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## Virological aspects of Epstein-Barr virus infections.

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## Abstract

Epstein-Barr virus (EBV) is usually maintained in an asymptomatic and latent form by the host immune system, and primarily by EBV-specific cytotoxic T cells (CTLs). However, EBV has been linked to several refractory diseases such as EBV-associated hemophagocytic syndrome (EBV-AHS) and chronic active EBV infection (CAEBV). In these ectopic diseases, EBV infects T/NK cells, causing severe immunodeficiency with a very high EBV load. In recent years, the laboratory procedure to assess these types of EBV infections has been improved. In particular, real-time polymerase chain reaction (PCR) has been used to quantify the EBV load, and the MHC: peptide tetramer assay has been used to quantitate EBV-specific CTLs; these tests have been employed for the management of the illnesses associated with EBV infection. Here, we have reviewed the recent progress in the clinical application of these assays. The pathogenesis of EBV-infected T/NK cells, and the host immune response to infection, including the roles carried out by innate immunity and inflammatory cytokines, are likely to be revealed in the future.

**KEYWORDS:** chronic active Epstein-Barr virus infection, Epstein-Barr virus-associated hemophagocytic syndrome, Real-time PCR, tetramer

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Review

## Virological Aspects of Epstein-Barr Virus Infections

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Epstein-Barr virus (EBV) is usually maintained in an asymptomatic and latent form by the host immune system, and primarily by EBV-specific cytotoxic T cells (CTLs). However, EBV has been linked to several refractory diseases such as EBV-associated hemophagocytic syndrome (EBV-AHS) and chronic active EBV infection (CAEBV). In these ectopic diseases, EBV infects T/NK cells, causing severe immunodeficiency with a very high EBV load. In recent years, the laboratory procedure to assess these types of EBV infections has been improved. In particular, real-time polymerase chain reaction (PCR) has been used to quantify the EBV load, and the MHC: peptide tetramer assay has been used to quantitate EBV-specific CTLs; these tests have been employed for the management of the illnesses associated with EBV infection. Here, we have reviewed the recent progress in the clinical application of these assays. The pathogenesis of EBV-infected T/NK cells, and the host immune response to infection, including the roles carried out by innate immunity and inflammatory cytokines, are likely to be revealed in the future.

**Key words:** chronic active Epstein-Barr virus infection, Epstein-Barr virus-associated hemophagocytic syndrome, Real-time PCR, tetramer

**E**pstein-Barr virus (EBV) is a ubiquitous herpes virus, but it can cause refractory diseases that can lead to the need for curative bone marrow transplantation. The prognosis of patients with such refractory EBV infection depends primarily on the EBV-infected cell type and the state of the host immunity.

During primary infection, EBV typically infects and replicates in B cells. After primary infection, EBV-specific T cells (cytotoxic T cells, or CTLs) are usually acquired, which regulate EBV-infected B cells at a rate of 1 to 10<sup>5</sup> B cells as memory cells. Thus, EBV infection is usually asymptomatic, or else is only occasionally

symptomatic as infectious mononucleosis (IM).

However, in a limited number of individuals, EBV infects T/ Natural killer (T/ NK) cells, which can induce EBV-persistent infection. For example, in patients with chronic active EBV infection (CAEBV), EBV infects primarily CD4<sup>+</sup> T cells or NK cells [1]. These patients fail to acquire a sufficient number of both EBV-specific and cytomegalovirus (CMV)-specific CTLs [2], which is suggestive of T/ NK cell dysfunction in the host immune system. This condition leads to a high EBV load and EBV-associated T/NK-cell lymphoproliferative disease. Patients with EBV-associated hemophagocytic syndrome (EBV-AHS) also have EBV-infected T cells primarily CD8<sup>+</sup> cells. EBV-AHS is characterized by the overproduction of inflammatory cytokines such as tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and interferon-gamma (IFN- $\gamma$ ), which

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play a central role in the rapid progress of the disease. EBV-AHS also carries a high mortality rate without immunosuppressive therapy using cyclosporine A and VP-16.

With the available technology, we analyzed cases of complicated EBV infection by monitoring EBV nucleic acid, EBV-specific CTLs, and virus-associated cytokines. We previously reported a high EBV load in patients with CAEBV [3] and EBV-AHS [4]. Until the present time, monitoring the EBV load has generally been employed for the management of cases involving complicated EBV infection. As regards EBV-specific CTLs, a major histocompatibility complex (MHC): tetramer assay has been developed as a revolutionary technology that renders it easy to quantitate virus-specific CTLs [5].

