Title: Clonal origin of Epstein-Barr virus-infected T/NK-cell subpopulations in chronic active Epstein-Barr virus infection

Authors: Shouichi Ohga, MD, PhD,1 Masataka Ishimura, MD,1 Goichi Yoshimoto, MD, PhD,2 Toshihiro Miyamoto, MD, PhD,2 Hidetoshi Takada, MD, PhD,1 Tamami Tanaka, PhD,1 Koichi Ohshima, MD, PhD,3 Ken-Ichi Imadome, PhD,4 Yasunobu Abe, MD, PhD,3 Koichi Akashi, MD, PhD,2 Toshiro Hara, MD, PhD1

Affiliations: 1. Department of Pediatrics, 2. Department of Medicine and Biosystemic Science, 3. Department of Medicine, and Bioregulatory Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, 4. Department of Infectious Diseases, National Research Institute for Child Health and Development, Tokyo, 5. Department of 2nd Pathology, Faculty of Medicine, Kurume University School of Medicine, Kurume, Japan

Correspondence: Shouichi Ohga, MD, PhD. Department of Pediatrics, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. TEL: +81-92-642-5421, FAX: +81-92-642-5435, E-mail: ohgas@pediatr.med.kyushu-u.ac.jp

Key words: Epstein-Barr virus, chronic active Epstein-Barr virus infection, lymphoproliferative disease, CD34+ cells

Running title: Clonal EBV-infected T/NK-cell subsets in CAEBV

**Conflicts of interest:** No conflicts of interest

Number of word count:  
Abstract 200, Text 2654

Number of Tables, Figures and Supplementary figures: 2, 4, 2

Number of references: 32
ABSRTACT

Clonal expansion of Epstein-Barr virus (EBV) infected B-cells occasionally occurs in immunocompromized subjects. EBV-infected T/natural killer (NK)-cells proliferate in patients with chronic active EBV infection (CAEBV) that is a rare mononucleosis syndrome. It is classified into either T-cell type or NK-cell type according to the primary target of infection, while the pathogenesis remains unclear. To search the clonal origin of EBV-infected T/NK-cells, virus distribution and clonotype were assessed by using highly purified cell fractions obtained from 6 patients. Patient 1 had a monoclonal proliferation of EBV-infected T-cell receptor Vδ2/Vγ9-expressing cells, and carried lower copy number of EBV in αβT-cells. Patients 2 and 3 had a clonal expansion of EBV-infected CD4+T-cells, and lower EBV load in CD56+cells. Patients 4, 5 and 6 had an expansion of CD56+cells with higher EBV load than CD3+cells. EBV-terminal repeats were determined as clonal bands in the minor targeted populations of 5 patients. The size of terminal repeats indicated the same clonotype in minor subsets as in major subsets of 4 patients. However, EBV was not detected in bone marrow-derived lineage negative CD34+cells of patients. These results suggested that EBV could infect T/NK-cells at differentiation stage, but spared bone marrow CD34+ hematopoietic stem cells in CAEBV patients.
INTRODUCTION

More than 90% adults are infected with Epstein-Barr virus (EBV), and the infection persists for life. The primary infection often causes acute infectious mononucleosis (IM) in adolescents or young adults. The γ herpes virus enters CD21+B cells, and establishes latent infection in the memory B-cell pools evading immune elimination by EBV-specific cytotoxic T-cells. EBV-infected B-cell lymphoproliferative disease (LPD) occurs in immunocompromized patients. Recently, novel EBV-driven T/natural killer (NK)-cell LPD has been identified in non-immunocompromised hosts such as chronic active EBV infection (CAEBV) and EBV-associated hemophagocytic lymphohistiocytosis (HLH). CAEBV is a rare mononucleosis syndrome typified by high circulating levels of EBV DNA and recurrent IM-like symptoms along with coronary artery lesions, chorioretinitis, and brain involvement. It was first described as a progressive EBV infection targeting B-cells, but most cases are reported from Asia as EBV-infected T/NK-cell LPD. The Asian type CAEBV is classified into T-cell type or NK-cell type according to the target of infection. The NK-cell type might be linked with hypersensitivity to mosquito bite (HMB) and slow progression. However, affected patients with either type result in fetal demise without allogeneic stem cell transplantation (SCT). CD8+ T cells are exclusively targeted in EBV-HLH patients, while the virus spreads over T/NK-cell subsets in CAEBV patients. The cell-type specific infection does not stipulate the transformation or tissue infiltration. Despite the increasing number of reports, the cause of T/NK-cell infection remains unclear.

