Table 3 Non-Hodgkin lymphoma and Kaposi sarcoma in AIDS-associated autopsies

		Total		ART (−) patients		ART (+) patients		*P* values	
		n	%	n	%	n	%		
All NHL cases		71	100.0%	41	100.0%	25	100.0%		
Histology	DLBCL	53	74.6%	30	73.2%	18	72.0%	0.917	
	BL	4	5.6%	3	7.3%	1	4.0%	0.987	Y
	PEL	5	7.0%	4	9.8%	1	4.0%	0.706	Y
	PBL	1	1.4%	1	2.4%	0	0.0%	0.801	Y
	Other	6	8.5%	2	4.9%	4	16.0%	0.279	Y
	Unknown	2	2.8%	1	2.4%	1	4.0%	0.703	Y
Site	Nodular	1	1.4%	0	0.0%	1	4.0%	0.801	Y
	Extranodular	45	63.4%	28	68.3%	12	48.0%	0.102	
	Both	21	29.6%	11	26.8%	10	40.0%	0.265	
	Unknown	4	5.6%	2	4.9%	2	8.0%	0.987	Y
PCNS	Yes	27	38.0%	18	43.9%	6	24.0%	0.103	
EBV	Positive	52	73.2%	35	85.4%	12	48.0%	**0.001**	
KSHV	Positive	6	8.5%	5	12.2%	1	4.0%	0.495	Y
Cause of death	Yes	50	70.4%	33	80.5%	16	64.0%	0.137	
All KS cases		38		22		10			
Site	Skin	32	84.2%	19	86.4%	9	90.0%	0.410	
	GI tract	27	71.1%	15	68.2%	8	80.0%	0.705	
	Lung	21	55.3%	11	50.0%	6	60.0%	0.799	
	Lymph node	20	52.6%	13	59.1%	6	60.0%	0.502	
	Other	16	42.1%	0	0.0%	0	0.0%	0.787	Y
Cause of death	Yes	11	29.0%	7	31.8%	2	20.0%	0.791	Y

NHL, Non-Hodgkin lymphoma; DLBCL, diffuse large B-cell lymphoma; BL, Burkitt lymphoma; PEL, primary effusion lymphoma; PBL, plasmablastic lymphoma; PCNS, primary central nervous system lymphoma; EBV, Epstein–Barr virus; KSHV, Kaposi sarcoma-associated herpes virus; KS, Kaposi sarcoma; GI, gastrointestinal. *P* values were calculated by Chi-square test. Y indicates the use of Chi-square test with Yates correction. Bold font indicates statistical significance.

Table 4 Lung disease in patients infected with HIV

Illness	n	% of total patients (n = 225)
Any illness	173	76.9%
Cytomegalovirus infection	93	41.3%
Pneumocystis jirovecii pneumonia	66	29.3%
Any bacterial pneumonia	31	13.8%
Aspergillus infection	23	10.2%
Kaposi sarcoma	21	9.3%
Non-tuberculous mycobacterium infection	14	6.2%
Cryptococcosis	11	4.9%
Candida infection	10	4.4%
Tuberculosis	4	1.8%

Because more than one illness was detected in patients, the numbers of all illness are greater than the total number.

HIV-infected patients identified from 1985–2012 at four central hospitals in Japan. CMV infection, PCP, NTM infection, NHL, and KS were frequently observed in the autopsy cases. The prevalence of CMV and PCP was lower in ART (−) patients compared with ART (+) patients. The prevalence of non-AIDS defining malignancies was higher among ART (+) patients than ART (−) patients, suggesting that the onset of various opportunistic infections and malignancies should be carefully monitored regardless of whether the patient is receiving ART.

The autopsy cases in the present study were predominantly male (95.1%, Table 1). Additionally, more than 70% of the autopsy cases in the present study had a CD4 count < 200 cells/μL at the last blood examination before death (Table 1). A recent clinical study demonstrated the incidence of AIDS-defining illnesses in patients with HIV infection was decreased by the introduction of ART, especially in patients with CD4 counts >200 cells/μL [2]. Thus, our findings at autopsy cannot be compared with previous clinical studies because many clinical study patients had a high range of CD4 counts and ART responses. Interestingly, there was no significant difference in the

Table 5 Brain disease in patients infected with HIV

Illness	n	% in total autopsied brains (n = 172)
Any illness	85	49.4%
Cytomegalovirus infection	45	26.1%
Malignant lymphoma	26	15.1%
HIV encephalopathy	21	12.2%
Progressive multifocal leukoencephalopathy	8	4.7%
Toxoplasmosis	8	4.7%
Non-tuberculous mycobacterium infection	4	2.3%
Aspergillus infection	2	1.2%
Varicella zoster virus infection	2	1.2%
Herpes simplex virus infection	1	0.6%
Glioblastoma	1	0.6%
Candida infection	1	0.6%

Because more than one illness was detected in patients, the numbers of all illness are greater than the total number.

cause of death between ART (+) and (−) patients, with the exception of those with cancer (Table 6), indicating the prevalence of lethal illness did not differ between ART (+) and (−) patients.

Malignancies were frequent causes of death in the present study regardless of ART status (Table 6). Several studies demonstrated that the introduction of ART reduced the incidence of NHL in patients with HIV infection [13,15,19-23]. The use of ART has also been associated with a decrease in the incidence of KS [15,24,25]. However, an association between the incidence of non-AIDS-defining cancers and ART remains controversial. An increase of non-AIDS-defining cancers in patients receiving ART was shown in previous clinical reports [26,27], but a separate study showed that, with the exception of long-term protease inhibitor usage, ART exposure was generally not associated with a risk of non-AIDS-defining cancers [28]. The reasons for increased risk of non-AIDS-defining cancers in patients on ART are unclear, but might reflect the concomitant increase of the mean age at autopsy during the study period. This suggests that life extension of HIV-infected patients by ART results in the increased chance of developing non-AIDS events and malignancies. It was also

Table 6 Cause of death in AIDS-associated autopsies

	All		ART (−) patients		ART (+) patients			
	n	%	n	%	n	%	*P* values	
Total*	225	100.0%	136	100.0%	66	100.0%		
Malignant lymphoma	50	22.2%	33	24.3%	16	24.2%	0.997	
Cytomegalovirus	44	19.6%	27	19.9%	9	13.6%	0.279	
Pneumonia	31	13.8%	19	14.0%	9	13.6%	0.949	
Pneumocystis jirovecii pneumonia	30	13.3%	21	15.4%	4	6.1%	0.058	
Non-tuberculous mycobacterium	12	5.3%	10	7.4%	2	3.0%	0.367	Y
Kaposi sarcoma	11	4.9%	7	5.1%	2	3.0%	0.749	Y
Progressive multifocal leukoencephalopathy	8	3.6%	4	2.9%	2	3.0%	0.684	Y
Cancer	8	3.6%	2	1.5%	6	9.1%	**0.026**	Y
Hepatitis	8	3.6%	3	2.2%	4	6.1%	0.320	Y
Cryptococcus	7	3.1%	6	4.4%	0	0.0%	0.197	Y
Kidney failure	7	3.1%	4	2.9%	3	4.5%	0.861	Y
HIV encephalopathy	7	3.1%	5	3.7%	2	3.0%	0.861	Y
Aspergillus	6	2.7%	5	3.7%	0	0.0%	0.274	Y
Toxoplasmosis	4	1.8%	3	2.2%	1	1.5%	0.835	Y
Tuberculosis	3	0.9%	2	1.5%	0	0.0%	0.816	Y
Sepsis	3	1.3%	2	1.5%	1	1.5%	0.551	Y
Candida	3	1.3%	2	1.5%	0	0.0%	0.816	Y
Varicella zoster virus	2	1.3%	1	0.7%	1	1.5%	0.816	Y
Nocardia	1	0.4%	1	0.7%	0	0.0%	0.711	Y
Histoplasma	1	0.4%	1	0.7%	0	0.0%	0.711	Y

*Because more than one illness was detected in patients, the numbers of all illness are greater than the total number.
HIV, human immunodeficiency virus. *P* values were calculated with the Chi-square test. Y indicates the use of Chi-square test with Yates correction. Bold font indicates statistical significance.

demonstrated that ART introduction changed the pathological features of lymphoma; for example, a decrease of Epstein–Barr virus-positive lymphoma in Japanese patients with AIDS was reported [29]. Although HL was rare in the general Japanese population compared with European countries and the United States [30], the incidence of HL increased in Japanese patients on ART [17]. Thus, the increased risk of malignancies during the clinical course of HIV infection in patients receiving ART was reflected as a cause of death in the autopsy cases used in our study.

