Accelerated production of multiple cytokines and chemokines by Epstein-Barr virus-infected natural killer cells and T cells

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[1]Department of Angiogenesis and Vascular Development, School of Medicine, and
[2]Department of Laboratory Sciences, School of Health Sciences, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University

Mika Inoue[1], Yoshihito Kasahara[1,2], Michiko Okajima[1], Taizo Wada[1], Tomoko Toma[1], Masaki Shimizu[1], Shoichi Koizumi[1], Akihiro Yachie[1]

Abstract: Ectopic Epstein-Barr virus (EBV) infection in natural killer (NK) cells and T cells is associated with hemophagocytic lymphohistiocytosis, chronic active EBV infection (CAEBV) with T/NK cell proliferation, and malignant lymphoma. We newly established seven EBV-infected NK cell lines and two T cell receptor (TCR) $\gamma\delta^+$ T cell lines from patients with CAEBV and NK cell leukemia. To identify the cytokines and chemokines produced by EBV^+ NK cells or T cells, 79 soluble factors were screened in the supernatant of theses cell lines by cytokine antibody array. EBV^+ NK cells and T cells and shortly cultured EBV^+ NK cells produced a significantly greater amount of four type 1 inflammatory cytokines, namely, interferon-γ, interleukin (IL)-6, oncostatin M, and IL-8, two type 2 cytokines, namely, IL-13 and granulocyte-macrophage colony-stimulating factor (GM-CSF), and three chemokines, namely, I-309/CC chemokine ligand (CCL)1, monocyte chemoattractant protein-1 (MCP-1)/CCL2, and regulated on activation, normal T cell expressed and secreted (RANTES)/CCL5, compared to normal activated EBV-negative NK cells or T cells and EBV-positive B cell lines. This accelerated production of multiple cytokines and chemokines in EBV-infected NK/T cells may be related to the pathogenesis of EBV-associated disease with proliferation of EBV-infected NK cells or T cells.

Key words Epstein-Barr virus, natural killer cells, TCR $\gamma\delta$ T cell, cytokines, chemokines

Introduction

Epstein-Barr virus (EBV) is a ubiquitous γ-herpes virus in humans. Primary EBV infection is usually asymptomatic in childhood and early adulthood, but sometimes results in symptomatic infection as infectious mononucleosis (IM). IM is the expansion and proliferation of cytotoxic T lymphocytes (CTLs) against EBV-infected B cells that shows a self-limited course[1]. EBV is usually transmitted orally and infects and replicates in oropharyngeal epithelial cells and B cells to produce life-long latent infection. However, ectopic EBV infection in natural killer (NK) cells or T cells has been found in diseases such as hemophagocytic lymphohistiocytosis (HLH)[2], chronic active EBV infection (CAEBV) with T or NK cell proliferation[3], and malignant T/NK lymphoma[1)4)]. The pathogenesis of CAEBV or T/NK cell proliferative diseases involves tissue infiltration of EBV-infected T/NK cells and tissue damage. HLH and CAEBV differ in prognosis from IM. Escape from EBV-specific immunity

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Corresponding author: M. Inoue, 13-1 Takaramachi, Kanazawa 920-8641, Ishikawa, Japan.
Tel.: +81-76-265-2314, Fax: +81-76-262-1866, E-mail: minishida04@yahoo.co.jp

Abbreviations: CAEBV, chronic active EBV infection; CTLs, cytotoxic T lymphocytes; EBER, EBV-encoded small non-polyadenylated RNA; EBV, Epstein-Barr virus; EBV pNK, EBV-positive activated natural killer; GM-CSF, granulocyte-macrophage colony-stimulating factor; HLH, hemophagocytic lymphohistiocytosis; IFN, interferon; IL, interleukin; IM, infectious mononucleosis; ISH, in situ hybridization; MCP-1, monocyte chemoattractant protein-1; MIP-1β, macrophage inflammatory protein 1β: NK, natural killer; OSM, oncostatin M; PHA, phytohemagglutinin; PMA, phorbol myristate acetate; RANTES, regulated on activation, normal T cell expressed and secreted; TCR, T cell receptor; TNF, tumor necrosis factor; TSA, trichostatin A

is one of the reasons for poor prognosis due to expansion and proliferation of EBV-infected T/NK cells. EBV-infected B cells show a type III latency pattern and express several EBV latent gene products, which are recognized by EBV-specific CTLs, such that EBV-infected B cells are eliminated by EBV-specific CTLs. On the other hand, EBV-infected T/NK cells show limited expression of EBV latent gene products, and therefore it is difficult for EBV-specific CTLs to recognize and eliminate EBV-infected T or NK cells[4)5)]. Replication gene products of EBV also are expressed in B cells in IM, which form the target for EBV-specific CTLs[4)]. Poor expression of EBV replication gene proteins such as BZLF1 was shown in EBV-positive malignant T/NK lymphoma[6)], which mediates the escape from EBV-specific immunity. However, the precise mechanism of EBV replication in EBV^+ NK/T cells from HLH or CAEBV is still unknown.

Cytokines or chemokines are important for the pathologenesis of EBV-associated diseases. Indeed, serum concentrations of interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-2, and IL-10 are significantly elevated in patients with HLH and CAEBV[4)7)8)]. Ectopic EBV infection induces the proliferation and activation of NK cells or T cells[4)9)] and may show a specific pattern of cytokine or chemokine production. HLH has been shown to be associated with a significant cytokine storm produced by activated EBV-infected $CD8^+$ T cells or NK cells, following cytopenia and organ dysfunction with endothelial damage[4)10)]. We investigated the pattern of cytokine and chemokine production in EBV^+ NK cells or T cells using EBV^+ NK cell lines and T cell lines, including newly established cell lines obtained from CAEBV and NK leukemia patients.

