genome Bioinfomatics (http://www.ncbi.nlm.nih.gov/), and obtained from the BACPAC Resource Center at the Children's Hospital (Oakland Research Institute, Oakland, CA, USA). Clones were ordered from chromosomes 1 to 22 and X within each chromosome on the basis of Ensembl Genome Data Resources from the Sanger Center Institute, February 2004 version. The locations of all the clones used for array CGH were confirmed by fluorescence *in situ* hybridization (FISH). Clone names and their chromosome locations are available on request. The template for degenerate oligonucleotide-primed PCR (DOP-PCR) consisted of 10 ng of BAC (or PAC) DNA. DOP-PCR products were ethanol precipitated and dissolved in DNA spotting solution DSP0050 (Matsunami, Osaka, Japan) and robotically spotted in duplicate onto CodeLink™ activated slides (Amersham Biosciences, Piscataway, NJ, USA) using the inkjet technique by a ceramic actuator (NGK, Nagoya, Japan). Fabrication and validation of the array, hybridization methods and analytical procedures have been described elsewhere in detail (Ota *et al.*, 2004). Briefly, 1 μg of tested (tumor or normal) and of referenced (normal) DNA was digested with *Dpn*II and labeled with the BioPrime DNA labeling system (Invitrogen Life Technologies, Inc., Tokyo, Japan) using Cy3-dUTP and Cy5-dUTP (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for the tested and referenced DNA, respectively. Test and reference DNAs were then mixed with 100 μg of Cot-1 DNA (Life Technologies, Inc., Gaithersburg, MD, USA), precipitated and resuspended in 45 μl of a hybridization solution (50% formamide, 10% dextran sulfate, 2 × SCC, 4% SDS and 100 μg tRNA) and hybridized onto a glass slide. After 48–66 h hybridization, the slide was washed and scanned with an Agilent Micro Array Scanner (Agilent Technologies, Palo Alto, CA, USA) and the acquired array images were analysed with Genepix Pro 4.1 (Axon Instruments, Inc., Foster City, CA, USA). After automatic segmentation of the DNA spots and subtraction of the local background, intensities of the signals were determined. Subsequently, ratios of the signal intensity of two dyes (Cy3 intensity/Cy5 intensity) were calculated for each spot and converted into $\log_2$ ratios on an Excel sheet in the order of chromosomal position. For the array, six simultaneous hybridizations of normal male versus normal male were performed to define the normal variation for the $\log_2$ ratio. A total of 113 clones with less than 10% of the mean fluorescence intensity of all the clones, with the most extreme average test over reference ratio deviations from 1.0 and with the largest s.d. in this set of normal controls were excluded from further analyses. Thus, we analysed a total of 2235 clones (covered 2988 Mb, 1.3 Mb of resolution) for further analysis. Out of 2235, 2176 clones (covered 2834 Mb) were from chromosome 1p telomere to 22q telomere. 59 out of 2235 clones were from chromosome X. Since more than 96% of the measured fluorescence $\log_2$ ratio values of each spot (2 × 2235 clones) ranged from +0.2 to −0.2, the thresholds for the $\log_2$ ratio of gains and losses were set at the $\log_2$ ratios of +0.2 and −0.2, respectively. Regions of low-level gain/amplification were defined as $\log_2$ ratio +0.2 to +1.0, those suggested of containing a heterozygous loss/deletion as $\log_2$ ratio −1.0 to −0.2, those showing high-level gain/amplification as $\log_2$ ratio $> +1.0$, and those suggested of containing a homozygous loss/deletion as $\log_2$ ratio < -1.0.

