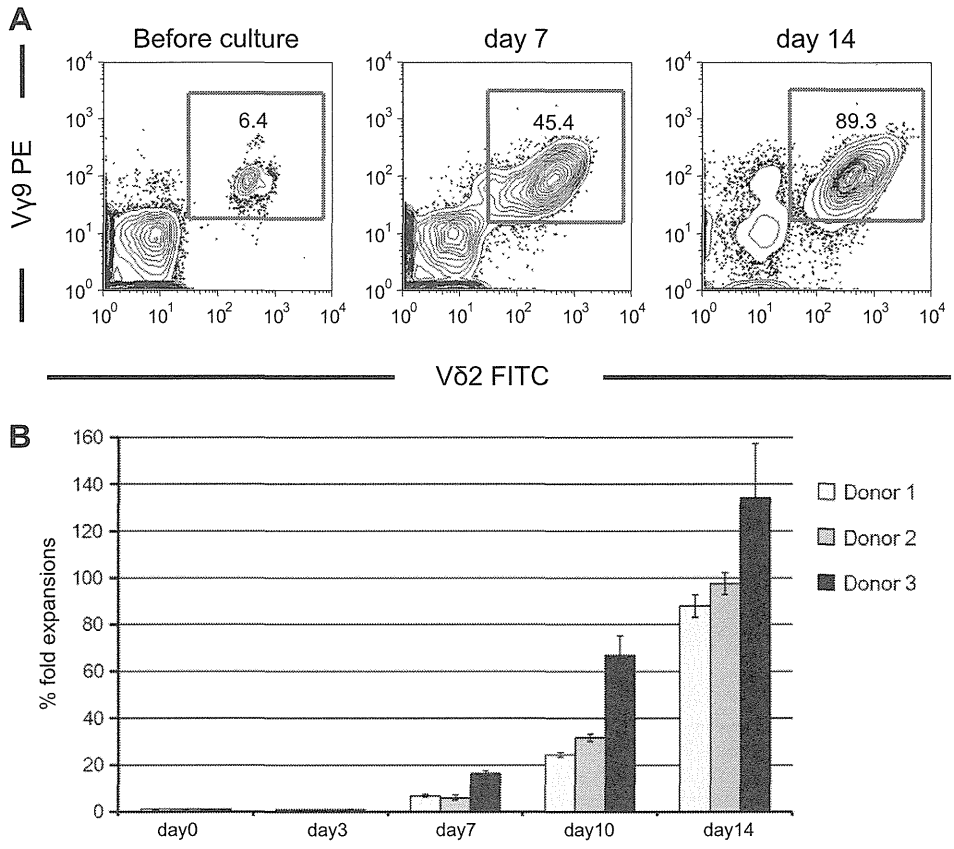

Fig. 1. Vγ9Vδ2 T cells were generated by expanding PBMC from healthy donors with Zol and IL-2. (A) Representative flow cytometric profiles with the ratio of Vγ9Vδ2 T cells before culture, on day 7, and 14. (B) Fold expansion (%) of Vγ9Vδ2 T cells following culture. Data are shown as the mean ± SD of three experiments from three healthy volunteers.

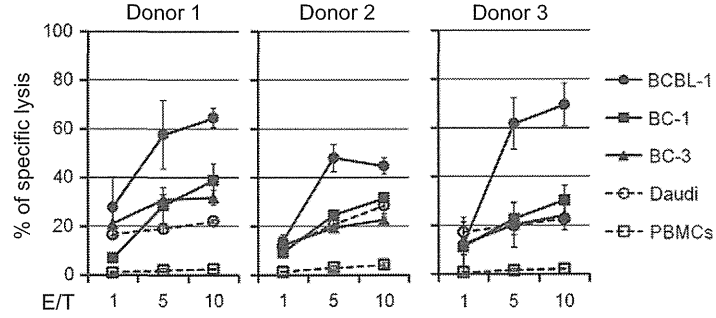

Fig. 2. Vγ9Vδ2 T cell-mediated cytotoxicity of PEL cell lines. Data are shown as the mean ± SD of three experiments from three healthy volunteers.

ranging 0–10 μM (Supplementary Fig. 1). Moreover, pretreatment of PEL cells with Zol (at concentrations up to 10 μM) for 24 h did not enhance the susceptibility to cytotoxicity by Vγ9Vδ2 T cells significantly (data not shown).