Childhood is the period during which humans form adaptive immunity to various viral infections. Thus, complicated EBV infection may be especially apparent in childhood. This review focuses on the clinical application of these assays mentioned above and on the host immune response to symptomatic EBV infection in childhood.

### Quantification of EBV DNA using real-time quantitative PCR

Due to the patient's insufficient serologic response to the EBV antigen, EBV antibodies are not useful in immunocompromised individuals such as AIDS patients or bone marrow transplant recipients. Thus, the detection and quantification of EBV nucleic acid is very important for assessing the EBV load in these patients. There are 3 methods available for analyzing EBV nucleic acid: Southern blotting to examine the monoclonality of EBV-infected cells, *in situ* hybridization of EBER to identify the EBV-infected cell type, and real-time polymerase chain reaction (PCR) to monitor the EBV load. Real-time PCR of EBV-DNA is especially useful because it can be repeatedly performed after diagnosis to determine the efficacy of therapy. This procedure has several advantages due to its rapidity and simplicity. Recently, real-time PCR assays using EBV-mRNA have been used in attempts at earlier detection and in the monitoring of reactivation [6], but the assessment of the actual need for real-time RT-PCR is an issue that will need to be explored in the future.

Our real-time PCR assay method has been described previously [3]. Briefly, peripheral blood mononuclear

cells (PBMNCs) were separated with Ficoll-Paque from EDTA-treated blood. To monitor the load of EBV-DNA, clinicians generally use whole blood [7-9] or PBMNCs [3, 9, 10], because EBV in peripheral blood is highly associated with leukocytes. However, cell-free EBV-DNA in serum [11-13] or plasma [14] has sometimes been examined, because it reflects the EBV load in terms of viral replication. We used a spin-column procedure to extract the DNA from PBMNCs (QIAamp Blood Kit; QIAGEN). We then chose  $10^{2.5}$  copies/ $\mu$ g DNA as a suitable cut-off level for distinguishing EBV-associated diseases from latent EBV infections or asymptomatic reactivation of the virus. Using a set volume of the DNA extraction solution from a fixed number of PBMNCs is simpler, but may produce a bias caused by differences in the extraction efficiency for each sample. If  $10^5$  lymphocytes produce  $0.5 \mu$ g DNA, as suggested in the manufacturer's handbook, then 500 copies/ $10^5$  PBMNCs should produce  $10^{3.0}$  copies/ $\mu$ g DNA, which is slightly greater than our criterion of  $10^{2.5}$  copies/ $\mu$ g DNA.

The differences between positive controls among laboratories is a shortcoming of the real-time PCR assay, as, for example, the control dilution error and the target region may vary from lab to lab. Standardization of a positive control is thus essential for improving the usefulness of real-time PCR. Our target region for the real-time PCR analysis is the BALF5 gene, which encodes the viral DNA polymerase. There remain some questions regarding the target region of EBV-DNA. Internal repeats and Bam HI fragments are not equivalent to variations of EBV. Thus, DNA polymerase regions such as BALF5 and EBNA are thought to be optimal target regions for PCR analysis. In addition, different levels of expression of EBV-mRNA between latency and replication should be considered via real-time PCR [6].

