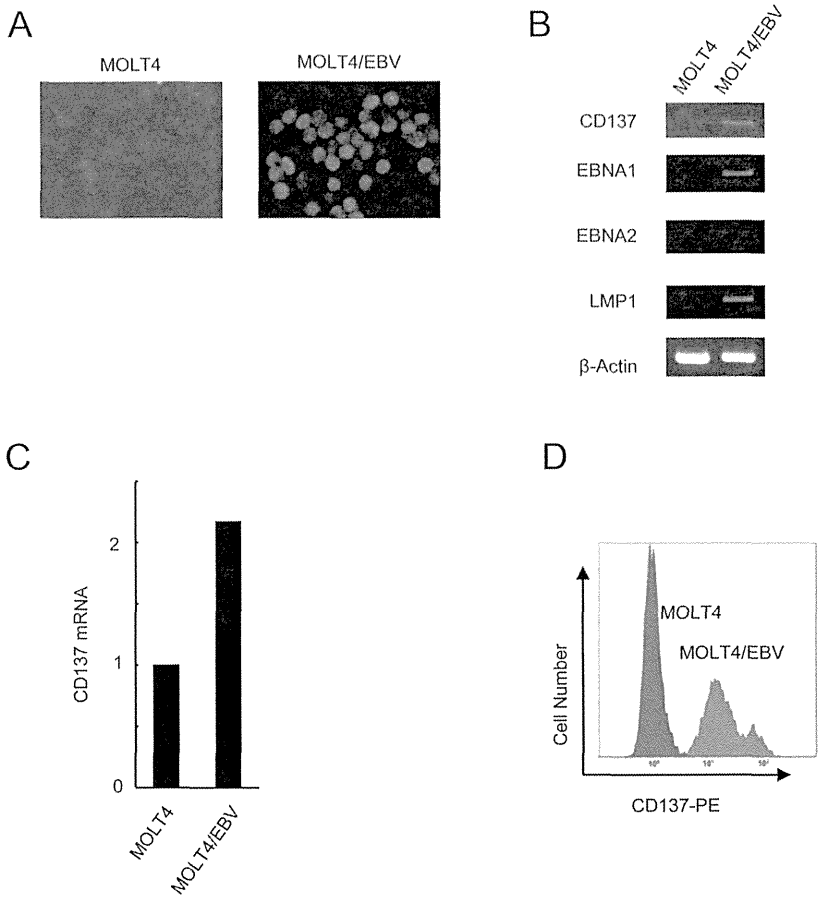

Figure 2. EBV induces CD137 expression in T cells. *In vitro* EBV infection assay performed in MOLT4 cells. (A) EBNA1 protein expression was examined by immune fluorescence staining, 48 hours after the infection, the time when CD137 expression was examined. (B) Expression of the *CD137* gene was examined by RT-PCR. The infection was confirmed by detecting mRNAs of the viral proteins, EBNA1 and LMP1. (C) Transcripts of *CD137* and *GAPDH* were quantified by real time RT-PCR. Relative copy number was obtained by normalizing the *CD137* transcripts to those of those of *GAPDH*. (D) Surface expression of CD137 of MOLT4 cells and EBV-infected MOLT4 cells was examined by flow cytometry.
doi:10.1371/journal.pone.0112564.g002

was confirmed in them (Figure S3). These results indicated that the infiltrating EBV-positive cells were both CD8- and CD137-positive.

Stimulation of CD137 decreases etoposide–induced cell death of EBV-T/NK cells

To explore the contribution of CD137 expression on EBV-T/NK cells to the development of EBV-T/NK-LPDs, we investigated the effects of CD137 on the survival. CHO-CD137L cells with stable expression of human CD137L on their surface were prepared for CD137 stimulation of EBV-T/NK cells (Figure 6A).

First we performed the assay for EBV-positive T- and NK-cell lines. We cocultured the cells with PKH-26-stained CHO cells in the presence of IL-2 with or without etoposide. Jurkat cells were used as a negative control. After the time indicated, we removed the cells and determined the number of living cells by detecting PKH-26 and DiOC6. PKH-26-negative cells were EBV-positive T/NK-cells and Jurkat cells. DiOC6-positive cells were living cells. In the presence of etoposide, the relative number of living EBV-positive T/NK-cells cultured with CHO-CD137L cells was significantly higher than that cultured with control CHO cells (Figure 6B). In contrast, T-cell line Jurkat cells, on which CD137 was not detected (Figure 1B), did not show a difference when cocultured with the 2 types of CHO cells (Figure 6B). In the absence of etoposide, CD137L had no significant effect on the viability of these cells (Figure 6B).

Next we performed the same assay for the primary cells from EBV-T/NK-LPDs patients. We cocultured PBMCs from 2 patients, CD4-2 and CD56-7 with PKH-26-stained CHO cells in the presence of IL-2 with or without etoposide. In the presence of etoposide, the relative number of living cells from EBV-T/NK-LPDs patients cultured with CHO-CD137L cells was significantly higher than that cultured with control CHO cells (Figure 6C). In contrast, cells form a healthy donor did not show a difference when cocultured with the 2 types of CHO cells (Figure 6C). These findings indicated that stimulation of CD137 significantly suppressed etoposide-induced cell death of the EBV-T/NK-LPDs cells.

Discussion

CD137 is expressed following activation of T or NK cells and mediates molecular signals for proliferation, survival, and cytokine production by acting as a costimulatory molecule of the CD3-TCR complex [11,16,17]. However, few data for its roles in

Table 1. Clinical information of the patients' samples subjected to the assay.

Case	Gender	Age	Infected cell	Clinical findings	EBV-DNA (copies/μgDNA) of PB (whole blood)	EBV-DNA (copies/μgDNA) of the EBV-infected cells fraction in PB	EBV-DNA (copies/μgDNA) of CD19-positive cells fraction in PB
CD4-1	M	45	CD4	sCAEBV	3.1×10^{2}	4.4×10^{4} (CD4)	4.4×10^{2}
CD4-2	F	25	CD4	HMB	7.0×10^{4}	2.2×10^{5} (CD4)	N.D.
CD4-3	F	62	CD4	sCAEBV	3.2×10^{4}	4.6×10^{5} (CD4)	N.D.
CD4-4	F	72	CD4	sCAEBV	9.4×10^{4}	6.4×10^{5} (CD4)	N.D.
CD8-1	F	38	CD8	sCAEBV	1.4×10^{5}	3.9×10^{5} (CD8)	N.D.
CD8-2	F	21	CD8	sCAEBV	1.9×10^{3}	4.2×10^{4} (CD8)	N.D.
CD8-3	F	64	CD8	sCAEBV	2.6×10^{5}	1.2×10^{6} (CD8)	4.6×10^{5}
CD8-4	M	28	CD8	sCAEBV	1.9×10^{3}	4.1×10^{5} (CD8)	2.0×10^{4}
CD8-5	M	13	CD8	sCAEBV	2.1×10^{3}	6.4×10^{4} (CD8)	N.D.
γδ	M	9	γδ	HV	8.0×10^{3}	2.6×10^{4} (γδ)	N.D.
CD56-1	F	18	CD56	sCAEBV	2.5×10^{2}	5.0×10^{4} (CD56)	N.D.
CD56-2	F	13	CD56	HMB	5.2×10^{4}	1.6×10^{6} (CD56)	7.5×10^{4}
CD56-3	F	23	CD56	sCAEBV	1.0×10^{4}	1.1×10^{5} (CD56)	N.D.
CD56-4	F	48	CD56	sCAEBV	8.6×10^{4}	1.6×10^{5} (CD56)	N.D.
CD56-5	M	9	CD56	sCAEBV	1.1×10^{4}	5.2×10^{5} (CD56)	N.D.
CD56-6	M	8	CD56	sCAEBV	5.1×10^{2}	3.5×10^{4} (CD56)	N.D.
CD56-7	M	24	CD56	sCAEBV	2.3×10^{3}	2.1×10^{4} (CD56)	N.D.

M: Male, F: Female.

EBV: Epstein-Barr virus, PB: peripheral blood.

sCAEBV: systemic chronic active Epstein-Barr virus infection, HMB: hypersensitivity to mosquito bites (HMB), HV: hydroa vacciniforme-like eruption.

*The clonality was detected by Southern blotting for EBV terminal repeat.