The prevalence of opportunistic infections differs among various regions and countries. In sub-Saharan African countries, more than 80% of HIV-positive patients die of infectious diseases, with disseminated tuberculosis being the most common (36%) [31]. Furthermore, there was no difference in the type of disease HIV patients succumbed to, regardless of ART status. In the USA and European countries, tuberculosis/NTM represented <10% of mortality in autopsy cases after 1996 [18]. In this study, tuberculosis was detected in only 2.7% of Japanese autopsy cases, but was the cause of death for 50% of afflicted patients. Mortality by PCP has decreased worldwide in patients with AIDS owing to prophylactic administration of an anti-PCP drug [16]. PCP was found in 36.4% (36/99 cases) of patients with AIDS before 1997, but was significantly reduced after 1997 (23.8%; 30/126 cases; $P = 0.04$; Chi-square test). This suggests that the decrease in PCP cases is associated with ART and anti-PCP prophylaxis.

Our study had several limitations. Bacterial culture was not available in this study owing to the use of formalin-fixed paraffin-embedded samples, and it was therefore difficult to identify the bacterial species responsible for many cases of pneumonia. Additionally, clinical information was limited. Information on HIV-RNA, an important indicator of ART effects, was not available for these patients. In addition, information regarding CD4 counts and the type, duration and possible interruption of ART were not available for a subset of patients. Therefore, we could not identify cases of immune reconstitution syndrome. Age at seroconversion and time living with HIV are also major predictors of HIV disease progression, however information of these parameters was limited. Thus, it should be noted that the conclusions in this study cannot be generally applied to the current HIV positive population in Japan. Furthermore, all findings in this study were obtained from autopsies.

Conclusions

Although further studies are required to demonstrate the association between ART and illness identified at autopsy, the present study demonstrates the prevalence of infectious diseases and malignancies in autopsy cases of HIV infection in Japan. While the prevalence of CMV infection and PCP at autopsy were lower in ART (+) patients than ART (−) patients, non-AIDS–defining malignancies were observed as a cause of death more frequently in ART (+) patients than ART (−) patients.

Abbreviations

HIV: Human immunodeficiency virus; ART: Antiretroviral therapy; AIDS: Acquired immunodeficiency syndrome; PCP: *Pneumocystis jirovecii* pneumonia; CMV: Cytomegalovirus; NTM: Non-tuberculous mycobacteria; NHL: Non-Hodgkin lymphoma; KS: Kaposi sarcoma.

Competing interests

The authors declare no conflicts of interests.

Authors' contributions

HK, S Okada and AY conceived this study; TH, MM, YKod, NO, YO, SM, TI, HH, and HK performed the autopsies, pathological analyses and reviews; AA, KT, JT, YKi, TU, TS, TK, AI, and S Oka collected clinical data; HK analyzed the data, performed statistical analyses, and drafted the manuscript. All authors read and approved the final manuscript.

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Review

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Current status of treatment for primary effusion lymphoma

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Summary **Primary effusion lymphoma (PEL) is a rare and aggressive B-cell non-Hodgkin's lymphoma that usually presents with malignant effusions without tumor masses. An extracavitary or solid variant of PEL has also been described. Human herpes virus 8/Kaposi sarcoma-associated herpes virus (HHV-8/KSHV) is universally associated with the pathogenesis of PEL. More than 70% of cases occur with concurrent Epstein-Barr virus infection, but its relation to the pathogenesis is unknown. Patients are found in the context of immunosuppressive states (HIV-1 infection, post-organ transplantation). PEL is usually treated with CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone)-like chemotherapy with antiretroviral therapy if HIV-1 is positive. However, it is generally resistant to chemotherapy with a short median survival of less than 6 months. The optimal treatment for PEL has not been established yet. More intensive chemotherapy, such as dose-adjusted EPOCH (DA-EPOCH; etoposide, prednisone, vincristine, cyclophosphamide and doxorubicin) and CDE (cyclophosphamide, doxorubicin, etoposide) are expected to show a favorable prognosis. Recently, the molecular steps in KSHV/HHV-8-driven oncogenesis have begun to be revealed, and molecular targeting therapies such as proteasome, NF-κB, cytokines and surface antigens would provide evidence for their clinical use.**

Keywords: Primary effusion lymphoma (PEL), Human herpes virus-8/Kaposi sarcoma-associated herpes virus (HHV-8/KSHV), HIV-1/AIDS, combination antiretroviral therapy (cART), NF-κB, PEL xenograft mouse model

1. Introduction

Primary effusion lymphoma (PEL) is defined as "a large B-cell neoplasm usually presenting as serious effusions without detectable tumor masses, and is universally associated with human herpes virus 8/Kaposi sarcoma-associated herpes virus (HHV-8/KSHV)" by the WHO Classification of Tumors of Haematopoietic and Lymphoid Tissues (4th edition) (*1*). Rare HHV-8-positive lymphomas indistinguishable from PEL present as solid tumor masses, named extracavitary PEL (*2*).

PEL was first described in 1989 as an AIDS-related lymphoma of uncertain lineage that demonstrated B-cell derivation and included Epstein-Barr virus (EBV) (*3*). In 1995, Cesarman *et al.* identified KSHV DNA sequences within a distinct subtype of AIDS-related lymphoma presenting with lymphomatous effusions (*4*). In 1996, Nador *et al.* designated this lymphoma as "primary effusion lymphoma", which is a distinct entity associated with HHV-8/KSHV (*5*). The majority of cases arise in young and middle-aged homosexual or bisexual men with HIV infection. The disease also occurs in elderly patients and post-transplantation patients (Table 1) (*6-9*). In the majority of PEL cases, co-infection with EBV has been detected. The latency of EBV is type I and the role of EBV in the PEL pathogenesis is still unclear. HIV-infected individuals have a 60-200-times higher relative risk of developing NHL than the HIV-negative population (*10*). Among HIV-associated lymphoma, PEL arises more frequently in the HIV-infected population. PEL accounts for approximately 4% of all HIV-associated NHL cases (*11,12*). PEL is described as a distinct entity and is also included in "lymphomas occurring more specifically

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Table 1. Etiology of primary effusion lymphoma (PEL)

Category	Characteristics
PEL in elderly persons	Especially in endemic areas of HHV-8/KSHV
Post-transplantation PEL	With immunosuppressive therapy
HIV-1-related PEL	Homosexuals have a high prevalence of HHV-8/KSHV

in HIV-positive patients" among HIV-associated lymphoma in the WHO classification (*13*).

In this review article, therapeutic evidence from case series and the potential use of drugs and novel therapeutic approaches from preclinical evaluation of this refractory lymphoma are discussed.

2. Clinical features

PEL is clinically characterized by lymphomatous effusions in body cavities (formerly called body cavity lymphoma) usually without extracavitary tumor masses, and the clinical symptoms depend on the cavities involved. The most common sites are the pleural, peritoneal and pericardical cavities, and joint space and meningeal space are rarely involved (*14*). Patients present with dyspnea from pleural or pericardial effusion, or abdominal distension from ascites, which are the results of mass effects of malignant effusions. Patients with PEL with more than one body cavity involved had a median overall survival (OS) of 4 months compared with 18 months in patients with only one cavity involved (*15*). PEL usually occurs in advanced AIDS patients with a decreased CD4 T-cell count at diagnosis. Approximately half of the patients have pre-existing or develop KS (*16*). HIV-negative patients with PEL are extremely rare but have been described in elderly men from the Mediterranean region (areas with high prevalence for HHV-8 infection) and immunocompromised patients after solid organ transplantation (*17,18*). Recently, rare cases of an extracavitary variant of PEL have been observed in the lymph nodes or extranodal sites, such as the gastrointestinal tract, skin, lung, and CNS without lymphomatous effusions (*2,19*). Since extracavitary PEL has immunoblastic-like and anaplastic features with CD30 expression, it is hard to diagnose without showing the existence of HHV-8/KSHV infection.

3. Laboratory features

Cytologic preparation (Cytospin) of the involved effusion fluid is used for pathological examination and diagnosis. PEL cells show nuclei that are large, round and irregular in shape, with prominent nuclei. The cytoplasm is deeply basophilic with occasional vacuolated cells.

PEL cells typically express a hematolymphoid marker, CD45, but they usually lack expressions of B-cell markers (CD19, CD20, CD79a, surface and cytoplasmic immunoglobulin) (*20*). PEL cells express plasma cell markers, including CD138, VS38c and MUM-1/IRF4. Moreover, the cells generally express various activation markers, such as CD30, CD38, CD71 and epithelial membrane antigen (EMA). They usually lack T-cell markers (CD2, CD3, CD4, CD5, CD7, CD8), although aberrant expression of T cell antigen may occur. *Bcl-6* and *c-myc* are usually absent, and immunoglobulin gene rearrangement shows monoclonality of B-cell origin. Thus, PEL is a post-germinal center tumor at a pre-terminal stage prior to plasma cell differentiation (*21*). Transcript profiling confirmed this genesis (*22*).