Southern blot analyses

To detect the target gene of 2q13 loss, probes 1–6 were designed from genomic DNA on BAC 438K19. The length of BAC 438K19 (Accession number: AC096670) is 179 497 bp. Probes 7 and 8 were designed from genomic DNA on BAC 368A17 (1.55 Mb telomeric to BAC438K19) and BAC537E18 (1.85 Mb centromeric to BAC 438K19), respectively. Probes 1–8 used by Southern blot analysis were amplified with the PCR method using eight primer pairs from human placenta DNA. The primer pairs used for PCR were Probe 1 (850 bp): sense (BAC438K19: 30 851–30 874 bp), 5′-ttgcacaagtaaagtggcaattac-3′; antisense (BAC438K19: 31 700–31 677 bp), 5′-atccctgacaactcagcgtttaga-3′, Probe 2 (837 bp): sense (BAC438K19: 34 214–34 237 bp), 5′-acgaatggttatcttacgactgtt-3′; antisense (BAC438K19: 35 050–35 027 bp), 5′-atctatgcatctgagtccagactg-3′, Probe 3 (850 bp): sense (BAC438K19: 49 071–49 094 bp), 5′-taccctccttgcatagtaa gcgtt-3′; antisense (BAC438K19: 49 920–49 897 bp), 5′-tagtgacag cttaatgaaagggca-3′, Probe 4 (811 bp): sense (BAC438K19: 75 127–75 150 bp), 5′-gggtttgtgttgatttgtcacaac-3′; antisense (BAC438K19: 75 937–75 914 bp), 5′-tgctgccctcagcattttcggcaa-3′, Probe 5 (1095 bp): sense (BAC438K19: 80 501–80 524 bp), 5′-ggg tttgtgtt gatttgtcacaac-3′; antisense (BAC438K19: 81 595–81 572 bp), 5′-cc gcgctggagttacaaactctat-3′ and Probe 6 (890 bp): sense (BAC438K19: 177 361–177 384 bp), 5′-cattccccagaaacagatctcgtt-3′; antisense (BAC438K19: 178 250–178 227 bp), 5′-catagcattat-caatgccatcgat-3′. Probe 7 (820 bp): sense (BAC368A17: 34 301–34 320 bp), 5′-ccatagttaatgtacacagc-3′; antisense (BAC368A17: 35 101–35 120 bp), 5′-tcgcaaaccattagga actg-3′. Probe 8 (500 bp): sense (BAC537E18: 191 071–191 094 bp), 5′-ttggagccaaggtaggat-taaaca-3′; antisense (BAC537E18: 191 487–191 570 bp), 5′-ctggag-gaatagtgcttccagatg-3′. Probes 2–4 included an open reading frame of *BIM*. *BIM* (*BIM EL*) has several splice variants such as *BIM L*, *BIM* alpha, *BIM* beta and *BIM* gamma (O'Connor *et al.*, 1998; U *et al.*, 2001; Liu *et al.*, 2002), and the open reading frame of *BIM EL* (597 bp) includes exons of these variants. Probe 4 includes the initiating codon (ATG) of *BIM* (*BIM EL* and *BIM L*), and Probe 2 the termination codon (TGA) of *BIM*. Amplifications were performed on a Thermal Cycler (Perkin-Elmer Corporation, Norwalk, CT, USA). PCR was conducted with the touchdown PCR method described elsewhere (Motegi *et al.*, 2000). Briefly, the reactions consisted of 10 cycles of denaturation (94°C, 0.5 min), annealing (63°C, 0.5 min, 1°C decrease per two cycles), and extension (72°C, 2.5 min), followed by 25 cycles of denaturation (94°C, 0.5 min), annealing (58°C, 0.5 min), and extension (72°C, 2.5 min), and a final extension of 5 min at 72°C. The basic annealing temperature of the reaction ranged from 63 to 58°C. All PCR products were separated by electrophoresis and purified with the QIA Quick™ Gel Extraction Kit (Qiagen). TA cloning to purified PCR products was performed with the aid of pBluescriptII SK (−), and sequenced with the ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems, Foster City, VA, USA). In all, 10 μg of each genomic DNA sample was restriction digested for 16 h with *Bam*H1 (for probes 1 and 6) or *Hin*dIII (for probes 2–4) and electrophoresed on a 0.8% agarose gel in 1 × TBE. Gels were sequentially immersed in 0.25 M HCl for 30 min, 1.5 M NaCl/0.5 M NaOH for 30 min and 0.5 M Tris (pH 7.4)/1.5 M NaCl for 30 min. Electrophoresed DNA was then transferred onto Hybond N^+ membranes (Amersham Pharmacia Biotech, Tokyo, Japan), washed and hybridized overnight at 65°C with [α-^{32}P]-dCTP-labeled probes 1–8. It was then washed, first with 2 × SSC and then with diminishing concentrations of SSC-0.1% *N*-lauryl sarcosine at 65°C, and finally exposed to BioMax™ MS films (EKC, Rochester, NY, USA).