doi:10.1371/journal.pone.0112564.t001

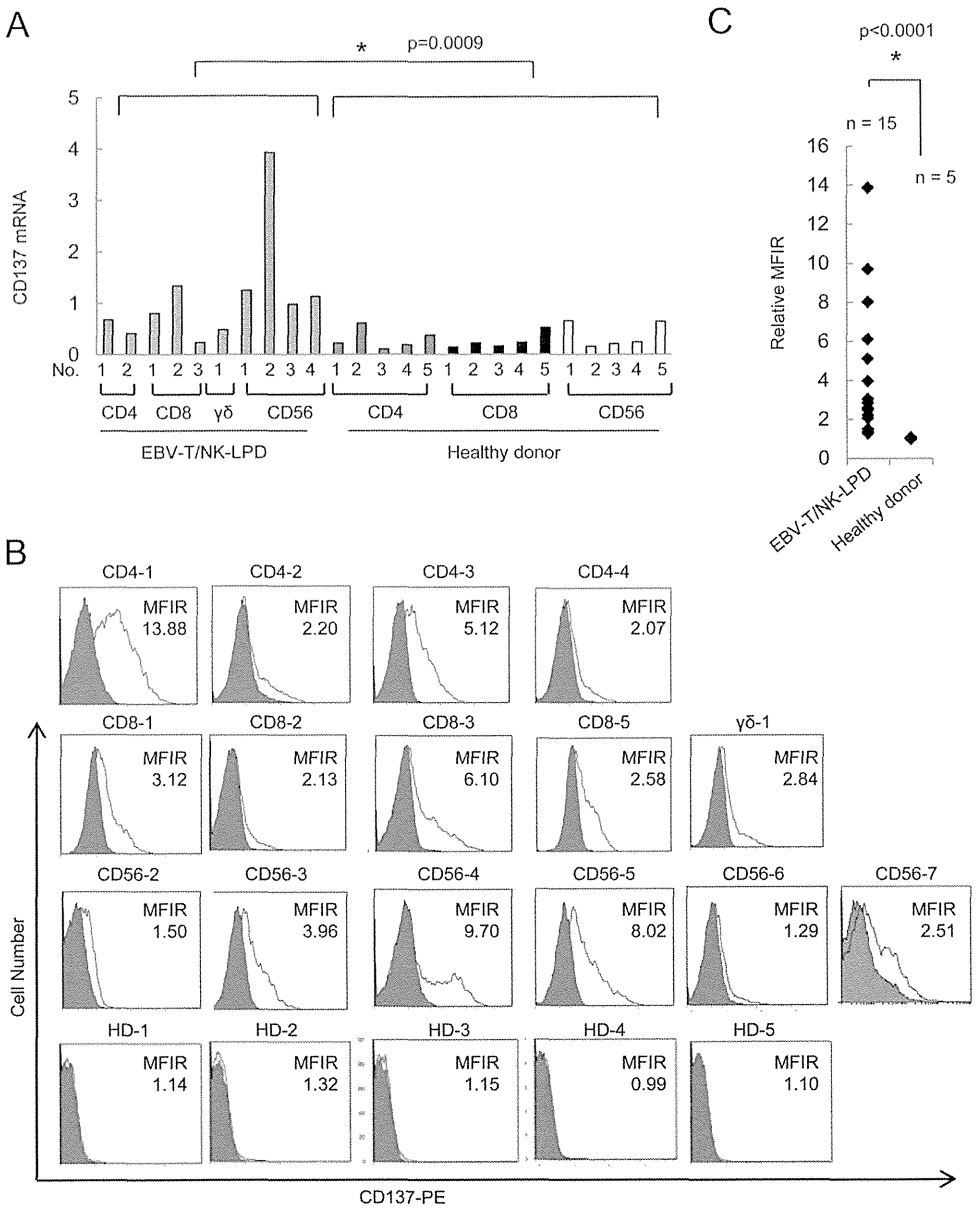

Figure 3. CD137 expression in EBV-positive T or NK cells of patients with EBV-T/NK-lymphoproliferative disorders (EBV-T/NK-LPDs). (A) Transcripts of *CD137* and *GAPDH* of freshly isolated EBV-positive cell fractions from 9 EBV-T/NK-LPDs patients, or cells of the same fractions from healthy donors were quantified by real-time RT-PCR. Relative copy number was obtained by normalizing the *CD137* transcripts to those of *GAPDH*. The relative copy number of the EBV-T/NK-LPDs patients' cells and healthy donor cells were compared. (B) CD137 protein expression in peripheral blood mononuclear cells (PBMCs) from 15 EBV-T/NK-LPDs patients or 5 healthy donors. PBMCs were cultured with IL-2 for 3 days and examined by flow cytometry. The mean fluorescent intensity of CD137 was normalized by that of isotype-matched control and expressed as MFIR (mean fluorescence intensity rate). (C) A bar graph for the relative MFIRs. Each point represents the MFIR of each sample.
doi:10.1371/journal.pone.0112564.g003

development of T or NK cell neoplasms have been reported to date. In this study we examined EBV-positive T or NK cells, and demonstrated that not only the cell lines but also freshly isolated cells of EBV-positive fractions from EBV-T/NK-LPDs patients expressed high levels of *CD137* mRNA. CD137 expression was also detected in EBV-positive cells isolated from the tissue lesions of EBV-T/NK-LPDs xenograft models. We demonstrated that EBV could directly induce CD137 expression most likely through LMP1 in T and NK cells. In addition, stimulation of CD137 by its ligand could suppress etoposide-induced cell death in EBV-positive and CD137-expressing T or NK cells. These results suggested that EBV could promote survival of T and NK cells by inducing CD137 and might be a cause for EBV-T/NK-neoplasms.

In the present study, *CD137* gene expression was significantly higher in freshly isolated EBV-positive T or NK cells from PB of patients compared with lymphocytes from healthy donors. *In vitro* IL-2 treatment enhanced CD137 expression in the EBV-infected

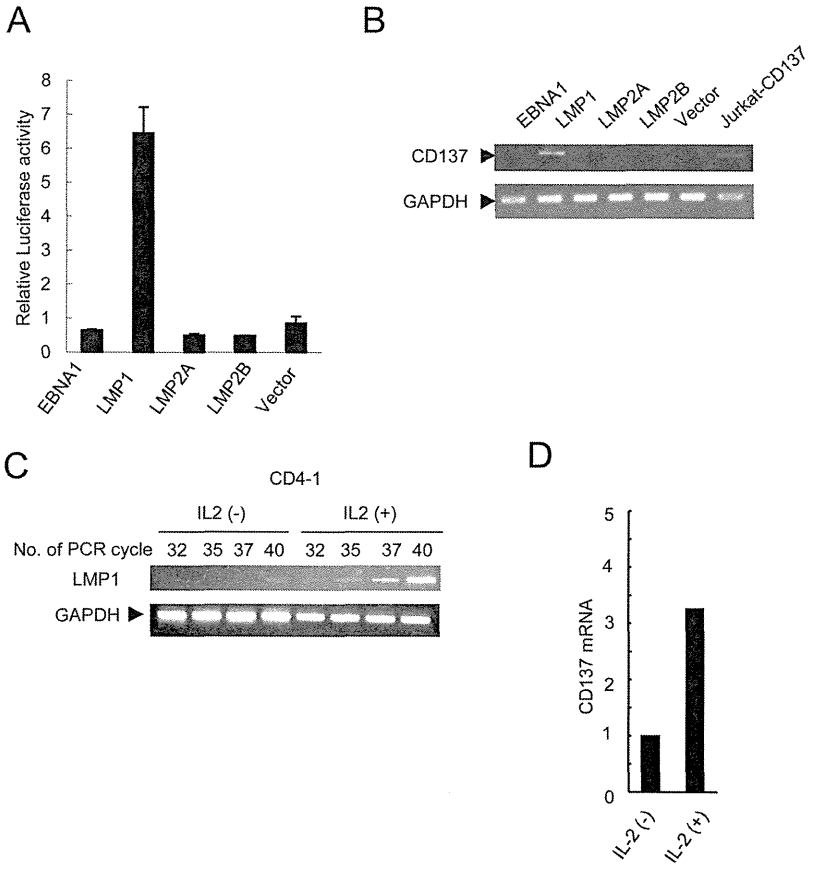

Figure 4. CD137 expression was upregulated by LMP1 whose expression was enhanced by IL-2 in EBV-T/NK-LPDs cells. (A) *CD137* transcription was examined using the assay described. Briefly, MOLT4 cells were transfected with 10 μg of the expression plasmids of the viral proteins, EBNA1, LMP1, LMP2A, LMP2B, or an empty vector as indicated, along with 10 μg of PGL3-4-1BB and 1 μg of pRLSV40. Twelve hours after transfection, the cells were harvested for a dual luciferase assay. Luciferase activity was normalized by *Renilla* luciferase activity and expressed in arbitrary units. The data are expressed as mean ± S.D. of 3 independent experiments. (B) MOLT4 cells were transfected with 10 μg of the expression plasmids of the viral proteins, EBNA1, LMP1, LMP2A, LMP2B, or an empty vector. Transcripts of CD137 (the upper panel) and GAPDH (the lower panel) in these cells were examined by RT-PCR. Jurkat-CD137 cells were used as a positive control. (C) RNAs were obtained from PBMCs from a EBV-T/NK-LPDs patient (CD4-1) which had been cultured with or without IL-2 for 3 days. Semi-quantitative RT-PCR assay for *LMP* was performed. Transcripts of *LMP1* (the upper panel) and *GAPDH* (the lower panel) were presented. (D) Transcripts of *CD137* and *GAPDH* were quantified by real time RT-PCR for the sample of 4C. Relative copy number was obtained by normalizing the *CD137* transcripts to those of *GAPDH*.
doi:10.1371/journal.pone.0112564.g004

Table 2. IL-2 concentration of the serum from EBV-T/NK-LPD patients.

EBV-T/NK-LPD (U/ml)		Healthy donor IL-2 (U/ml)
Case	**IL-2 (U/ml)**	
CD4-2	<0.8	<0.8
CD4-3	1.9	<0.8
CD4-5	0.9	<0.8
CD4-6	2.4	<0.8
CD8-2	2.1	1
CD8-3	1.1	
CD56-2	0.9	
CD56-3	0.9	

The concentration of IL-2 of the serum from EBV-T/NK-LPDs patients and from healthy donors. The lowest detection limit was 0.8 U/ml.
doi:10.1371/journal.pone.0112564.t002