The detection of HHV-8 infection in neoplastic cells is needed for definitive diagnosis of PEL (*1*). Immunohistochemistry for latent nuclear antigen-1 (LANA-1) is currently the standard method to detect the presence of HHV-8/KSHV in lymphoma cells (*14*). Typically positive results are characterized by a nuclear dot-like pattern. Polymerase chain reaction (PCR) amplification using a DNA extract from lymphoma cells is also useful to detect HHV-8/KSHV and measure peripheral blood HHV-8/KSHV viral load (*23*) as HHV-8 can be detected in the plasma at the onset of PEL (*24*). Evidence of EBV infection is most reliably detected by in situ hybridization for EBV-encoded small RNA (EBER), while immunohistochemical staining for EBV latent membrane protein-1 (LMP-1) is negative (*25*).

High levels of interleukins (IL-6, IL-10) and soluble forms of antigens such as soluble CD30 might also help in the identification of a clinical marker for treatment (*26,27*). Onset of PEL is mostly related with immunosuppression (*6*) and is associated with HIV load and CD4 cell count in HIV-1 related PEL (*28*).

4. Molecular genetics

The HHV-8/KSHV genome has a 145 kb gene and PEL cells usually contain 40-80 copies of HHV-8/KSHV episomes per cell and express HHV-8/KSHV latent genes (Table 2, Figures 1 and 2). Five latent gene products, which are thought to play significant roles in PEL, are latency-associated nuclear antigen-1 (LANA-1), LANA-2/vIRF-3, viral cyclin (v-Cyclin), viral FLICE inhibitory protein (v-FLIP) and Kaposin (K12). LANA-1 binds to p53 and RB protein, inhibits their function, and impairs the apoptosis of HHV-8/KSHV-infected cells (*29,30*). v-Cyclin (viral homologue of cyclin D), binds to cyclin-dependent kinase 6 (CDK6) and inactivates RB protein (*31*). v-FLIP, a viral homologue of FLICE inhibitory protein (c-FLIP), inhibits apoptosis by blocking Fas-and TNF-mediated caspase activation and activates NF-κB thorough activation of IKKγ (*32,33*). Kaposin A has

Table 2. HHV-8/KSHV-encoded protein implicated in tumorigenesis

HHV-8/KSHV -encoded protein	Host cell homologue	Possible function
LANA-1		Inhibition of p53, Rb and GSK3β Induce hTERT, Id-1 and IL-6
LANA-2/ vIRF-3	Interferon regulatory factor	Inhibition of p53
v-Cyclin	D-Type cyclin	Inactivation of pRB promotes G1 to S phase transition
v-FLIP	FLICE inhibitory protein (c-FLIP)	Activation of NF-κB pathway, Inhibition of CD95L (FasL) and TNF induced apoptosis
Kaposin (K12)		Kaposin A: oncogenic potential Kaposin B: stabilize cytokine expression
K1		Transformation
v-MIPs	CC chemokines	Chemoattraction, angiogenesis
v-IL-6	IL-6	Growth factor
v-Bcl2	Bcl-2 family proteins	Inhibition of apoptosis
v-GPCR	IL-8 GPCR	Cellar growth signal
v-Ox-2	N-CAM family proteins	Cellular adhesion molecule
ORF4	CD21/CR2 complement binding protein	Escape form host immune response

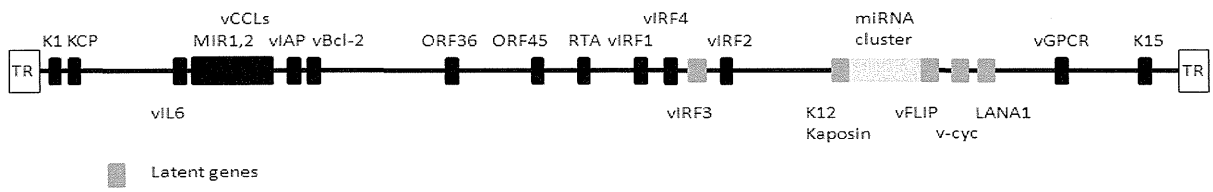

Figure 1. HHV-8/KSHV genome and viral gene expression in PEL. The latent HHV-8 genes LANA, v-cyclin, vFLIP, K12/ Kaposine, and vIRF3 are shown as grey boxes.

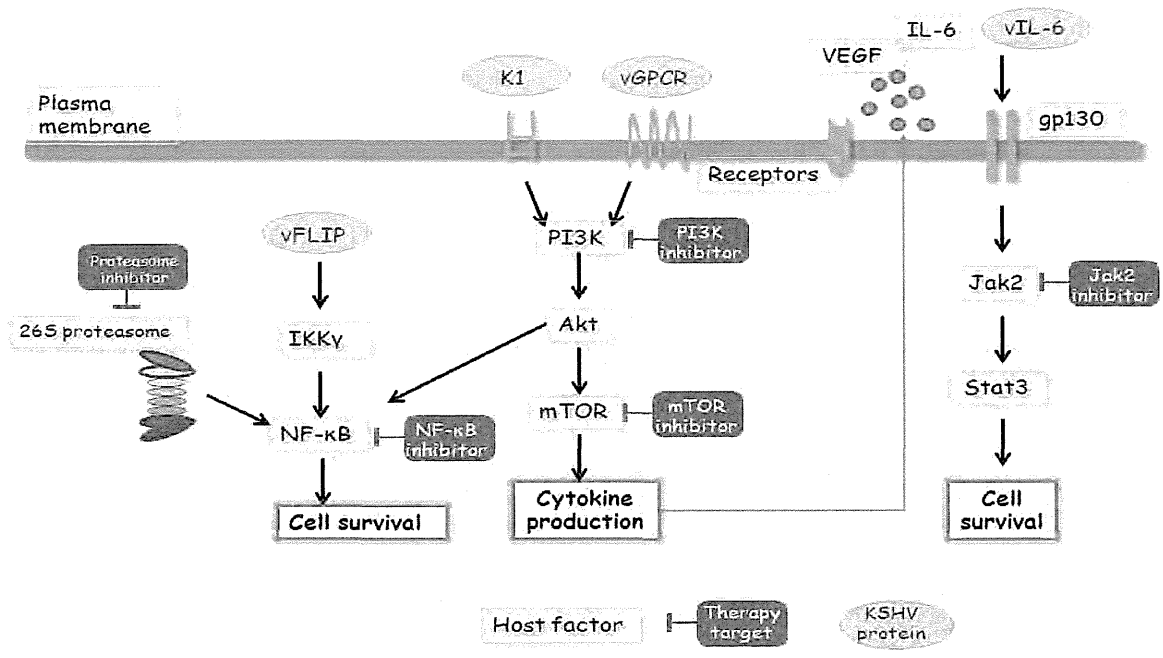

Figure 2. Potential candidate targeting molecules for the treatment of PEL. PEL constitutively activates NF-κB, JAK/STAT and PI3K/AKT/mTOR pathways, which are essential for the survival of PEL cells. These signaling pathways, cytokines and surface antigens are considered as targeting molecules for treatment.

oncogenic potential through cytokesin-1 (*34*). Kaposin B stabilizes cytokine expressions, such as IL-6 and granulocyte-macrophage colony stimulating factor (GM-CSF), by stabilizing cytokine mRNA containing AU-rich elements, which plays a role in latent HHV-8/KSHV infection (*35*). vIL-6 is a homologue of cellular IL-6 (24.6% amino acid sequence identity), directly binds to gp130 without the cooperation of the IL-6 high affinity receptor, and triggers the JAK/STAT (Janus tyrosine kinases/signal transducers and activators of transcription) pathway (*36*). LANA-2/vIRF-3 has a potential role in developing drug resistance by binding to polymerized microtubules, reducing their stability (*37*). Furthermore, HHV-8/KSHV encodes homologous human interferon response factors (IRF), which inhibit interferon-mediated effects (*38*). These viral proteins are essential for the survival of PEL cells and could be a target of PEL treatment. The major latency-associated region of the HHV-8/KSHV genome also encodes 12 micro (mi) RNA genes. Of note, miR-K12-11 is a HHV-8/KSHV miRNA sharing full seed sequence homology with human miRNA, miR-155. Given that miR-155 promotes plasma cell differentiation, miR-K12-11 might contribute to HHV-8/KSHV lymphomagenesis (*39,40*).

Approximately 50%-80% of PEL are co-infected with EBV (*5*). EBV gene expression in dually infected PEL cells is restricted to EBNA-1 and EBER (latency I). Although EBV-positive PEL exhibits a different pattern of gene expression from EBV-negative PEL, there is no evidence that EBV-positive PEL presents with the characteristic clinical manifestation, and the contribution of PEL features is unknown (*13*).

5. Differential diagnosis

The most common differential diagnoses in cases of PEL are other types of non-Hodgkin's lymphoma with lymphomatous effusion, such as diffuse large B cell lymphoma (DLBCL) and Burkitt's lymphoma (BL) with secondary effusion (Table 3). Recently classified HHV-8-negative PEL-like lymphoma shows similar clinical and laboratory features, except for being HHV-8/KSHV negative and CD20 positive (*41*), and the term "HHV-8/KSHV-negative effusion-based lymphoma" was proposed (*42*). This lymphoma also presents with lymphomatous effusion without detectable masses. HHV-8/KSHV is negative in all cases. Hepatitis C virus (HCV) and EBV are positive in nearly 30% of cases, respectively. Patients are generally elderly and have underlying medical conditions, such as cirrhosis or cardiovascular dysfunction. It is also considered to be associated with fluid overload states. Confirmation of the typical morphology and immunophenotype described previously and evidence of HHV-8/KSHV infection are required for the diagnosis of PEL (*1*).