The episome of EBV DNA has a highly variable reiteration of terminal repeat (TR) sequences that are joined by random recombination upon circularization of the linear genome at entry into cells. As the TR number is inherent in a round of EBV
reactivation and reinfection, the diversification denotes the latently infected cell clones. Nonetheless, cell purity and blotting sensitivity have hampered the precise analysis of CAEBV T/NK-cells. EBV-producing T/NK-cell lines established from patients do not represent the in vivo clonal nature.

In the present study, EBV load and clonality of T/NK-cell subsets were analyzed in CAEBV patients by using the high-accuracy cell sorting. EBV DNA was detected in the major targeted subsets as well as in the other subsets with the evidence of clonotypic infection. These results first implied the infection of T/NK-cell precursors. Whereas, bone marrow (BM)-derived CD34+ cells, supposed to include common lymphoid progenitors, were not involved in the infection. The pathogenesis of CAEBV was discussed with special reference to the cellular origin of EBV infection.

MATERIALS and METHODS

Patients

Six Japanese patients treated in Kyushu University Hospital between 2002 and 2008, were recruited. All fulfilled the diagnostic criteria.11 Clinical profiles are shown in Table 1. Median age at the onset of illness was 3.5 years (range: 1~8). The unique symptoms included HMB (n=4), hydroa vacciniforme (n=4), and coronary artery lesion (n=1). Three patients had abnormal patterns of anti EBV-antibodies (Abs). Five had increased number of activated T-cells and/or high CD4/CD8 ratio. Primary target was T-cell in 3 and NK-cell in 3 patients. Clonality was screened for peripheral blood (PB) mononuclear cells (MNCs) by EBV-TR and immunoglobulin (Ig)/T-cell receptor (TCR) genes. No patients received cytotoxic agents at the time of study. Controls included EBV-seropositive subjects or patients with immune thrombocytopenia.
**Antibodies, cell staining, and sorting**

Mononuclear cells were separated by centrifugation using separation medium (Cappel, Aurora, OH). Each cell fraction was positively selected by immunomagnetic beads with Vario-MACS columns (Miltenyi Biotec, Bergisch Gladbach, Germany) after the staining with monoclonal Abs (mAbs) against CD3, CD4, CD8 and CD56. TCR Vδ2+ cells and TCRαβ+ cells were procured by using MACS anti-FITC or anti-PE microbeads (Miltenyi Biotec) after the staining with FITC-conjugated anti-Vδ2 mAb (Immunotech/Beckman Coulter, Marseille, France), or PE-conjugated TCRαβ mAb. CD56+ cells were positively collected using Lymphocyte Separation column (Miltenyi Biotec, Bergisch Gladbach, Germany), after the depletion of CD3+ cells by Lymphocyte Depleting column. The procedures yielded cell samples with more than 97% of purity, which was determined by the flow cytometry using EPICS XL (Immunotech Coulter [IC], Miami, FL). To raise the maximum purity (>99.9%) of CD3+ cells, Vδ2+ cells, TCRαβ+ cells, and CD34+ cells, all sorting and analyses were performed on FACS Aria (BD Biosciences, Oxnard, PA, USA). CD3+ cells, Vδ2+ cells, and TCRαβ+ cells were positively sorted after staining with each FITC-conjugated mAbs. BM CD34+ cells were sorted as follows; CD34+ cells were enriched from BM cells by using the Indirect CD34 MicroBead Kit (Miltenyi Biotec) as described previously.12 For analyses and sorting, cell samples were stained with Cy5-PE- or PC5-conjugated lineage mixture, including Cy5-PE- or PC5-conjugated anti-CD3, CD4, CD8, CD10, CD20, CD11b, CD14, GPA, FITC-conjugated anti-CD3, CD4 or Vδ2, PE-conjugated anti-Vαβ, CD16, or CD56, allophycocyanin (APC)-conjugated anti-CD34, and nonviable cells were excluded by propidium iodide (PI) staining. CD34+ cells were isolated from BM cells by FACS Aria. Appropriate isotype-matched control mAbs were used to determine the
background staining level in each channel. To avoid any contamination, the second round of sorting was performed routinely with the same sorting gates as the first round. FACS data were analyzed with FlowJo software (Tree Star, Inc.). The re-analyses warranted the consistent purity of > 99.9% procured cells (Supplementary figure 1).