3.3. PEL cell lines constitutively express death receptors and MICA/B

To determine the possible mechanisms involved in Vγ9Vδ2 T cell-mediated cytotoxicity, we examined PEL cell surface expression of Fas (CD95), DR4 (TRAIL-R1), DR5 (TRAIL-R2) and MICA/B. As depicted in Fig. 3A, PEL cell lines constitutively expressed all these molecules, although the level of expression varied among the cell lines.

3.4. Vγ9Vδ2 T cells recognize endogenous mevalonate metabolites and MICA/B of PEL cell lines, inducing cytotoxicity via the perforin and TRAIL-mediated pathway

It has been reported that Vγ9Vδ2 T cells recognize endogenous mevalonate metabolites and NKG2D ligands, such as MICA/B, of tumor targets [8,14,15]. The mechanism of cytotoxicity includes death receptor/ligand interactions and the release of perforin/granzymes. As shown in Fig. 3B, killing-inhibition experiments using CMA, Mev and Ab against MICA/B revealed Vγ9Vδ2 T cells recognized PEL cells via endogenous metabolites (means of 34.0–65.8% inhibition) and MICA/B (means of 25.5–39.7% inhibition), and killed them by the perforin pathway (means of 28.2–43.7% inhibition). Addition of Ab against TRAIL caused significant killing

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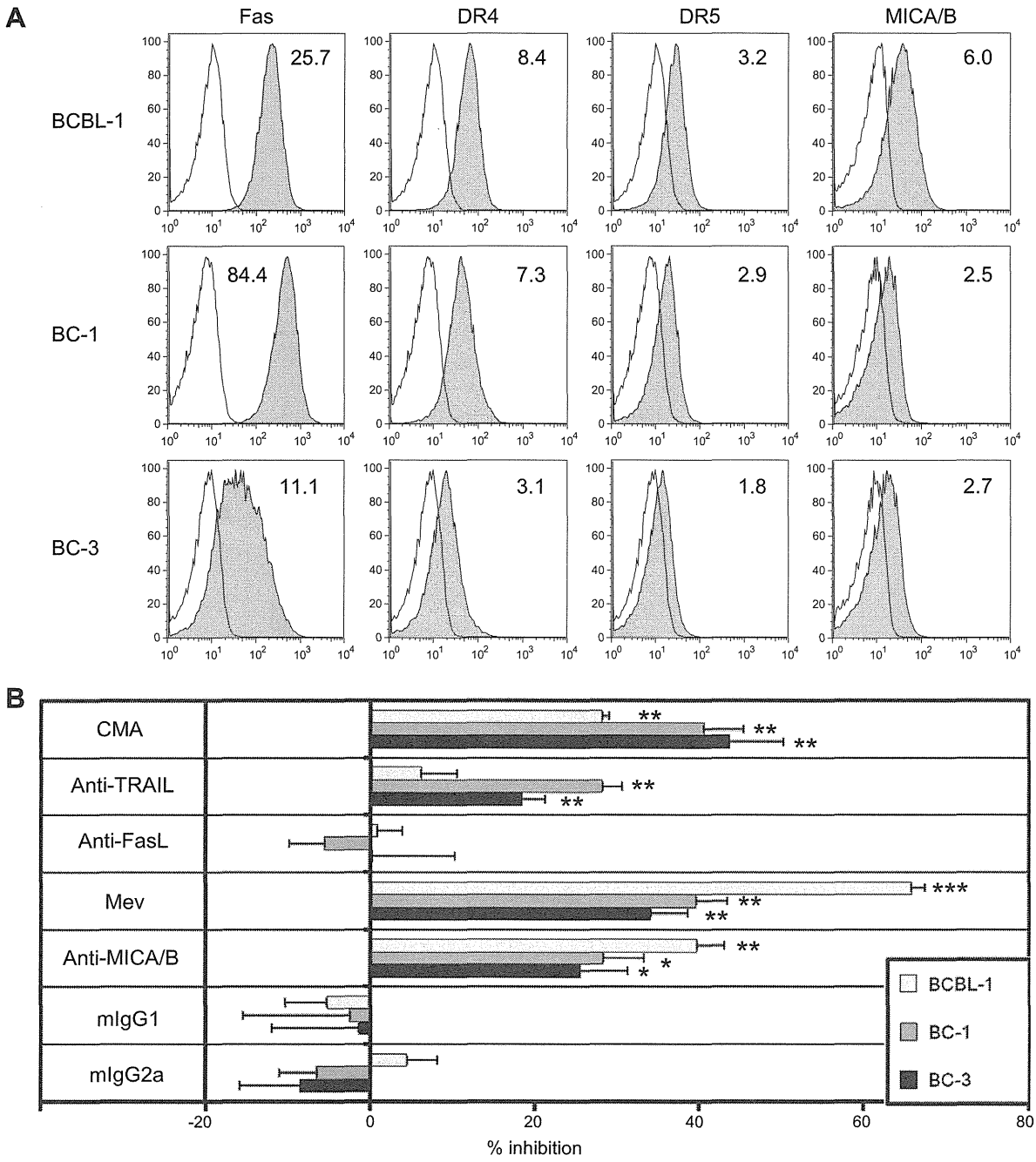