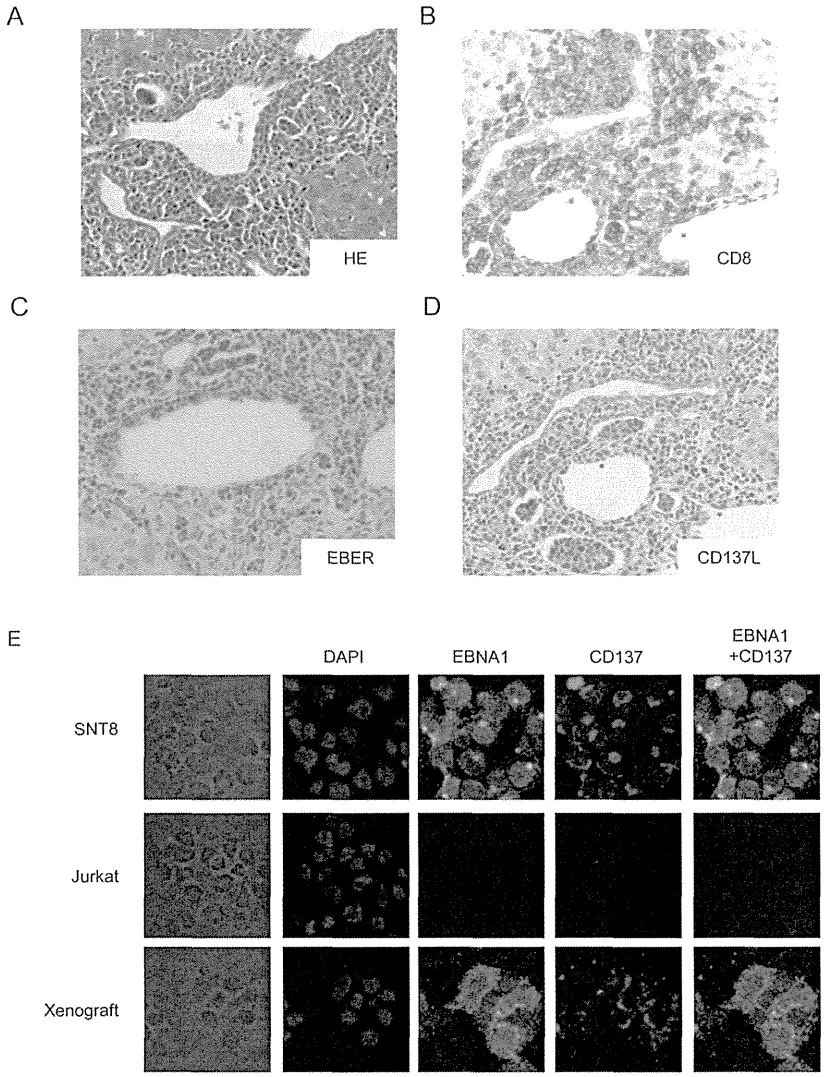

Figure 5. Histopathological specimen from the liver of the xenograft models. We generated the models by transplanting the cells from CD8-3 patient. Nine mice were examined and the representative data were shown. (A) Hematoxylin and eosin staining showed periportal infiltration of lymphocytes. (B) Immunochemical staining with anti-CD8 antibody (brown) showed that the infiltrating lymphocytes were positive for CD8. (C) *In situ* hybridization of Epstein–Barr virus-encoded mRNA (EBER) (brown). Infiltration of EBV-positive cells was detected in the periportal space. (D) Immunochemical staining with anti-CD137L antibody (brown) showed that CD137L-positive cells existed in the periportal space although the number of the cells was smaller than that of EBER positive cells. (original magnification, ×400). (E) Immune-fluorescent staining with anti-EBNA1 and anti-CD137 antibodies of cells isolated from the lesions. Mononuclear cells were obtained from the tissue lesions of a model mouse, stained with the antibodies. The cells were analyzed by confocal microscopy.
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cells of the patients, whereas not in control cells of the healthy donors. IL-2 treatment also increased *LMP1* gene expression in EBV-positive cells of EBV-T/NK-LPDs. Takahara and colleagues previously reported that IL-2 enhanced LMP1 expression in EBV-positive ENKL cell lines [18]. Since *CD137* promoter activity was enhanced by LMP1, we suggested that IL-2-induced CD137 protein expression was mediated by LMP1. In addition, the concentration of IL-2 in the serum of EBV-T/NK-LPDs patients was higher than that of healthy donors. Actually the concentration was lower than that of the culture medium, which we used in the assay. Ohga and colleagues, however, reported that the transcription of *IL-2* gene was upregulated in EBV-positive T- or NK-cells [19]. This finding suggested that the level might be high in the tissue lesion where large amount of EBV-positive T- or NK-cell were infiltrating. We detected CD137 protein expression in EBV-positive cells isolated from the lesion. The high expression level of *CD137* mRNA in the circulating EBV-positive cells may contribute to rapid and strong induction of the protein expression in the lesions.

We suggested that EBV enhanced *CD137* mRNA expression through LMP1. Expression level of LMP1 in ENKL is actually variable and other factors, such as miRNA, may play roles for lymphomagenesis in EBV-positive T- or NK-neoplasms [20]. However, all EBV-positive T- or NK-cell lines examined in the present study, expressed LMP1 according to our results (data not shown) and the report [14]. LMP1 activates c-JUN N-terminal

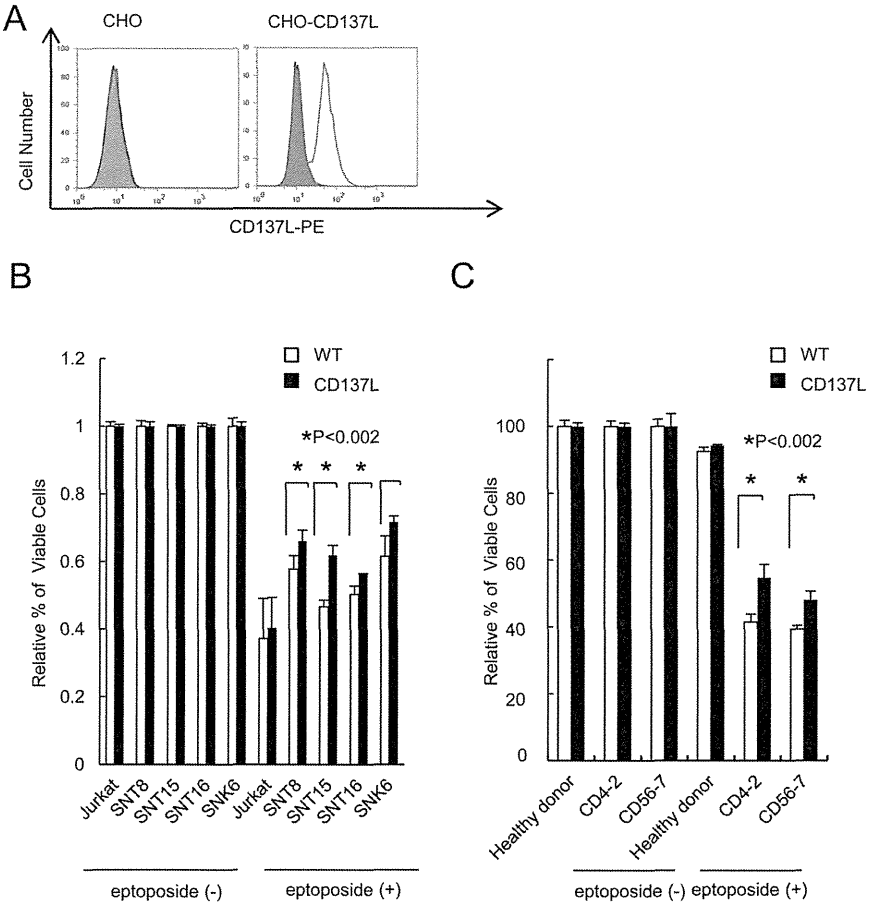

Figure 6. Stimulation of CD137 decreases etoposide-induced cell death of cells from patients with EBV-T/NK-LPDs. (A) CD137L expression on control Chinese Hamster Ovary (CHO) and CHO-CD137L cells. The expression was analyzed by flow cytometry using an antibody to CD137L (open histogram) or isotype-matched control immunoglobulin (gray, shaded histogram). (B) Jurkat cells and EBV- positive T- or NK-cell lines were cultured with 175 U/ml of IL-2 for 48 hours. Then they were cultured on control CHO or CHO-CD137L cells, which had been stained with PKH-26, with or without 2 μM of etoposide for 48 hours. They were then removed for assessment of viability. The cells were stained with DiOC6 and living EBV-T/NK-LPDs cells were detected as PKH-26-negative and DiOC6-positive cells by flow cytometry. The graph chart represents the relative numbers of living cells normalized by those of control cells which were cultured without etoposide. The data are expressed as mean ± S.D. of 3 independent experiments. (C) The PBMCs of EBV-T/NK-LPDs patients and healthy donors were cultured with 175 U/ml of IL-2 for 48 hours. Then they were cultured on control CHO or CHO-CD137L cells. They were then removed for assessment of viability as in B. The graph chart represents the relative numbers of living cells normalized by those of control cells which were cultured without etoposide. The data are expressed as mean ± S.D. of 3 independent experiments.

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kinase (JNK) [21], p38 mitogen-activated kinase (p38) [22], and Erk [23], which mediate the AP-1-activating pathway, and also activates NF-κB [24]. It was reported that CD137 expression was regulated by AP-1 and NF-κB in activated T cells [25]. LMP1 can, therefore, induce CD137 expression through AP-1 and NF-κB in T cells. In addition, we reported previously that EBV infection induced ectopic CD40 expression in T-cells [26,27]. CD40 is known to activate NF-κB, JNK, p38 and Erk [28,29]. Also, CD40-induced CD137 expression was recently reported [30]. These results indicate that EBV-induced CD137 expression can be mediated by LMP1, directly as well as through CD40.

Some questions, however, remain to be answered. The first concerns the localization of the CD137L. CD137L expression is induced in T cells when they are activated. [10] Its expression is also detected on various cancer cells [31]. Furthermore, expression of CD137 and CD137L is induced by the viral protein, Tax in ATLL cells and mediates autocrine survival signals, leading to proliferation of the infected cells and tumor development [12]. We therefore investigated CD137L expression on EBV-T/NK-cells themselves. However, we could not detect CD137L expression clearly on the surface of EBV-T/NK-LPDs cells. CD137L expression is usually detected not only on the surface of activated B and T cells, but also on antigen-presenting cells (APCs) such as dendritic cells, monocytes, and macrophages [32,33]. EBV-negative cells, including histiocytes and macrophages are detected in EBV-T/NK-LPDs lesions surrounding EBV-infected cells [3]. These cells may express CD137L on their surface. Interestingly, CD137L-positive cells were certainly present in the lesions of EBV-T/NK-LPDs (Figure 5D). Since the number of CD137L-positive cells was markedly smaller than that of EBV-positive cells, they were considered to be different cell types. As we previously described, we generated the models by injection of the PBMCs from the patients [15]. Further investigations is required to determine the phenotype of the CD137L-positive cells in the

lesions and to clarify whether these cells have some effects on EBV-positive cells, thereby contributing to disease progression. In addition, soluble CD137L (sCD137L) needs to be investigated. sCD137L is produced by lymphocytes or monocytes, with studies showing that it is present in PB of healthy donors and its level is increased in that of patients with hematological malignancies [34] and autoimmune diseases [35]. sCD137L may also have a role in hematopoietic neoplasm development, with its serum levels potentially being a prognostic factor in acute myeloid leukemia and myelodysplastic syndrome [36].