Pyothorax-associated lymphoma (PAL) is a non-Hodgkin's B-cell lymphoma developing in the pleural cavity of patients after a long-term history of pyothorax resulting from an artificial pneumothorax for the treatment of pulmonary tuberculosis or tuberculous pleuritis (*43*). PAL is more common in Japan and usually occurs in elderly men with a history of pulmonary tuberculosis or tuberculous pleuritis. PAL usually shows the diffuse proliferation of large cells of B-cell type (diffuse large B-cell lymphoma; DLBL), and is strongly associated with EBV infection with the expression of EBV latent genes such as EBNA-2, LMP-1, together with EBNA-1 (latency III) and HHV-8/KSHV negative (*44*).

Plasmablastic lymphoma (PBL) is an aggressive non-Hodgkin's B-cell lymphoma that presents at both oral and extra-oral sites (especially the gastrointestinal tract) of chronically HIV-infected immunosuppressed young men. The morphology shows plasmablastic differentiation and plasma cell markers (CD20-, CD38+, CD138+) in all cases. EBV is detected in most cases, but HHV-8/KSHV is negative.

Because of their similar morphology and lack of a B-cell marker, T-cell anaplastic large cell lymphoma is sometimes confused with PEL (*45*). Immunohistochemistry for anaplastic lymphoma kinase (ALK) and the TCR gene rearrangement would be helpful in these cases.

If the morphological findings show large immunoblastic to plasmablastic with anaplastic

Table 3. Classification and differential diagnosis of non-Hodgkin's lymphomas involving the serous body cavities and presenting as effusion lymphomas

Type of lymphoma	Primary effusion lymphoma	HHV-8/KSHV-unrelated PEL-like lymphoma	Extranodal large cell lymphoma	Extranodal Burkitt's lymphoma	Systemic lymphomas or body cavity-based mass-forming lymphomas
effusion	primary	primary	primary	primary	secondary
HHV-8/KSHV	+	-	-	-	-
EBV	+	+/-	+/-	+/-	various
CD20	-	+ (70-80%)	+	+	+
c-*myc*	-	-	-	+	-
Morphology	IBL/ALCL		IBL/DLBCL	BL	Various histoypes

Attenuated from (*13*). IBL: Immunoblastic lymphoma, ALCL: Anaplastic large cell lymphoma, DLBCL: diffuse large B-cell lymphoma, BL: Burkitt's lymphoma.

morphology, virological analysis of HHV-8/KSHV and EBV is essential for diagnosis. HHV-8/KSHV can be demonstrated by PCR, *in situ* hybridization or by immuno-histochemistry against LANA-1, which is consistently expressed in HHV-8/KSHV infected cells.

6. Treatment

The prognosis of PEL is extremely poor with few long-term survivors. Owing to the rarity of the disease, there are very few longitudinal observational series of patients and prospective randomized clinical studies are not feasible; thus, treatment is mostly based on expert consensus opinion and small case series.

6.1. *Chemotherapy*

Traditional chemotherapy with cyclophosphamide, doxorubicin, vincristine and predonisolone (CHOP) is the most common chemotherapy regimen for treating non-Hodgkin's lymphoma (NHL), and has been attempted for the treatment of PEL; however, the prognosis of patients with PEL remains extremely poor. Boulanger *et al.* showed a median survival of 6.2 months and a 1-year overall survival rate of approximately 40% (*46*). Studies using CHOP-like regimens resulted in similar outcomes. Recently, an anti-CD20 monoclonal antibody (Rituximab) -containing regimen became the standard therapy for CD20-positive B cell NHL. Although most PEL cases do not express CD20, Rituximab can be considered for the treatment of rare cases of CD20-positive PEL (*47,48*).

Methotrexate-containing regimens, such as high-dose methotrexate and CHOP with methotrexate, have been studied. However, methotrexate accumulates in effusions, resulting in delayed clearance and an increased risk of systemic toxicity. Infusion therapy such as dose-adjusted EPOCH (DA-EPOCH; etoposide, prednisone, vincristine, cyclophosphamide and doxorubicin) and CDE (cyclophosphamide, doxorubicin, etoposide) has been shown to be well tolerated and effective in the treatment of AIDS-related aggressive B-cell lymphomas, and can be applied for the treatment of PEL (*21*).

6.2. *Stem cell transplantation*

The efficacy of high-dose chemotherapy with autologous stem cell transplantation (ASCT) for chemotherapy-sensitive relapsed disease in HIV-associated lymphoma has been reported (*49,50*). Only two cases have been reported in PEL (*51,52*): one failed to recover from PEL, while the other was successfully treated with high-dose chemotherapy with ASCT following complete remission 12 months post-transplantation. Successful treatment with reduced-intensity conditioning allogeneic hematopoietic stem cell transplantation in second remission has been reported (*53*). This patient remained in complete remission 31 months post-transplantation only on cART with an undetectable HIV viral load.

6.3. *Combination antiretroviral therapy (cART)*

Prior to the administration of cART, the therapeutic results with chemotherapy were unsatisfactory in HIV-1 associated lymphomas. The prognostic impact of cART in combination with chemotherapy has been reported in PEL (*46*), although the impact of cART is lower than for other HIV-1 associated lymphomas such as DLBCL and BL (*54,55*). In addition, complete remission of PEL patients with cART but without chemotherapeutic drugs has been reported (*56-58*). Thus, implementation of cART is recommended when treating PEL patients with HIV-1 infection.

It is important to avoid major drug-drug interactions during chemotherapy (Table 4). Among antiretroviral agents, protease inhibitors modify the metabolism of cytotoxic drugs and potentiate myelotoxicity by inhibiting the CYP3A4 enzyme to various extents (*59*). Thus, anticancer drugs, which rely on Cytochrome P450, should be used carefully with protease inhibitor-based regimens to avoid inadvertent toxicity. Currently, integrase strand transfer inhibitors (INSTI), raltegravir and dolutegravir, are recommended by many experts

Table 4. Adverse effects of anti-HIV-1 reagents during chemotherapy

Agents	Adverse effects
AZT	Bone marrow suppression, contraindication
d4T/ddI	Peripheral nerve disorder/ileus (avoid with VCR) Liver dysfunction (toxic for mitochondria)
Protease inhibitor (PI) RTV > IDV = APV > NFV> = SQV	High blood level of anti-cancer agents (inhibition of CYP450-3A4)
NNRTI Efavirenz (EFV) Nevirapine (NVP)	Reduced function of anti-cancer agents (activate CYP450)
Abacavir (ABC)	Hypersensitivity
Tenofovir (TDF)	Renal dysfunction

Table 5. Anti-HIV-1 treatment during cancer chemotherapy

Recommended therapy	EFV + TDF/FTC RAL + TDF/FTC
Alternative therapy	EFV + ABC/3TC RAL + ABC/3TC

EFV, efavirenz; TDF, tenofovir; FTC, emtricitabine; RAL, raltegravir; ABC, abacavir; 3TC, lamivudine. Summarized from lines for the Use of Antiretroviral Agents in HIV-1-Infected Adults and Adolescents (*http://aidsinfo.nih.gov/contentfiles/lvguidelines/adultandadolescentgl.pdf*).

to anchor cART regimens in patients receiving chemotherapy (Table 5). Another INSTI, elvitegravir is only available as a component of four-drug combination product, which contains potent CYP3A inhibitor.

6.4. *Treatment of opportunistic infections*

Supportive treatment of opportunistic infections is important in HIV-infected patients and post-organ transplantation patients with PEL. Granulocyte-colony stimulating factor (G-CSF) helps reduce chemotherapy-induced neutropenic complications. All patients need to receive prophylaxis for *Pneumocystis carinii* pneumonia with trimethoprim-sulfamethoxazole, regardless of the CD4 cell count. For patients who have severe neutropenia with chemotherapy, alternation of trimethoprim-sulfamethoxazole for *Pneumocystis carinii* prophylaxis can be considered, including dapsone or aerosolized pentamidine. Infectious complications may be minimized by using prophylactic fluroquinolone antibiotics and azoles during periods of protracted neutropenia. Prophylaxis for *Mycobacterium avium complex* (MAC), *Toxoplasmosis* and other opportunistic infection should be also considered as PEL usually arises in an immunodeficient state and chemotherapy induces myelosuppression. Prophylaxis against infection during chemotherapy may include drugs that interact with cART and anticancer agents. Careful attention must be paid for the adverse effects and drug-drug interaction among these agents (*60*).