Fig. 3. Phenotype of PEL cell lines and mechanisms of Vγ9Vδ2 T cell cytotoxicity of PEL target cells. (A) Expression of Fas, DR4, DR5 and MICA/B on the surface of PEL cell lines. Numbers in histograms indicate the fold increase in the mean fluorescence intensity. (B) Vγ9Vδ2 T cells were cultured with PEL cells at an E:T ratio of 10:1 in the presence of CMA, Mev or blocking Abs to FasL, TRAIL, MICA/B or isotype Ctrl. Data are shown as the mean ± SD of three experiments from one healthy volunteer. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ when compared with cytotoxicity carried out in the absence of inhibitors.

inhibition of BC-1 (mean of 28.1% inhibition) and BC-3 (mean of 18.4% inhibition), respectively, but had no significant effect on the death of BCBL-1 (mean of 6.2% inhibition), indicating that the cytotoxicity of Vγ9Vδ2 T cells against PEL cells was partially mediated by the TRAIL pathway. Addition of Ab against FasL or isotype Ctrl caused no significant inhibition against all tested PEL cells. Next, we evaluated the expression of CD107a (lysosome-associated membrane protein-1), a marker associated with the degranulation of cytotoxic $CD8^+$ T cell [16] and NK cell [17]. Following coculture of Vγ9Vδ2 T cells with PEL cell lines, CD107a expression on the surface of Vγ9Vδ2 T cells was up-regulated 2.1- to 2.8-fold, indicating the degranulation of Vγ9Vδ2 T cells against PEL cells (Fig. 4).

3.5. In vivo effect of Vγ9Vδ2 T cells

To evaluate the potential of the immunotherapy strategy, we used a previously established model of PEL transplantation into severe immunodeficient, NOD/Rag-2/Jak3-deficient (NRJ) mice [18]. NRJ mice were inoculated intraperitoneally with 7×10^6 BCBL-1 cells or BC-3 cells. BCBL-1 or BC-3 produced profuse ascites within 4 weeks of inoculation (Fig. 5A). Then, 3×10^7 Vγ9Vδ2 T cells or PBS alone was administrated via intraperitoneal injection on day 3 after cell inoculation and once a week; 2 μg Zol (on day 3) and 3000 U IL-2 (twice a week) were also injected intraperitoneally. Vγ9Vδ2 T cell-treated mice seemed to show no apparent change,