**DNA extraction and real-time PCR for EBV-DNA**

High molecular DNA was extracted from cell samples. DNA was used for PCR and Southern blotting. The real-time PCR for EBV DNA was performed according to the established method. Briefly, TaqMan probe was labeled with the reporter dye molecule at the 5' end, and with the quencher flour at the 3' end via a linker arm nucleotide. Gene dosages were analyzed by ABI PRISM 7700 (PE Biosystems, Foster City, CA). DNA was mixed with TaqMan Universal PCR Master Mix (PE Biosystems), primers, and TaqMan probe. PCR conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 50 cycles at 95 °C for 15 sec, and 60 °C for 1 min. Healthy persons with past EBV infection show <200 copies/ml and <40 copies/μgDNA in PB and MNCs, respectively.

**Southern blotting probed with EBV-tandem repeats (TR) or TCR/Ig genes**

Southern blotting was performed as the established method. Briefly, 5μg of high molecular weight DNA were digested with EcoRI and/or BamHI. The digested DNA was electrophoresed on 0.9% agarose gel and transferred to Byodine-B, which was hybridized with a ^32^P-labeled probe. Clonality of lymphocytes was assessed probed with a 5.2-kb BamHI-EcoRI fragment containing the TR sequence of the EBV genome, and/or TCR/immunoglobulin (Ig) gene probes of Cβ1, Jβ1, Jβ2, Jγ, and JH. Normal control DNA was extracted from MNC of healthy EBV-seropositive adults.

**Gene analysis of γδT-cells**
Genomic DNA was extracted from highly purified PB panTCRγδ positive PB MNCs by the conventional methods. Direct sequencing of PCR product of N-regions was completed for TCRδ-gene and TCRγ-gene. The exon and exon–intron boundary regions of each gene were amplified by PCR, and the products were then subjected to direct sequencing using 3130xl Genetic Analyzer (Life Technologies Corp., CA, USA).

**Immunohistology**

BM clot samples fixed in buffered formalin and embedded in paraffin wax, were immunostained with Abs against CD3, CD4, CD8, CD56, latent membrane protein (LMP1) and EBNA-2 (Dako, Copenhagen, Denmark). For *in situ* hybridization (ISH) to detect EBV-RNA, the samples was hybridized with an oligo-probe (EBER-1 region) and a sense probe (negative control), and then was labeled with digoxigenin as our methods. Briefly, after de-waxing, dehydration and proteinase-K digestion, the sections were hybridized overnight in a solution of 50% formamide containing 5 ng DIG-labeled probes. After washing, detection was accomplished using an avidin-alkaline-phosphatase conjugate.

**RESULTS**

**Target cells and clinical expression**

**T-cell type** Patient (Pt) 1 presented fever and skin eruption. CD4CD8γδ T cells increased to 30% of PB MNC, which expressed Vδ2/Vγ9 *(Figure 1)*. PCR products amplified by Vδ2 or Vγ9 primer indicated clonal proliferation of Vδ2/Vγ9 T-cells. Sequencing of Vγ9-JγP1 junctional transcripts revealed an N-region sequence *(Table 2)*. Vδ2+ cells and TCRαβ+ cells had 4×10^5 and 4×10^3 copies EBV/μgDNA, respectively. Screening blotting of MNC showed a single EBV-TR and TCRγ gene rearrangement.
The clonal expansion of EBV-infected Vδ2/Vγ9 T-cells has continued for 6 years. Pt 2 suffered hydroa vacciniforme. CD3⁺ cells, CD8⁺ cells and CD56⁺ cells carried 2×10⁵, 5×10³ and 9×10³ copies/μgDNA, respectively. Screening blotting indicated diclonal expansion of EBV⁺CD4⁺T-cells. At 25 years of age, she underwent a sibling donor SCT because of cutaneous lymphoma with EBER1⁺ CD4⁺T-cells or CD56⁺ cells. She attained EBV-free remission, but died of graft-versus-host disease. Pt 3 had fever and hepatitis. CD4⁺ cells, CD8⁺ cells and CD56⁺ cells had 3×10⁴, 90 and <40 copies/μgDNA, respectively. He attained remission after an HLA-matched sibling donor BMT.