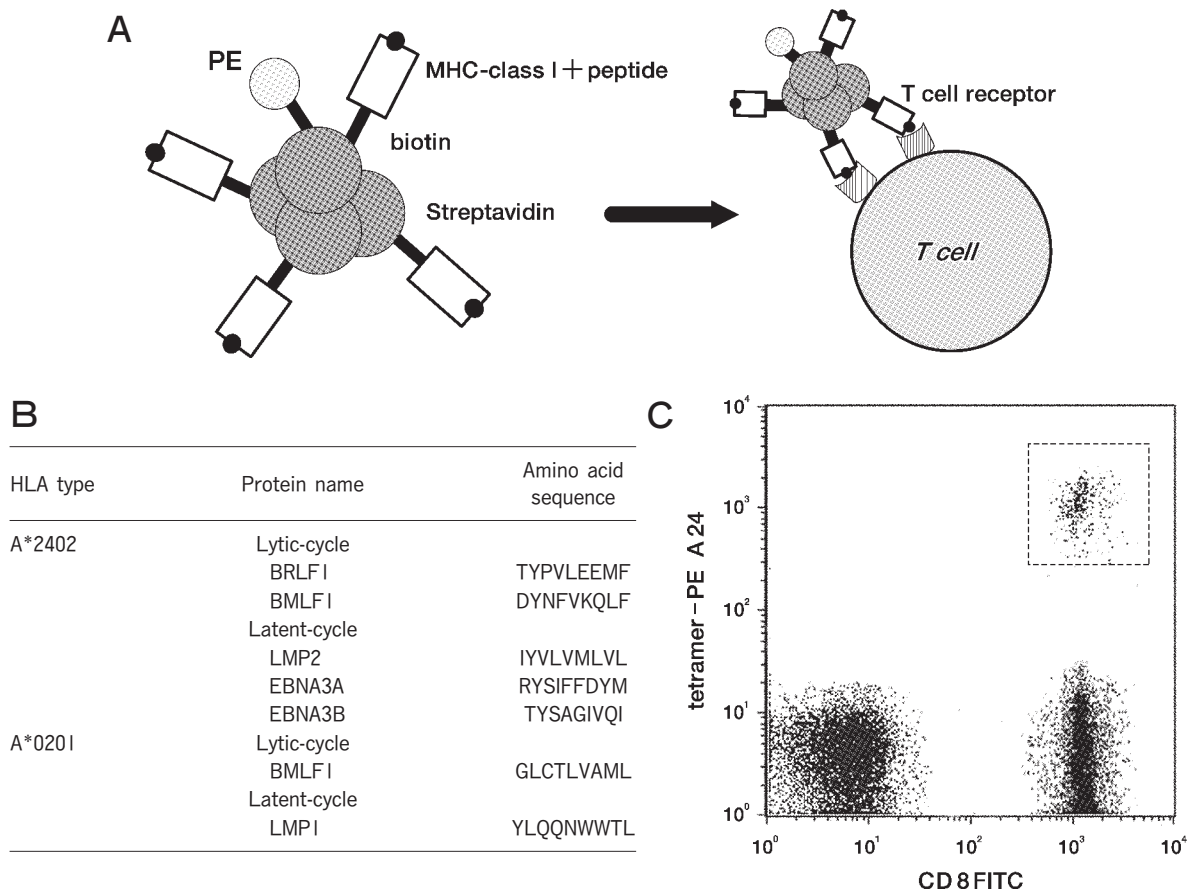
### Quantification of EBV-specific cytotoxic T cells

Host cellular immunity, especially via virus-specific CTLs, is closely associated with EBV load. To identify and quantify EBV-specific CTLs, we have used a major histocompatibility complex (MHC): peptide tetramer assay, an IFN- $\gamma$  enzyme-linked immunospot (ELISPOT) assay, and intracellular cytokine staining. The antigen-specific T-cell receptor recognizes a complex of an antigenic peptide and a molecule of self-MHC, and

the affinity of interaction between the T cell and the MHC: peptide is very low. Thus, before it was possible to use the MHC: peptide tetramer (*i.e.*, the 4 specific MHC: peptide complexes bound to a single molecule of streptavidin labeled with a fluorochrome), it was not possible to directly count the number of antigen-specific T cells (Fig. 1A) [5]. Kuzushima *et al.* reported 3 EBV epitopes that were recognized by human leukocyte antigen (HLA)-A\* 2402-restricted EBV-specific CTLs [15]. Based on their data, MHC: EBV-peptide tetramers of HLA-A\*0201 and 2402 are commercially available in Japan (Medical and Biological Laboratories Co., Ltd., Nagoya, Japan) (Fig. 1B). HLA-A\*0201 is observed in approximately 10–20% of Japanese, and HLA-A\*2402 is seen in approximately 37% of Japanese [16, 17]. These mounting EBV-peptide tetramers are gene prod-

ucts during latency (LMP, EBNA) or replication (BRLF1, BMLF1). With an MHC: peptide tetramer assay, virus-specific CTLs can be measured easily and within a few hours using a flowcytometer (Fig. 1C). One shortcoming of the tetramer assay is that a patient's HLA haplotype is restricted. However, due to the convenience of this approach, the MHC: peptide tetramer assay is widely used when treating patients with other viral infections, such as HIV, CMV, and HBV.