Materials and Methods

Cells

Peripheral blood mononuclear cells were isolated from one patient with NK cell leukemia and six patients with CAEBV. Informed consent was obtained from all of the patients or their parents. EBV^+ NK cell lines and EBV^+ T cell lines were established by culture in RPMI 1640 medium supplemented with 2ME (2-mercaptoethanol) 5×10^{-5} M, amphotericin B 1 μg/mL, 10% heat-inactivated fetal calf serum (Nichirei Biosciences Inc., Tokyo, Japan), 5% heat-inactivated pooled human serum, and 50 U/mL recombinant human IL-2 (Shionogi Co, Osaka, Japan). We obtained seven EBV^+ NK cell lines, designated KUP-TS, KUP-YM, KUP-TE, KUP-MK, KUP-KB, TTM, and TTL, and two TCR $\gamma\delta^+$ EBV^+ T cell lines, designated P2HL1 and P2HL2. P2HL1 and P2HL2 were obtained after stimulation of EBV^+ TCR $\gamma\delta$ T cells with pamidornate (Novartis Pharma, Tokyo, Japan). KUP-YM, P2HL1, and P2HL2 were infected with herpesvirus saimiri at four days after initial stimulation. KAI03, an already established EBV^+ NK cell line, was a kind gift from Dr. Tsuge[11)], and AIK-T4, AIK-T8, SKN, and SIS were EBV^+ T cell lines from Dr. Imai[6)]. EBV^+ activated NK (EBV pNK) cells were obtained from peripheral blood containing increased EBV-infected NK cells from six CAEBV patients by culture with IL-2 for 4-6 weeks, but these cells could not proliferate for more than 6-8 weeks. More than 98% of EBV pNK cells were CD56-positive and CD3-negative, and EBV infection in these cells was confirmed by EBV-encoded small non-polyadenylated RNA 1 (EBER1) in situ hybridization (ISH). IL-2-cultured normal NK cells were obtained from normal healthy volunteers with phytohemagglutinin (PHA) (Sigma, St. Louis, USA) and IL-2 activation of enriched NK cells, from which $CD3^+$T cells, $CD19^+$B cells, and $CD14^+$ monocytes were deleted by an immunobead method (Dynal Biotech ASA, Oslo, Norway). Contaminated T cells were deleted with CD3-immunobeads during culture, and more than 95% of normal NK cells cultured for 2-3 weeks were CD56-positive and CD3-negative. These cells were used as EBV-negative NK cells. Normal activated T cells were obtained with PHA and IL-2 activation from E-rosette positive cells, and more than 98% were CD3-positive. EBV^+ B cell lymphoblastoid cell lines were obtained from B cells isolated from normal volunteers by infection with the B95-8 strain of EBV[12)]. Daudi, Jijoye, and IM-9 cells were obtained from the ATCC (American Type Culture Collection) (Manassas, USA). These cells were cultured in RPMI 1640 supplemented with 2ME 5×10^{-5} M and 10% heat-inactivated fetal calf serum.

Cell morphology and cell surface antigen expression

Newly established cells were examined their morphology by May-Giemsa staining. Cell surface antigens were investigated by direct immunofluorescent staining with monoclonal antibodies. Stained cells were analyzed by flow cytometry (FACScan Calibur; Becton Dickinson Immunocytometry System, Mountain View, CA).

Detection of EBV infection by EBER1 ISH

EBV infection was determined by EBER1 ISH. The probe for EBER1 was obtained from Dako (Copenhagen, Denmark), and ISH for EBER1 was performed using the EBV-ISH kit from Dako, as reported previously[13)].

Analysis of the number of EBV-terminal repeats

To determine the clonality of EBV^+ cell clones, the number of EBV-terminal repeats was examined by Southern blot analysis, as reported previously[11)].

Detection of viral replication by BZLF1 expression

Induction of viral replication was estimated by EBV immediate-early protein, BZLF1, expression, which indicates the switch from latent infection to the replicative cycle[14)]. Cells were stimulated with 20 ng/mL phorbol myristate acetate (PMA) (Sigma), 1.0 mM *n*-butyric acid (Sigma), 10 ng/mL transforming growth factor (TGF)-β

(R&D systems, Minneapolis, USA), or 50 nM trichostatin A (TSA) (Sigma)[15)-18)] for 48 hours. Cytospined cells on slides were stained with anti-BZLF1 antibody (Dako) and alkaline phosphatase-anti-alkaline phosphatase (APAAP) (DAKO) method[19)]. The frequency of BZLF1-positive and -negative cells was counted in 3 different experiments.

Determination of cytokines and chemokines in supernatant

Cells at a concentration of 1×10^5 cells/mL were cultured in RPMI medium without human serum. After 48 hours, culture supernatants were collected and analyzed with the RayBio Human Cytokine Antibody Array V (RayBiotech Inc, Norcross, GA) according to the manufacturer's instructions. Signal intensities of each dot blot were analyzed with BioCapt MW software (Vilber Lourmat, Cedex, France), and the ratio of the density in each dot to the density in the positive control was quantified. Eleven cytokines and chemokines, namely, IFN-γ, IL-6, oncostatin M (OSM), IL-8, IL-13, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-10, I-309/CC chemokine ligand (CCL)1, monocyte chemoattractant protein-1 (MCP-1)/CCL2, macrophage inflammatory protein 1β (MIP-1β)/CCL4, and regulated on activation, normal T cell expressed and secreted (RANTES)/CCL5, which showed an increase in signal intensity, were selected, and concentrations in supernatants were measured by enzyme-linked immunosorbent assay (ELISA). ELISA kits for IFN-γ, OSM, IL-8, IL-13, GM-CSF, I-309/CCL1, MCP-1/CCL2, MIP-1β/CCL4, and RANTES/CCL5 were purchased from R&D systems, that for IL-6 from Beckman Coulter, and for IL-10 from eBioscience.

Statistical analysis

The above parameters were converted to logarithmic values and compared between EBV$^+$ NK cell lines or EBV$^+$ T cell lines and normal activated NK cells or T cells using the Student's t-test. $P < 0.05$ indicated statistical significance.

Results

Newly established cell lines

Seven EBV$^+$ NK cell lines, designated KUP-TS, KUP-YM, KUP-TE, KUP-MK, KUP-KB, TTM, and TTL, were newly established from six patients with EBV-infected NK cells, and 2 TCR $\gamma\delta^+$ EBV$^+$ T cell lines, P2HL1 and P2HL2, were established from one patient. The clinical features of these patients are described in Table 1. These EBV$^+$ NK cell lines and EBV$^+$ T cell lines showed constant growth with a doubling time of 24-36 hours. Morphologically, EBV$^+$ NK cell lines and EBV$^+$ T cell lines showed typical lymphoblasts (Fig. 1). EBV genome carriage was confirmed by EBER1 RNA expression (Fig. 2). The clonality of EBV was confirmed by southern blot analysis of the terminal repeat (Fig. 3). All cell lines have been growing stably for more than 24 months in the presence of IL-2, and IL-2 deprivation from the culture medium stopped cell proliferation and induced apoptosis.