**NK-cell type**  Pt 4 showed aggressive course with cardiac and enteral involvement. EBV-infected CD56⁺ cells infiltrated vesicular HMB lesions. CD4⁺ cells, CD8⁺ cells and CD56⁺ cells carried 2×10³, 1×10³, and 4×10⁵ copies/μgDNA, respectively. Screening of MNC showed diclonal TR bands. She attained EBV-free remission after cord blood transplantation. Pt 5 had fever and HMB. CD3⁺ cells and CD56⁺ cells carried 3×10⁴ and 8×10⁵ copies/μgDNA, respectively. Skin infiltrations with EBER1⁺ CD4⁺ cells subsided by low dose prednisolone. Pt 6 showed fever and HMB. CD4⁺ cells, CD8⁺ cells and CD56⁺ cells had 8×10³, 4×10⁴, and 4×10⁵ copies/μgDNA, respectively.

**Clonality of EBV-infected subsets**

The clonotype of EBV-infected subsets were assessed by Southern blotting with TR probe for highly purified cell fractions. The blotting sensitivity was estimated to detect more than 0.5% contamination of EBV-infected cells by the add-back experiments (Supplementary figure 2). As shown in Figure 2, TCRαβ cell fraction (>99.9%) showed the same sized single band as Vδ2⁺ cells in Pt 1 after either EcoRI or BamHI digestion (Figure 2). Pt 2 and Pt 3 having high EBV load in CD4⁺ T-cells showed a single TR band in CD56⁺ cells and no detectable band in CD8⁺ T-cells, respectively.
The TR size in CD56⁺ cells of Pt 2 was same as one of diclonal bands in CD3⁺ T-cells. The clonotype in highly purified T-cells was assessed in NK-cell type CAEBV. Clonal TR bands were detected in CD3⁺ cells (>99.5%) in all 3 patients. The diclonal bands and monoclonal band in CD3⁺ cells of Pt 4 and Pt 5 were in the same size as those in the CD56⁺ cells, respectively. On the other hand, in Pt 6, CD3⁺ cells showed a distinct monoclonal band in larger size than CD56⁺ cells (Figure 2). The screening of MNC from Pt 6 showed the same sized TR band as CD56⁺ cells.

**EBV load in BM CD34⁺ cells**

To further assess the cellular origin of EBV-infection, the virus load was re-quantified using highly purified cells. EBV DNA was undetectable in BM derived lin CD34⁺ cells of 5 patients studied, and seropositive controls (Figure 3). Immunohistochemical staining and/or *in situ* hybridization of BM clot samples revealed no detectable CD34⁺ cells positive for LMP1, EBNA or EBER (Figure 4).

**DISCUSSION**

Clonal expansion of EBV-infected T/NK-cells was determined not only in the major targeted subsets but also in the less infected subsets of CAEBV patients. The clonotype of EBV in the minor targeted cells notably coincided with that in the major EBV-infected T/NK-cell fractions. On the other hand, BM-derived CD34⁺ cells did not carry EBV DNA. These suggested that EBV infection could occur at differentiation stages of lymphocytes and then clonally expand to diverse subsets of T/NK-cells, but not involve CD34⁺ hematopoietic stem cells in CAEBV patients.