Two other methods can be used without restriction regarding the patient's HLA haplotype. The ELISPOT assay is a variant of the ELISA assay, in which the number of cytokine-induced antigen-specific T cells is counted on a plastic surface. Intracellular cytokine staining using a flowcytometer can be used to detect cytokine production at the single-cell level. The producing cytokine



**Fig. 1** MHC: peptide tetramer assay. **A**, Structure of MHC class I: peptide tetramer. Recombinant MHC class I molecules with an antigen-specific peptide are bound to each subunit of streptavidin and are labeled with phycoerythrin (PE), which binds stably to T cells. **B**, EBV-specific MHC tetramers that are commercially available in Japan; **C**, Staining of cells with the HLA A\*2402-restricted EBV tetramers. The tetramer-positive CD8<sup>+</sup> T cells were detected by flow cytometry.

accumulates in the endoplasmic reticulum by a metabolic inhibitor, and cytokine-specific antibodies penetrate the cell to bind to the intracellular cytokine. Intracellular cytokine staining could be applied to so-called double-positive T cells, such as CD25<sup>+</sup>CD4 T cells.

### Clinical application for EBV-associated diseases

**Infectious mononucleosis (IM).** The EBV-infected cell type is B cells in cases of IM, and the primary EBV infection in childhood is usually asymptomatic, although some children and younger adults manifest IM, which occasionally causes serious complications such as pneumonitis, hepatic failure, rupture of the spleen, and central nervous system involvement. Primary EBV infection can develop into EBV-AHS in a limited number of individuals and may be the first symptom of CAEBV [14].

We previously compared EBV-DNA among patients with IM, CAEBV, and lymphoproliferative disease (LPD) (Fig. 2) [3]. The mean copies of EBV-DNA in the PBMNCs was  $10^{2.2}$  copies/ $\mu$ g DNA in patients with IM,  $10^{4.1}$  copies/ $\mu$ g DNA in patients with CAEBV, and  $10^{3.7}$  copies/ $\mu$ g DNA in patients with LPD. These numbers were significantly larger than those in EBV-seropositive controls. The reason why patients with IM showed a lower viral load compared with patients with CAEBV or LPD may be a difference of host immunity. Patients with IM can acquire virus-specific CTLs, and the virus load decreases gradually.

During primary EBV infections, it is important to distinguish EBV-AHS from IM. It has been reported that there is a greater EBV load in patients with EBV-AHS than in those with IM [11]. Cell-free viral DNA, which reflects the EBV load in replication, had been considered to be a useful marker. However, some patients with the onset of EBV-AHS were found to have  $10^2$  to  $10^3$  copies/ml of EBV-DNA in their plasma, which is the average viral load in patients with IM. EBV-DNA in the PBMNCs is apparently higher in these patients, and thus it is considered to be a better marker for distinguishing between these 2 diseases [4].

**EBV-associated hemophagocytic syndrome (EBV-AHS).** EBV-AHS progresses very rapidly and becomes a life-threatening disease without immunosuppressive therapy. In EBV-AHS, the hyperactivation of EBV-infected T cells leads to the subsequent activation of macrophages. The EBV-infected cells are usually CD8<sup>+</sup> cells, but occasionally they are CD4<sup>+</sup> T cells or both T-cell populations [18]. The EBV-infected cell type and a high EBV load are considered as the main triggers for EBV-AHS.

These activating cells produce cytokines in too great a quantity, which is referred to as a "cytokine storm". The cytokine storm causes coagulopathy and multiple organ failure, both of which progress very rapidly. The postulated cytokine network in EBV-AHS is shown Fig. 3. Th1-type cytokines (IL-12, IL-2, IFN- $\gamma$ , and IL-18) are highly elevated, and in contrast, IL-4 is suppressed [19, 20]. Two major inflammatory cytokines, IFN- $\gamma$  and TNF- $\alpha$ , play a central role in the resistance of the