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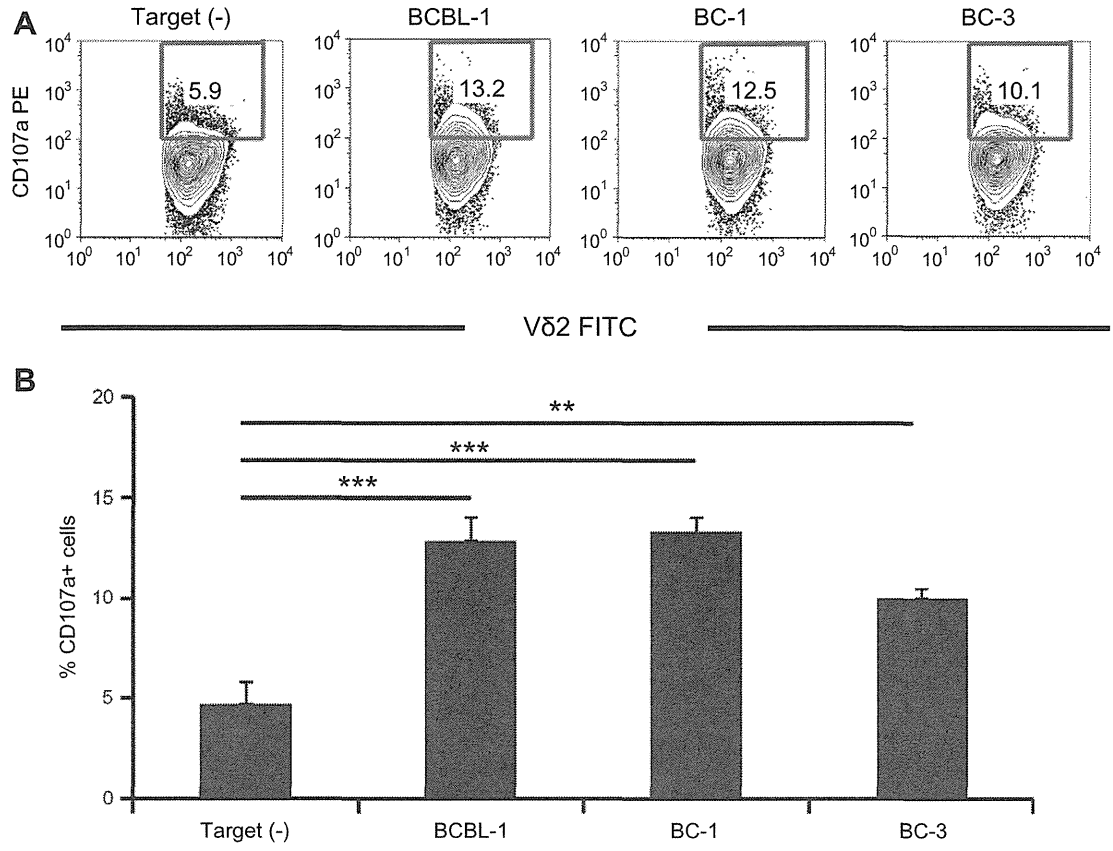

Fig. 4. Expression of CD107a of γδ T cells following coculture with PEL cells. (A) Representative flow cytometric profiles of CD107a$^+$ cells among Vδ2$^+$ cells following coculture with PEL cell lines. (B) Levels of CD107a$^+$ cells among Vδ2$^+$ cells following coculture with or without PEL cell lines at an E:T ratio of 1:1. Data are shown as the mean ± SD of three experiments from one healthy volunteer. $^{**}P < 0.01$; $^{***}P < 0.001$ when compared with CD107a expression of Vδ2$^+$ cells in the absence of PEL cells.

and the body weight gain of untreated mice significantly increased compared to that of Vγ9Vδ2 T cell-treated mice on day 31 (7.5 ± 2.7 g *vs.* 4.1 ± 3.1 g in BCBL-1 xenografts, 6.5 ± 2.6 g *vs.* 2.8 ± 2.0 g in BC-3 xenografts, $n = 7$ for each group; Fig. 5B). The body weight gain of treated mice was not significantly different from that of age matched control mice on day 31. In addition, the volume of ascites was significantly lower than in untreated mice on day 31 (2.8 ± 1.0 ml *vs.* 0.9 ± 1.0 ml in BCBL-1 xenografts, 3.3 ± 1.5 ml *vs.* 0.6 ± 0.8 ml in BC-3 xenografts, $n = 7$ for each group; Fig. 5C).

