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Brief Communication

# High frequencies of asymptomatic Epstein-Barr virus viremia in affected and unaffected individuals with *CTLA4* mutations

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ABSTRACT

Patients with *CTLA4* mutations present with autoimmune diseases, lymphoproliferation, and hypogammaglobulinemia, and a subset of patients developed Epstein-Barr virus (EBV)-associated malignancies, suggesting an impaired immune function against EBV. Here we investigated EBV infection in individuals with *CTLA4* mutations. We measured EBV viral DNA in healthy individuals, individuals with autoimmune diseases, and individuals with *CTLA4* mutations. In addition, we evaluated the numbers and function of EBV-specific T cells, invariant NKT cells, and NK cells. More than half of individuals with *CTLA4* mutations including asymptomatic ones had detectable EBV DNA, which is a significantly higher frequency with higher viral loads compared with healthy and disease controls. However, individuals with *CTLA4* mutations had almost normal immunity against EBV. Individuals with *CTLA4* mutations have an increased susceptibility to Epstein-Barr virus infections. Asymptomatic viremia occurs at high frequencies, which can be persistent and can occur in unaffected individuals.

## 1. Introduction

Heterozygous germline mutations in *CTLA4* are associated with various organ-specific autoimmune diseases, lymphoproliferation, and hypogammaglobulinemia, although one-third of individuals with *CTLA4* mutations are asymptomatic [1, 2]. A worldwide study also revealed that individuals with *CTLA4* mutations are susceptible to developing malignancy: in a cohort of 133 individuals, 8 and 3 patients developed lymphoma and gastric adenocarcinoma, respectively [3]. Six of these individuals were associated with Epstein-Barr virus (EBV), suggesting that they had an impaired immunity against EBV. However, previous reports have focused only on clinically apparent EBV infection, which is a relatively rare setting. Herein, we describe high frequencies of asymptomatic EBV viremia in individuals with *CTLA4* mutations, which may affect treatment options for patients.

## 2. Methods

### 2.1. Study approval

This study was conducted in accordance with the Helsinki Declaration and approved by the ethics boards of Tokyo Medical and Dental University.

*Abbreviations:* ALPS, autoimmune lymphoproliferative syndrome; APDS, activated phosphoinositide 3-kinese δ syndrome; BENTA, B-cell expansion with nuclear factor-κB and T-cell anergy; EBV, Epstein-Barr virus; ITP, immune thrombocytopenic purpura; LCLs, lymphoblastoid cell lines; MMF, mycophenolate mofetil; NF-κB, nuclear factor-κB; PBMCs, peripheral mononuclear cells; PI3K, phosphoinositide 3-kinase; PIDs, primary immunodeficiency diseases

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### 2.2. Diagnosis of CTLA4 deficiency

The methods of genomic analysis including whole exome sequencing and flow cytometric analysis were described previously [4]. For *CTLA4* mRNA analysis, real-time PCR was performed using cDNA from sorted $CD4^+$ T cells. The TaqMan probe (Applied Biosystems, Waltham, MA, *CTLA4* transcript variant 1; full length *CTLA4*) were used. Data were normalized to *GAPDH* and then normalized to those of control for relative gene expression.

### 2.3. Quantification of EBV viral DNA

Quantitative polymerase chain reaction was performed based on the TaqMan system (Applied Biosystems) using DNA which isolated from peripheral mononuclear cells (PBMCs) and plasma, as described elsewhere [3,5].

### 2.4. Flow cytometric analysis

PBMCs or lymphoblastoid cell lines (LCLs) were stained with fluorochrome-conjugated antibodies and analyzed using BD LSRFortessa (BD Biosciences, Franklin Lake, NJ). For lymphocyte phenotyping, monoclonal antibodies were used as follows: anti-CD3, CD4, CD8, CD19, CD45RO, CD57 (Miltenyi Biotec, Bergisch Gladbach, Germany), CD16, CD27, CD56, CD62L, TCR Vα24, TCR Vβ11 (Beckman Coulter, Brea, CA), CD70, NKG2D and 2B4 (BioLegend, San Diego, CA) [4]. For EBV-specific $CD8^+$ T cells, PBMCs were incubated with Clear Back (MBL, Nagoya, Japan) and stained with HLA-A*24:02 EBV mix Tetramer-phycoerythrin (MBL), anti-CD3 and anti-CD8 [6].