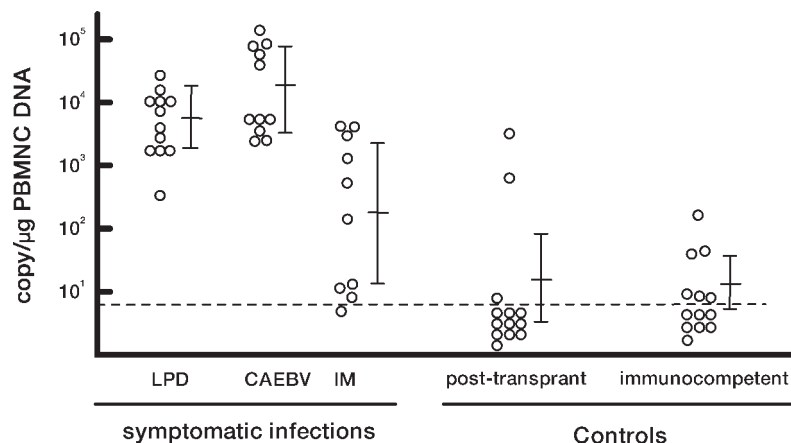


Fig. 2 Quantitation of EBV DNA by real-time PCR (cited from [3]).



host to EBV infection, as well as in the activation of macrophages. However, EBV has a mechanism for evading the antiviral function of these cytokines. For example, when EBV infects NK cells, IFN- $\gamma$  may prevent EBV-infected cells from apoptosis [21]. As regards TNF- $\alpha$ , immediate-early protein BZLF1 inhibits TNF- $\alpha$ -induced signaling and apoptosis by the downregulation of TNF receptor 1 [22].

These inflammatory cytokines are also associated with liver dysfunction, which is a common feature of EBV infection. TNF- $\alpha$  itself can cause liver dysfunction [23], but the Fas-ligand greatly affects EBV-associated liver dysfunction [24]. We experienced 1 case with severe hepatitis. This patient's liver biopsy showed that infiltrating CD8<sup>+</sup> cells, and not hepatocytes, were positive for EBV, and these EBV-infected lymphocytes expressed Fas-ligand, whereas the hepatocytes expressed Fas-antigens [24]. As regards other cytokines, the elevation of IL-18, which is produced by activated macrophages, has been reported as a useful marker of disease activity in EBV-AHS [25].

The cytokine profile in EBV-AHS may be slightly different from that in CAEBV (Table 1)[20, 26]. For example, transforming growth factor (TGF)- $\beta$ , which might activate EBV from latency through BZLF1, was elevated in one of our patients with CAEBV [27]. In addition, there are differences between the 2 types of CAEBV (T cell-type and NK cell-type). That is, the

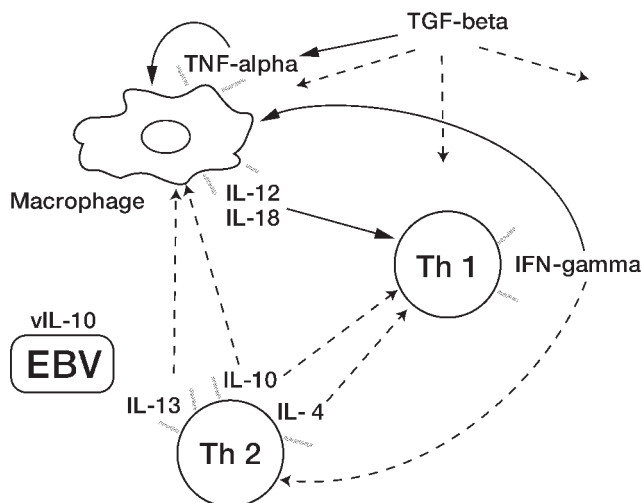
levels of IL-13 are significantly higher in patients with NK cell-type CAEBV than in those with T cell-type CAEBV [26]. IL-13 is a Th2-type cytokine that induces a class switch to IgE and suppresses the cytotoxic function of macrophages.

IL-10, which is an inhibitory factor for IFN- $\gamma$ , TNF- $\alpha$ , and IL-6, is also elevated in patients with symptomatic EBV infection. However, its role in EBV infection is complex [28]. EBV contains a homolog of IL-10 (viral IL-10), and its function differs slightly from that of cellular IL-10 [29]. Viral IL-10 shares many of the immunosuppressive properties of cellular IL-10, but lacks several of its immunostimulatory features, such as increasing the activity of both NK cells as well as CTLs [28]. Therefore, viral IL-10 would be elevated in patients with CAEBV [30]. Beyond that, allelic polymorphism in the IL-10 gene induces a variation in IL-10 production, and variation of the IL-10 level may influence the host immunity to EBV infection [31].

