Induction of Cytokines and Cytotoxic Molecules with the Expression of Latent Epstein-Barr Virus Infection Genes in Human T-cell Lines

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In chronic active Epstein-Barr virus (EBV) (CAEBV) disease, EBV infects T or natural killer cells, and patients frequently develop life-threatening complications, such as EBV-associated hemophagocytic lymphohistiocytosis, which is characterized by the excessive release of cytokines such as interleukins (ILs), interferons, and chemokines and leads to multiple organ failure. We have introduced a plasmid containing the coding sequences of EBV-encoded small RNAs (EBERs) or latent membrane protein (LMP) 1, or both plasmids into human T-lymphocyte virus-I-negative human T-cell lines using an expression plasmid vector harboring EBV nuclear antigen 1 and established stable transformants. Here, we evaluated the modification of cytokine expression by the latent EBV genes, EBERs and LMP1, in these stable transformants. The expression of tumor necrosis factor α, perforin, and IL-10 increased with the introduction of both EBERs and LMP1 at both the mRNA and protein levels in CCRF-HSB2 cells, an immature T-cell line without cell surface protein expression of both the TCRα/β and TCRγ/δ. EBV latent infection genes, EBERs and LMP1, would be responsible for the pathogenesis of CAEBV disease. CCRF-HSB2 would be useful for the further analyses of these intracellular mechanisms in T-lineage cells and the pathogenesis of CAEBV disease. *Shinshu Med J 74 : 101—109, 2026*

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Key words : chronic active Epstein-Barr virus (EBV) disease, cytokines, EBV-encoded small RNAs, latent membrane protein 1, T-cell lines

Abbreviations : CAEBV, chronic active EBV ; EBERs, EBV-encoded small RNAs ; EBNAs, EBV nuclear antigens ; EBV, Epstein-Barr virus ; EBV-HLH, EBV-associated hemophagocytic lymphohistiocytosis ; ELISA, enzyme-linked immunosorbent assay ; FBS, fetal bovine serum ; GAPDH, glyceraldehyde 3-phosphate dehydrogenase ; GFP, green fluorescence protein ; HTLV-I, human T-lymphocyte virus-I ; IFNs, interferons ; ILs, interleukins ; LMPs, latent membrane proteins ; NK, natural killer ; RT-PCR, reverse transcriptase-polymerase chain reaction ; TGF, transforming growth factor ; TNF, tumor necrosis factor

I Introduction

Epstein-Barr virus (EBV) is a gamma herpesvirus and ubiquitous in humans. Primary EBV infection is basically asymptomatic but sometimes develops infectious mononucleosis, which is usually self-limiting[1]. However, some individuals in east Asia develop chronic infection with EBV. Chronic active EBV (CAEBV) disease is characterized by chronic infectious mononucleosis-like symptoms and by high viral loads in the peripheral blood of patients[2)-5)]. CAEBV disease patients frequently exhibit EBV-associated T/natural killer (NK)-cell lymphoproliferative disorders[6)] and life-threatening complications including EBV-associated hemophagocytic lymphohistiocytosis (EBV-HLH), EBV-positive apparent lymphoid neoplasms, mainly in the T- and NK-cell lineages, and cardiovascular diseases and arteritis with the infiltration of EBV-infected lymphocytes[2)-8)]. While some patients with CAEBV disease remain stable without interven-

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tion, others exhibit rapid disease progression with fatal outcomes. Currently, hematopoietic stem cell transplantation is assumed to be the only curative treatment option[9].