6.5. *Clinical trial*

On the basis of recent preclinical data and translational studies, several new targeted therapies are being explored, and several clinical trials have been performed based on expert consensus opinions and evidence in preclinical studies.

A proteasome inhibitor, bortezomib, is expected to show clinical effects against PEL. Despite the promising results of *in vitro* experiments and a mouse model (*61,62*), bortezomib treatment either alone or in combination with chemotherapy showed no clinical improvement (*20,63*). The optimization of treatment protocol and combination therapy with bortezomib may be needed to show the preferable effects of bortezomib.

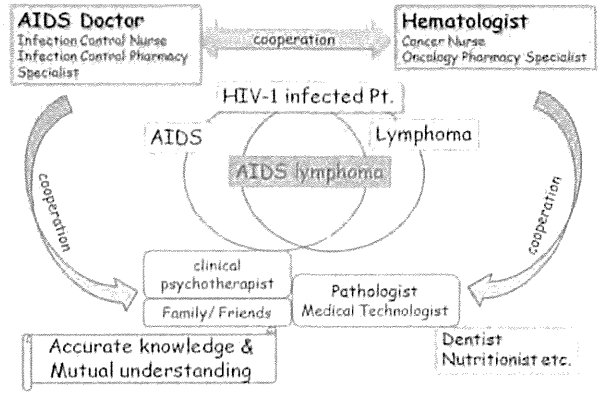

Figure 3. Treatment and support of AIDS-related malignant lymphoma -Team Medical Care-

Lenalidomide is an immunomodulatory drug that is commonly used to treat newly diagnosed and relapsed multiple myeloma as well as a variety of hematological malignancies. It exerts its antitumor action through various mechanisms, such as activation of the immune system, inhibition of angiogenesis and direct antineoplastic effects. Treatment with lenalidomide has never been reported in PEL patients with favorable results (*64*). As lenalidomide was also successfully used to treat three patients with advanced refractory Kaposi sarcoma, this novel agent is expected to be used in prospective studies.

Antiviral treatment can be induced to effect the lytic phase of HHV-8/KSHV viral replication. Complete remission has been reported after the administration of an antiviral nucleotide analogue, cidofovir (*57,65,66*), an antiviral agent with broad activity against multiple DNA viruses, inducing lytic replication of HHV-8/KSHV.

PEL cells are quite sensitive to irradiation in culture and in a xenograft mouse model (*67*). It was reported that chemotherapy-refractory PEL patients achieved remission and survived for more than 12 months with radiation therapy (*68*). Irradiation therapy should be considered as part of the treatment recommendation for patients with chemotherapy-refractory PEL-associated solid masses or localized effusions.

6.6. *Mental support*

There are considerable difficulties in the treatment of AIDS-related lymphoma, including the mental care of patients. The close cooperation of AIDS doctors and hematologists, intensive care by nurse specialists, support from pharmacy specialists, and other co-medical staff is essential. Mental care from a psychiatrist, clinical psychotherapist, and the patient's family and friends are quite supportive for patients. It is especially important to ensure an organic link with the specialist as well as family and friends for treatment (Figure 3).

6.7. *Molecular-targeted preclinical studies*

Since PEL cells display constitutive activity of many signaling pathways and survival, including NF-κB, JAK/STAT and PI3K/AKT pathways, these molecules and HHV-8/KSHV latent proteins are considered ideal for targeted therapy (Figure 2). In particular, vFLIP has the ability to activate the NF-κB pathway by binding to the IκB kinase (IKK) complex (*32,33*), and NF-κB activation is known to be the key player in PEL oncogenesis, so various NF-κB and proteosome inhibitors have been investigated in a preclinical trial. Xenograft PEL mouse models and *in vitro* culture of PEL cell lines were used in preclinical studies, and promising preclinical results were reported with multiple NF-κB inhibitors, such as cepharanthine (*69*), diethyldithiocarbamate (*70*), berberine (*71*), and heat-shock protein 90 (*72,73*). Xenograft mouse models using severe immunodeficient mice are a powerful tool to confirm the effects and adverse effects of candidate reagents in a preclinical study.

The PI3K/AKT pathway, JAK2/STAT3 pathway and mTOR are also activated in PEL cell lines and could be promising targets (74-76). Several inhibitors are currently undergoing clinical trials in patients with hematological malignancies and can be used for the treatment of PEL in the near future (*77*).

Interferon-α and AZT induced TRAIL-mediated apoptosis of PEL (78,79). IFN-α upregulates TRAIL in PEL cells while AZT sensitizes them to TRAIL, resulting in the activation of a suicide program. The efficacy of this approach needs to be validated in clinical trials.

6.8. *Immunotherapy*

Although rituximab, a chimeric anti-CD20 antibody, has provided a significant survival advantage for B-cell NHL in combination with standard chemotherapy, rituximab does not play a significant therapeutic role in PEL because CD20 is not usually expressed on the surface of PEL cells. Rare cases expressing CD20 have been reported to respond to rituximab (*47*).

CD30 is expressed significantly in case of PEL. Brentuximab vedotin (SGN-35) is an antibody–drug conjugate in which a chimeric anti-CD30 antibody is combined with the synthetic microtubule-disrupting agent monomethylauristatin E (MMAE) (*80*). Since treatment with brentuximab vedotin also prolonged the survival of a PEL xenograft mouse model (*81*), brentuximab is expected to be a candidate for the treatment of PEL.

PEL cells secrete vascular endothelial growth factor (VEGF)-A (*82*), and treatment with mouse anti-human VEGF-A monoclonal antibody inhibited the development of ascites in a xenograft mouse model. Because bevacizumab, a humanized VEGF-A monoclonal antibody, is clinically used for the treatment of a variety of human cancers, including colorectal, non-small-cell lung, ovarian and metastatic renal cell carcinoma (*83*), it is also expected to be a novel target of treatment.

7. Conclusion

PEL is a rare but aggressive form of NHL, mostly arising in immunodeficient patients. PEL is commonly resistant to conventional chemotherapy and has a poor prognosis. Currently, more intensive chemotherapy with cART is recommended. The management of opportunistic infection is also needed since PEL arises in immunodeficient states. Drug interaction between anti-cancer reagents and cART, especially protease inhibitors, should be carefully monitored in HIV-1-positive individuals. Close communication among the oncologist, the patient's primary HIV-treating physician, and co-medical staff is needed for the intensive treatment of AIDS-related PEL patients. It is also important to avoid drug interactions in chemotherapy. Several molecular-targeted therapies are in clinical trial and preclinical stages, and their clinical use is anticipated. Since PEL is mostly associated with immunodeficient states, early diagnosis and treatment of HIV-1 may prevent the onset of PEL.

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ORIGINAL ARTICLE - CANCER RESEARCH

Targeting VEGF and interleukin-6 for controlling malignant effusion of primary effusion lymphoma

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Abstract
Purpose Primary effusion lymphoma (PEL) is an aggressive subtype of non-Hodgkin lymphoma that shows malignant effusion most commonly seen in advanced AIDS patients. In this study, we clarified the potential role of VEGF and IL-6 in PEL fluid retention and evaluated the efficacy of humanized anti-VEGF monoclonal antibody (mAb), bevacizumab, and humanized anti-IL-6 receptor mAb, tocilizumab, against PEL.
Methods The production of VEGF and IL-6, and the expression of IL-6Rα in PEL cell lines were examined. The antiproliferative effect of bevacizumab and tocilizumab on PEL cells was evaluated in vitro. The effect of tocilizumab on VEGF was also examined. An intraperitoneal xenograft mouse model was used for in vivo efficacy.
Results Although we found the production of VEGF and IL-6, and the expression of IL-6Rα in PEL cell lines, bevacizumab and tocilizumab did not inhibit the proliferation of PEL cells in vitro. Tocilizumab decreased VEGF mRNA and VEGF production by inhibiting Stat3 phosphorylation and Stat3 binding to VEGF promoter. In a PEL xenograft mouse model that showed profuse ascites, bevacizumab suppressed ascites formation completely, indicating the critical role of VEGF for PEL fluid retention. Tocilizumab also significantly inhibited ascites formation in vivo. Moreover, these mAbs improved the overall survival of treated mice.
Conclusions IL-6-VEGF axis contributed to fluid retention, and bevacizumab and tocilizumab could be effective molecular targeting therapies for PEL.