The next question is the actual role of CD137 in the disorders. EBV-T/NK neoplasms are not only lymphoid malignancies, but also have aspects of severe inflammatory diseases accompanied by high fever, cytokinemia, hemophagocytic syndrome and so on [3,18,37-39]. As CD137 mediates survival, proliferation, and cytokine production of CD137-expressing T cells, it may cause inflammation associated with the disease. In addition, CD137 acts as a "ligand" for CD137L. CD137L stimulation by CD137 also mediates intracellular signaling in CD137L-expressing cells [40]. In monocytes expressing CD137L, stimulation of the molecule induces proliferation and differentiation into DCs [41,42]. In B cells expressing CD137L, the stimulation induces proliferation, differentiation and production of immunoglobulins [43,44]. EBV-T/NK-neoplasms are associated with local and systemic inflammation, cytokinemia, or polyclonal gammopathy [38,39]. CD137 may therefore contribute to disease development by inducing not only survival of the infected cells but also inflammation. Inhibition of CD137-mediating signals by targeting CD137 or CD137L should be conducted in order to clarify their roles.

It is well known that CD137 activates survival-promoting molecules including NF-kB in activated T cells [10]. However, the role of the CD137-CD137L interaction *in vivo* is still controversial. Recently, an agonistic CD137 antibody was created and used for xenograft models of human disease, cancer, or autoimmune diseases. In some mouse cancer models, agonistic CD137 antibody induces tumor suppression by upregulating the immune reaction of cytotoxic T-cells against tumor cells [45,46]. On the other hand, in disease models of hyperimmune reactions such as asthma, GVHD, and autoimmune disease, the same antibody had the effect of suppressing T cells [47]. These findings show that CD137 regulates T-cell reactions both positively and negatively, and that the mechanism of the action *in vivo* is extremely complicated. As mentioned previously, EBV-T/NK-LPDs have two aspects: suppressed immune-reaction against EBV-T/NK-cells and a hyper-immune reaction as an inflammatory disease. The conflicting roles of the CD137–CD137L axis may be compatible with these clinical findings of EBV-T/NK-LPDs.

Our results indicate that upregulation of CD137 expression through LMP1 by EBV promotes cell survival in T or NK cells. This effect may contribute to the development of EBV-T/NK-neoplasms and suggests an attractive therapeutic target for the diseases.

Materials and Methods

Cells and reagents

The EBV-positive T/NK-cell (EBV-T/NK cell) lines SNT8, SNT15, SNT16, SNK1, SNK6, and SNK10 were cultured in RPMI containing 10% FCS and 175 U/ml of human IL-2 [14]. The EBV-negative T- and NK-cell lines, Jurkat, MOLT4, HPB-ALL, and MTA were cultured in RPMI containing 10% fetal calf serum (10% FCS-RPMI), whereas the EBV-negative NK-cell line, KHYG1 was cultured in 10% FCS-RPMI containing 175 U/ml of human interleukin-2 (IL-2). The B- cell lines, BJAB, Ramos, Raji, MD901 [48], HS-Sultan, and LCL were cultured in RPMI containing 10% FCS-RPMI. Jurkat, MOLT4, BJAB, Ramos, HS-Sultan and Raji cells were obtained from the American Type Culture Collection. LCL was established as previously described [26]. The expression of the viral proteins in LCL was demonstrated in Figure S4. MTA cells were obtained from Japanese Collection of Research Bioresources Cell Bank. Jurkat-CD137 and Chinese Hamster Ovary (CHO)-CD137L were generated as previously described [30]. Human recombinant IL-2 was purchased from R&D systems (Abington, UK) and etoposide from Wako (Osaka, Japan).

PCR assay for CD137

The sequences of the PCR primers used for detection of the CD137 gene were as follows: forward, 5′-GTGCCAGATTT-CATCATGGG-3′ (exon 2 of CD137) and reverse, 5′-CAA-CAGCCCTATTGACTTCC-3′ (exon 9 of CD137). The expression levels of the CD137 gene were determined by quantitative PCR, as described previously [13].

Diagnosis of EBV-T/NK-LPDs

EBV-T/NK-LPDs was diagnosed according to the following criteria: the presence of characteristic symptoms, an increase in EBV DNA load in peripheral blood (PB), and the detection of clonally proliferating EBV-positive T or NK cells [4,49].

Detection and isolation of EBV-positive cells in EBV-T/NK-LPDs patients

Detection and isolation of EBV-infected cells were performed as described previously [27]. Briefly, peripheral blood mononuclear cells (PBMCs) from EBV-T/NK-LPDs patients were isolated by density gradient centrifugation using Separate-L (Muto Pure Chemical, Tokyo, Japan) and sorted into CD19-, CD4-, CD8-, or CD56-positive fractions by antibody-conjugated magnetic beads (IMag Human CD19, 4, 8, and 56 Particles-DM; BD Biosciences, Sparks, MD, USA). The fraction which was negative for these markers was considered γδ T cell fraction. The EBV DNA load in each fraction was then measured by the real-time RT-PCR [50] on the basis of the TaqMan system (Applied Biosystems, Foster City, CA, USA). The fraction with the highest titer was assumed to be that with EBV-positive cells. In order to examine *CD137* mRNA expression in the infected cell, we isolated EBV-positive cells from PBMCs by magnetic beads conjugating antibodies for the surface markers of the infected cells.

Antibodies

Mouse antihuman CD137-PE, CD4-FITC, CD8-FITC, CD56-FITC and CD137L-PE as well as their control isotype antibodies were purchased from Becton, Dickinson and Company (Franklin Lakes, NJ, USA).

In vitro EBV infection assay

MOLT4 cells were infected with EBV as described previously [26]. Briefly, EBV was prepared from culture medium of B95-8 cells as described [51], and then concentrated (200-fold) in RPMI medium 1640 supplemented with 10% FCS. The virus suspension was filtered (0.45 μm) and the recipient cells (2×10^6 to 1×10^7) were incubated in 1 or 5 ml of the suspension for 1 h, and then rinsed twice with culture medium (10% RPMI). The efficiency of infection was >90% as judged by EBNA1 staining. For inactivation of the EBV genome, 1 ml of virus suspension in a 100-mm dish was irradiated with UV (254 nm) at 1 Jμcm^2 using a FUNA-UV-LINKER FS-800 (Funakoshi, Tokyo). Infection was

verified by EBV DNA quantification, and immune fluorescence staining of EBNA1 staining of the cells as described using Polyclonal Rabbit Anti-Human C3c Complement/FITC antibody (Dako, Glostrup, Denmark) [52].

PCR assay for EBV proteins

RT-PCR for detection of mRNA for the viral proteins, *LMP1, LMP2A, LMP2B* and *EBNA1* was performed according to the previous report [15].

Plasmids

The reporter plasmid PGL3-4-1BB for the detection of *CD137* promoter activation was kindly provided by Dr. Pichler [12]. The reporter plasmid for detection of NF-κB activation, pNF-κB-Luc, was purchased from Stratagene (Santa Clara, CA, USA), and the control *Renilla* luciferase plasmid pRL-SV40 from Promega (Madison, WI, USA). Plasmids containing EBV-encoded proteins, LMP1, LMP2A, LMP2B and EBNA1 were generated from the EBV-infected cell line B95-8 [53].

Luciferase reporter assays

The assays of transiently transfected cells were performed as described previously [54].

Measurement of serum IL-2

The concentration of IL-2 in the serum was examined by SRL, Inc. (Tokyo, Japan) using enzyme-linked immunosorbent assay (ELISA). The lowest detection limit was 0.8 U/ml.

Generation of the xenograft model of EBV-T/NK-LPDs

Male NOD/Shi-scid/IL-2Rγnull (NOG) mice were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan) and maintained under specific pathogen-free conditions. The model was generated by injection of PBMCs from patients to six weeks old mice through the tail vein as described previously [15]. Intravenous anesthesia by tribromoethanol was performed in order to minimize suffering. Engraftment was determined by detecting EBV DNA in the peripheral blood. After engraftment, mice were euthanized via CO_2 inhalation and applied for pathological and virological analyses.

Immunohistochemistry

The 4 μm thick paraffin-embedded formalin-fixed tissue sections were de-paraffinized, and heat-based antigen retrieval was performed in 0.1 M citrate buffer (pH 6.0). Endogeneous peroxidase activity was inhibited using hydrogen peroxide. The primary antibodies for CD137 (ab3169) and CD137L (ab64912) were purchased from Abcam (Cambridge, MA, USA). The detection system was the streptavidin-biotin-peroxidase complex technique (ABC kit; Vector Laboratories, Burlingame, CA, USA) with diaminobenzidine (DAB; Nichirei Bioscience, Tokyo, Japan) as the chromogen. *In situ* hybridization (ISH) of Epstein–Barr virus-encoded mRNA (EBER) was performed for detection of EBV in tissue sections by Epstein-Barr Virus (EBER) PNA Probe/Fluorescein (DAKO, Carpinteria, CA, USA) and second antibody for Fluorescein (Dako, Glostrup, Denmark).

Immune-fluorescent staining

The expression of CD137 protein on EBV-infected cells was examined by immune-fluorescent staining. Cells were fixed on slides by immersing in 4% paraformaldehyde for 10 min, followed by washing three times in PBS and incubation with mouse monoclonal anti-CD137, goat polyclonal anti-EBNA1) antibodies (Abcam, Cambridge, MA, USA), Cy5-conjugated Affinipure donkey anti-mouse antibody, and FITC-conjugated donkey anti-goat antibody (Jackson ImmunoResearch Laboratories, Inc. PA, USA). Nuclei were counterstained with ProLong Gold and DAPI (Invitrogen, Carlsbad, CA, USA), and the cells were analyzed by confocal microscopy (Fluoview FV10i, Olympus).

Stimulation of CD137 by ligand-expressing cells and detection of cell viability

The PBMCs were isolated from patients of EBV-T/NK-LPDs. Control CHO or CHO-CD137L cells were stained with PKH-26 (PKH-26 Red Fluorescent Cell Linker Kit; Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions, and plated on the wells. The PBMCs were then overlaid on pre-seeded control CHO or CHO-CD137L cells, and cultured with or without etoposide in 10% FCS-RPMI containing 175 U/ml of IL-2. After 48 h incubation, the cells were stained with DiOC6 (Invitrogen, Carlsbad, CA, USA) and removed. The cells were analyzed using a FACS Calibur flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ USA), with PKH-26–negative and DiOC6-positive cells considered as living EBV-T/NK cells.

Statistical analysis

For statistical analyses of Figure 3A and 3B, Mann-Whitney test was performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Student *t* test was performed for Figure 6B and 6C.