EBV-HLH is strongly associated with cytokine storms (hypercytokinemia), characterized by the excessive release of cytokines such as interleukins (ILs), interferons (IFNs), and chemokines, and leads to multiple organ failure. In addition to inflammatory cytokines, including tumor necrosis factor (TNF)α, IFNγ, and IL-6, immunoregulatory cytokines, including IL-10 and transforming growth factor (TGF)β, are also produced in patients with EBV-HLH or CAEBV disease[10][11]. In CAEBV disease, EBV infects T or NK cells[4], and typically, EBV infection of these cells results in a latency II pattern, namely, EBV nuclear antigen 1 (EBNA1), EBV-encoded small RNAs (EBERs), *Bam*HI-A rightward transcripts, latent membrane protein (LMP) 1, and LMP2A/B are expressed in infected T and NK cells[1]. However, the profiles of latent EBV gene expression show vary among the cases[12][13]. For cell model analyses of EBV infection of T-lineage cells, the *in vitro* infection of human T-cell lines with EBV after the forced expression of the EBV receptor in B-lineage cells, CD21, enhances the production of the macrophage-activating cytokine TNFα[14]. However, the EBV gene responsible for the upregulation of TNFα expression in T-lineage cells remains unclear. On the other hand, EBV infects a human T-lymphocyte virus-I (HTLV-I)-positive human T-cell line, MT-2[15], and EBERs induce IL-9 in EBV-infected MT-2 cells[16]. However, HTLV-I-infection activates human T cells and induces various cytokines[17] ; thus, the alteration of cytokine expression in HTLV-I-positive T-cell lines with EBV superinfection might not reflect the role of EBV *in vivo*. In non-T-lineage cells, on the other hand, the EBV latent genes responsible for cytokine induction have been identified, namely, EBERs and LMP1 upregulate IL-10 expression in Burkitt's lymphoma B-cell lines[18][19], and LMP1 also induces IL-1, IL-6, and IL-10 in epithelial cell lines[20]-[22].

On the basis of the previous reports mentioned above, we focused on EBERs and LMP1 as possible candidates for the EBV latent infection gene responsible for cytokine induction in CAEBV disease and attempted to establish cell models. EBNA1 enables efficient EBV episomal replication, transcription, and maintenance in latently infected dividing cells[23], and EBNA1 has been shown to activate transcription from episomes but not integrated DNA and does not affect cellular gene expression[24]. Therefore, a plasmid vector harboring EBNA1[25] would mimic latent EBV infection and be useful for analyses of the role of latent EBV genes. We have introduced a plasmid containing the coding sequences of EBERs or LMP1, or both plasmids into HTLV-I-negative human T-cell lines using an expression plasmid vector harboring EBNA1 and established stable transformants[26].

In the present study, we evaluated the modification of cytokine expression by the EBV latent genes, EBERs and LMP1, in these stable transformants, which might lead to the pathogenesis of CAEBV.

Ⅱ Materials and Methods

A Cell lines and culture

An HTLV-I-negative human T-cell line, MOLT14[27], was a gift from Fujisaki Cell Center, Hayashibara Biochemical Labs., Inc. (Okayama, Japan). CCRF-HSB2[28], also an HTLV-I-negative human T-cell line, was a gift from the Health Science Research Resources Bank (Sennan, Japan). The human T-cell lines Jurkat[29] and MOLT4[29] (both HTLV-I-negative) were also used. These cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS ; MP Biomedicals, LLC, Solon, OH). MOLT14/G, MOLT14/E6, and MOLT14/L are stable transformants of MOLT14 cells expressing green fluorescence protein (GFP), EBERs, and LMP1, respectively, generated via the transfection of the EBNA1-harboring episomal expression vector containing the GFP gene, the six tandemly connected EBV DNA fragments containing EBER1 and EBER2 (E6 fragment), and the LMP1 gene, respectively, and subsequent cloning[26]. MOLT14/E6+L is the stable transformant of MOLT14 cells coexpressing EBERs and LMP1 and was established via the transfection of both the EBNA1-harboring episomal expression vector containing the E6 fragment and that containing

the LMP1 gene[26]. Stable transformants of CCRF-HSB2 (CCRF-HSB2/G, CCRF-HSB2/E6, CCRF-HSB2/L, and CCRF-HSB2/E6+L), Jurkat (Jurkat/G, Jurkat/E6, Jurkat/L, and Jurkat/E6+L), and MOLT4 (MOLT4/G, MOLT4/E6, MOLT4/L, and MOLT4/E6+L) are established in the same manner as those in MOLT14 cells[26]. In these stable transformants, the introduced EBV genes (EBERs and/or LMP1) are expressed[26]. Geneticin (G418 sulfate ; Life Technologies, Grand Island, NY) was added to the culture medium (RPMI1640 with 10% FBS) at concentrations of 1 mg/mL and 800 μg/mL in the transformants of Jurkat cells and those of the other cells, respectively, to maintain the vector. Before sample preparation, these transformants were cultured for one week without Geneticin, and for RNA preparation, the cells were harvested. The cell pellets were quickly frozen in liquid nitrogen and stored at −80℃ until RNA extraction.