In the early phase of EBV-AHS, EBV infects CD8<sup>+</sup> cells, but the role played by EBV-specific CD8<sup>+</sup> CTLs remains unclear. Use of the MHC: peptide tetramer assay may help elucidate this role in the future.

**Chronic active EBV infection (CAEBV).**

In CAEBV, there are more EBV-infected CD4<sup>+</sup> T cells or NK cells than EBV-infected CD8<sup>+</sup> T cells. CAEBV is characterized by chronic or recurrent IM-like symptoms persisting over a long period of time and by an unusual pattern of anti-EBV antibodies. The 3 main criteria for CAEBV are: (I) severe illness lasting more than 6 months, which began as a primary EBV infection and



**Fig. 3** The putative cytokine network in cases of EBV infection. Solid arrows indicate stimulatory effects, and dotted arrows indicate inhibitory effects.

**Table 1** Comparison of cytokine profiles in patients with EBV-AHS and CAEBV

Cytokine	EBV-AHS	CAEBV
<i>Th1 type</i>		
IL-12	↑	↓
IL-18	↑	↑
TNF- $\alpha$	↑	↑
IFN- $\gamma$	↑	↑
<i>Th2 type</i>		
IL-10	↑	↑ (T > NK)
IL-4	↓	↓
IL-13	?	↑ (T < NK)
<i>Others</i>		
TGF- $\beta$	?	↑
viral IL-10	?	↑

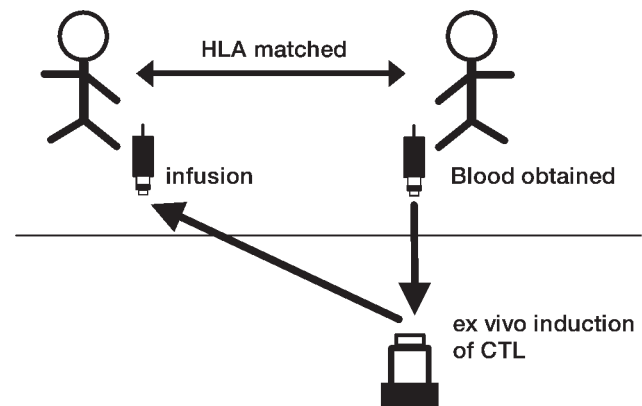
was associated with grossly abnormal EBV antibody titers, anti-viral caused antigens (VCA) IgG  $\geq 5120$ , anti-early antigens (EA) IgG  $\geq 640$ , and anti-EB nuclear antigens (EBNA)  $< 2$ ; (II) histological evidence of major organ involvement such as interstitial pneumonia, hyperplasia of some bone marrow elements, ileitis, lymphadenitis, persistent hepatitis, or splenomegaly; and (III) increased quantities of EBV in affected tissues [32]. However, in some cases, patients lacking these abnormal patterns of EBV-related antibodies and major organ involvement have only dermatological symptoms, such as hypersensitivity to mosquito bites or hydroa vacciniforme-like eruptions [33–35].

In addition to these reported findings regarding the clinical criteria, we previously reported the new virologic characteristics of CAEBV [36]. First, we observed a high viral load in the peripheral blood of CAEBV patients (Fig. 2). Moreover, patients with life-threatening symptoms showed a higher viral load than did patients with mild disease severity. The EBV load was found to be associated with the severity of EBV-related disease. Second, we observed chromosomal aberrations and monoclonality of EBV in more than half of the patients with CAEBV. There was no chromosomal specificity among the patterns of aberrations that we observed. Chromosomal aberration was more frequent in NK-cell CAEBV than in T-cell CAEBV. Patients with chromosomal aberrations showed a higher rate of malignancy. However, the probability of survival at 5 years was  $0.41 \pm 0.18$  for patients with T-cell CAEBV and  $0.83 \pm 0.15$  for patients with NK-cell CAEBV, in patients who did not undergo bone marrow transplantation. The survival rate was also associated with the age of the patient upon the onset of disease. Patients who were older than 8 years at the time of disease onset had a significantly poorer prognosis [37]. Prognostic factors for CAEBV are summarized in Table 2.