### 2.5. Functional assay

The capacity of T cells which respond to EBV was examined by assessing CD137 expression using flow cytometry. PBMCs were cultured with autologous EBV-LCLs at different effector-to-target ratios (1:1 and 10:1) for 24 h, and were stained for CD8 and CD137 (Miltenyi Biotec).

## 3. Results

### 3.1. Case reports

An 11-year-old boy (P1.2) was born to non-consanguineous parents (Supplementary Table 1). His father (P1.1) had atopic dermatitis and alopecia areata. His younger brother (P1.2) was healthy, but his younger sister (P1.4) was diagnosed with insulin-dependent diabetes mellitus at the age of 5 years. The patient consulted a hospital due to urticaria, and mild thrombocytopenia was unexpected detected at the age of 3 years. Based on the positive anti-EBV antibody titer, the thrombocytopenia was supposed to be associated with an EBV infection. The platelet count was spontaneously recovered without any medication. At the age of 4 years, the patient developed skin purpura with severe thrombocytopenia when he was infected with herpes zoster, and he was diagnosed as having immune thrombocytopenic purpura (ITP). He has been treated with high-dose immunoglobulin therapy and immunosuppressive agents, including prednisolone, cyclosporine A, tacrolimus, and mycophenolate mofetil (MMF) (Fig. 1A). At the age of 7 years, flow cytometric analysis revealed the increased level of T cell receptor $\alpha/\beta^+CD4^-CD8^-$ double negative T cells, suggestive of autoimmune lymphoproliferative syndrome (ALPS). However, the mutations in ALPS-related genes including *FAS, FALSG, KRAS* and *NRAS* were not found. The whole exome sequencing identified a heterozygous mutation in *CTLA4* (c.G494A, p.W165X), and the reduced level of CTLA4 in $CD4^+FOXP3^+$ T cells and *CTLA4* mRNA in $CD4^+$ T cells were detected (Supplementary Fig. 1) [4]. Therefore, the patient was diagnosed with CTLA4 haploinsufficiency, and his father and two siblings had the same *CTLA4* mutations. Although MMF proved to be effective, it was discontinued because of the development of agranulocytosis. The association between thrombocytopenia and EBV infection has been reported previously; hence, the EBV viral load was measured after the second round of high-dose immunoglobulin therapy. EBV DNA was detected in PBMCs, and low copy numbers of EBV DNA continue to be present (Fig. 1A). EBV DNA in plasma was detected at several time points.

P2.2 is a 16-year-old girl with a *CTLA4* mutation (*CTLA4* c.34C > T, p.Q12X) (Supplementary Fig. 1) who developed granulomatous lymphocytic interstitial lung disease at 9 years of age. Computed tomography incidentally revealed paraaortic lymphadenopathy. A histopathologic analysis of the paraaortic lymph node showed follicular hyperplasia with a dominance of T cells. Moreover, a few scattered cells positive for EBV-encoded small RNA were found. The patient was successfully treated with immunosuppressive agents, including prednisolone, cyclosporine A, and tacrolimus. Although the patient did not develop symptomatic EBV infection, low copy numbers of EBV DNA were still detected in PBMCs (Fig. 1B). The patient developed the complication of autoimmune hemolytic anemia and underwent bone marrow transplantation at the age of 14 years.