Keywords Fluid retention · IL-6 · Primary effusion lymphoma · VEGF

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Introduction

Primary effusion lymphoma (PEL) is an infrequent and aggressive subtype of non-Hodgkin lymphoma that occurs in an immunodeficient status, most commonly in human immunodeficiency virus (HIV)-infected patients (Greene et al. 2007), and is universally associated with infection by Kaposi's sarcoma-associated herpes virus (KSHV)/human herpesvirus-8 (HHV-8) (Cesarman et al. 1995). PEL presents with pleural, peritoneal, or pericardial effusion without tumor masses. Malignant effusion helps tumor invasion and changes the blood concentration of anticancer agents. The accumulation of effusion is an important cause of morbidity and mortality in patients with PEL (Castillo et al. 2012). Moreover, PEL is generally resistant to conventional chemotherapy and has a poor prognosis (Boulanger et al. 2005). Thus, there is a need for novel agents targeting the characteristics of PEL, such as malignant effusion. PEL has been reported to secrete both vascular endothelial growth factor (VEGF) and interleukin-6 (IL-6) (Aoki and Tosato 1999; Drexler et al. 1999). These molecules are considered to be related to PEL progression. However, the biological activity of IL-6 signaling in PEL cells and whether VEGF and IL-6R-mediated signaling can be a therapeutic target for PEL have not been fully clarified. In addition,

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no preclinical evaluation of the activity of humanized anti-VEGF monoclonal antibody (mAb), bevacizumab, and humanized anti-IL-6 receptor (IL-6R) mAb, tocilizumab, in PEL is available. Therefore, the aim of this study was to identify the antitumor effect of both mAbs on PEL using a PEL xenotransplantation model and to clarify the potential role of VEGF and IL-6 in fluid retention.

VEGF is an angiogenic factor that induces endothelial cell proliferation and angiogenesis (Ferrara 2002). Bevacizumab inhibits the angiogenesis of tumors and is now clinically used for the treatment of a variety of human cancers, including colorectal, non-small cell lung, ovarian, and metastatic renal cell carcinoma (Shih and Lindley 2006). VEGF also enhances vascular permeability and may play a role in the pathogenesis of certain ascites tumors (Senger et al. 1983; Nagy et al. 1995). IL-6 is a multifunctional cytokine that regulates immune and inflammatory responses (Kishimoto 2005). Serum IL-6 is elevated in inflammatory diseases and certain tumors and is correlated with disease progression (Trikha et al. 2003; Hong et al. 2007). A series of studies has shown that inhibition of IL-6 signaling by tocilizumab is therapeutically effective in rheumatoid arthritis, juvenile idiopathic arthritis, Castleman's disease, Crohn's disease, and IL-6-related malignancy, such as multiple myeloma (Tanaka et al. 2012).

In this study, we investigated the potential role of VEGF and IL-6 in PEL fluid retention using specific humanized mAbs.

Materials and methods

Cell growth conditions

BCBL-1, BC-1, BC-3, TY-1, and Raji cells were maintained in RPMI 1640 supplemented with 10 % fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified incubator at 37 °C and 5 % CO_2. BC-2, BCP-1, and RM-P1 cells were maintained in RPMI 1640 supplemented with 20 % FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified incubator at 37 °C and 5 % CO_2.

Reagents

Bevacizumab and tocilizumab were obtained from Chugai Pharmaceutical Co. Ltd. (Tokyo, Japan).

Measurement of VEGF and IL-6

VEGF was measured by human VEGF-A Platinum ELISA (e-Bioscience, San Diego, CA, USA), and IL-6 was measured by human IL-6 ELISA (e-Bioscience), following the manufacturer's instructions.

Flow cytometry

Cells were stained with anti-IL-6Rα (CD126) PE (BioLegend, San Diego, CA, USA). 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, USA). Data were analyzed with FlowJo software (Tree Star, San Carlos, CA, USA).

Tetrazolium dye methylthiotetrazole (MTT) assay

The antiproliferative activities of bevacizumab and tocilizumab against PEL cell lines were measured by the methylthiotetrazole (MTT) method (Sigma-Aldrich, St. Louis, MO, USA). Briefly, 1×10^4 cells were incubated in triplicate in a 96-well microculture plate in the presence of different concentrations of bevacizumab or tocilizumab in a final volume of 0.1 ml for 72 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 acidified isopropanol (HCl 34 μl/10 ml isopropanol) was added to dissolve the crystals. Absorption values at 595 nm were determined with an automatic ELISA plate reader (Multiskan; Thermo ElectronVantaa, Finland). Values were normalized to untreated (control) samples.

RT-PCR analysis

Total RNA was isolated from cells using RNAiso plus (Takara Bio, Ohtsu, Japan) according to the manufacturer's instructions. Real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis for human VEGF and internal control β2-microglobulin (β2 M) was carried out with SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA, USA) following the manufacturer's instructions. PCR amplifications were performed using the StepOne real-time PCR system (Applied Biosystems) with the following amplification conditions: 95 °C for 3 min, 40 cycles at 95 °C for 10 s, at 55 °C for 30 s. The Ct values for each gene amplification were normalized by subtracting the Ct value calculated for β2 M. The normalized gene expression values were expressed as the relative quantity of VEGF gene-specific messenger RNA (mRNA).

The oligonucleotide primers used in this study are as shown below. VEGF-A 5′-ATGACGAGGGCCTGGAGTG TG-3′ and 5′-CCTATGTGCTGGCCTTGGTGAG-3′, β2 M 5′-CGGGCATTCCTGAAGCTGA-3′ and 5′-GGATGGA TGAAACCCAGACACATAG-3′.

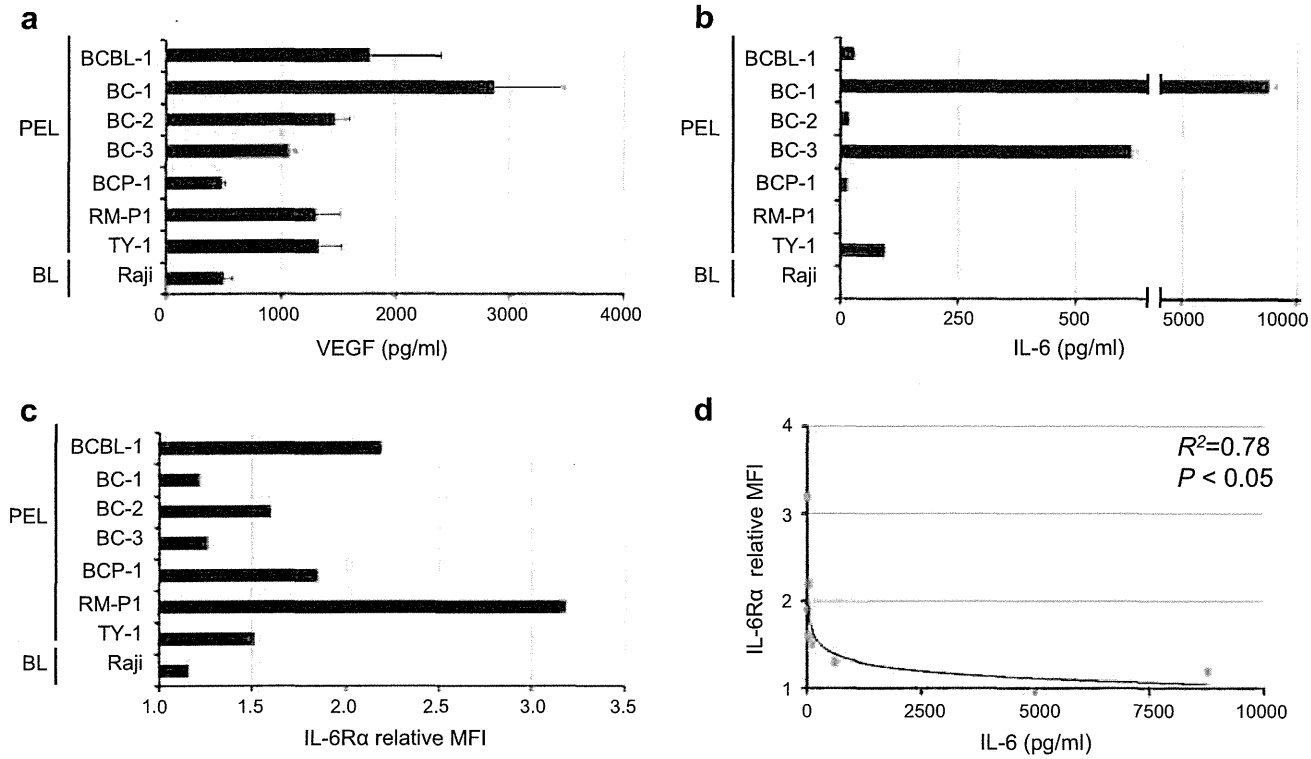

Fig. 1 PEL cell lines produce VEGF and IL-6, and express IL-6Rα. **a** Production of VEGF in PEL cell lines. **b** Production of IL-6 in PEL cell lines. Levels of VEGF and IL-6 in the culture supernatants were determined by ELISA. Results are the means of three independent experiments. **c** Expression of IL-6Rα on the surface of PEL cell lines. Numbers of IL-6Rα expression indicate the fold increase in the mean fluorescence intensity (MFI) by flow cytometry. Raji cells were used as a negative control. **d** Inverse correlation between IL-6 production and IL-6Rα expression in PEL cell lines

Western blot analysis

For whole cell extraction, BCBL-1 cells treated with 10 μM tocilizumab for 48 h were collected and washed in cold PBS before the addition of 100 μl cold lysis buffer (25 mM HEPES, 10 mM $Na_4P_2O_7 \cdot 10H_2O$, 100 mM NaF, 5 mM EDTA, 2 mM Na_3VO_4, 1 % Triton X-100). After rotation for 2 h at 4 °C, whole cell extracts were obtained by centrifugation at 15 000 rpm for 15 min. Whole cell extracts (30 μg protein) were separated by 10 % SDS-PAGE and blotted onto a PVDF membrane (GE Healthcare, Tokyo, Japan). Blots were probed with the indicated antibodies and detected using Chemi-Lumi One Super (Nacalai Tesque, Kyoto, Japan).