Phenotypic analysis showed that all EBV$^+$ NK cell lines and EBV$^+$ T cell lines were positive for CD2, CD25, CD45, CD30, HLA-DR, and CD95 antigens (Fig. 4). CD38 and CD44 expression was positive in more than half of the NK cell lines, and CD16 expression was negative in KUP-TS, KUP-YM, TTM, and TTL, and CD56 expression was negative in TTM and TTL. TTM and TTL were

Table 1. Clinical features of patients from whom Epstein-Barr virus (EBV)-positive cell lines were obtained.

Cell line	KUP-TS	KUP-YM	KUP-TE	KUP-MK	KUP-KB	TTM TTL	P2HL1 P2HL2
Age(years)/sex	18/M	13/F	17/F	12/F	9/M	7/M	4/F
Clinical diagnosis	Leukemia	CAEBV	CAEBV	CAEBV	CAEBV	CAEBV	CAEBV
Hypersensitivity to mosquito bites	−	−	+	+	+	+	−
Hydroa vacciniforme	−	−	−	−	−	−	+
EBV-related antibody							
VCA-IgG	160	2560	>10240	640	160	40	320
VCA-IgA	ND	80	160	ND	ND	ND	ND
EA-DR-IgG	40	160	5120	10	ND	<10	<10
EBNA	10	640	160	40	<40	80	40
EBV TR clonality	Mono	Mono	Mono	Mono	Mono	Mono	Mono
EBV-infected cell	NK	NK	NK	NK	NK	NK	TCR$\gamma\delta$Vδ2
CD16$^+$ in PBMNC (%)	5.3	55.1	77.5	48.6	71.2	68.5	
CD56$^+$ in PBMNC (%)	62.2	46.8	66.8	ND	72.3	51.7	

Serum EBV-related antibodies were assayed by indirect immunofluorescence assay. EBV clonality was assayed by southern blot analysis of the EBV terminal repeat. Cell CD16 and CD56 antigen expression in PBMNC was investigated by flow cytometric analysis. CAEBV, chronic active EBV infection; ND, not done; PBMNC, peripheral blood mononuclear cells.

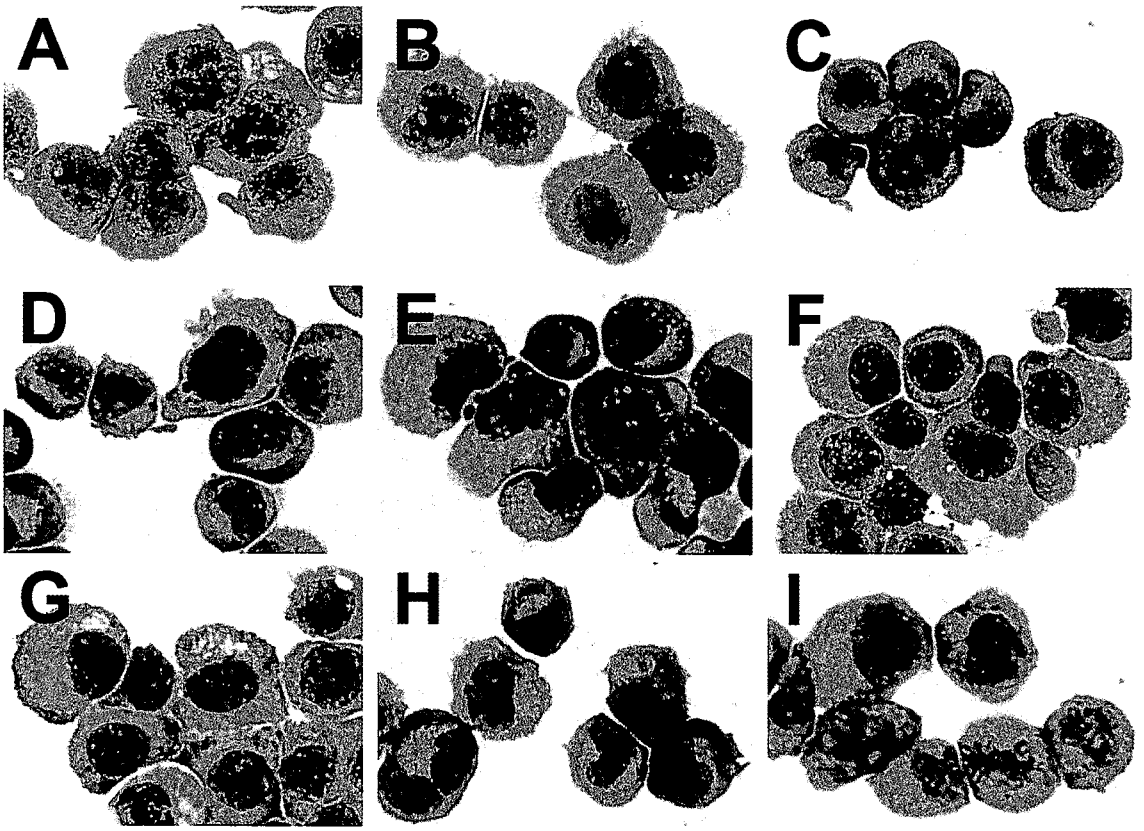

Fig. 1. Morphology of cell lines. The morphology of nine newly established Epstein-Barr virus (EBV)-positive cell lines was shown by May-Giemsa staining. A: KUP-TS; B: KUP-YM; C: KUP-TE; D: KUP-MK; E: KUP-KB; F: TTM; G: TTL; H: P2HL1; I: P2HL2.