### 3.2. EBV copy numbers in peripheral blood mononuclear cells

We measured EBV viral loads in PBMCs at an arbitrary time point from the following three groups of individuals: healthy individuals (healthy control; $n = 32$), individuals with autoimmune diseases (disease controls; $n = 31$) and individuals with *CTLA4* mutations ($n = 15$) (Supplementary Table 2 and Supplementary Fig. 2). Individuals with *CTLA4* mutations comprised 12 affected and 3 unaffected individuals (Supplementary Table 1). All individuals had no symptoms associated with EBV infection at the time of evaluation. EBV DNA was detected in healthy controls (21.9%) and disease controls (12.9%), a result similar to that described previously [7]. Interestingly, 53.3% of individuals with *CTLA4* mutations had detectable EBV DNA, which is a significantly higher frequency with higher viral loads (Fig. 2). In 3 individuals with *CTLA4* mutations, EBV viral loads could retrospectively be measured at several time points (Fig. 1C, D and E) and were detectable at multiple points in one patient. EBV viral loads were not associated with clinical presentations or therapeutic agents. EBV viremia was also observed in unaffected individuals. Intriguingly, one of them had very high viral loads (Fig. 2 and Supplementary Table 1).

### 3.3. Anti-EBV IgG responses in the individuals with CTLA-4 mutations

Anti-EBV IgG responses were tested in 10 individuals (Supplementary Table 1). All of the individuals but one were seropositive. Of the 9 individuals, 9 individuals were positive for VCA-IgG, but only 4 individuals were positive for EBNA, although not primary infection judging from the clinical symptoms and VCA-IgM. There was no tendency between those findings and EBV viral loads.

### 3.4. Lymphocyte subpopulations and EBV-specific $CD8^+$ T cells in the individuals with CTLA4 deficiency

To investigate the effect of *CTLA4* mutations in EBV infection, we added *in vitro* analyses using lymphocytes from the affected and unaffected individuals. The proportions of lymphocyte subsets including T-cell subsets were largely normal (Supplementary Fig. 3). But decreased B cells, especially memory B cells, and decreased NK cells were observed in some individuals, consistent with the previous reports [3]. Some individuals showed decreased invariant NKT (iNKT) cells. Because NK/iNKT cells play important roles in anti-EBV immunity, decreased NK/iNKT cells could contribute to the EBV viremia. However, those findings were not related to whether having the EBV viremia or not.