EBV-positive NK-cell lines, SNK1[30] and SNK6[31] (gifts from Dr. Norio Shimizu ; Tokyo Medical and Dental University, Tokyo, Japan), were grown in Artemis-2 medium (Nihon Techno Service, Ushiku, Japan).

B RNA extraction and real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the cell lines, quantified, and subsequently treated with DNase as previously described[26]. For the reverse transcription of RNA samples, 4 μg of DNase-treated total RNA was converted to cDNA in a 50-μL reaction volume, as previously described[26]. For real-time PCR analyses, a 0.5-μL aliquot of the cDNA sample (cDNA converted from 40 ng of total RNA) was diluted to 20 μL of a mixture containing TaqMan™ Fast Advanced master mix (Thermo Fisher Scientific, Vilnius, Lithuania) and each predeveloped TaqMan assay reagent (TaqMan Gene Expression Assays ; #Hs00174125_m1 for IL-9, #Hs99999035_m1 for IL-10, #Hs00989291_m1 for IFNγ, #99999043_m1 for TNFα, #Hs00998133_m1 for TGFβ1, #Hs01554355_m1 for granzyme B, #Hs00169473_m1 for perforin 1 ; Applied Biosystems, Foster City, CA). Predeveloped TaqMan assay reagent for human glyceraldehyde 3-phosphate dehydrogenase (GAPDH ; #Hs99999905_m1 ; Applied Biosystems) was used as an internal control for mRNA quantification. Serial fivefold dilutions of cDNA samples of SNK1 and SNK6 were used to obtain the standard curve of relative amounts of cytokine mRNA. PCR of the cDNA samples was performed in triplicate, including serially diluted cDNA samples of SNK1 and SNK6 as standards. Real-time PCR amplification was performed using the QuantStudio 3 real-time PCR system (Applied Biosystems) according to the manufacturer's protocol, and the relative amounts of cytokine mRNAs in each cDNA sample to those in the corresponding mother cell line were estimated.

C Measurement of the protein concentration of the culture supernatant

After one week of culture without Geneticin, a 3-mL culture of transformants of T-cell lines was prepared in a well of six-well tissue culture plates at a density of 2.0×10^5 cells/mL. After 72 hrs of incubation, the culture supernatants were harvested by centrifugation, and a 500-μL aliquot of each supernatant was made and stored at −25℃ until use. The amounts of human IL-10 and TNFα in the culture supernatants of the cell lines were quantified by enzyme-linked immunosorbent assay (ELISA) with human IL-10 and TNFα ELISA kits (both from R & D Systems, Minneapolis, MN), respectively. The amount of human perforin in the culture supernatants was also measured by ELISA with a human perforin ELISA kit (Arigo Biolaboratories, Hsinchu, Taiwan).

D Statistical analysis

Statistical significance between two samples was determined by the two-tailed Student's t-test. $P<0.05$ was considered statistically significant.

Ⅲ Results

A Expression of cytokine mRNAs

Considering the pathophysiology of CAEBV disease, we first examined the expression of the macrophage-activating cytokines TNFα and IFNγ. As shown in **Fig. 1A**, the expression levels of TNFα mRNA in the current four T-cell lines increased with the expression of the EBV latent genes EBERs and/or LMP1. On the other hand, the apparent expression of IFNγ mRNA was not detected in any of the four cell lines,

even after 35 cycles of amplification (data not shown).