Primary antibodies were as follows: anti-IκBα (9242), anti-phospho(Ser32/36)-IκBα (9246), anti-Erk (4695), anti-phospho(Thr202/Tyr203)-Erk (4370) (Cell Signaling Technology, Danvers, MA, USA), anti- Stat3 (sc-8019), anti-phospho(Tyr705)-Stat3 (sc-7993-R) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-Hsc70 (SPA-815) (Stressgen Bioreagents, Ann Arbor, MI, USA).

Chromatin immunoprecipitation (ChIP) assay

To examine the binding of Stat3 to VEGF promoter in PEL cells, nuclear extracts of BCBL-1 cells were used for the ChIP assay. BCBL-1 cells were cross-linked using formaldehyde (1 % final concentration) added directly to the cell culture media at 37 °C for 15 min, and the reaction was stopped by adding glycine (0.125 M final concentration). Cells were rinsed with cold PBS and resuspended in cell lysis buffer, consisting of 5 mM PIPES [piperazine-*N,N'*-bis(ethanesulfonic acid), pH 8.0], 85 mM KCl, 0.5 % NP-40, and 1 % protease inhibitor (PI) cocktail (Nacalai Tesque). This mixture was incubated on ice for 10 min and then homogenized. The nuclei were resuspended in nucleus lysis buffer [1 % sodium dodecyl sulfate (SDS), 10 mM EDTA, 50 mM Tris–HCl (pH 8.1), and 1 % PI cocktail] and incubated on ice for 10 min. The samples were sonicated on ice with the ultrasonic homogenizer VP-050 (TAITEC, Saitama, Japan) to an average length of up to 1,000 bp and microcentrifuged. The chromatin solution was precleared using *Staphylococcus aureus* protein A-positive cells (Pansorbin, #507862; Calbiochem). Precleared chromatin was incubated with 2 μg anti-Stat3 antibody (sc-8019) (Santa

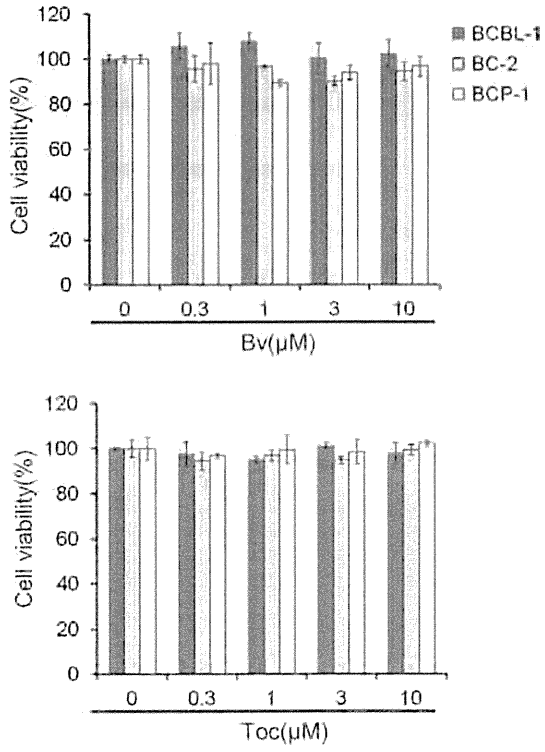

Fig. 2 Bevacizumab (Bv) or tocilizumab (Toc) does not inhibit the proliferation of PEL cells. PEL cell lines (BCBL-1, BC-2, and BCP-1) were incubated with 0, 0.3, 1, 3, 10 μM Bv or Toc for 72 h. A cell proliferation assay was carried out using MTT as described in "Materials and methods" section

Cruz Biotechnology) or mouse IgG at 4 °C for 24 h and microcentrifuged. After washing, we performed cross-link reversal and DNA extraction. PCR was performed using Ex *Taq* polymerase (Takara Bio) according to the recommended protocol.

The primers recognized the putative Stat-binding sites located at −848 and −630 in the human VEGF promoter region (accession no. AF095785). The oligonucleotide primers used in this study were VEGF promoter 5′-TTGGTGCCAAATTCTTCTCC-3′ and 5′-CACACGTCCTCACTCTCGAA-3′ (Cheranov et al. 2008).

Xenograft mouse model

NOD Rag-2/Jak3 double-deficient (Rag-$2^{-/-}$Jak$3^{-/-}$) mice (NRJ mice) were established as described previously (Goto et al. 2012) 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. In an intraperitoneal xenograft mouse model, 10- to 12-week-old NRJ male mice were intraperitoneally inoculated with 7×10^6 BCBL-1 cells suspended in 200 μl phosphate-buffered saline (PBS). The mice were then treated with intraperitoneal injections of PBS, bevacizumab, or tocilizumab (100 μg/mouse, 3 times a week). Tumor burden was evaluated by measuring the volume of ascites on day 28. For assessment of overall survival, Kaplan–Meier analysis was performed and *P* values were determined by two-tailed analysis with the log-rank test.

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 PA1-73 N antibody, 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.

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.

Results

Production of VEGF and IL-6 in PEL cell lines

Production of VEGF and IL-6 in culture supernatants of seven PEL cell lines (BCBL-1, BC-1, BC-2, BC-3, BCP-1, RM-P1, TY-1) and the Burkitt lymphoma cell line Raji was analyzed. After cell lines (5×10^5/ml) had been cultured for 48 h, the production of VEGF and IL-6 was quantified by enzyme-linked immunosorbent assay (ELISA). High levels of VEGF and IL-6 were detected in PEL cell lines (means of 489.7–2,861.7 pg/ml and 1.4–8,777.7 pg/ml, respectively) compared with Raji (means of 504.6 pg/ml and 0.2 pg/ml, respectively) (Fig. 1a, b).

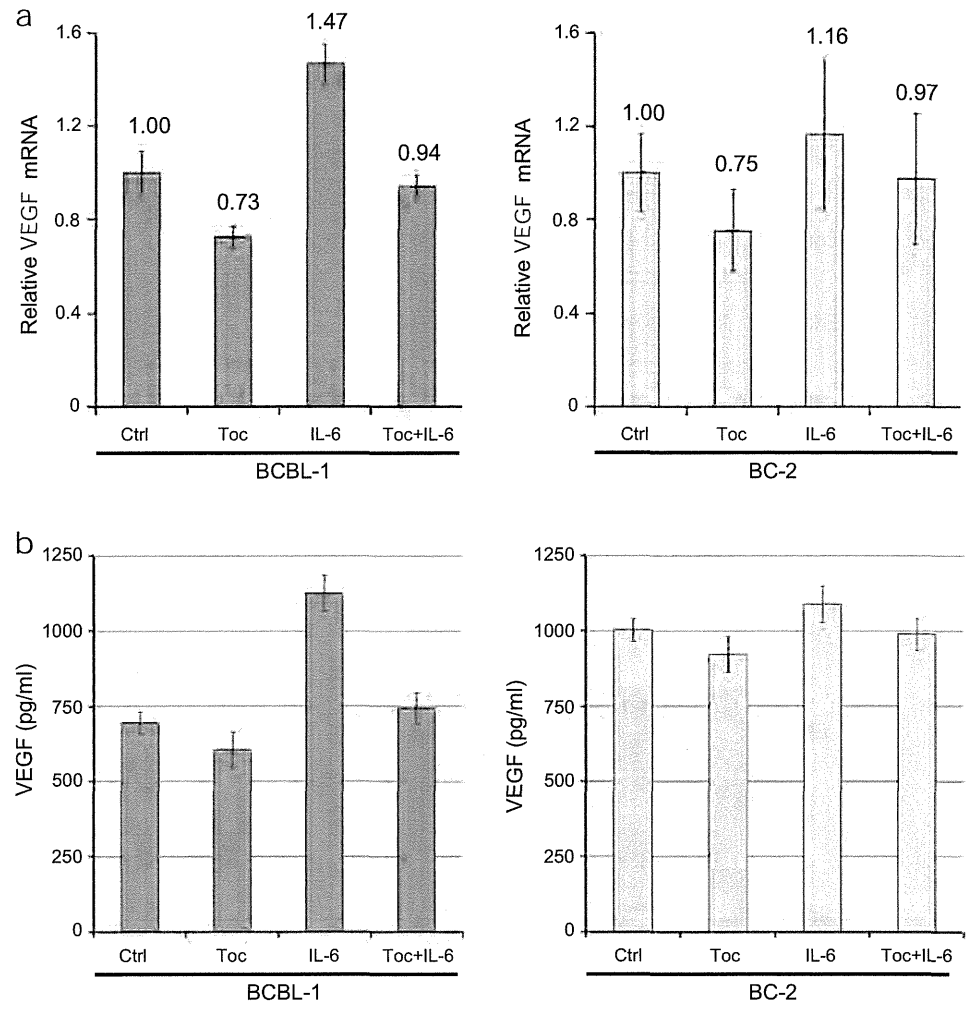

Fig. 3 Tocilizumab (Toc) inhibits VEGF in BCBL-1 and BC-2. **a** BCBL-1 cells or BC-2 cells were treated with 10 μM Toc for 72 h in the absence or presence of 100 ng/ml IL-6. VEGF mRNA expression level was measured by quantitative RT-PCR. **b** 5×10^4/ml BCBL-1 cells or BC-2 cells were treated with 10 μM Toc and cultured for 72 h in the absence or presence of 100 ng/ml IL-6. Production of VEGF was measured by ELISA