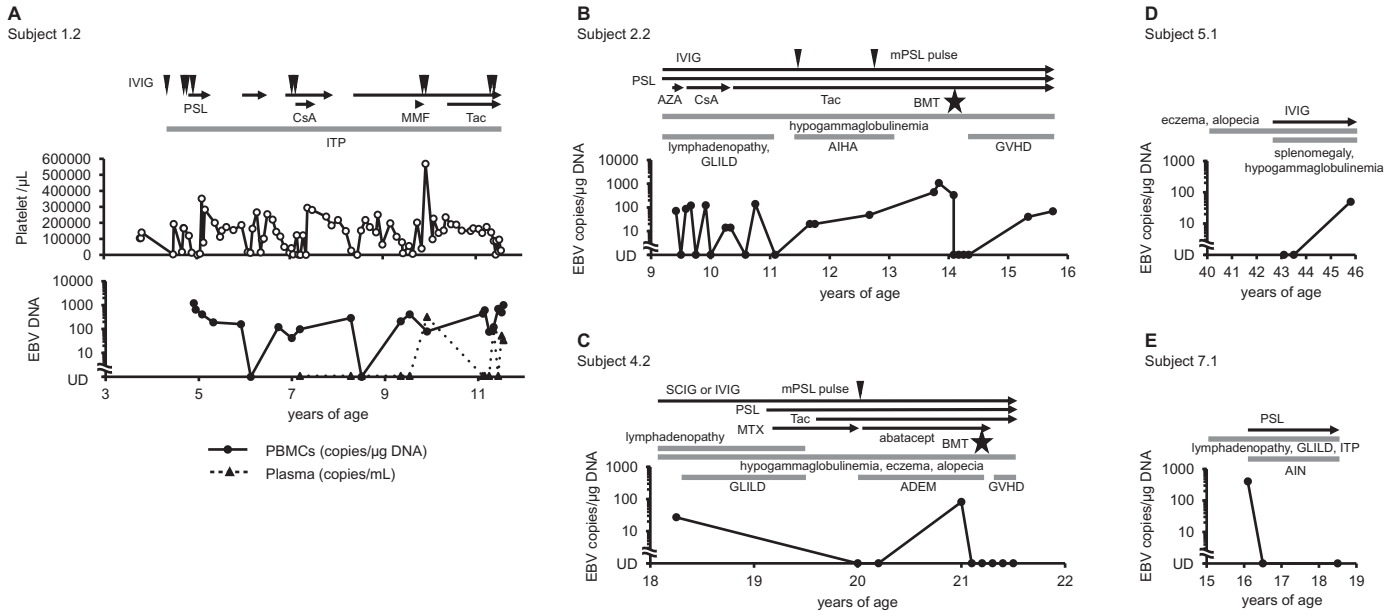


**Fig. 1.** Disease courses and EBV copy numbers of the patients.
The upper panel shows the treatment regimens and clinical presentations. The arrows indicate the duration of the treatment. The arrowheads indicate the time point of the treatment. The stars indicate the bone marrow transplantation. ADEM, acute disseminated encephalomyelitis; AIHA, autoimmune hemolytic anemia; AIN, autoimmune neutropenia; AZA, azathioprine; BMT, bone marrow transplantation; CsA, cyclosporine A; GLILD, granulomatous lymphocytic interstitial lung disease; GVHD, graft *versus* host disease; ITP, immune thrombocytopenic purpura; IVIG, intravenous immunoglobulin; MMF, mycophenolate mofetil; mPSL, methylprednisolone; MTX, methotrexate; PBMCs, peripheral blood mononuclear cells; PSL, prednisolone; SCIG, subcutaneous immunoglobulin; Tac, Tacrolimus; UD, undetectable.

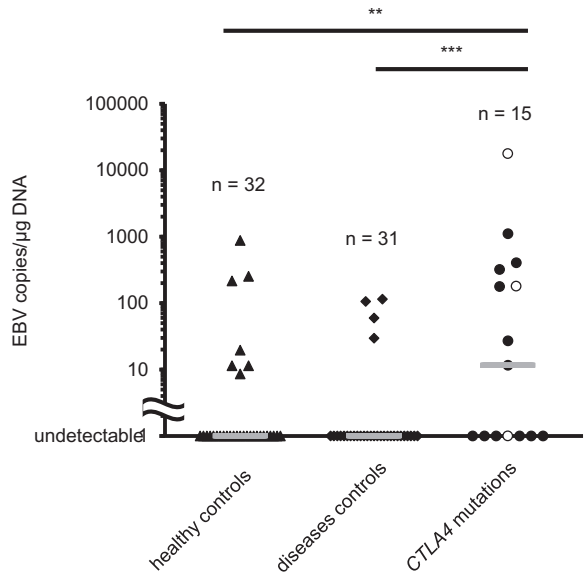


**Fig. 2.** EBV copy numbers in peripheral blood mononuclear cells in three groups of individuals.
Black circles indicate affected individuals with *CTLA4* mutations. Open circles indicate unaffected individuals with *CTLA4* mutations. Gray bars indicate median values. $^{**}p = .0085$, $^{***}p = .00074$ (Student's *t*-test).

The control of EBV infection by T cells requires the recognition of EBV-infected cells and cell to cell interaction. We evaluated the cell surface molecules; NKG2D and 2B4, which are receptors for the EBV-infected cells, and CD70, which is expressed on EBV-infected cells, were normally expressed on $CD8^+$ T cells or EBV-LCLs (Supplementary Fig. 4). The immunosenescence can cause impaired T-cell function and the uncontrol of EBV infection. There was a trend, although not statistically significant, for increased CD57 expression on $CD8^+$ T cells, which is a senescence marker (Supplementary Fig. 4).