Next, we examined the cytotoxic proteins in lymphocytes, granzyme B and perforin, which might be involved in tissue injury, such as vasculitis, in CAEBV disease patients. The expression of perforin 1 mRNA was enhanced by LMP1 in CCRF-HSB2 and MOLT14 cells **(Fig. 1B)**. In Jurkat cells, the expression of perforin 1 mRNA decreased with the introduction of EBERs and/or LMP1 **(Fig. 1B)**. On the other hand, the expression of granzyme B mRNA was not detected in CCRF-HSB2, MOLT14, or MOLT4 cells, even after 35 cycles of amplification (data not shown). In Jurkat cells, granzyme B mRNA was clearly expressed ; however, alterations of the mRNA expression with latent EBV genes were not obvious (data not shown).

In addition to these cytokines, we also examined the expression of the immunosuppressive cytokines IL-10 and TGFβ, which might be involved in the establishment of CAEBV disease. The expression of IL-10 mRNA was enhanced with EBERs in CCRF-HSB2 cells and with EBERs and LMP1 in both CCRF-HSB2 and MOLT14 cells **(Fig. 1C)**. In Jurkat cells, the expression of IL-10 mRNA decreased with EBERs and/or LMP1 **(Fig. 1C)**. Furthermore, all four T-cell lines clearly expressed TGFβ mRNA ; however, alterations in the expression levels of TGFβ mRNA with EBV latent infection genes were not obvious (data not shown). Finally, we examined the expression of a T-cell growth factor, IL-9[32]. In our cell culture system, the apparent expression of IL-9 mRNA was not detected in any of the four cell lines, even after 35 cycles of amplification (data not shown).

B Protein concentration in the culture supernatant measured by ELISA

The protein concentration of TNFα in the culture supernatant slightly increased in CCRF-HSB2 cells following the introduction of both EBERs and LMP1 **(Fig. 2A)**. On the other hand, in the other three cell lines, TNFα protein was not detected **(Fig. 2A)**, although the expression of TNFα mRNA was upregulated with EBERs and/or LMP1 **(Fig. 1A)**. The protein concentration of perforin increased in CCRF-HSB2 cells with EBERs and/or LMP1. On the other hand, in Jurkat cells, the protein concentration of perforin decreased with EBERs and/or LMP1 **(Fig. 2B)**. In MOLT14 and MOLT4 cells, the protein concentration of perforin also decreased with EBERs and/or LMP1 **(Fig. 2B)**, although perforin mRNA expression was generally unchanged **(Fig. 1B)**. The protein concentration of IL-10 increased in CCRF-HSB2 cells with EBERs and/or LMP1. On the other hand, in Jurkat cells, the protein concentration of IL-10 decreased with EBERs and/or LMP1 **(Fig. 2C)**. In MOLT14 cells, the protein concentration of IL-10 greatly decreased with the coexpression of EBERs and LMP1 **(Fig. 2C)**, in contrast to the increased expression of IL-10 mRNA **(Fig. 1C)**. In MOLT4 cells, the IL-10 protein was not detected in the culture supernatant **(Fig. 2C)**.

Ⅳ Discussion

In the present study, we examined the alteration of cytokine production in human T-cell lines with the forced expression of the latent EBV infection genes EBERs and LMP1. Changes in cytokine expression were induced in some T-cell lines with latent EBV infection genes ; however, changes in cytokine expression varied depending on the cell lines, the introduced EBV genes, and the examined cytokines. Furthermore, the production of cytokine proteins did not uniformly correlate with the level of the expression of each mRNA.

During the induction of TNFα expression, the upregulation of both the mRNA and protein levels was observed in CCRF-HSB2 cells with the introduction of both EBERs and LMP1. On the other hand, in the other three T-cell lines, protein expression was not observed under any conditions, irrespective of EBV gene expression. In these three cell lines, the TNFα gene or transcript might exhibit some alterations, mainly in the 3'-downstream region, such as splicing variants, since the real-time RT-PCR primers (Applied Biosystems) used in this study are designed to anneal to exons 1 and 2 of TNFα gene ; subsequently the ELISA kit employed in this study might not be able to detect the mutated TNFα protein.