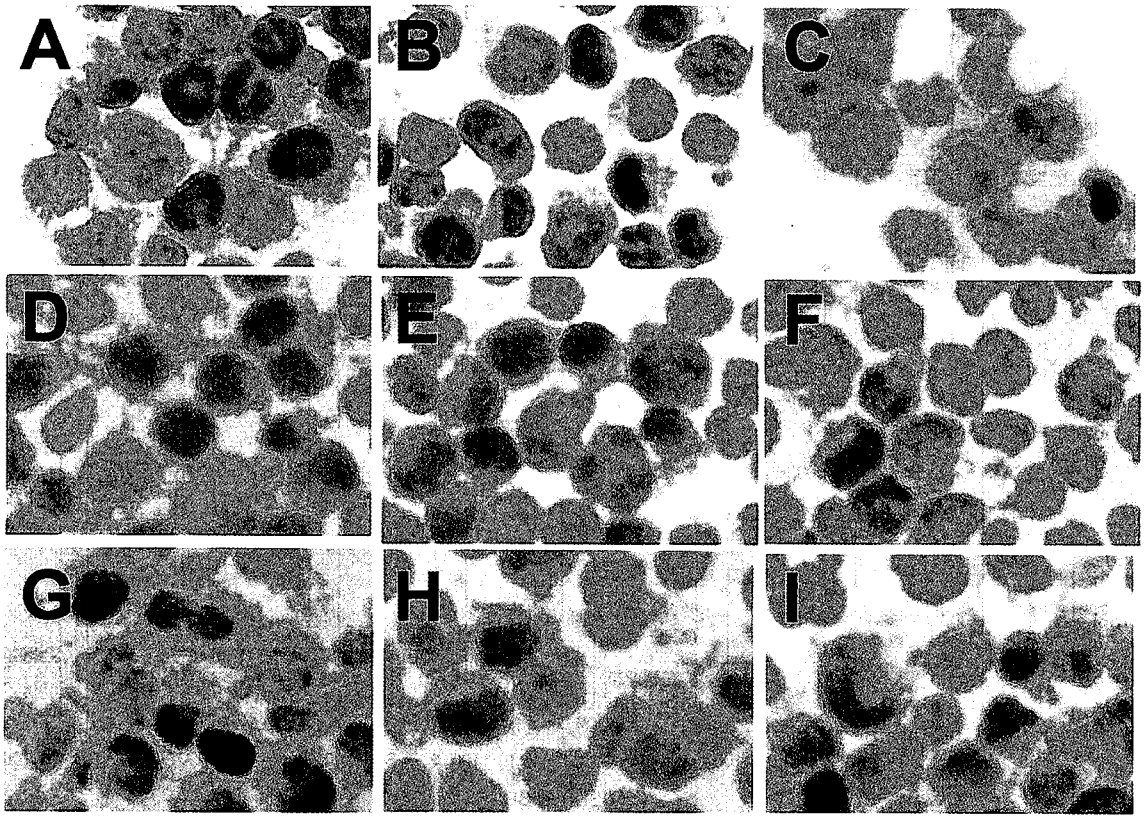

Fig. 2. In situ hybridization with the EBER1 probe. A: KUP-TS; B: KUP-YM; C: KUP-TE; D: KUP-MK; E: KUP-KB; F: TTM; G: TTL; H: P2HL1; I: P2HL2.

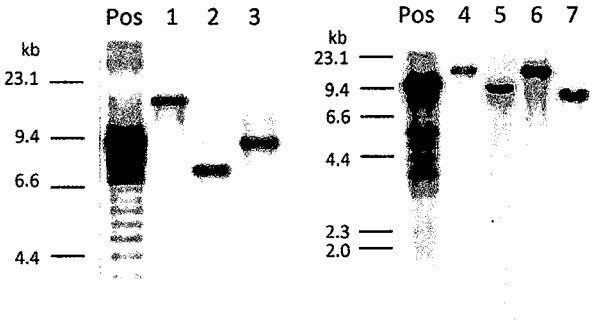

Fig. 3. Southern blot analysis of terminal fragments of the EBV genome. Positive control (Pos) was obtained from B95-8 cells. lane 1: KUP-TS; lane 2: KUP-YM; lane 3: KUP-TE; lane 4: KUP-MK; lane 5: KUP-KB; lane 6: TTL; lane 7: P2HL1.

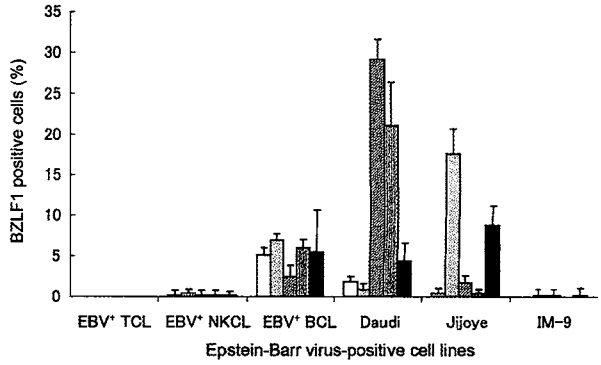

Fig. 5. Induction of EBV replication in EBV+ cells. Each cell line was stimulated with phorbol myristate acetate (PMA; 20 ng/mL), *n*-butyrate (1.0 mM), transforming growth factor (TGF)- β (10 ng/mL), or trichostatin A (TSA; 50 nM) for 48 hours, and BZLF1 protein expression was analyzed by immunostaining with monoclonal antibodies against BZLF1. BZLF1-positive cells were counted for 3 different experiments. Each bar indicates the mean (SD). □: no stimulation; ▨: PMA; ▨: *n*-butyrate; ▥: TGF-β; ■: TSA. TCL: T cell line; NKCL: NK cell line; BCL: B cell lymphoblastoid cell line.