Next, EBV-specific $CD8^+$ T cells were evaluated using an HLA-A*2402/EBV peptide tetramer. Adequate frequencies of EBV-specific $CD8^+$ T cells were identified. Their subsets and the expression of NKG2D, 2B4 and CD57 were similar to those of healthy controls (Supplementary Fig. 5).

Finally, we analyzed anti-EBV immunity of $CD8^+$ T cells. CD137, which is expressed in recently activated T cells, was expressed after co-culture with autologous EBV-LCLs (Supplementary Fig. 6).

## 4. Discussion

These two case studies raised several possibilities regarding the causes of persistent EBV viremia, such as autoimmune diseases, lymphadenopathy, therapeutic agents, and *CTLA4* mutation itself. Individuals with CTLA4 mutations had significantly higher frequency with higher loads of EBV viral DNA. In this context, we concluded that EBV viremia might be caused by the *CTLA4* mutations.

We demonstrated that individuals with *CTLA4* mutation had an increased susceptibility to EBV infection. Previous studies have described clinically apparent EBV infections including lymphoma, lymphoproliferative disease, HLH, and gastric adenocarcinoma in patients with CTLA4 deficiency [1–3, 8]. Our study provides additional evidence that asymptomatic viremia can occur at high frequencies, can be persistent, and can occur in unaffected individuals.

Several primary immunodeficiency diseases (PIDs) are associated with high susceptibility to EBV infection and have high frequencies of symptomatic EBV infections. Some PIDs include EBV-associated HLH (up to 70% of X-linked lymphoproliferative disease 1 patients) and EBV-positive lymphoma (up to 70%, 70%, and 50% of ITK deficiency, X-MEN disease, and CD27 deficiency patients, respectively) [9]. These features are attributed to the absence or impaired activation of EBV-specific T cells, iNKT cells, and NK cells. However, individuals with *CTLA4* mutations had almost normal levels and function of these cells, possibly contributing to the unique clinical features of these

individuals. Several patients had undetectable EBV DNA, indicating incomplete but functional anti-EBV immunity or pre-infection with EBV.

While the above-mentioned PIDs are caused by loss-of-function mutations of the critical genes involved in anti-EBV immunity, activated phosphoinositide 3-kinase (PI3K) δ syndrome (APDS), which is caused by a gain-of-function in the PI3K signaling pathway, results in EBV viremia (29.5%) and EBV-positive lymphoma (5.8%) [10]. B-cell expansion with nuclear factor-κB (NF-κB) and T-cell anergy (BENTA), which shows hyperactivation of NF-κB signaling pathway caused by gain-of-function mutation in *CARD11*, also results in EBV viremia [11]. CTLA4 deficiency also shows hyperactivation of signaling pathways, likely including PI3K and NF-κB signaling, because of impaired suppression of T-cell activation signaling [12, 13]. Our observations and previous reports revealed that the hyperactivation of some signaling pathways can cause the susceptibility to EBV. Previous studies showed T cell exhaustion and senescence, which could lead to impaired T-cell function, in patients with APDS and CTLA4 deficiency [14, 15]. Although our study showed the senescence tendency and limiting anti-EBV IgG responses in individuals with *CTLA4* mutations, the mechanism underlying the susceptibility to EBV remain to be fully understood [10, 14].

Our observations raise two important clinical implications. First, symptomatic EBV infection may be easily induced, especially by immunosuppression, through therapeutic agents. In P1.2, EBV DNA was detected in plasma after taking MMF, suggesting an EBV lytic cycle (Fig. 1A). It has also been reported that two siblings experienced uncontrolled EBV infection under abatacept therapy [8]. Discussions of appropriate therapeutic agents for patients with CTLA4 deficiency may have to be different from those for autoimmune disease patients without an underlying disease. Second, B-cell transformation may occur in not only affected but also unaffected individuals with *CTLA4* mutations. Careful monitoring of EBV infection may be useful. Further studies are required to define whether the frequencies of symptomatic EBV infection are increased in affected as well as unaffected individuals in the long term.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2018.07.012.

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