The study complied with the principles of the Declaration of Helsinki and was approved by the ethical committee of Tokyo Medical and Dental University (TMDU). Written informed consent was obtained from each patient. The experiments with NOG mice are in accordance with the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science, as well as ARRIVE guidelines [55]. The experiments were approved by the Institutional Animal Care and Use Committee of TMDU (No. 0140087A).

Supporting Information

Figure S1 CD137L expression in EBV-positive cell lines. Surface expression of CD137L was examined by flow cytometry using an antibody to CD137L (open histogram) or isotype-matched control immunoglobulin (gray, shaded histogram). The mean fluorescent intensity of CD137 was normalized by that of isotype-matched control and expressed as MFIR (mean fluorescence intensity rate) in arbitrary units. CHO-CD137L cells were used as positive control.
(TIF)

Figure S2 CD137 expression in PBMCs from EBV-positive T-NK-lymphoproliferative patients and those from healthy donors (HD). After collection, the cells were cultured with IL-2 for 3 days. The expression was analyzed by flow cytometry using an antibody to CD137 and to surface protein expressed on EBV-positive cells.
(TIF)

Figure S3 Immune-fluorescent staining with anti-LMP1 antibody of cells isolated from the lesions. Mononuclear cells were obtained from the tissue lesions of a model mouse, stained with the antibody. The cells were analyzed by confocal microscopy.
(TIF)

Figure S4 LCL that we used in the study was established as previously described [26]**.** The infection was confirmed by

RT-PCR for EBNA. We also examined and detected the expression of the lytic protein, BZLF1 [56]. Akata cells [57] stimulated with IgG were used as a positive control for BZLF1 expression. Since BZLF1 was not expressed in them, we concluded that the infection was latent.
(TIF)

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Author Contributions

Conceived and designed the experiments: MY KII SY SF OM AA. Performed the experiments: MY KII LW HK YS TF MK TK NS AA. Analyzed the data: MY KII LW HK YS TF MK TK NS SF OM AA. Contributed reagents/materials/analysis tools: MY KII YS SY TF TK SF OM AA. Wrote the paper: MY KII SY TK SF OM AA. Contributed to the modification of the draft and approved the final submission: MY KII HK LW YS SY TF MK TK NS SF OM AA.

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Clinical Investigations

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Detection of Herpes Viruses by Multiplex and Real-Time Polymerase Chain Reaction in Bronchoalveolar Lavage Fluid of Patients with Acute Lung Injury or Acute Respiratory Distress Syndrome

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Key Words

Acute lung injury · Acute respiratory distress syndrome · Human herpes virus · Multiplex polymerase chain reaction · Real-time polymerase chain reaction

Abstract

Background: Human herpes viruses (HHVs) are important pathogens in acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). Rapid and efficient diagnostic tools are needed to detect HHVs in the lung in ALI/ARDS patients. ***Objectives:*** This study aimed to evaluate the usefulness of multiplex and real-time polymerase chain reaction (PCR) analysis of bronchoalveolar lavage fluid (BALF) for detecting HHV reactivation in ALI/ARDS patients. ***Methods:*** Between August 2008 and July 2012, eighty-seven BALF samples were obtained from ALI/ARDS patients with unknown etiology and analyzed for HHVs. The types of HHVs in the BALF samples were determined using qualitative multiplex PCR followed by quantitative real-time PCR. ***Results:*** Multiplex PCR identified herpes simplex virus type 1 (HSV-1) (n = 11), Epstein-Barr virus (EBV) (n = 16), cytomegalovirus (CMV) (n = 21), HHV type 6 (HHV-6) (n = 2), and HHV-7 (n = 1) genomic DNA in 35 (40%) of the BALF samples, including 14 (16%) samples containing 2 or 3 HHV types. CMV and EBV reactivation was rare in immunocompetent patients, whereas reactivation of HSV-1 was predominantly observed in intubated patients regardless of their immune status. Overall, HHVs were almost exclusively found in patients with immunosuppression or endotracheal intubation. Real-time PCR detected 0.95–1.59 × 10^6 copies of viral DNA/μg human genome DNA, and HSV-1 (n = 4), CMV (n = 9), and HHV-6 (n = 1) were identified as potentially pathogenic agents. ***Conclusions:*** The implementation of multiplex and real-time PCR of BALF was feasible in ALI/ARDS patients, which allowed efficient detection and quantification of HHV DNA.

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Introduction

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) remain critical illnesses with substantial morbidity and mortality [1]. Despite advances in understanding the pathophysiology of ALI/ARDS, the

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mainstay of treatment for these diseases primarily involves supportive care and management of the underlying clinical disorder. Therefore, the pathogenic factors underlying these medical conditions need to be identified to indicate potential therapeutic interventions.

Human herpes viruses (HHVs) have been recognized as clinically important pathogens of pulmonary infections that can result in ALI/ARDS in immunocompromised patients [2–4], and they have also been recognized as potential pathogens in nonimmunocompromised critically ill patients [5–9]. However, there have been few comprehensive studies to date on the prevalence and pathogenic role of HHVs in ALI/ARDS patients, partly due to the limited diagnostic tools available for identifying different HHVs in a clinical sample.

In recent years, development of the multiplex polymerase chain reaction (PCR) assay has enabled simultaneous detection of a wide range of viruses [10]. Although a positive PCR for HHVs does not distinguish asymptomatic shedding from an active infection, quantification of the viral load in bronchoalveolar lavage fluid (BALF) could potentially differentiate between these two conditions [6, 11–13]. Therefore, rapid screening of BALF by qualitative multiplex PCR to identify virus-positive samples followed by quantitative PCR of the positive samples may be a useful diagnostic approach for evaluating lung diseases possibly caused by HHVs.

The aim of the present study was to evaluate the usefulness of multiplex and real-time PCR analysis of BALF for detecting HHV reactivation in ALI/ARDS patients.

Methods

Patients and Clinical Samples

Between August 2008 and July 2012, a total of 134 diagnostic bronchoalveolar lavage (BAL) procedures were performed in new-onset ALI/ARDS patients when the etiology of ALI/ARDS was unknown and was unlikely to be caused by bacterial pneumonia based on the clinical presentation, radiological findings, or the response to antibiotics. Of these, 87 BALF samples were analyzed for HHVs using multiplex PCR as part of the diagnostic examination, and the data were retrospectively examined in this study. BAL sampling was carried out via standard techniques, usually instilling five 30-ml aliquots of normal saline, and the specimens were stored at –80°C until the PCR was performed. The BALF was routinely tested for common pathogens (bacteria, fungi, and *Mycobacterium*) and for viral inclusion bodies by cytology. Additional microbiological studies, including quantitative PCR for *Pneumocyctis jirovecii* in BALF and cytomegalovirus (CMV) pp65 antigen levels in blood were performed as appropriate. Clinical and laboratory data were also collected from medical records. This study was approved by our institutional review board.

Classification of Viral Pneumonia Cases and Clinical Definitions

Classification of viral pneumonia cases was based on viral detection in BALF and on the following criteria: (1) proven viral pneumonia: specific cytopathic effects (inclusion bodies) in cells from BALF or transbronchial biopsy, (2) probable viral pneumonia: otherwise unexplained ALI/ARDS with a clinical response to specific antiviral agents, and (3) possible viral pneumonia: viral load $>10^4$ copies/μg DNA in BALF, otherwise unexplained ALI/ARDS, and antiviral agents ineffective or not administered. In possible viral pneumonia cases, there may be a clinical response to specific antiviral agents administered concurrently with efficacious treatments for known causes of ALI/ARDS. ALI/ARDS was defined according to previously reported criteria [14]. An immunocompromised patient was defined as one receiving immunosuppressants or corticosteroid therapy for more than 1 month or having AIDS.

PCR Analysis

HHV genomic DNA was measured by two independent PCR assays: qualitative multiplex PCR followed by quantitative real-time PCR of the HHV-positive samples detected by multiplex PCR.

The multiplex PCR was designed to identify the genomic DNA of 8 HHVs: herpes simplex virus type 1 (HSV-1), HSV-2, varicella zoster virus (VZV), Epstein-Barr virus (EBV), CMV, HHV type 6 (HHV-6), HHV-7, and HHV-8. DNA extraction was performed on 200- to 400-μl BALF samples using E21 virus minikits (Qiagen Inc., Valencia, Calif., USA). The multiplex PCR amplifications were set up in 2 capillaries (capillary 1: HSV-1, HSV-2, VZV, CMV, and HHV-6; capillary 2: EBV, HHV-7, and HHV-8), each containing 5 μl DNA extract, specific primers, hybridization probe mix, and Accuprime Taq (Invitrogen, Carlsbad, Calif., USA). The primers and probes for HHVs have been described previously [15] and are shown in table 1. The reactions were performed using the LightCycler PCR System (Roche, Switzerland) with the following conditions: an initial denaturation step at 95°C for 2 min, followed by 40 cycles at 95°C for 2 s, 58°C for 15 s, and 72°C for 15 s, with a final extension at 40°C for 30 s. Hybridization probes were then mixed with the PCR products and melting curves were analyzed using the LightCycler System. The sensitivity of the multiplex PCR analysis was 50 copies/tube.

Subsequently, for HHV-positive samples, real-time PCR was performed to measure the viral load using Amplitaq Gold and the Real-Time PCR 7300 System (ABI, Foster City, Calif., USA). The sequences of the primers and probes for the HHVs and the PCR conditions used in this study have been previously reported [16]. The BALF samples were also analyzed by PCR for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control, and the number of viral DNA copies was calculated as copies per microgram of human genome DNA. The viral DNA in the blood samples of selected patients was measured using the same multiplex PCR conditions, and the results were calculated as copies per milliliter of whole blood.