It has been controversial whether CAEBV is defined as chronic infection or clonal disorder. Diverse subsets of T-cells and/or NK-cells of CAEBV patients carried EBV
DNA as expected. T-cell and NK-cell lines shedding EBV have been established from CAEBV patients.\textsuperscript{15,16} EBER1\textsuperscript{+} T-cells and NK-cells are found in the tonsils of patients with acute IM.\textsuperscript{17} EBV could infect T/NK-cells during the primary infection. If the excessive viral load raises the chance of ectopic infection, the diverse infection in T/NK-cell subsets might be explained by the frequent reactivation and reinfection of lytic virus during the prolonged course of CAEBV. Reactive T-cells oligoclonally expand in CD8\textsuperscript{+} but not CD4\textsuperscript{+} subsets of patients with acute IM.\textsuperscript{18} In the current study, EBV-infected T/NK-cells clonally proliferated in the major targeted populations (non-CD8\textsuperscript{+} T-cells) as well as other subsets of CAEBV patients. The primary targeted cells (V\(\delta\)2 in Pt 1, NK-cells in Pt 2 and Pt 3) and other minor subsets (\(\alpha\beta\)T cells in Pt 1, T-cells in Pt 4 and Pt 6) shared the same sized TR. PB \(\gamma\delta\)T-cells of healthy human consist entirely of polyclonal V\(\delta\)2/V\(\gamma\)9 T-cells, while those of Pt 1 originate from a single EBV-carrying V\(\delta\)2/V\(\gamma\)9 clone. Toyobe \textit{et al.}\textsuperscript{19} reported a CAEBV patient who had biclonal expansion of V\(\beta\)7\textsuperscript{+} and V\(\beta\)9\textsuperscript{+} cells in PB CD4\textsuperscript{+} T-cells. These T-cell clones were primarily targeted by EBV, and the strain was indistinguishable from each other by genotype analyses. Endo \textit{et al.}\textsuperscript{20} analyzed the clonotype of EBV in 2 CAEBV patients who had biphenotypic expansions of CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells. Both T-cell subsets carried the same TR sized clone of EBV with hypermethylated status in the Cp region. Germinal center B-cells retaining latent EBV could be the source of B-cell LPD.\textsuperscript{21} In this line, the diverse T/NK-cell infection in CAEBV patients might not stem from the spread of lytic infection but the differentiation and expansion of EBV-infected T/NK-cells retaining the latent episome. Alternative explanation of the clonotypic virus is that EBV gene was integrated into the host genome.\textsuperscript{22} Proliferating EBV-infected T/NK-cells of CAEBV patients lack malignant morphology. EBV latent genes are
expressed in heterogeneous and restricted patterns in the EBV-infected T/NK-cells of CAEBV patients.\textsuperscript{23} The integration of a specific site of EBV genome is unlikely in CAEBV T/NK-cells. Taken together, it may raise the possibility that EBV infection occurs at an early stage of differentiation, and the clonotypic EBV-infected T/NK-cells enter the circulating lymphocytes. Multiple EBV strains are differently associated with EBV-associated tumors in a single host.\textsuperscript{24} Further study on the site specific clonality might provide a clue to the pathophysiology of CAEBV.

The major concern is how clonal EBV-infected T/NK-cells are allowed in patients. The etiological analogy was inferred between CAEBV and X-linked LPD, while EBV\textsuperscript{+} B-cell LPD arises in the latter. Only a Caucasian patient with the B-cell type CAEBV had defective cytotoxicity due to \textit{PRF1} mutations.\textsuperscript{25} However, no genetic defects have been identified in T/NK-cell type CAEBV or EBV-HLH.\textsuperscript{26,27} Cytotoxic defect has been implicated in CAEBV patients,\textsuperscript{28} though EBV-infected T/NK-cells may have altered cytotoxicity. No familial occurrence was determined in the cases with T/NK-cell type CAEBV reported in Asians and Latin Americans. To the best of our knowledge, in two Japanese pairs of identical twins, one suffered from CAEBV but each counterpart was healthy carrier. The end-to-end length of fused TRs may itself drive clonal selection consequent to the opposing growth advantages and disadvantages of LMP1.\textsuperscript{29} The dynamics of primary infection may provide a clue to the evolution of CAEBV.