Organ invasion by PEL cells on day 31 was evaluated by hematoxylin–eosin staining and LANA immunostaining. We found that mice inoculated intraperitoneally with BCBL-1 exhibited invasion into the liver and lung without macroscopic lymphoma formation (Fig. 6). The number of LANA-positive cells in Vγ9Vδ2 T cell-treated mice was significantly reduced (0–1 cells per field magnification, ×40) compared to untreated mice (50–100 cells per field magnification, ×40). The presence of BCBL-1 cells and Vγ9Vδ2 T cells was assessed by flow cytometry on day 31. The ratio of BCBL-1 cells in the spleen of treated mice was reduced significantly compared with untreated mice (0.1 ± 0.1% *vs.* 2.6 ± 1.8%, $n = 5$ for each group, $P < 0.05$; Fig. 7A and B).

These results demonstrated that Vγ9Vδ2 T cells significantly inhibit the growth and infiltration of PEL cells *in vivo* and could be a potential therapy for patients with PEL.

3.6. Engraftment of Vγ9Vδ2 T cells in the xenograft model

The percentage of Vγ9Vδ2 T cells in treated mice averaged 4.3 ± 2.6% in the spleen (Fig. 7A and C) and 2.2 ± 2.0% in the peripheral blood on day 31. Vγ9Vδ2 T cells were present and persisted in the xenograft model in the spleen and peripheral blood of NRJ mice for at least 7 days after the final i.p. administration of Vγ9Vδ2 T cells. The xenograft model using NRJ mice could be a potential tool to evaluate not only the therapeutic effect of PEL cells but also the dynamics of Vγ9Vδ2 T cells *in vivo*.

4. Discussion

PEL is characterized by clinical aggressiveness and resistance to conventional chemotherapy; therefore, there is a need to develop new therapies. Recently, several new therapeutic strategies for PEL have been proposed. These strategies involve activating TRAIL-mediated apoptosis by IFN-α and azidothymidine [19,20], inhibition of NF-κB [21], or inducing lytic replication of HHV-8 while blocking virus production [22]. Despite *in vitro* and *in vivo* studies of therapy for PEL, to date there is no standard treatment.

Although Vγ9Vδ2 T cells represent a minor subset (1–10%) of human peripheral lymphocytes, they exhibit potent MHC-unrestricted lytic activity against different tumor cell and play a crucial role in tumor immunosurveillance. Vγ9Vδ2 T cell number and reactivity are rapidly impaired after HIV-1 infection [23]; however, highly active antiretroviral therapy (HAART) is able to restore Vγ9Vδ2 T cell number and function [24]. HIV-mediated depletion of Vγ9Vδ2 T cells has the possibility to contribute to HIV-related malignancy [25]. Induction of Vγ9Vδ2 T cells is considered to be potential immunotherapy against HIV-related malignancies including PEL. Zoledronic acid (Zol), currently the most potent third generation bisphosphonate, is safe and widely used to treat

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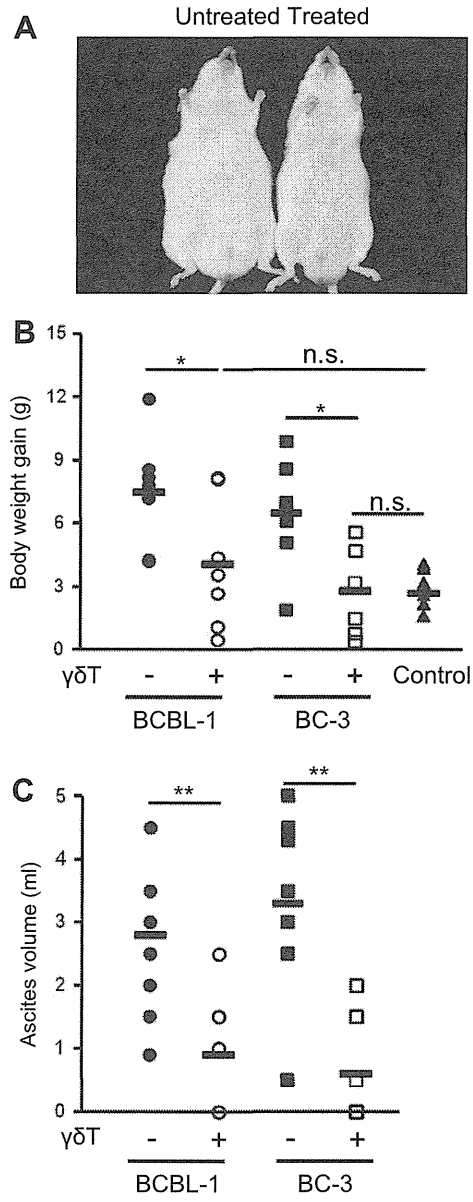