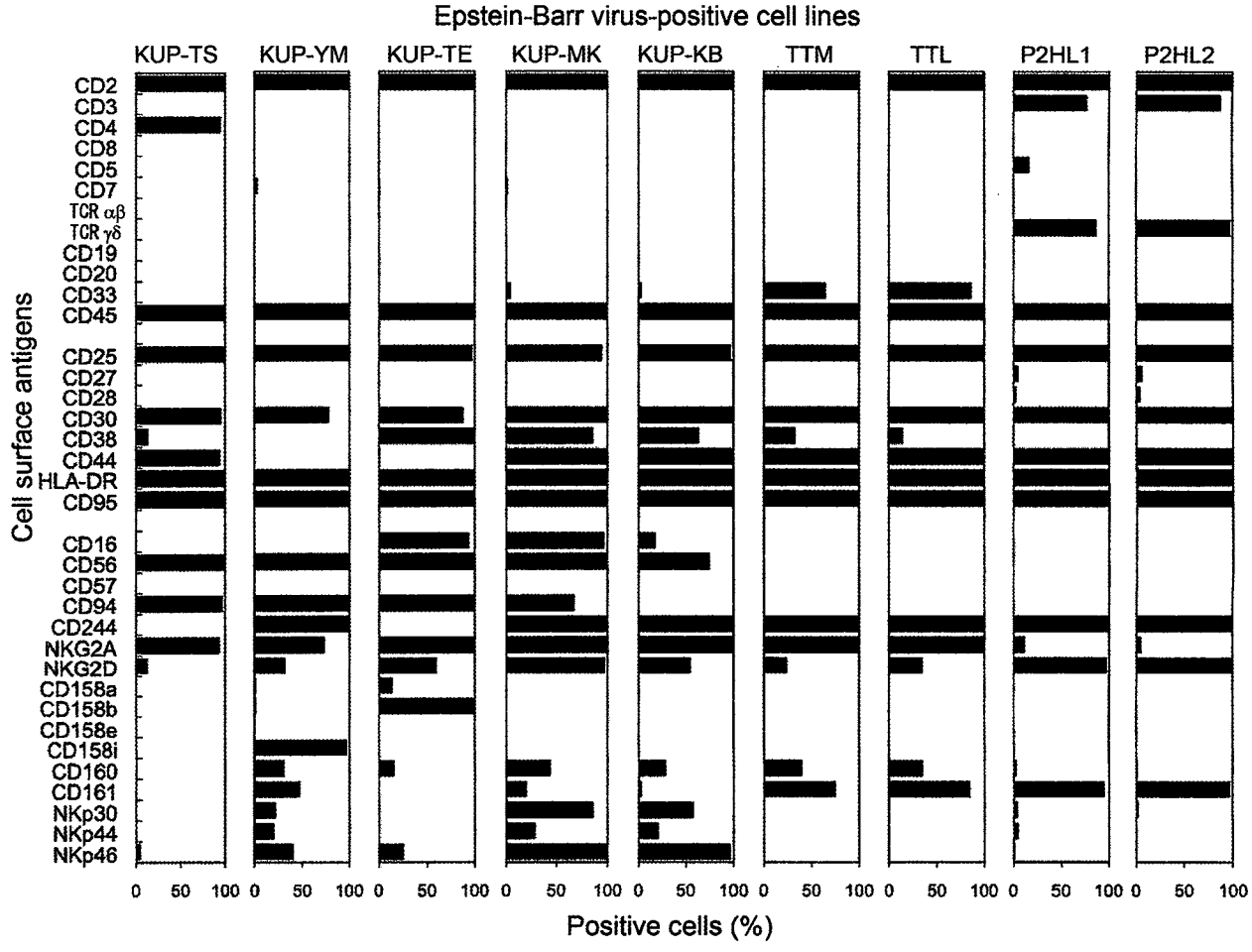

Fig. 4. Cell surface antigens of seven natural killer (NK) cell lines and two T cell lines. Expression of each antigen was investigated by flow-cytometric analysis. The width of each bar exhibits the percentage of positive cells.

CD33-positive. All NK cell lines were positive for NKG2A, but T cell lines were negative for NKG2A and positive for NKG2D. KUP-YM was CD158i-positive, KUP-TE was CD158b1-positive, and KUP-TS was CD4-positive.

EBV$^+$ NK cell lines and T cell lines showed no induction of BZLF1 protein expression

To examine the capacity for replication in EBV$^+$ NK cell lines and T cell lines, we analyzed BZLF1 protein expression by immunohistochemistry. Although the level of BZLF1 expression induced by different stimuli, namely, PMA, *n*-butyric acid, TGF-β, and TSA, differed between Daudi, Jijoye, IM-9, and EBV$^+$ B cell lymphoblastoid cell

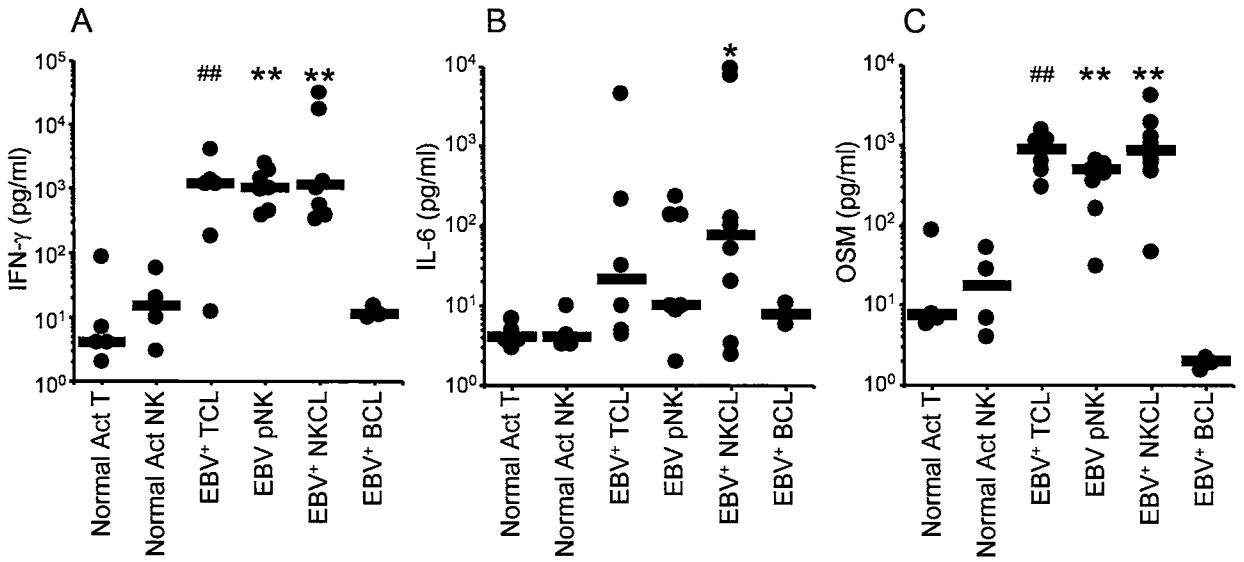

Fig. 6. Cytokine production in EBV-infected cells. EBV-infected and -non-infected cells were cultured for 48 hours, and the protein concentration of interferon (IFN)-γ, (A), interleukin (IL)-6 (B), and oncostatin M (OSM) (C) in the supernatant was measured by enzyme-linked immunosorbent assay (ELISA). Each dot represents the mean value of 3 independent experiments. The differences in cytokine concentrations converted to logarithmic values were analyzed by Student's *t*-test. # $P < 0.05$ and ## $P < 0.01$ compared with normal T cells. * $P < 0.05$ and ** $P < 0.01$ compared with normal NK cells. TCL: T cell line; NKCL: NK cell line; BCL: B cell lymphoblastoid cell line.