In CCRF-HSB2, various T-cell factors were upregulated with the introduction of latent EBV genes,

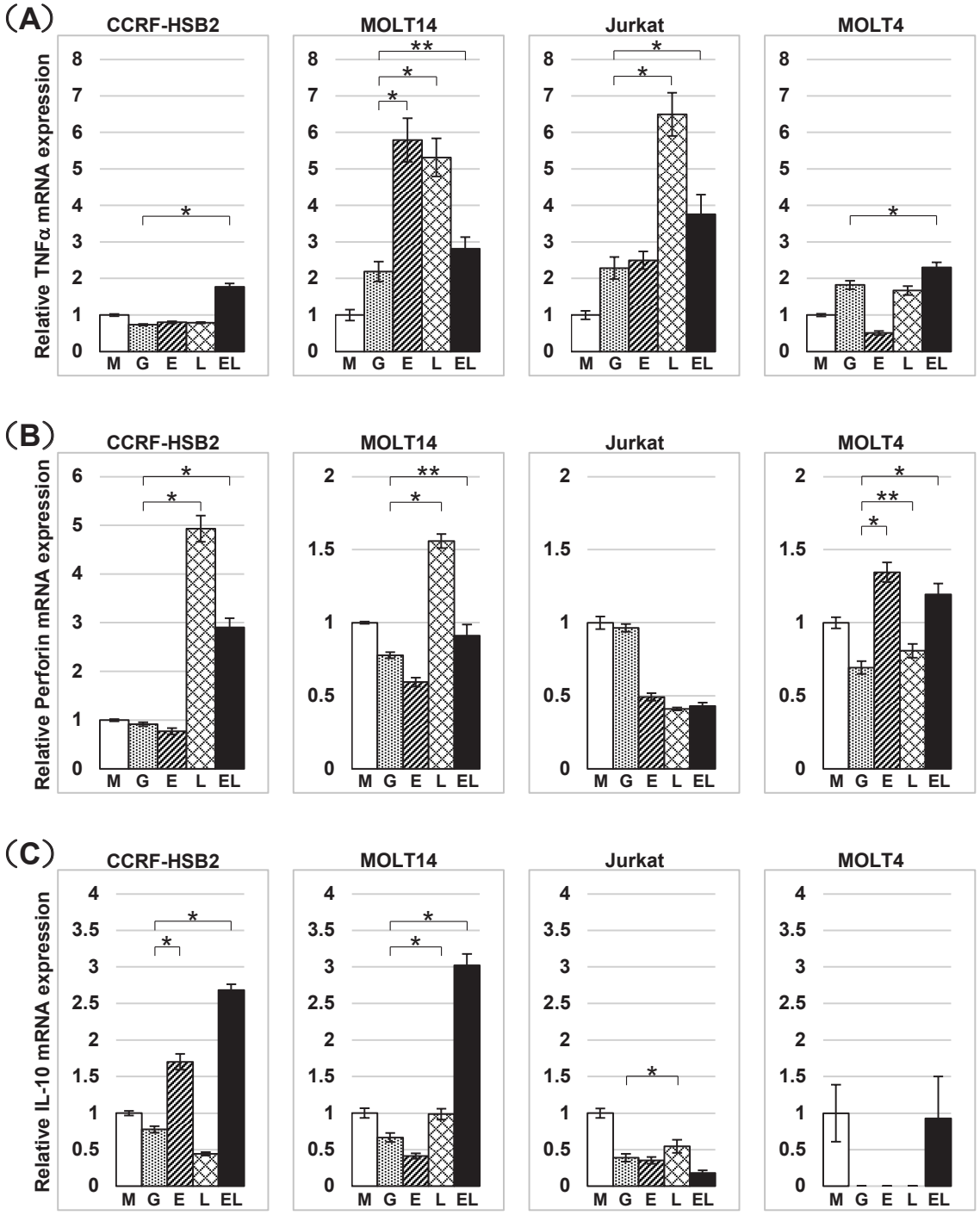

Fig. 1 Expression of TNFα (A), perforin (B), and IL-10 (C) mRNAs in human T-cell lines and their stable transformants. Real-time RT-PCR was performed in triplicate as described in the Materials and methods. The relative expression level of mRNA compared with that in each corresponding mother cell line, which was standardized with the expression of mRNA of GAPDH, was estimated. Each real-time RT-PCR was repeated twice, and the bars indicate the mean value plus/minus SD of a total of six reactions in each sample. Statistical significance to the value of GFP-transfected cells was determined by the two-tailed Student's *t*-test. $^{*}P<0.01$. $^{**}P<0.05$. M : Open bar, mother cells ; G : dotted bar, transformants with the GFP gene ; E : hatched bar, transformants with the E6 fragment ; L : latticed bar, transformants with the LMP1 gene ; EL : closed bar, transformants with both the E6 fragment and LMP1 gene.