Fig. 5. Treatment of NOD/Rag-2/Jak3-deficient mice with Vγ9Vδ2 T cells suppresses the development of PEL *in vivo*. (A) Photograph of Vγ9Vδ2 T cell-treated and -untreated ascites-bearing mice 31 days after inoculation with BCBL-1 intraperitoneally. (B) Body weight gain of mice 31 days after inoculation with BCBL-1 cells or BC-3 cells in Vγ9Vδ2 T cell-treated or untreated mice is shown as the mean ± SD of seven mice. Control indicates the group of age matched mice without PEL cell injection and Zol/IL-2 treatment. (C) Volume of ascites 31 days after inoculation with BCBL-1 cells or BC-3 cells in the mice is shown as the mean ± SD of seven mice. $^{*}P < 0.05$; $^{**}P < 0.01$ when compared with body weight gain or ascites volume.

osteoporosis, hypercalcemia and bone metastases in patients with breast and prostate cancer [26]. Zol induces Vγ9Vδ2 T cell expansion more efficiently by inhibiting farnesyl diphosphate synthase compared with other bisphosphonates [27]. In this study, we investigated the cytotoxic activity of Zol-induced Vγ9Vδ2 T cells against PEL cell lines *in vitro* and *in vivo*. Our results show that Vγ9Vδ2 T cells exhibit potent antitumor effects against PEL cells and give evidence to support a clinical study of immunotherapy using Vγ9Vδ2 T cells.

The result of blocking studies revealed that Vγ9Vδ2 T cells recognized endogenous mevalonate metabolites and MICA/B of PEL cell lines (Fig. 3B). The recognition of these antigens is considered to exert the difference of cytotoxicity among PEL cell lines. The mechanism responsible for Vγ9Vδ2 T cell killing of PEL cells was via perforin and TRAIL, but not the FasL-mediated pathway (Fig. 3B), although PEL cell lines expressed both Fas and TRAIL-R (Fig. 3A). It has been proposed that v-FLIP, viral analogs of FLIP encoded by HHV-8, binds to FADD and caspase-8, and thereby inhibits Fas-mediated apoptosis [28]. The presence of some decoy receptors, which are related to Fas but lack a functional death domain [29,30], might also influence resistance to death receptor-mediated apoptosis. Although DR4 and DR5 expressing cells are much more in BCBL-1 (8.4 and 3.2 respectively) compared with BC-3 (3.1 and 1.8 respectively) in Fig. 3A, blocking TRAIL caused significant killing inhibition of BC-3 (means of 18.4% inhibition) instead of BCBL-1 (means of 6.2% inhibition) in Fig. 3B. Resistance to TRAIL-induced apoptosis or some decoy receptors might be related to the difference of killing inhibition [14].

Previous studies have shown that CD107a expression on the cell surface is a marker associated with the degranulation of cytotoxic lymphocytes [16,17]. We also confirmed the upregulation of surface CD107a expression on Vγ9Vδ2 T cells following coculture of Vγ9Vδ2 T cells with PEL cell lines (Fig. 4). Our study demonstrates that Vγ9Vδ2 T cells induced efficient apoptosis of PEL cells via the release of cytotoxic granules, and CD107a expression on the cell surface could be a marker associated with the degranulation of Vγ9Vδ2 T cells.