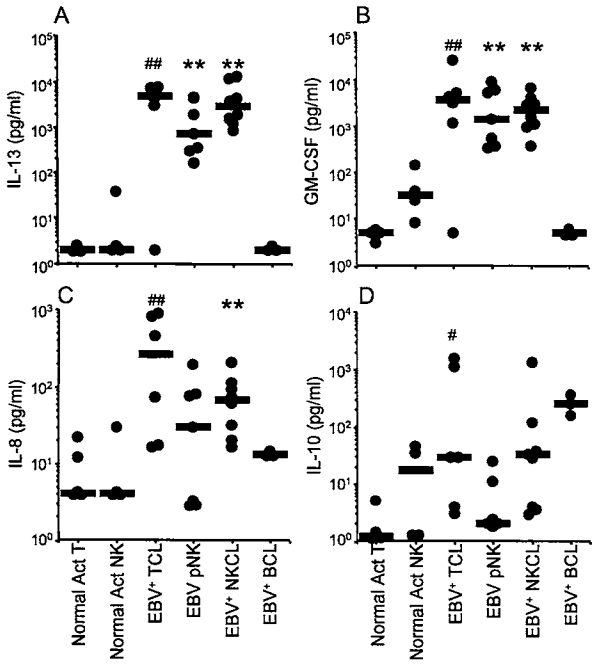

Fig. 7. Cytokine production in EBV-infected cells. IL-13 (A), granulocyte-macrophage colony-stimulating factor (GM-CSF) (B), IL-8 (C), and IL-10 (D) concentrations were analyzed by ELISA.

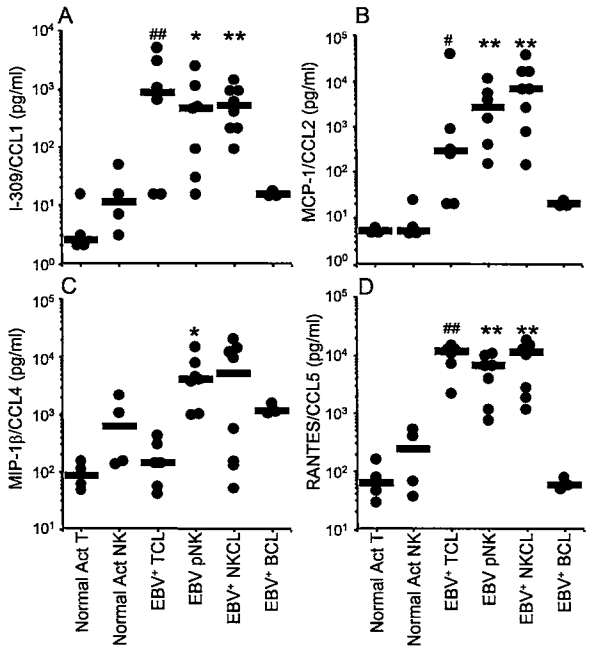

Fig. 8. Chemokine production in EBV-infected cells. CCL1 (A), CCL2 (B), CCL4 (C), and CCL5 (D) concentrations were analyzed by ELISA.

lines cells, apparent expression was induced in Daudi, Jijoye, and EBV$^+$ B cell lymphoblastoid cell lines. On the other hand, no BZLF1 expression was seen in EBV$^+$ NK cell lines and EBV$^+$ T cell lines with any stimuli (Fig. 5).

Cytokine and chemokine production by EBV$^+$ NK cell lines and EBV$^+$ T cell lines

To examine differences in cytokine and chemokine production by EBV$^+$ NK cell lines and EBV$^+$ T cell lines, we used a cytokine antibody array system. Among the 79 cytokines and chemokines, a significant increase in signal intensity was found for 11 cytokines or chemokines in the supernatant of EBV$^+$ NK cell lines and EBV$^+$ T cell lines compared to that of EBV$^+$ B cell lymphoblastoid cell lines. The production of cytokines and chemokines such as IFN-γ, IL-6, OSM, IL-8, IL-13, GM-CSF, IL-10, I-309/CCL1, MCP-1/CCL2, MIP-1β/CCL4, and RANTES/CCL5 was quantified in EBV-positive cells and EBV-negative normal cells by ELISA.