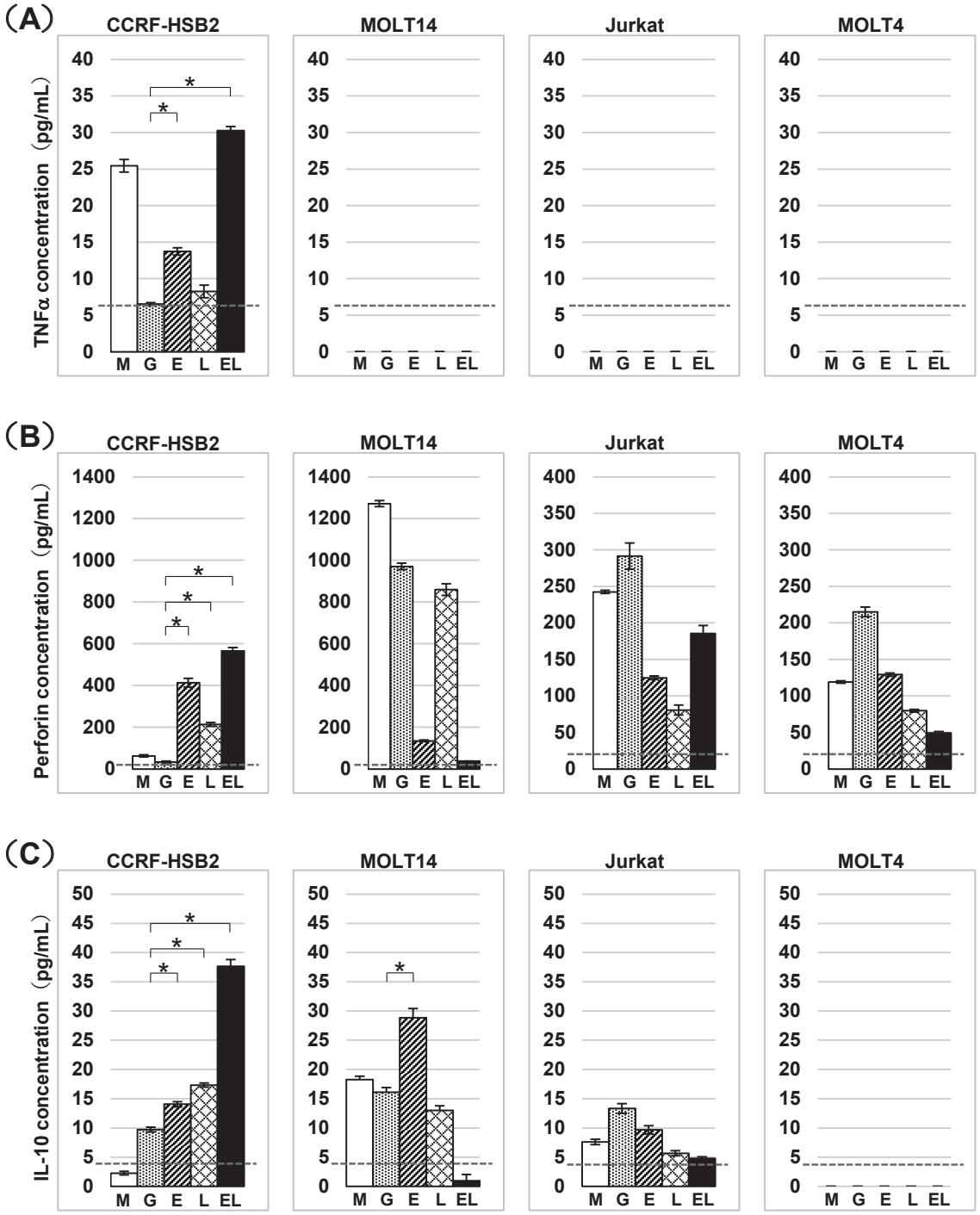

Fig. 2 Concentration of TNFα (A), perforin (B), and IL-10 (C) proteins in the culture of transformants. After 72 h of culture, the supernatants were harvested by centrifugation and stored at −25℃. The protein concentration was measured by ELISA. The bars represent the mean values plus/minus SD of triplicate wells. The dotted line indicates the sensitivity of each ELISA. Statistical significance to the value of GFP-transfected cells was determined by the two-tailed Student's *t*-test. $^{*}P<0.01$. M : Open bar, mother cells ; G : dotted bar, transformants with the GFP gene ; E : hatched bar, transformants with the E6 fragment ; L : latticed bar, transformants with the LMP1 gene ; EL : closed bar, transformants with both the E6 fragment and LMP1 gene.

and the protein production of these factors generally correlated with the expression levels of the corresponding mRNA. We noted that the expression of all three T-cell factors examined in the present study, TNFα, perforin, and IL-10, increased with the introduction of both EBERs and LMP1 at both the mRNA and protein levels in CCRF-HSB2 cells **(Fig. 1, 2)**. Thus, CCRF-HSB2 would be a suitable cell model for analyzing alterations in T-cell function associated with latent EBV infection genes. On the other hand, in Jurkat cells, protein production was generally correlated with the expression level of corresponding mRNA, with the exception of TNFα ; however, T-cell factors were not upregulated with latent EBV infection genes. In MOLT4 and MOLT14 cells, the correlation between mRNA expression and protein synthesis was disrupted. We previously introduced a single copy of the EBERs gene into MOLT14 cells in a site-directed manner with a Flip recombinase-mediated integration kit, the Flip-In^{TM} system (Invitrogen, Carlsbad, CA), and observed upregulated expression of the IL-10 protein in MOLT14 cells