Northern blot analysis

Northern blotting was performed with *BIM EL* cDNA against seven MCL cell lines, Karpas 231 (FCL) and Raji (Burkitt's lymphoma). Probes used for Northern blot analysis were amplified with the RT–PCR method using a primer pair: sense, 5′-atggcaaagcaaccttctgatgta-3′; antisense, 5′-tcaatgcattctccacac-caggcg-3′. cDNA (open reading frame, 597 bp) of *BIM EL* was

generated from fetal brain cDNA. Total cellular RNA (10 μg) was size-fractioned on a 1% agarose/0.66 M formaldehyde gel and transferred onto a Hybond-N$^+$ nylon membrane (Amersham Pharmacia Biotech, Tokyo, Japan). The membranes were then hybridized overnight at 42°C with [α-^{32}P]-dCTP-labeled probes, washed and finally exposed to BioMax™ MS films.

Fluorescence in situ *hybridization*

Interphase chromosomes were prepared from paraffin-embedded sample (G468) and cell lines. Dual-color FISH analysis was conducted as described previously (Nomura *et al.*, 2003; Zhang *et al.*, 2004). Probes used in this experiment were probe A: BAC438K19 (green) and probe B: BAC368A17 (red).

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Generation of tumor-specific, HLA class I–restricted human Th1 and Tc1 cells by cell engineering with tumor peptide–specific T-cell receptor genes

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Tumor antigen–specific CD4+ and CD8+ T lymphocytes, especially interferon-γ (IFN-γ)–producing type-1 helper T (Th1) and type-1 cytotoxic T (Tc1) cells, play a crucial role in tumor eradication. Adoptive transfer using tumor-specific Th1 and Tc1 cells is a promising therapeutic strategy for tumor immunotherapy. However, its clinical application has been hampered because of difficulties in generating tumor-specific Th1 cells from patients with tumors. To overcome this problem, we have developed an efficient method to prepare tumor-specific Th1 and Tc1 cells. T-cell receptor (TCR) α and β genes obtained from an HLA-A24–restricted, Wilms tumor 1 (WT1) peptide–specific Tc clone were lentivirally transduced to polyclonally activated Th1 and Tc1 cells. As expected, TCR gene-modified Tc1 cells showed cytotoxicity and IFN-γ production in response to peptide-loaded lymphoblastoid cell lines, *WT1* gene–transduced cells, and freshly isolated leukemia cells expressing both WT1 and HLA-A24. Surprisingly, we further demonstrated that Th1 cells transduced with HLA-class I–restricted TCR genes also showed both cytotoxicity and cytokine production in an HLA-A24–restricted manner. In contrast to gene-modified Tc1 cells, Th1 cells produced high amounts of interleukin-2 (IL-2) in addition to IFN-γ, which is beneficial for induction of antitumor cellular immunity. Thus, TCR gene–modified HLA-class I–restricted Th1 and Tc1 cells are a powerful strategy for the application to adoptive immunotherapy of human cancer. (Blood. 2005;106:470-476)

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Introduction

It has been shown that tumor-specific type-1 immunity, which is controlled mainly by tumor antigen–specific type-1 helper T (Th1) and type-1 cytotoxic T (Tc1) cells, play a critical role in tumor eradication.[1-3] Tumor-specific Tc1 cells can directly destroy tumor cells when they recognize tumor antigenic peptide bound by major histocompatibility complex (MHC) class I molecules. On the other hand, Th1 cells produce interleukin-2 (IL-2) and interferon-γ (IFN-γ) by recognition of antigenic peptide presented on MHC class II molecules. Although few tumor cells express MHC class II molecules essential for Th cell activation, it is widely accepted that Th cells, especially Th1 cells, play an important role in eradication of tumor cells via cellular immunity.[4-6] This may be because tumor-specific Th1 cells provide local help to enhance antitumor cellular immunity by interacting with tumor antigen-bound MHC class II molecules on antigen-presenting cells (APCs) such as dendritic cells (DCs). In addition, direct interaction of Th cells with MHC class II molecules expressed on tumor cells may result in stronger induction of tumor-specific Tc cells.[7-9]