Statistics

Continuous variables are expressed as means ± SD unless stated otherwise. Categorical variables were compared using the χ^2 test or Fisher's exact test as appropriate. The CMV viral load in BALF was assessed as a diagnostic test for CMV pneumonia us-

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Table 1. Sequences of primers and probes used in the multiplex PCR

Virus	Target gene	Primer sequence	Probe sequence
HSV-1, HSV-2[a]	polymerase	F: GCTCGAGTGCGAAAAAACGTTC R: TGCGGTTGATAAACGCGCAGT	3′FITC: GCGCACCAGATCCACGCCCTTGATGAGC LcRed604-5′: CTTGCCCCCGCAGATGACGCC
VZV	gene 29	F: TGTCCTAGAGGAGGTTTTATCTG R: CATCGTCTGTAAAGACTTAACCAG	3′FITC: GGGAAATCGAGAAACCACCCTATCCGAC LcRed640-5′: AAGTTCGCGGTATAATTGTCAGT
EBV	BamH1	F: CGCATAATGGCGGACCTAG R: CAAACAAGCCCACTCCCC	3′FITC: AAAGATAGCAGCAGCGCAGC LcRed640-5′: AACCATAGACCCGCTTCCTG
CMV	CMV glycoprotein	F: TACCCCTATCGCGTGTGTTC R: ATAGGAGGCGCCACGTATTC	3′FITC: TCGTCGTAGCTACGCTTACAT LcRed705-5′: ACACCACTTATCTGCTGGGCAGC
HHV-6	101k gene region	F: ACCCGAGAGATGATTTTGCG R: GCAGAAGACAGCAGCGAGTA	3′FITC: TAAGTAACCGTTTTCGTCCCA LcRed705-5′: GGGTCATTTATGTTATAGA
HHV-7	U57	F: GAAAAATCCGCCATAATAGC R: ATGGAACACCTATTAACGGC	3′FITC: GCCATAAGAAACAGGTACAGACATTGTCA LcRed705-5′: TTGTGAAATGTGTTGCG
HHV-8	EB BDLF1 ORF26	F: AGCCGAAAGGATTCCACCAT R: TCCGTGTTGTCTACGTCCAG	3′FITC: CCGGATGATGTAAATATGGCGGAAC LcRed705-5′: TGATCTATATACCACCAATGTGTCATTTATG

F = Forward primer; R = reverse primer.

[a] The primer sequences were the same for both HSV-1 and HSV-2, while the probe sequences were a complete match with the HSV-2 genome sequence but had a two-base mismatch with the HSV-1 genome sequence. Therefore, there was a difference in melting temperature between HSV-1 and HSV-2, enabling discrimination of these two viruses.

ing a receiver operating characteristic plot. $p < 0.05$ was considered statistically significant. All statistical analyses were performed using JMP 7.0.2 software (SAS Institute Inc., Cary, N.C., USA).

Results

Study Population

The clinical characteristics of the patients in this study are summarized in table 2. The underlying conditions were diverse and 49 (56%) patients were immunocompromised. ALI/ARDS developed in a nosocomial setting in 31 (35%) patients. Predominant ground-glass opacity mixed with focal air space consolidation was the most frequent radiological finding.

Viral Detection in BALF

The HHV virology results are summarized in table 3. Multiplex and real-time PCR detected HSV-1 in 11 (13%) BALF samples (2.92×10^2–6.22×10^5 copies/μg DNA), EBV in 16 (18%) samples (9.50×10^{-1}–6.62×10^4 copies/μg DNA), CMV in 21 (24%) samples (2.55–1.59×10^6 copies/μg DNA), HHV-6 in 2 (2%) samples (6.90×10^4–6.90×10^6 copies/μg DNA), and HHV-7 in 1 (1%) sample (3.12×10^3 copies/μg DNA). HSV-2, VZV, and HHV-8 were not detected. Overall, 35 (40%) patients were positive for at least one type of HHV, and multiple HHV types were detected in 14 (16%) patients.

The prevalence of HSV-1 was higher in intubated patients than in nonintubated patients (41 vs. 3%, $p < 0.0001$), but it was not significantly different between immunocompromised and immunocompetent patients (12 vs. 13%, $p = 0.90$) (fig. 1). In contrast, in CMV- and EBV-positive patients, the prevalence of CMV (fig. 2) and EBV (fig. 3) was not significantly different between intubated patients (31 and 18%, respectively) and nonintubated patients (22 and 18%, respectively) ($p = 0.33$ and $p = 1.00$, respectively), but it was significantly higher in immunocompromised patients (39 and 29%, respectively) than in immunocompetent patients (5 and 5%, respectively) ($p = 0.0003$ and $p = 0.006$, respectively). HHV-6- and HHV-7-positive BALF was obtained from immunocompromised patients. Overall, the prevalence of HHVs was higher in immunocompromised and/or intubated patients (55%; 32/58) than in immunocompetent patients without endotracheal intubation (10%; 3/29) ($p < 0.0001$).

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Table 2. Baseline characteristics of the 87 patients in this study

Age, years	64.3±14.3
Male gender, n (%)	59 (68)
Underlying disease, n (%)	
Connective tissue disease	19 (22)
Posttransplant	9 (10)
Hematologic malignancy	8 (9)
Solid cancer	8 (9)
Postoperative	3 (3)
Hemodialysis	3 (3)
AIDS	2 (2)
Underlying pulmonary disease, n (%)	
IIP	21 (24)
CTD-IP	6 (7)
Immunocompromised, n (%)	49 (56)
Nosocomial onset, n (%)	31 (35)
Mechanical ventilation, n (%)	
At the time of BAL	27 (31)
Over the course of hospitalization	61 (70)
PaO_2/FIO_2, mm Hg	163±68.7
ALI, n (%)	28 (32)
ARDS, n (%)	59 (68)
Duration of symptoms, days	9.7±9.6
LDH, IU/l	543±363
CRP, mg/dl	10.3±3.3
CT findings (%)	
GGO only	12 (14)
GGO predominant	62 (71)
Consolidation predominant	13 (15)
BALF samples[a]	
Median total cell count (range), n × 10^5/mm^3	2.35 (0.1–88.8)
Median neutrophils (range), %	28 (0–93)
Median lymphocytes (range), %	15 (1–82)
Median eosinophils (range), %	1 (0–46)
Median macrophages (range), %	32 (0–84)

IIP = Idiopathic interstitial pneumonia; CRP = C-reactive protein; LDH = lactate dehydrogenase; CTD-IP = connective tissue disease-associated interstitial pneumonia; GGO = ground-glass opacity.

[a] Fiberoptic bronchoscopy was performed with supplemental oxygen (n = 23), under noninvasive ventilation (n = 42), or under endotracheal intubation (n = 22).

Diagnosis

Fourteen patients in this study were diagnosed with viral pneumonia: 9 with CMV pneumonia (5 proven, 3 probable, 1 possible viral pneumonia), 4 with HSV-1 pneumonia (all possible viral pneumonia), and 1 with HHV-6 pneumonia (probable viral pneumonia) (table 4). Diagnoses of the other patients included acute exacerbation of interstitial pneumonia (n = 18), pneumocystis pneumonia (n = 13), other pulmonary infections (n = 7), diffuse alveolar hemorrhage (n = 7), septic ARDS (n = 3), drug-induced pneumonia (n = 3), other causes of ALI/ARDS (n = 11), and unidentified etiology (n = 12). HHVs were not detected in the BALF of patients with acute exacerbation of interstitial pneumonia, except in one BALF that had a low EBV viral load (278 copies/μg DNA). For a diagnosis of proven or probable CMV pneumonia, a cutoff value for the viral load in BALF of 1.39×10^4 copies/μg DNA had 87.5% sensitivity and 84.6% specificity.

Treatment and Outcome

Antiviral agents were administered to 13 patients with a diagnosis of viral pneumonia. Although respiratory improvement was seen, at least temporally, in 9 of the patients diagnosed with CMV or HHV-6 pneumonia, 10 (77%) patients eventually died due to a newly acquired infection, sustained respiratory failure, or multiorgan dysfunction (table 4). The overall in-hospital mortality in this cohort was 51%.

Discussion

This study showed that multiplex PCR and real-time PCR analysis of HHVs in BALF provided informative data regarding the epidemiologic characteristics of HHVs in ALI/ARDS patients with unknown etiology. To our knowledge, this is the first study to conduct comprehensive multiplex and real-time PCR analyses of HHVs in BALF of ALI/ARDS patients.

Viruses in the Herpesviridae family are the most common viral pathogens causing pulmonary infection in immunocompromised patients. Although CMV is best known for its propensity to cause pneumonia [2], other herpes viruses (e.g. HSV-1 [3, 17], VZV [18], EBV [19], and HHV-6 [20, 21]) have also been implicated as etiologic agents. Moreover, recent studies have documented frequent pulmonary reactivation and/or infection of HSV-1 [6, 7] and CMV [8, 9] in critically ill patients with no known immunocompromise. Therefore, ALI/ARDS patients with unknown etiology should be examined for possible viral pneumonia caused by HHVs, which often presents with bilateral ground-glass opacification and/or consolidation [17, 21, 22]. In this setting, the diagnosis must be broadened to a wide range of HHVs that can be efficiently examined by multiplex PCR instead of conventional microbiological tests for specific pathogens. In fact, this study found reactivation of 5 types of herpes viruses in 40% of ALI/ARDS patients, including 16% of patients with multiple types of HHVs in their BALF.

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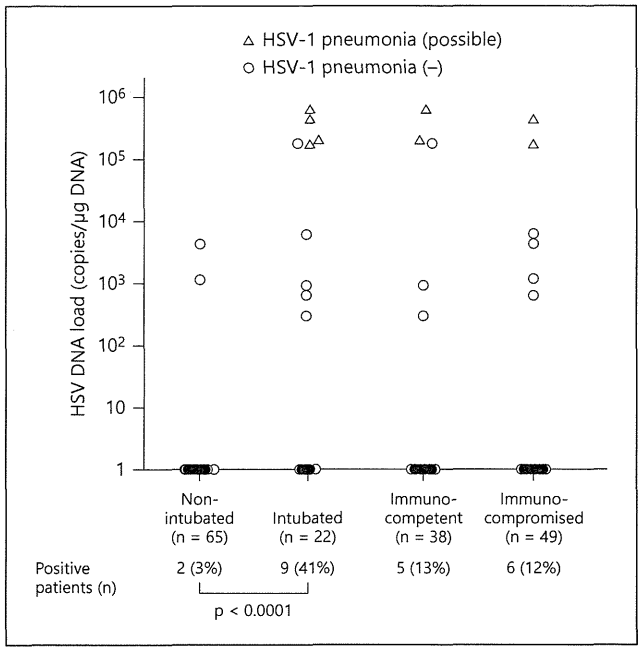

Fig. 1. HSV-1 prevalence and semi-log plot of the HSV-1 DNA load in BALF as a function of patient intubation or immune status. Negative PCR results were set to 1 ($\log_{10} 1 = 0$).