In the present study, we investigated the direct involvement of Vγ9Vδ2 T cells in the growth and invasion of PEL cells using NOD/Rag-2/Jak3 double-deficient (NRJ) mice [18], which displayed rapid and efficient engraftment of PEL cells and Vγ9Vδ2 T cells, as a small animal model. NRJ mice display not only complete deficiency in mature T/B lymphocytes and complement protein but also complete deficiency of NK cells, indicating that they are appropriate recipients of human hematopoietic and tumor cells, as are NOD/Scid/common γ-deficient (NOG) mice [31]. While the efficacy of Vγ9Vδ2 T cells in tumor xenograft mouse models has been described in the immunodeficient mice bearing s.c. or i.v. tumor xenografts [32–37], we evaluated the effect of Vγ9Vδ2 T cells using an orthotopic PEL xenograft mouse model. Transfer of Vγ9Vδ2 T cells to NRJ mice showed significant inhibition of ascites formation as well as organ invasion (Fig. 5). Previously, Lozupone et al. reported that γδ T cells began to be depleted at 24 h and were undetectable in the spleen of the SCID mouse model 48 h after the i.v. administration of γδ T cells [38]. We confirmed that Vγ9Vδ2 T cells were present and persisted in the spleen and peripheral blood of NRJ mice for at least 7 days after the final i.p. administration of Vγ9Vδ2 T cells (Fig. 7A and C). Depletion of NK cell activity has been reported to facilitate the engraftment of human mature T-lymphocytes [39–41], indicating the contribution to the engraftment of Vγ9Vδ2 T cells. Taken together, NRJ mice are expected to be more convenient recipients of both human tumor cells and Vγ9Vδ2 T cell xeno-transplantation.

As previously reported, HAART is able to improve the prognosis of AIDS-related lymphomas [42,43]. Moreover, antiretroviral therapy could provide potential benefits for the treatment of PEL [44,45], indicating the importance of immunocompetence in the treatment of PEL. Considering the immunodeficient status at the onset in HIV-1-infected patients with PEL, induction of Vγ9Vδ2 T cells is expected to be a potent therapeutic approach instead of conventional therapy. Occasionally, the proliferative responses of Vγ9Vδ2 T cells from patients with cancer were impaired [46]. Although Zol and IL-2 treatment has been reported to improve immunocompetence in HIV-1-infected persons by activating Vγ9Vδ2 T cells [47], developing more efficient expansion and administration of Vγ9Vδ2 T cells in patients with malignancy could lead to the clinical use of immunotherapy by Vγ9Vδ2 T cells for a variety of malignancies as well as PEL.

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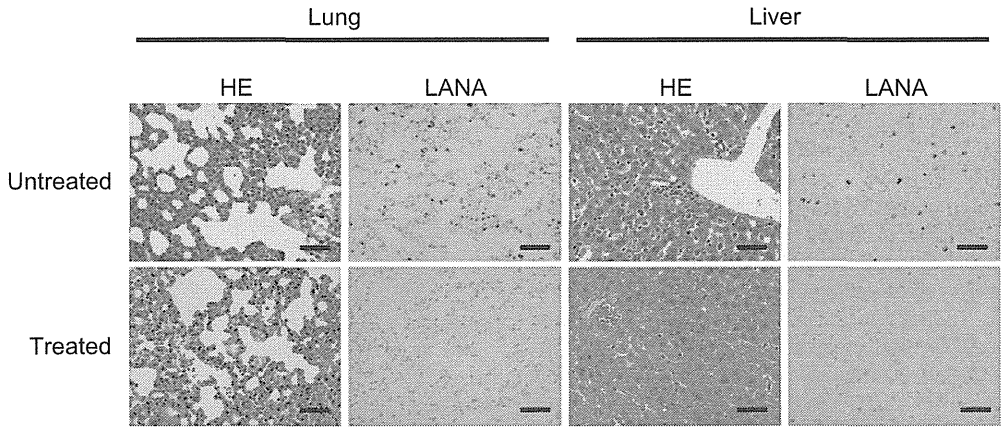

Fig. 6. Invasion of PEL cells into the organs of BCBL-1-inoculated mice on day 31. Hematoxylin–eosin staining and immunohistochemical staining using anti-LANA (PA1-73 N antibody) were performed to detect BCBL-1 in the lung and liver. All panels are the same magnification, bar scale, 100 μm.