Many tumor-associated antigens derived from various types of tumors have been characterized.[10,11] The identification of tumor-associated peptides, which are bound by MHC class I molecules and recognized by CD8+ Tc cells, make it possible to induce tumor-specific Tc cells from peripheral blood mononuclear cells (PBMCs) of healthy donors as well as patients with tumors. In contrast to Tc cells, tumor antigen–specific Th cells can be generated only for a limited number of patients with cancer because HLA class II–binding peptides were generally determined for only a few human leukocyte antigen (HLA) types such as DRB1*0401. Moreover, there are significant difficulties in the generation and propagation of tumor-specific Th cells from patients with tumors. Therefore, it is critically important to develop an efficient method to prepare tumor-specific Th1 cells in vitro.

Recently, it has been shown that antigen specificity can be transferred to nonspecific T cells by transducing both T-cell receptor (TCR) α and TCR β genes obtained from antigen-specific T cells.[12-19] In most experiments, MHC class I–restricted TCR genes, which were obtained from MHC class I–restricted antigen-specific Tc cells, were transduced into nonspecific Tc cells.[12-17] Some investigators also reported successful transduction of MHC class II–restricted TCR genes to nonspecific Th cells to transfer antigen specificity.[18,19] Recently, we have established an efficient method to generate antigen-specific mouse Th1 cells from polyclonally activated Th1 cells by transducing MHC class II–restricted TCR genes. We also demonstrated that the gene-modified, antigen-specific Th1 cells exhibited potent antitumor activity both in vitro and in vivo.[19] Here, we tried to extend our findings to establish a

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method for preparing human tumor–specific Th1 cells. However, it is difficult to induce tumor-specific Th cells, which are an essential source for preparing MHC class II–restricted TCR genes, because few MHC class II–binding tumor peptides have been identified. To overcome this problem, we developed an alternative approach for preparing tumor-specific Th1 cells. Our working hypothesis is that transfer of tumor antigen specificity into nonspecifically activated Th cells from patients by gene transduction with MHC class I–restricted TCR genes is an effective method for preparing tumor antigen–specific Th1 cells from many patients. Our working hypothesis is based on the following evidence: (1) it is easy to induce MHC class I–restricted tumor-specific Tc cells, because hundreds of MHC class I–binding tumor peptides from a variety of tumor cells have been identified; (2) MHC class I–restricted TCR genes recognizing tumor-antigen peptide bound by various HLA genotypes are available from these Tc clones; and (3) it has already been shown that MHC class I–restricted tumor antigen specificity can be transferred to $CD4^+$ T cells as well as to $CD8^+$ T cells.[20]

In the present study, we developed a novel strategy to induce both tumor-specific Th1 and Tc1 cells by lentiviral transduction of HLA-A24–restricted TCR α and β chain genes isolated from a WT1-specific Tc clone. TCR gene-transduced Th1 and Tc1 cells, expressing tumor-specific TCR complex on the cell surface, exhibited both cytotoxicity and cytokine production in response to WT1 tumor peptide–pulsed HLA-$A24^+$ lymphoblastoid cell lines (LCLs) and freshly isolated HLA-$A24^+$ $WT1^+$ leukemia cells. Thus, it is feasible to prepare human tumor–specific Th1 cells from polyclonally activated Th cells if we obtain MHC class I–restricted TCR genes. We believe that our established method will contribute to the development of a novel tailor-made immunotherapy of human tumors using gene-modified tumor-specific Th1 and Tc1 cells.