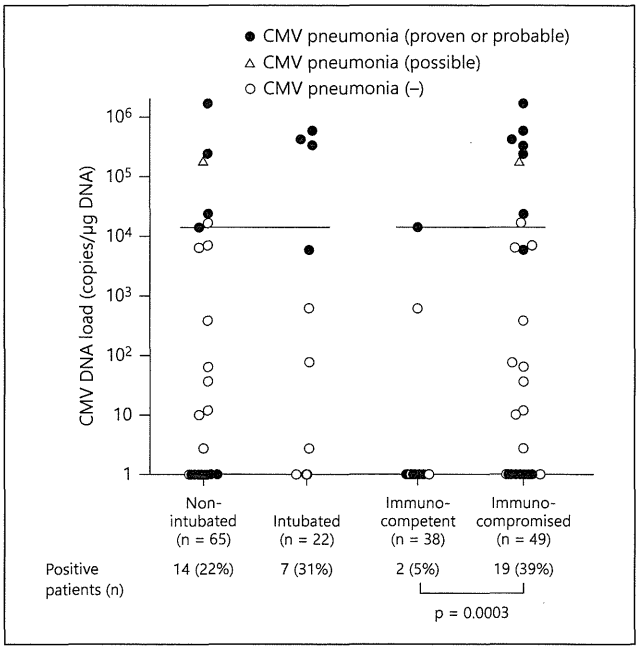

Fig. 2. CMV prevalence and semi-log plot of the CMV DNA load in BALF as a function of patient intubation or immune status. Negative PCR results were set to 1 ($\log_{10} 1 = 0$). The bar represents the cutoff value indicating CMV pneumonia (1.39×10^4 copies/μg DNA).

Table 3. HHV prevalence and viral load in BALF samples from the 87 patients in this study

	HSV-1	HSV-2	VZV	EBV	CMV	HHV-6	HHV-7	HHV-8
Positive BALF[a], n (%)	11 (13)	0	0	16 (18)	21 (24)	2 (2)	1 (1)	0
Viral load, copies/μg DNA	2.92×10^2–6.22×10^5	–	–	9.50×10^{-1}–6.62×10^4	2.55–1.59×10^6	6.90×10^4–6.90×10^6	3.12×10^3	–
BALF samples with a viral load, n								
≥0	0	–	–	2	3	0	0	–
≥10	0	–	–	2	4	0	0	–
$\geq10^2$	3	–	–	5	2	0	0	–
$\geq10^3$	3	–	–	3	3	0	1	–
$\geq10^4$	0	–	–	4	3	1	0	–
$\geq10^5$	5	–	–	0	5	0	0	–
$\geq10^6$	0	–	–	0	1	1	0	–

[a] Two types of HHVs were detected in 12 samples (EBV and CMV in 6, HSV-1 and EBV in 3, HSV-1 and CMV in 2, and CMV and HHV-6 in 1). Three types of HHVs were detected in 2 samples (EBV, CMV, and HHV-7 in 1 and HSV-1, EBV, and CMV in 1).

Considering the diversity of HHVs detected in this study, multiplex PCR has the additional advantage of being able to identify unexpected agents that might otherwise be overlooked, thereby enabling early therapeutic intervention.

This study provided insights into the epidemiologic features of herpes viruses in ALI/ARDS. Of note, reactivation of HSV-1 was predominantly observed in intubated patients regardless of their immune status, and a high HSV-1 DNA load in BALF was not associated with high-

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Table 4. Laboratory and clinical findings of 14 patients diagnosed with or suspected of having viral pneumonia

Patient No.	Underlying disease	Virus	Diagnosis	DNA load		pp65 (+) cells	Other HHVs in BALF	Dominant CT finding	Antiviral agent	Outcome
				BAL copies/μg DNA	copies/ml blood					
1	SLE	CMV	proven	1.59×10^6	NA	82	none	GGO	ganciclovir	dead
2	posttransplant	CMV	proven	4.03×10^5	NA	206	EBV	GGO	ganciclovir	dead
3	SLE	CMV	proven	3.12×10^5	NA	34	EBV, HHV-7	GGO	ganciclovir	dead
4	MPA	CMV	proven	2.22×10^4	NA	0	none	GGO	ganciclovir	alive
5	DIHS	CMV	proven	1.39×10^4	NA	31	none	GGO	ganciclovir	dead
6	posttransplant	CMV	probable	5.65×10^5	NA	2	none	consolidation	ganciclovir	alive
7	SLE	CMV	probable	2.26×10^5	NA	30	none	GGO	ganciclovir	alive
8	DM-ILD	CMV	probable	5.70×10^3	NA	1	none	GGO	ganciclovir	dead
9	HIV-PCP	CMV	possible	1.70×10^5	NA	74	none	GGO	ganciclovir	alive
10	sepsis	HSV-1	possible	6.22×10^5	ND	NA	none	GGO	aciclovir	dead
11	posttransplant	HSV-1	possible	4.30×10^5	ND	0	none	GGO	none	dead
12	trauma	HSV-1	possible	2.00×10^5	NA	NA	none	GGO	aciclovir	dead
13	ML	HSV-1	possible	1.72×10^5	3.30×10^2	0	CMV	GGO	aciclovir	dead
14	posttransplant	HHV-6	probable	6.90×10^6	1.39×10^5	NA	none	GGO	foscarnet	dead

SLE = Systemic lupus erythematosus; MPA = microscopic polyangiitis; DIHS = drug-induced hypersensitivity syndrome; DM-ILD = dermatomyositis-associated interstitial lung disease; PCP = pneumocystis pneumonia; ML = malignant lymphoma; NA = not assessed; ND = not detected; GGO = ground-glass opacity.

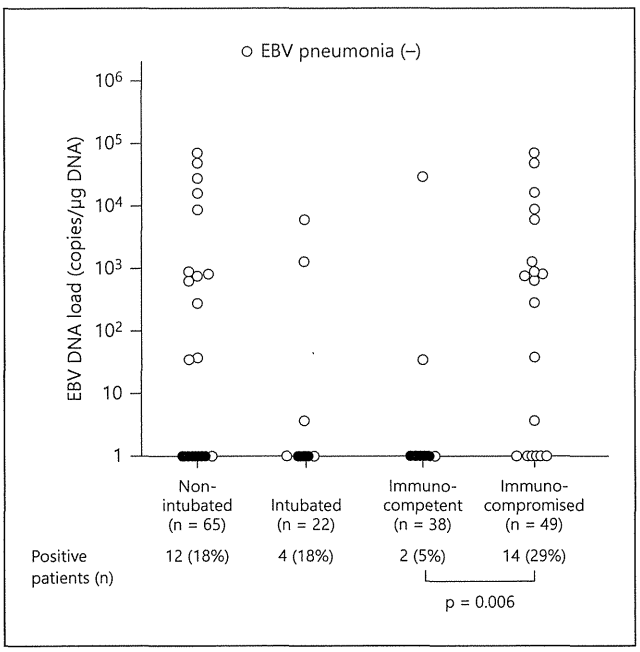

Fig. 3. EBV prevalence and semi-log plot of the EBV DNA load in BALF as a function of patient intubation or immune status. Negative PCR results were set to 1 ($\log_{10}$ 1 = 0).

level viremia. These findings are in agreement with previous reports that HSV-1 pneumonia frequently presents as late-onset ventilator-associated pneumonia due to aspiration from the upper respiratory tract rather than pulmonary dissemination secondary to systemic viral infection [5, 6]. We also found that reactivation of HHVs other than HSV-1 mostly occurred in classically high-risk immunocompromised patients. Although recent studies have shown that CMV reactivation is common in nonimmunosuppressed, critically ill patients [8, 9], our results showed that CMV reactivation of the lung was rare in this patient population. Overall, clinically significant pulmonary reactivation of HHVs was almost exclusively observed in patients with endotracheal intubation or known immunocompromised status, indicating that patients with this clinical picture merit careful investigation for HHVs in the lung.

Viral pneumonia caused by HHVs still represents a diagnostic challenge. Demonstration of cytopathic effects by HHVs, indicating viral pathogenicity, is not a sensitive diagnostic tool and is often hard to obtain [23], while detection of HHVs via viral culture or PCR does not differentiate active infection from asymptomatic shedding. Therefore, combined use of real-time PCR with multiplex PCR is an integral part of the evaluation of HHV patho-

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genicity. Since the amount of recovered epithelial lining fluid in BALF can vary considerably, the quantitative methodology in this study used data normalization to GAPDH, a housekeeping gene, to correct for this variation [24]. In agreement with previous studies [11–13], we found that quantification of the viral load in BALF may be useful in diagnosing CMV pneumonia. We noted that 3 patients with proven or probable CMV pneumonia had negative or clinically insignificant pp65 antigenemia, highlighting the importance of obtaining samples from the site of infection. Meanwhile, the pathogenicity of HSV-1 was unclear in this study because antiviral therapy did not lead to clinical improvement in 3 of 4 patients diagnosed with possible HSV-1 pneumonia. Previous studies also showed that HSV-1 in BALF may be a marker for underlying clinical conditions rather than a cause of mortality [25, 26]. In clinical practice, however, when there is a high viral load in BALF samples from ALI/ARDS of otherwise unexplained etiology, administration of antiviral agents against HHVs with possible lung pathogenicity (e.g. CMV, HSV-1, and HHV-6) would be an option for treatment.

We must acknowledge that it is difficult to extrapolate our results to all ALI/ARDS patients or to elucidate the etiologic role of HHVs in ALI/ARDS due to the selected group of patients and the retrospective nature of our study without a standardized diagnostic procedure. In addition, we must note that the pathogenic significance of HHVs is often difficult to determine via quantification of the viral DNA in BALF because of the substantial overlap between viral loads in symptomatic and asymptomatic patients [6, 11, 13]. Accordingly, although we have demonstrated that our diagnostic approach was technically feasible and potentially useful in ALI/ARDS patients to detect HHVs in the lung, further studies prospectively collecting all de novo ALI/ARDS patients in order to assess the exact prevalence of HHVs in ALI/ARDS or to investigate the possible impact of common bacterial infections on the reactivation of HHVs are needed. Also, a controlled antiviral treatment trial is warranted to draw conclusions regarding the etiologic role of the detected viruses in the development or worsening of ALI/ARDS.