Expression of IL-6Rα on the surface of PEL cell lines

We examined PEL cell surface expression of IL-6Rα. As a negative control in flow cytometry analysis, Raji cells were used. As shown in Fig. 1c, PEL cell lines expressed IL-6Rα. Although the production of IL-6 was low in some PEL cell lines such as RM-P1 (1.4 pg/ml) (Fig. 1b) and the level of IL-6Rα expression varied among the cell lines, lower production of IL-6 was significantly correlated with higher IL-6Rα expression ($R^2 = 0.78$, $P < 0.05$) (Fig. 1d). Some PEL cell lines may compensate for the low production of IL-6 by the high expression of IL-6R.

Direct anti-proliferative effect of bevacizumab or tocilizumab on PEL cells

We determined whether treatment with bevacizumab or tocilizumab leads to the inhibition of PEL cell proliferation using the MTT assay. Three PEL cell lines (BCBL-1, BC-2, BCP-1) were cultured with varying concentrations of bevacizumab or tocilizumab (0, 0.3, 1, 3, 10 μM) for 72 h, and proliferation was analyzed by the MTT assay. Figure 2 shows that bevacizumab or tocilizumab did not inhibit the proliferation of PEL cells significantly.

Tocilizumab reduces the level of VEGF in PEL cells

To investigate the mechanism underlying the effect of tocilizumab on PEL, we assessed VEGF levels by quantitative RT-PCR and ELISA. As shown in Fig. 3a, tocilizumab reduced VEGF mRNA of BCBL-1 and BC-2 in the absence or presence of IL-6. The production of VEGF was also decreased by tocilizumab treatment (Fig. 3b).

Tocilizumab suppresses IL-6-induced Stat3 activity and Stat3 binding to VEGF promoter in PEL cells

Signal transduction through IL-6R is mediated by the JAK/STAT (Janus family tyrosine kinase/signal transducer and activator of transcription) and MAPK/ERK

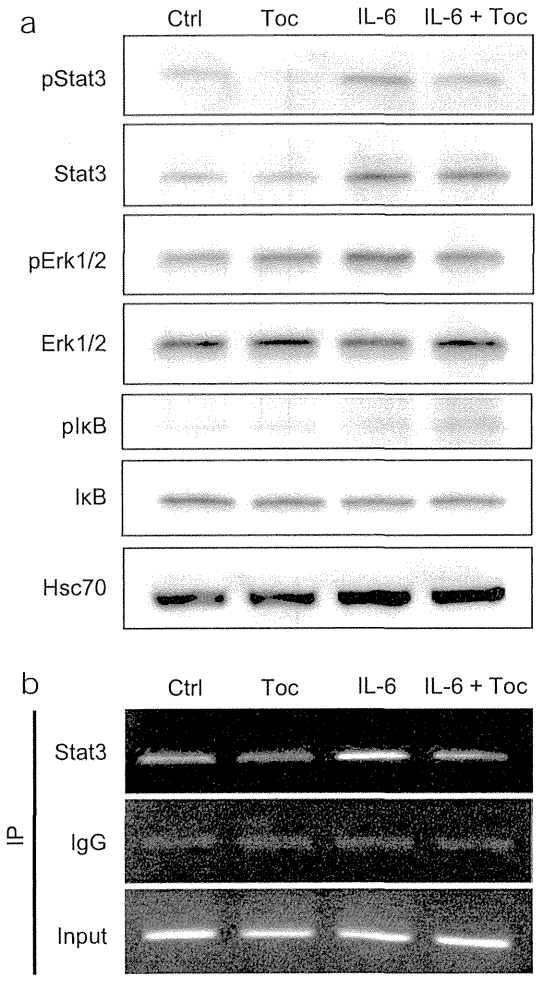

Fig. 4 Tocilizumab (Toc) inhibits IL-6-induced Stat3 phosphorylation and Stat3 binding to VEGF promoter. **a** Inhibitory effects of Toc on IL-6-induced Stat3 phosphorylation. BCBL-1 cells were treated with 10 μM Toc for 48 h in the absence or presence of 100 ng/ml IL-6. Total proteins were extracted for Western blotting. **b** Suppression of Stat3 recruitment to VEGF promoter by Toc. BCBL-1 cells were treated with 10 μM Toc for 48 h in the absence or presence of 100 ng/ml IL-6. Stat3 binding on the VEGF promoter was determined by the ChIP assay using nuclear extracts of BCBL-1 cells

(mitogen-activated protein kinase/extracellular-regulated kinase) pathways (Akira et al. 1990, 1994; Heinrich et al. 2003). Stat3, a member of the JAK/STAT pathway, and nuclear factor (NF)-κB are constitutively activated and related to the pathogenesis of PEL cells (Aoki et al. 2003; Keller et al. 2000); therefore, we examined whether tocilizumab inhibited the phosphorylated form of Stat3, Erk, and IκB. When BCBL-1 cells were treated with 10 μM tocilizumab for 48 h, treatment with tocilizumab reduced IL-6-induced Stat3 phosphorylation, whereas phospho-Erk and phospho-IκB were not significantly changed (Fig. 4a), suggesting that IL-6 maintains Stat3 activation and exerts a beneficial effect on PEL cells, including the production of VEGF. Next, we determined Stat3 binding to the VEGF promoter by the ChIP assay using nuclear extracts of BCBL-1 cells. When BCBL-1 cells were treated with 10 μM tocilizumab for 48 h, tocilizumab suppressed IL-6-induced Stat 3 binding to VEGF promoter in BCBL-1 cells (Fig. 4b). These results show that IL-6 modulates transcription factor Stat3, which directly binds to the promoter of VEGF, resulting in increased VEGF transcription in PEL cells.

In vivo effect of bevacizumab or tocilizumab in severely immunodeficient mice inoculated intraperitoneally with BCBL-1

Although bevacizumab or tocilizumab did not show direct antiproliferative effects of both mAbs on PEL, we assessed the in vivo effects of both mAbs using PEL xenograft NOD/Rag-2/Jak3-deficient (NRJ) mice (Goto et al. 2012). NRJ mice display not only complete deficiency in mature T/B lymphocytes and complement protein but also complete deficiency of NK cells, providing efficient engraftment of PEL cells. BCBL-1 cells (7×10^6/mouse) were inoculated intraperitoneally into NRJ mice. BCBL-1 xenograft mice showed profuse ascites within 4 weeks. As patients with PEL show lymphomatous effusion in body cavities without a definable tumor mass, these mice could be clinically equivalent to the PEL model. A dose of 100 μg/mouse bevacizumab, tocilizumab, or PBS alone was administrated via intraperitoneal injection on day 3 after cell inoculation and 3 times a week. Bevacizumab- or tocilizumab-treated mice apparently seemed to stay healthy, and the body weight did not change, whereas the volume of ascites was significantly lower than in untreated mice on day 28 (0.0 ± 0.0, 0.4 ± 0.7, 2.6 ± 1.0 ml, respectively, $n = 7$, $P < 0.001$; Fig. 5a, b). Organ invasion by PEL cells on day 28 was evaluated by hematoxylin–eosin staining and LANA immunostaining. We found that mice inoculated intraperitoneally with BCBL-1 exhibited invasion into the liver and lungs without macroscopic lymphoma formation (Fig. 5c). The number of LANA-positive cells in treated mice was significantly reduced (0–1 cells per field magnification, 40×) compared with untreated mice (10–20 cells per field magnification, 40×). As shown in Fig. 6, treatment with bevacizumab or tocilizumab significantly prolonged the survival of the mice (48.4 ± 6.9 and 45.5 ± 7.7 days, respectively) compared with PBS (32.6 ± 3.8 days) (log-rank test, $P < 0.01$). Inhibiting the effect of human VEGF on mouse cells was considered to suppress vascular permeability. These results indicate that treatment with bevacizumab or tocilizumab inhibits the development of malignant effusion and provides a survival benefit. These mAbs could be potentially therapeutic agents in patients with PEL.