Materials and methods

Construction of lentiviral vector containing WT1-specific TCR genes

The generation and characterization of the HLA-A24–restricted WT1 peptide (CMTWNQMNL)–specific Tc clone, TAK-1, were described previously.[21,22] Total RNA of TAK-1 was extracted by using Isogene reagent (Nippon Gene, Tokyo, Japan) and converted to cDNA by reverse transcription using oligo-dT primer and superscript II reagent (Invitrogen, Carlsbad, CA). 5′-rapid amplification of cDNA ends–polymerase chain reaction (RACE-PCR) was performed by SMART RACE cDNA Amplification Kit (Clontech Laboratories, Palo Alto, CA) using gene-specific antisense primers for the constant region of TCR α and β chain genes. PCR products were inserted into pCR II-TOPO vector (Invitrogen, Carlsbad, CA) and sequenced. Coding regions of TCR α and β chain genes were amplified with primers containing restriction enzyme sites and inserted between *Bst*XI and *Xba*I sites and *Eco*RI and *Not*I sites, respectively, of a lentiviral self-inactivating vector (CSII-EF-MCS-IRES-hrGFP).[23,24] A schematic of the lentivirus plasmid vector carrying TCR α and β chain genes (CSII-β-IRES-α) is shown in Figure 1. Other constructs used to produce the lentivirus vector have been described elsewhere.[25]

Production of recombinant lentivirus particles

Production of lentivirus vectors was carried out as described previously.[23-25] Briefly, 293T cells were transfected with CSII-β-IRES-α together with packaging construct (pMDLg/p RRE), REV expression construct (pRSV-rev), and envelope construct (pMD.G) by the calcium phosphate method. After 16 hours, culture medium was replaced with fresh medium containing 10 μM forskolin (Sigma, St Louis, MO), and the cells were incubated for 48 hours. Lentivirus containing supernatant was cleared by 0.45-μm filter and concentrated to about 1/100 by ultracentrifugation. The concentrated lentivirus stock was stored at −80°C until use.

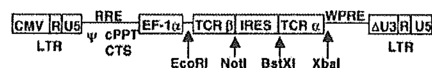

Figure 1. Schematic of a self-inactivating vector carrying WT1-specific TCR α and β chain genes. The coding region of WT1-specific TCR genes was obtained from cDNA of the WT1-specific Tc clone, TAK-1, by a 5′-RACE–PCR method. Genes encoding WT1-specific TCR α and β chains were inserted into self-inactivating lentivirus vector (CS II-MCS-IRES-GFP) as described in "Materials and methods," and CS II-β-IRES-α was constructed. Ψ indicates packaging signal; RRE, rev responsive element; cPPT, central polypurine tract; CTS, central termination sequence; EF-1α, human elongation factor 1α subunit gene promoter; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element; LTR, long terminal repeat; and IRES, internal ribosome entry site.

Generation of TCR gene–transduced Th1 and Tc1 cells

PBMCs were isolated from healthy donors by density gradient using Ficoll-Paque reagent (Amersham Biosciences AB, Uppsala, Sweden) and were activated with 20 μg/mL phytohemagglutinin-P (PHA; Honen, Tokyo, Japan). After 36 hours, cells were harvested, washed, and infected with lentivirus vector on a 96-well plate precoated with both RetroNectin (25 μg/mL; Takara, Ohtu, Japan) and anti-CD3 monoclonal antibody (mAb; 5 μg/mL; Pharmingen, San Diego, CA). Cells were expanded for about 10 days, and a fraction of the cells was stained with phycoerythrin (PE)–conjugated anti-CD4 or anti-CD8 mAb (Nichirei, Tokyo, Japan) together with fluorescein isothiocyanate (FITC)–conjugated Vβ5.1 mAb (Immunotech, Marseille, France) to determine transduction efficiency. Fluorescence intensity of the cells was measured by a FACSCalibur instrument and analyzed by CellQuest software (BD Biosciences, San Jose, CA). The remaining cells were stained with PE-conjugated anti-CD4 mAb and FITC-conjugated anti-CD8 mAb and sorted into $CD4^+$ and $CD8^+$ T cells by a FACSVantage instrument (BD Biosciences). In some experiments, sorted $CD4^+$ and $CD8^+$ cells were restimulated by LCLs pulsed with WT1 peptide and expanded. To polarize cells into type-1 T cells, interleukin-12 (IL-12; 50 U/mL; kindly donated from Genetics Institute, Cambridge, MA), IFN-γ (10 ng/mL; PeproTech, Rocky Hill, NJ), IL-2 (100 U/mL; kindly donated from Shionogi Pharmaceutical Institute, Osaka, Japan), and anti–IL-4 mAb (5 μg/mL; Pharmingen) were added for 10 days.