CAEBV patients die without successful allogeneic SCT, which eradicates EBV-infected hematopoietic cells and reconstitutes the normal EBV-specific immunity. Based on the clonotypic EBV in CAEBV patients, we hypothesized that CD34\textsuperscript+ hematopoietic stem cells might be targeted by the infection. HIV-1-infected CD34\textsuperscript+ hematopoietic stem cells have been detected in a subpopulation of HIV-positive
patients, ranging from 14 to 36%.\textsuperscript{30} HTLV-1 or HHV8 infects CD34\textsuperscript{+} hematopoietic stem cells,\textsuperscript{31} although those cells appeared not to be the major reservoir in carriers. The current results first indicated that BM CD34\textsuperscript{+} cells were not a niche of EBV infection in CAEBV patients. The primitive hematopoietic stem cells are resistant to HIV-1 infection, although once these cells begin to differentiate and become committed hematopoietic stem cells they become increasingly susceptible to HIV-1 infection and permissive to viral gene expression and infectious virus production.\textsuperscript{32} EBV might target CD34 negative T-cell progenitors prior to the differentiation into αβ T cells and γδ T cells. Quiescent hematopoietic stem cells are resistant to \textit{in vitro} infection of pathogens. Dormant CD34\textsuperscript{+} cells in BM might be protected from infection, or promptly eliminated after infection. CAEBV may be a clonal disease of EBV-infected lymphoid progenitor cells, arising from cytotoxic defect of unknown cause. It explains the rationale of the allogeneic SCT that is necessary for complete recovery of CAEBV patients. Further study on the origin of EBV-infected T/NK-cells may shed some lights not only on the relationship between infection and clonal disease, but also on the therapeutic target of EBV-associated T/NK-cell LPD/lymphoma.

**Acknowledgments:** We thank to Drs. Shima T. (Kyushu Univ., Fukuoka, Japan), Wada M. (Otsuka Assay Lab., Tokyo, Japan), Ogawa Y. and Yamamori S. (Mitsubishi Chem. Med. Corp, Tokyo, Japan) for the quality control of experiments. This work was supported by a Grant-in-Aid for Scientific Research (C2: 60233053) to Ohga S from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

**Potential conflicts of interest.** All authors: no conflicts.
REFERENCES


Clinical and virological characteristics of 15 patients with chronic active Epstein-Barr virus infection treated with hematopoietic stem cell transplantation. *Clin Infect Dis* 2008; **46**: 1525-1534.


Differential chemokine, chemokine receptor and cytokine expression in


29. Repic AM, Shi M, Scott RS, Sixbey JW. Augmented latent membrane protein 1 expression from Epstein-Barr virus episomes with minimal terminal repeats. *J*


FIGURE LEGENDS

Figure 1. Representative flow cytometry for lymphocytes of Patient 1. TCRγδ cells increased to ~30% of peripheral blood lymphocytes, exclusively expressing Vγ9/Vδ2 but negligible expression of HLA-DR. Vγ4- and Vδ3-expressing cells were <1% of lymphocytes. More than 99% of Vδ2⁺ lymphocytes were CD4⁻CD8⁻ T cells.

Figure 2. Southern blotting analysis of high molecular DNA obtained from highly purified cell fractions (>99.5%) probed with the EBV-terminal repeated (TR) sequence. EcoRI (E) or BamHI (B)-digested DNA was electrophoresed (2~5 μg/lane) and hybridized with a 3²P-labeled 5.2-kb BamHI-EcoRI fragment containing the TR repeat sequences. The blotting sensitivity was confirmed to detect >0.5% contamination of EBV-infected cells. Arrow, a clonal band. * non-specific band in each digestion. NC: normal control of healthy human lymphocytes, PC: positive control of Raji cell lines.

Figure 3. Quantification of EBV DNA in highly purified cell fractions (>99.9%) obtained from CAEBV patients assessed by real-time PCR. High molecular DNA was extracted from Vδ2, αβT, CD4, CD3, and CD56-expressing cells fractionated from peripheral blood, and CD34⁺ cells (hatched bar) obtained from bone marrow cells. Solid bars represent the major infected subsets with high EBV load (black bar), and the minor infected subsets with low EBV load (gray bar).