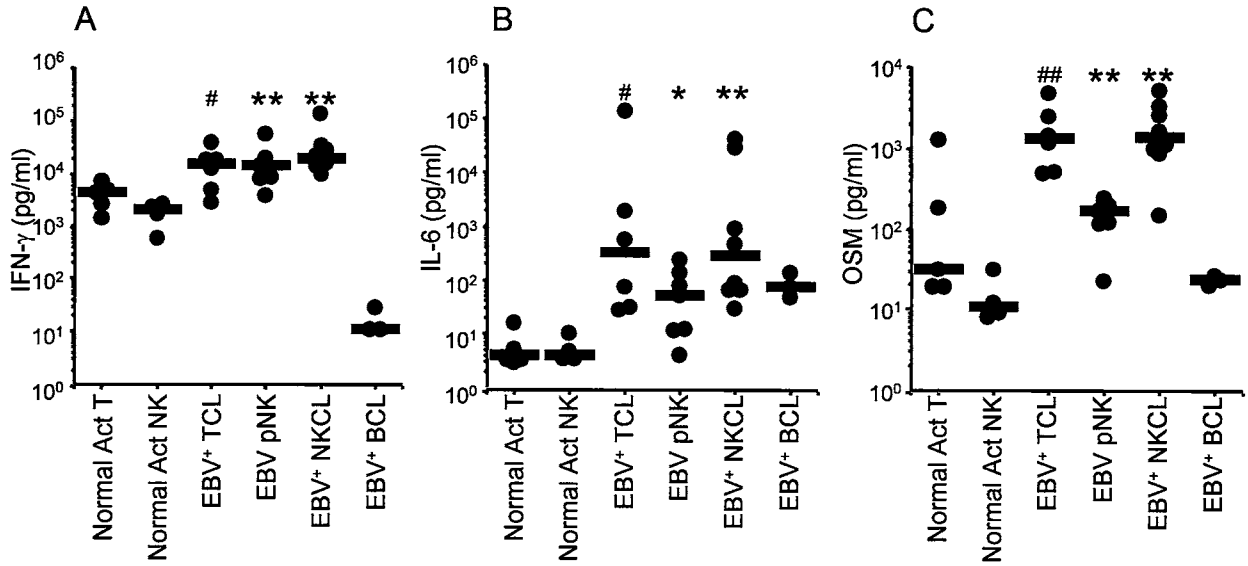

Fig. 9. Cytokine production in EBV-infected cells stimulated in the presence of PMA and Ca ionophore at final concentrations of 10 ng/mL and 500 ng/mL, respectively. IFN-γ (A), IL-6 (B), and OSM (C) concentrations were analyzed by ELISA.

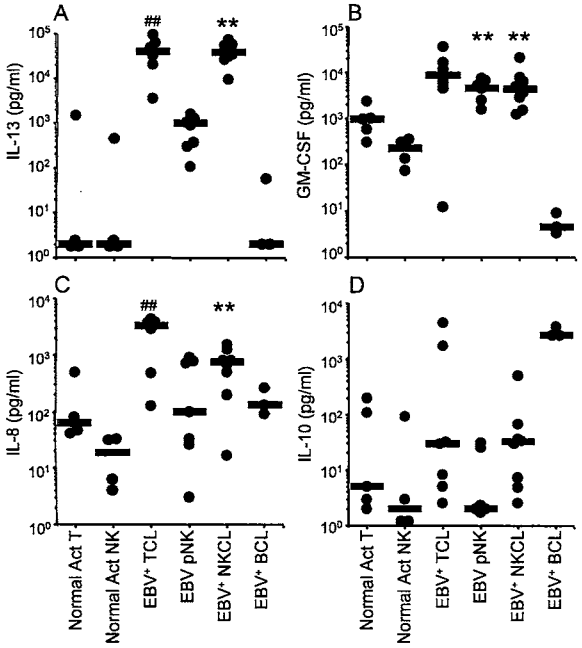

Fig. 10. Cytokine production in EBV-infected cells stimulated in the presence of PMA and Ca ionophore. IL-13 (A), GM-CSF (B), IL-8 (C), and IL-10 (D) concentrations were analyzed by ELISA.

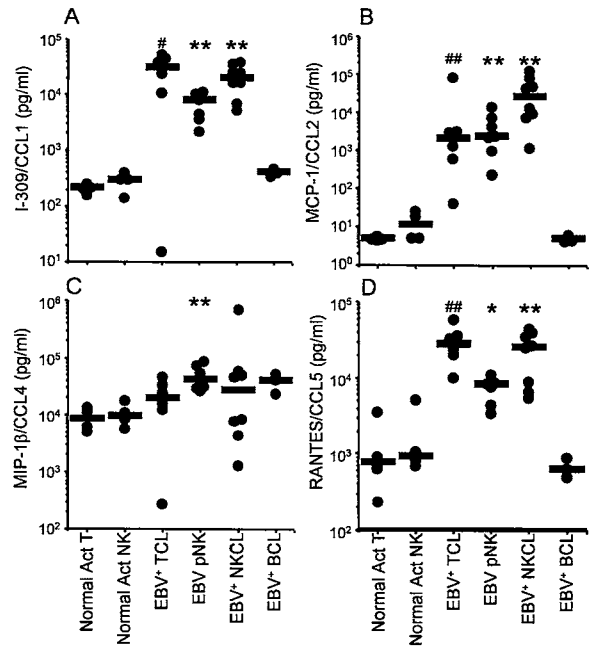

Fig. 11. Chemokine production in EBV-infected cells stimulated in the presence of PMA and Ca ionophore. CCL1 (A), CCL2 (B), CCL4 (C), and CCL5 (D) concentrations were analyzed by ELISA.

The production of IFN-γ and OSM was significantly higher in EBV$^+$ NK/T cells than in normal activated NK/T cells or EBV$^+$ B cell lymphoblastoid cell lines (Fig. 6). Two type 2 cytokines, IL-13 and GM-CSF, also were significantly increased in EBV$^+$ NK/T cells, except one EBV$^+$ T cell lines (Fig. 7). The production of these cytokines did not differ significantly between EBV$^+$ T cell lines and EBV$^+$ NK cell lines or EBV pNK cells. IL-6 and IL-8 levels in EBV$^+$ NK/T cells were higher than those in the other cells, and these cytokines showed a wide distribution among different cell lines (Fig. 6, 7). IL-10 production was high in EBV$^+$ B cell lymphoblastoid cell lines, but some EBV$^+$ NK/T cells also produced large quantities of IL-10, whereas others did not. Patterns for the production of I-309/CCL1, MCP-1/CCL2, and RANTES/CCL5 were the same as those for IFN-γ, OSM, IL-13, and GM-CSF (Fig. 8). MIP-1β/CCL4 levels tended to be high in EBV$^+$ NK cell lines and EBV$^+$ T cell lines. A difference in the production of the 11 cytokines and chemokines between EBV-infected cells and non-infected cells was also found after PMA and Ca ionophore stimulation, especially for IL-13 and CCL1, CCL2, and CCL5 (Fig. 9- Fig. 11).