Evaluation of antitumor activity

LCLs with various combinations of HLA types were generated and maintained in our laboratory. HLA-$A24^+$ LCLs, which express WT1, were generated by retroviral transduction of *WT1* gene. Reverse transcription (RT)–PCR analysis showed that *WT1* gene–transduced LCLs expressed strong level of WT1 mRNA, while their parental LCLs showed no WT1 mRNA expression (data not shown). Primary leukemia cells were obtained from patients with leukemia by a density gradient method. Antitumor activity of TCR gene-transduced Th1 and Tc1 cells was investigated against HLA-$A24^+$ LCLs pulsed with 10 μg/mL WT1 peptide (CMTWNQMNL), which binds to HLA-A24 and is recognized by the TAK-1 Tc clone. HLA-A24 binding peptide derived from human cytomegalovirus (CMV) pp65 protein (QYDPVAALF)[26] was used as control irrelevant peptide. Cytotoxicity against peptide-loaded or unloaded LCLs or leukemia cells was measured by 4-hour ^{51}Cr-release assay. To evaluate antigen-specific cytokine production, 1×10^5 TCR gene-transduced or control T cells were cocultured with 5×10^4 stimulator cells for 20 hours. Cytokine levels in the supernatant were measured by enzyme-linked immunosorbent assay (ELISA). In some experiments, 10 μg/mL mAb against HLA-A2 (BB7-2) or -A24 (A11.1M) were added to determine MHC restriction.

Approval was obtained from the institutional review board of the Institute for Genetic Medicine, Hokkaido University, Hokkaido University School of Medicine, and Ehime University School of Medicine for these studies. Informed consent was provided according to the Declaration of Helsinki.

Results

Lentiviral transduction of TCR genes into T cells

PBMCs from healthy donors were nonspecifically activated with PHA and plate-bound anti-CD3 mAb under type-1 T-cell–inducing conditions (IL-12, IFN-γ, IL-2, and anti–IL-4 mAb) and infected with a lentivirus carrying HLA-A24–restricted WT1-specific TCR α and β chain genes. The subtypes of the variable region of the transduced TCR α and β chain genes were determined as Vα30 and Vβ5.1, respectively, from their DNA sequences. Cells were expanded for 10 days under type-1 conditions, and expression of the transduced TCR β chain was determined by staining with FITC-conjugated anti-Vβ5.1 mAb to evaluate transduction efficiency. As shown in Figure 2A, the percentage of Vβ5.1 expressing $CD4^+$ and $CD8^+$ T cells was remarkably increased after TCR gene transfer. Transduction efficiency was estimated at about 70% in $CD4^+$ T cells and 60% in $CD8^+$ T cells. Expression of Vβ5.1 and Vα30 mRNA was also investigated by RT-PCR using $CD4^+$ and $CD8^+$ T cells isolated from control or TCR gene–transduced T cells (Figure 2B). Consistent with the intensity of cell-surface expression of TCR Vβ5.1, the mRNA expression levels of TCR Vβ5.1 were increased in TCR gene–transduced Th1 and Tc1 cells compared with control cells. We also confirmed that TCR gene–transduced Th1 and Tc1 cells show increased levels of Vα30 mRNA expression compared with control cells. Expression of WT1 peptide–specific TCR complex was investigated by staining with HLA-A24 tetramer loaded with WT1 peptide, which reacted to the original HLA-A24–restricted WT1-specific CTL clone, TAK-1 (data not shown). As shown in Figure 2C, TCR gene–transduced Tc1 cells were stained with WT1 tetramer, demonstrating that the transduced TCR α and β chains recognized HLA-A24–restricted WT1 peptide as well as native TCR complex. On the other hand, such positive staining was not observed in TCR gene–transduced Tc1 cells treated with HLA-A24 tetramer loaded with unrelated HLA-A24–binding peptide derived from HIV envelope (Figure 2C). Control Tc1 cells were not stained with WT1 or HIV tetramer (data not shown). These data indicated that class I–restricted TCR α and β chain genes of the WT1-specific Tc clone were successfully expressed by nonspecific Th1 and Tc1 cells by lentiviral transduction of TCR genes.