Our study has some more limitations. First, there was a large heterogeneity in the studied population that precluded assessment of the impact of HHVs on the prognosis. Second, we may have underestimated the prevalence of HHVs because an HHV type with a low viral load in BALF could have been missed in the multiplex PCR assays if the BALF also contained a high viral load of another HHV type. Third, the impact of virus-bacteria coinfections could not be assessed because two thirds of our patients had received antimicrobial agents at the time of the BAL. Forth, oral contamination cannot be excluded because BALF samples obtained without a tracheal tube were possibly contaminated with oropharyngeal secretions by virtue of the technical procedure.

In conclusion, the implementation of multiplex and real-time PCR for HHVs allowed efficient detection and quantification of viral genomic DNA in BALF in selected ALI/ARDS patients of unknown etiology, especially in patients with immunosuppression or endotracheal intubation. In this setting, where clinicians must consider a wide differential diagnosis, the combination of multiplex and real-time PCR for HHVs may represent a useful diagnostic tool for the management of ALI/ARDS.

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Review Article

Current research on chronic active Epstein–Barr virus infection in Japan

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Abstract Epstein–Barr virus (EBV) infection is usually asymptomatic and persists lifelong. Although EBV-infected B cells have the potential for unlimited proliferation, they are effectively removed by the virus-specific cytotoxic T cells, and EBV-associated lymphoproliferative disease develops only in immunocompromised hosts. Rarely, however, individuals without apparent immunodeficiency develop chronic EBV infection with persistent infectious mononucleosis-like symptoms. These patients have high EBV-DNA load in the peripheral blood and systemic clonal expansion of EBV-infected T cells or natural killer (NK) cells. Their prognosis is poor with life-threatening complications including hemophagocytic lymphohistiocytosis, organ failure, and malignant lymphomas. The term "chronic active EBV infection" (CAEBV) is now generally used for this disease. The geographical distribution of CAEBV is markedly uneven and most cases have been reported from Japan and other East Asian countries. Here we summarize the current understanding of CAEBV and describe the recent progress of CAEBV research in Japan.

Key words chronic active EBV infection, EBV-associated hemophagocytic lymphohistiocytosis, EBV-associated T/NK-cell lymphoproliferative disease, Epstein–Barr virus, flow-cytometric *in situ* hybridization, hydroa vacciniforme, hypersensitivity to mosquito bites, mouse model.

Epstein–Barr virus (EBV) was discovered in cultured cells of Burkitt lymphoma as the first human tumor virus.[1] Since then EBV has been found to be associated with a number of malignancies, including Hodgkin lymphoma, nasopharyngeal carcinoma, and gastric carcinoma.[2] Despite this close association with these malignancies, EBV was found to be a ubiquitous virus infecting >90% of the adult population worldwide. EBV-associated malignancies thus develop in a restricted fraction of hosts through collective effects of various factors, including host genetic background and environmental factors, as well as functions of EBV genes. EBV infection in humans is usually asymptomatic and persists lifelong as a latent infection, although primary infection later than adolescence frequently results in infectious mononucleosis (IM). IM is caused by transient proliferation of EBV-infected B cells accompanied by excessive response of EBV-specific cytotoxic T cells (CTL). The main target of EBV is B cells and epithelial cells, and EBV has a unique biological activity to transform B cells and establish immortalized lymphoblastoid cell lines. Given that EBV-transformed cells express at least nine viral proteins including the highly immunogenic EBV nuclear antigen 3 (EBNA3) and EBNA2 (the latency III type EBV gene expression), they are readily removed by the virus-specific CTL and the virus does not cause lymphoproliferative disease (LPD) in normal immunocompetent hosts.[3] In immunocompromised hosts such as transplant recipients and AIDS patients, however, EBV-transformed cells are not efficiently removed and may cause EBV-associated B-cell LPD.

Rare EBV-infected individuals without apparent immunodeficiency present with persistent or recurring IM-like symptoms including fever, hepatosplenomegaly, lymphadenopathy, and liver dysfunction, as well as high EBV-DNA load in the peripheral blood.[4–7] The term "chronic active EBV infection" (CAEBV) is now generally used to describe this disease. Patients with CAEBV encountered in Japan and other East Asian countries have poor prognosis and are characterized by clonal expansion of EBV-infected T cells or natural killer (NK) cells.[8–11] In contrast, a similar disease with less morbidity and mortality has been reported from Western countries and it is usually associated with

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proliferation of EBV-infected B cells.[12] In this review, focused on CAEBV as an EBV-associated T/NK-cell LPD (EBV$^+$ T/NK-LPD), we summarize the current understanding of the disease and describe the authors' own recent work subsidized by grants from the Ministry of Health Labour and Welfare of Japan.

Clinical characteristics of CAEBV and other EBV-associated T/NK-LPD

As described in the previous section, IM-like symptoms are the main symptoms of CAEBV.[4–7] Other clinical manifestations include thrombocytopenia, anemia, pancytopenia, diarrhea, and uveitis. Peripheral blood EBV-DNA load regularly exceeds $10^{2.5}$ copies/μg DNA.[13] High-level production of various cytokines, including interleukin (IL)-1β, IL-10, and interferon (IFN)-γ has been detected in CAEBV patients and is thought to play an important role in inflammatory symptoms of the disease.[14–16] CAEBV can be classified into the T-cell and NK-cell types, depending on which lymphocyte subset is mainly infected with EBV. A survey of Japanese CAEBV patients found that the T-cell type is associated with less favorable prognosis than the NK-cell type.[17,18] CAEBV was included in the 2008 World Health Organization (WHO) classification of lymphomas as the systemic EBV$^+$ T-cell LPD of childhood.[19]

Although the clinical course of CAEBV is chronic, patients often develop fatal complications such as multi-organ failure, disseminated intravascular coagulopathy (DIC), digestive tract ulcer/perforation, coronary artery aneurysms, and malignant lymphomas, as well as EBV-associated hemophagocytic lymphohistiocytosis (EBV-HLH).[7] HLH is a hyper-inflammatory condition caused by overproduction of cytokines by excessively activated T cells and macrophages. Clinical characteristics of HLH include fever, hepatosplenomegaly, pancytopenia, hypertriglyceridemia, DIC, and liver dysfunction.[20]EBV-HLH usually occurs following primary EBV infection and is itself characterized by clonal proliferation of EBV-infected T or NK cells (most often CD8$^+$ T cells).[21,22] EBV-HLH can also occur in association with X-linked lymphoproliferative disease (XLP) and XIAP deficiency.[23]

Patients with CAEBV may have characteristic cutaneous complications, namely hypersensitivity to mosquito bites (HMB) and hydroa vacciniforme (HV), that are themselves distinct EBV$^+$ T/NK-LPD characterized by clonal proliferation of EBV-infected T or NK cells. Both HMB and HV can occur independently or in association with CAEBV. HV is a childhood photosensitivity disorder, characterized by necrotic vesiculopapules on sun-exposed areas.[24] EBV-DNA level is elevated in patients' peripheral blood, and histochemical analysis of skin lesions indicates infiltration of T cells expressing EBV-encoded small RNA (EBER).[25]Although most cases of HV resolve by early adulthood, HV overlapping with CAEBV may eventually develop into EBV-positive malignant lymphoma, which was included in the 2008 WHO classification of lymphoma as the hydroa vacciniforme-like lymphoma.[19,26] HMB is characterized by severe local skin reactions to mosquito bites including erythematous swelling with bullae, necrotic ulcerations, and depressed scars.[27] These local reactions may be accompanied by general symptoms such as high fever, lymphadenopathy, and liver dysfunction. Most HMB patients have EBV infection in NK cells in skin lesions and peripheral blood.[28,29] HMB patients without systemic symptoms may eventually develop CAEBV.[28]

Prospective clinicopathologic analysis of CAEBV and other EBV$^+$ T/NK-LPD

Chronic active EBV infection, EBV-HLH, HMB, and HV are thus distinct but overlapping entities categorized as EBV$^+$ T/NK-LPD. The higher incidence of these diseases in East Asian countries and their occasional coincidence in a single patient imply a common pathogenesis.[7,30] Kimura *et al.* performed a large-scale prospective study of Japanese EBV$^+$ T/NK-LPD.[31] A total of 108 cases of EBV$^+$ T/NK-LPD (80 cases of CAEBV, 15 cases of EBV-HLH, nine cases of HMB, and four cases of HV) were analyzed. They found that the clinical profile of EBV$^+$ T/NK-LPD is closely linked with the lineage of EBV-infected cells. More than half (53%) of EBV-HLH patients had EBV in the CD8$^+$ T-cell subset, in contrast to the low incidence of EBV infection in this subset in the other EBV$^+$ T/NK-LPD. Most HMB patients (89%) had EBV-infected NK cells, whereas the majority (75%) of HV patients had EBV-infected γδT cells. In a median follow-up period of 46 months, 47 patients (44%) died of severe organ complications and 13 (12%) developed overt lymphoma or leukemia. Age of onset ≥8 years and liver dysfunction were risk factors for mortality, and transplant patients had better prognosis. Patients with CD4$^+$ T-cell infection had shorter survival as compared with those with NK-cell infection. Because shorter time from onset to hematopoietic stem cell transplantation (HSCT) and inactive disease at HSCT were associated with longer survival, earlier HSCT in good condition was considered preferable. Among the 108 patients enrolled, four patients developed aggressive NK-cell leukemia (ANKL) and six patients developed extranodal NK/T-cell lymphoma (ENKL). It is thus conceivable that a certain fraction of patients with ANKL and ENKL developed these malignancies as a consequence of CAEBV.[32,33]

Characteristics of adult CAEBV

Chronic active EBV infection has been described mainly as a disease of childhood and young adulthood; the mean age of onset was estimated to be 11.3 years.[18] Recently, however, an increasing number of adult patients fulfilling the criteria of CAEBV has been reported. This may be a true increase in the incidence of adult-onset CAEBV or reflect improved recognition of this disease by physicians. Arai *et al.* reviewed 23 cases of adult-onset CAEBV and described the characteristics.[34] In 87% of adult cases, T cells were infected with EBV, whereas in childhood-onset cases, the T- and NK-cell types were equally frequent. Adult-onset cases appeared rapidly progressive and more aggressive, although the number of patients analyzed was limited. Further investigation with a larger number of patients is required to elucidate the characteristics of adulthood CAEBV and its relation to the childhood counterpart.

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