with EBERs[33], which was reproducible in our current EBV gene introduction system. CCRF-HSB2 is an immature T-cell line without cell surface protein expression of both the TCRα/β and the TCRγ/δ[28]. On the other hand, MOLT14 is a γ/δ T-cell line[27], and MOLT4 and Jurkat are α/β T-cell lines[29]. The mechanism of EBV infection of T-lineage cells remains unclear, although a previous report suggested that EBV may infect human thymocytes[34]. Thymocytes include the most immature T cells, double negative T cells, which do not express any cell surface TCRs. Therefore, the fact that EBV preferentially infects thymocytes, not mature peripheral T cells, may support that CCRF-HSB2, an immature T-cell line, is a good cell model for alterations in T-cell function with latent EBV infection genes.

In the present study, we evaluated the cytokine-modulating effects of EBERs and/or LMP1 in human T-cell lines. However, these effects were largely dependent on the cell lines, and we showed the upregulated expression of TNFα, perforin, and IL-10 at least in CCRF-HSB2 cells with forced expression of both EBERs and LMP1, which might contribute to the development of EBV-HLH, vasculitis, and persistent EBV infection, respectively. This finding indicates that the latent EBV infection genes EBERs and LMP1 would be responsible for the pathogenesis of CAEBV disease, although the mechanisms by which latent EBV infection genes induce functional alterations in T-lineage cells remain unclear. CCRF-HSB2 would be useful for further analyses of these intracellular mechanisms in T-lineage cells and the pathogenesis of CAEBV disease. These analyses could lead to the development of effective therapeutic approaches for CAEBV disease, such as targeted suppression of the expression of latent EBV infection genes responsible for functional alterations in EBV-infected T cells.

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