CAEBV is not only a premalignant condition, but is also a condition involving severe immunodeficiency. Most CAEBV patients examined had an insufficient number of EBV-specific CTLs, as determined by a MHC: peptide tetramer assay using both lytic and latent proteins [2]. In particular, latent membrane 2 (LMP-2)-specific CD8<sup>+</sup> T cells were not detected in any of these patients. LMP-2 is expressed in EBV-infected cells in CAEBV patients, and thus LMP-2 expression may be involved in the mechanism that enables EBV to evade host immunity [2]. A sufficient number of EBV-specific CTLs is the

**Table 2** Factors associated with prognosis for patients with CAEBV (modified from [37])

Factor	5-years survival
Age at disease onset	
< 8 years	$0.94 \pm 0.04\%$
$\geq 8$ years	$0.45 \pm 0.09\%$
Thrombocytopenia at diagnosis	
$\geq 12 \times 10^4/\mu\text{l}$	$0.76 \pm 0.06\%$
$< 12 \times 10^4/\mu\text{l}$	$0.38 \pm 0.13\%$
EBV-infected cell type	
NK cell type	$0.87 \pm 0.07\%$
T cell type	$0.59 \pm 0.09\%$



**Fig. 4** Scheme for CTL adoption (cited from [38]).

most important factor in the cure of CAEBV infection. We previously reported our observation that EBV-specific CTL adoptive immune therapy is potentially useful in cases of severe CAEBV infection [38]. Adoptive immune therapy is defined as the infusion of immune effector cells, such as EBV-specific CTLs, for the treatment and/or prevention of disease [39]. Briefly, an EBV-specific CTL line, which is established from PBMCs obtained from an HLA-matched donor, is transfused to the patient (Fig. 4). In our experience, after the transfusion of EBV-specific CTLs, cell-free EBV-DNA showed transient but repeated decreases associated with increases in anti-EBV CTL activity.

The pathogenesis of CAEBV remains largely obscure. Initially, CMV-specific CTLs were not observed in CMV-seropositive CAEBV patients [2]. The reason why the host immunity to CMV is also suppressed in CAEBV patients is also unclear. In addition, little is



**Table 3** Summary of EBV loads and host immunity in patients with IM, EBV-AHS, and CAEBV

Disease	EBV-infected cell	EBV load and Host immunity
Infectious Mononucleosis (IM)	B cell	EBV load at onset is about $10^{2-3}$ copy/ug/DNA (PBMC), and it disappears within 4–5 weeks after normal increase of EBV-CTL.
EBV-associated hemophagocytic lymphoproliferative syndrome (EBV-AHS)	T cell (mainly CD8 <sup>+</sup> , occasionally CD4 <sup>+</sup> or both phenotypes)	EBV load is usually more than $10^{3-6}$ copy/ug/DNA(PBMC), but sometimes at the same level as IM. It gradually decrease, although can be detected 3 months after remission. An overproduction IFN- $\gamma$ , TNF- $\alpha$ , IL-10 and IL-12 which is called a cytokine storm causes a rapid progress of EBV-AHS. The role of EBV- CTL has been unknown yet.
Chronic active EBV infection (CAEBV)	T cell (mainly CD4 <sup>+</sup> ), NK cell	The average of EBV is $10^4$ copy/ug/DNA (PBMC) that is the same level as LPD. EBV load doesn't decrease because of insufficient EBV-CTLs. Host immunodeficiency are not only related to EBV but other viruses, such as CMV.

known about how EBV is capable of infecting T/NK cells that do not express CD21. It is possible that after the early activation of T/NK cells by conjugation to CD21<sup>+</sup> B-EBV cells, these cells transiently acquire a weak CD21<sup>+</sup> phenotype [40]. Yet another factor that remains unclear is why CAEBV occurs more often in Asian countries than in Western countries. The perforin gene mutation revealed in EBV-AHS and CAEBV patients may be related to this racial difference [41, 42]. Calcification of the bilateral basal ganglia, which is a common feature in HIV patients, has also been reported in patients with CAEBV [43]. NK cell proliferation and TNF- $\alpha$  may also be involved in its pathogenesis [44].

Here, we reviewed recent prognoses in order to evaluate complicated EBV infection (Table 3). In addition to EBV-induced tumorigenesis, an overproduction of cytokines in cases of EBV-AHS and a weak host immune response such as CAEBV can be fatal, especially in children. Future analysis of host immunity, including innate immunity, may help establish the optimum therapy for these complicated and refractory EBV infections.

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