Figure 4. Immunohistochemical staining of bone marrow (BM) cells obtained from Patient 5 and Patient 6. BM clot samples were stained with anti-CD34 antibody using fuchsin as a chromogen for visualization of alkaline phosphatase activity (positive: red cytosol, open arrow), and were then employed for in situ hybridization of EBER1 using diaminobenzidine as a chromogen of peroxidase (positive: brown nucleus, closed arrow). There were no double positive cells for CD34 and EBER1 in BM cells.
Table 1. Clinical profiles of patients with chronic active EBV infection

<table>
<thead>
<tr>
<th>Patient</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>f</td>
<td>f</td>
<td>m</td>
<td>f</td>
<td>m</td>
<td>m</td>
</tr>
<tr>
<td>Age (yrs) at onset, at the study</td>
<td>6, 7</td>
<td>8, 25</td>
<td>2, 5</td>
<td>4, 4</td>
<td>1, 10</td>
<td>3, 8</td>
</tr>
</tbody>
</table>

**Involvement**
- CAL: no no no yes no no
- Enteral: no no no yes no no
- HMB: no yes no yes yes yes
- HV: yes yes no no yes yes

**Anti EBV-Abs**
- VCA-IgG: 160 640 1280 640 160 160
- IgM: <10 <10 <10 <10 <10 <10
- IgA: <10 40 20 <10 <10 <10
- EADR-IgG: <10 160 640 40 10 10
- IgA: na na <10 160 <10 <10
- EBNA: 40 10 40 40 80 80
- EBV-DNA1) PB/MNC: 100/106 300/104 600/na 3000/na 300/105 2000/na

**Major targeted subsets**
- γδT: CD4 CD4 CD56 CD56 CD56 CD56
- CD3/CD19/CD56 (%): 70/28/2 75/18/7 69/24/7 82/5/13 47/17/36 58/15/27
- CD3+HLA-DR+ (%): 14.4 21.5 15.8 14.2 0.6 5.9
- CD4/CD8: 1.6 0.8 5.3 2.0 3.3 2.9

**Clonality2) EBV-TR**
- M D M D M M

**TCR**
- R R na G G G

**Outcome**
- AOD Death ADF ADF AOD AOD
- post-SCT post-SCT post-SCT

---

1) Each value means the copy number of EBV DNA (PB: /ml, MNC: /μgDNA).
2) Clonality was screened by Southern blotting for PB-MNC-derived DNA probed with EBV-TR, TCR, and Ig genes. There was no evidence of clonally proliferating B cells. CAEBV: chronic active Epstein-Barr virus infection, EBV: Epstein-Barr virus, CAL: coronary artery lesion, CNS: central nervous system, HMB: hypersensitivity to mosquito bite, HV: hydroa vacciniforme, VCA: viral capsid antigen, EBNA: EBV nuclear antigen, PB: peripheral blood, MNC: mononuclear cells, TR: terminal repeat, TCR: T-cell receptor, M: monoclonal, D: diclonal, R: rearrangement, G: germ line, AOD: alive on disease, ADF: alive on disease free state, SCT: stem cell transplantation
Table 2. Nucleotide Sequence of Vγ9-JγP1 Junctional Transcripts Expressed by γδT-Cells

<table>
<thead>
<tr>
<th>Vγ9</th>
<th>N region</th>
<th>JγP1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Germline ATT CCG TCA GCC ****TACTGT GCC TTG TGG GAG GTG</td>
<td>ACC ACT GGT TGG ATC</td>
<td></td>
</tr>
<tr>
<td>Vγ9 primer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pt</td>
<td>TGT GCC TTG TGG GAG GTG</td>
<td>CAG</td>
</tr>
<tr>
<td></td>
<td>C A L W E V Q T T G W</td>
<td></td>
</tr>
</tbody>
</table>

The germline sequences of the 3’ end of Vγ9, N region, and the 5’ end of JγP1 gene segments are at the top.
Figure 2
Figure 3

- Pt 1
  - V82
  - αβ T
  - CD34
  - CD4
  - CD3
  - CD56
  - CD34
- Pt 2
- Pt 3
- Pt 5
- Pt 6
- Cont. (n=5)

Log scale: Copies/μgDNA