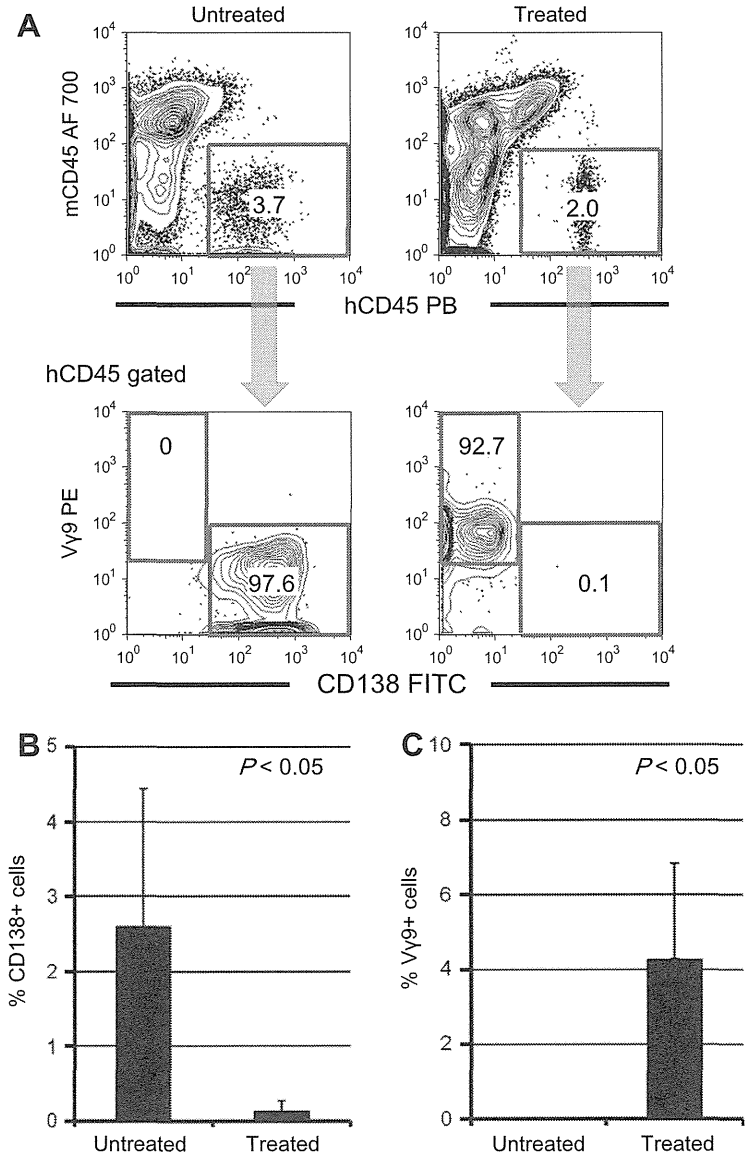

Fig. 7. Percentage of PEL cells and Vγ9Vδ2 T cells in the spleen of BCBL-1 xenograft mouse model. (A) Representative flow cytometric profiles with the ratio of human CD45 gated CD138$^+$ cells and human CD45 gated Vγ9$^+$ T cells in the spleen of untreated and treated mice. (B) Percentage (mean ± SD of five mice) of human CD45 gated CD138$^+$ cells in the spleen of untreated and treated mice. (C) Percentage (mean ± SD of five mice) of human CD45 gated Vγ9$^+$ T cells in the spleen of untreated and treated mice.

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In summary, our data have shown the potent antitumor activity of Vγ9Vδ2 T cells against PEL for the first time, and immunotherapy with Zol-induced Vγ9Vδ2 T cells could be an efficient strategy for PEL.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.canlet.2012.12.021.

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