Discussion

We established seven EBV$^+$ NK cell lines and two TCR $\gamma\delta^+$ EBV$^+$ T cell lines from the peripheral blood of patients with CAEBV and NK cell leukemia. All cell lines required IL-2 supplementation in the culture medium for constant growth, as described for previously established NK cell lines and T cell lines[6)11)20)21)]. We attempted to establish NK cell lines or T cell lines from many samples of CAEBV patients, but more than half of these cells could not grow for more than 2 months, such as EBV pNK cells, although all EBV$^+$ NK cells showed strong proliferation with IL-2 stimulation at first. It has previously been shown that IL-9 contributes to the growth of EBV$^+$ T/NK cells in an autocrine manner[22)23)]. Except for IL-10 production, there were no clear differences in cytokine and chemokine production between EBV$^+$ NK cell lines and EBV pNK cells in this study, suggesting that these other cytokines do not contribute to cell growth. In a preliminary experiment in which we blocked IL-13, GM-CSF, and OSM activity by the addition of antibodies in culture, we found no clear inhibition of cell growth. Although we could not determine the cytokine profiles and clinical features related to the constant proliferation of EBV$^+$ cells, some other factors such as gene arrangement may be responsible for this constant proliferation.

P2HL1 and P2HL2 were the first EBV$^+$ TCR $\gamma\delta^+$ T cell lines obtained from a CAEBV patient with hydroa vacciniforme with EBV$^+$ TCR $\gamma\delta^+$ proliferation using herpesvirus saimiri, although EBV$^+$ TCR $\gamma\delta^+$ T cell lines obtained from malignant lymphoma tissues have been reported[24)]. This finding is of particular importance, as the number of EBV$^+$ T cell lines is limited compared to EBV$^+$ NK cell lines. The combination of pamidornate stimulation and herpesvirus saimiri infection that we used in this study may be useful for the establishment of EBV$^+$ TCR $\gamma\delta$ T cell lines.

We used a cytokine antibody array system to identify cytokines and chemokines that were significantly increased in EBV$^+$ NK/T cells. We found that the production of 11 cytokines and chemokines was increased in EBV$^+$ NK cell lines and EBV$^+$ T cell lines in comparison with EBV$^+$ B cell lymphoblastoid cell lines. Quantification by ELISA showed a significant increase in four cytokines, namely, IFN-γ, OSM, IL-13, and GM-CSF, and three chemokines, namely, CCL1, CCL2, and CCL5. Although it was previously reported that EBV-infected NK cells or T cells produced a significant amount of IFN-γ or TNF-α [20)25)], this is the first report showing that EBV$^+$ NK cell lines and T cell lines markedly increased the production of multiple cytokines and chemokines at a cellular level. In EBV-associated diseases, serum concentrations of cytokines or chemokines and the transcription of cytokine genes have been analyzed[26)27)]. In CAEBV patients, an increase in serum concentration and gene transcription of IFN-γ, IL-2, IL-10, IL-13, TNF-α, and TGF-β has been reported[3)26)-28)]. However, these analyses could not show whether EBV-infected T/NK cells or secondary reacting inflammatory cells produce these cytokines and chemokines. As we used cell lines in our study, our results directly show that EBV-infected cells produce these cytokines and chemokines.

Normal NK cells are differentiated into type 1 NK cells and type 2 NK cells under culture conditions with type 1 and type 2 cytokines, respectively, as has been shown for T cells[29)]. Although the cell lines used in the present study were cultured only with IL-2, these cells produced both type 1 and type 2 cytokines in large quantities. This finding indicates that these cell lines are type 0 cells with an inflammatory feature that may be induced by ectopic EBV infection.

OSM is an IL-6 family cytokine produced mainly by activated T cells and macrophages in vivo. OSM induces growth inhibition, differentiation, and apoptosis in tumor cells, as well as the production of growth-related oncogene-α and -β and eotaxin in endothelial cells, and the expression of adhesion molecules such as intracellular adhesion molecule-1. OSM may contribute to local inflammation in EBV-associated disease such as CAEBV through induction of chemokine production and augmentation of adhesion molecule expression.

A recent report[30)] showed that EBV infection in normal B cells induced IL-13 production, mediated by expression of the EBV immediate-early protein, namely BZLF1, and that IL-13 promotes proliferation of EBV-infected B cells

and EBV+ B cell lymphoblastoid cell lines. Replication in EBV+ NK/T cells was not induced by any stimuli, although the production of IL-13 was markedly increased. These results suggest that IL-13 production by EBV+ NK/T cells was independent of EBV replication mechanisms. Further study is needed to identify the mechanisms of IL-13 production by EBV infection, especially the influence of the EBV gene. Our findings suggest that an increase in IL-13 and GM-CSF, both of which are type 2 cytokines, is involved in allergic disorders, particularly hypersensitivity to mosquito bites in CAEBV patients with NK cell-type infection. Indeed, high concentrations of IL-13 in CAEBV patients with NK cell-type infection have been reported, which may contribute to high serum immunoglobulin E levels and the hypersensitivity to mosquito bites[27].

Receptors for the chemokines that were elevated in EBV+ NK/T cells (CCL1, CCL2, and CCL5), including CCR1, CCR2, CCR3, CCR5, and CCR8 are mainly expressed on immature dendritic cells and inflammatory monocytes[31][32]. It has been suggested that these receptors are associated with the pathogenesis of HLH that sometimes develops during the clinical course of EBV infection. Chemokine overproduction by activated EBV+ NK/T cells may activate immature dendritic cells and inflammatory monocytes, resulting in the cytokine storm. Indeed, the serum concentration of CCL2 and CCL4 has been shown to be elevated in HLH[33]. The expression of CXCL10 and CXCL9 has also been reported to be elevated in lymph node tissue in patients with EBV-associated NK cell lymphoma[28], although no increase in CXCL10 and CXCL9 production was detected in our study. These results indicate that these chemokine proteins are produced by monocytes/macrophages activated by EBV-infected NK/T cells through IFN-γ and IL-6, as well as CCL1, CCL2, and CCL5.

In vivo, the relationship between EBV+ NK/T cells and other immune cells such as monocytes, dendritic cells, B cells, and non-immune cells is complex, and cytokines and chemokines play an important role in the clinical symptoms of EBV-related disease. Treatment of EBV-related HLH at an early stage with suppression of the cytokine storm improves clinical prognosis. As there is a possibility for treatment of these diseases by reducing cytokine and chemokine activity, thereby blocking the activation of inflammatory monocytes and macrophages, further study will be required to examine the role of cytokines and chemokines in the relationship between EBV+ NK/T cells and the innate immune system.

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