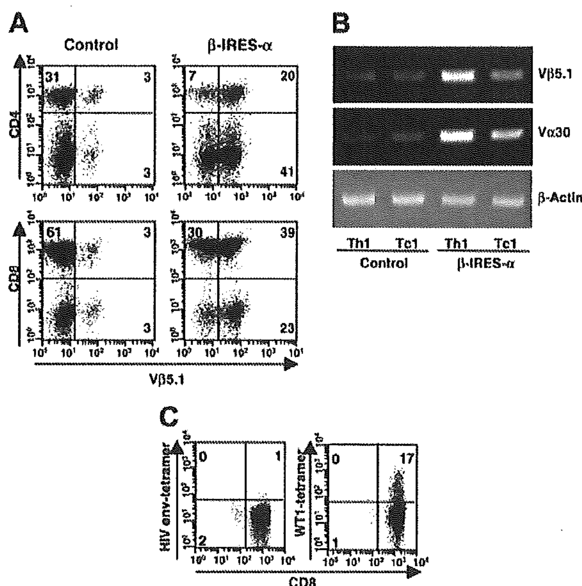

Figure 2. Expression of transduced WT1-specific TCR. PBMCs were activated by PHA and plate-bound anti-CD3 mAb in the presence of type-1–inducing conditions and infected with a lentivirus carrying WT1-specific TCR α and β chain genes or mock virus. Ten days after infection, expression of the transduced TCR (Vα30 and Vβ5.1) was examined. (A) Cells were stained with FITC-conjugated anti-TCR Vβ5.1 mAb and either PE-conjugated anti-CD4 or anti-CD8 mAb. Fluorescence intensity of the cells was measured by a FACSCalibur instrument and analyzed by CellQuest software. (B) After isolation of CD4+ and CD8+ T cells by a FACSVantage instrument, mRNA was extracted and converted to cDNA by reverse transcription. TCR Vα30 and Vβ5.1 cDNA were amplified by PCR, separated on 1% agarose gel, and visualized with ethidium bromide. (C) TCR gene–transduced Tc1 cells were stained with PE-conjugated HLA-A24 tetramer loaded with WT1 peptide or HIV envelope peptide. Then, cells were stained with FITC-conjugated anti-CD8 mAb. Staining profile of the cells was measured by a FACSCalibur instrument and analyzed by CellQuest software. In panels A and C, percentage of cells in each quadrant is indicated in cytometer plots.

WT1 peptide–specific cytotoxicity mediated by TCR gene–transduced Th1 and Tc1 cells

To investigate WT1-specific reactivity of TCR gene–transduced $CD4^+$ Th1 and $CD8^+$ Tc1 cells, $CD4^+$ Th1 and $CD8^+$ Tc1 cells were enriched from heterogeneous T-cell populations, which had been transduced with WT1-specific TCR genes and expanded under type-1 T-cell–inducing conditions. Fluorescence-activated cell sorter (FACS)–isolated Th1 and Tc1 cells were examined for their CD4 and CD8 expression as shown in Figure 3A before functional assay. The purity of $CD4^+$ Th1 and $CD8^+$ Tc1 cells was greater than 98% in all experiments. Then, the ability of the cells to lyse WT1-expressing target cells was determined by ^{51}Cr-release assay. TCR gene–transduced Tc1 cells showed strong cytotoxicity against WT1 peptide–pulsed HLA-$A24^+$ LCLs but not irrelevant HLA-A24 binding CMV peptides pulsed or peptide–unloaded LCLs (Figure 3B). Control Tc1 cells exhibited no significant cytotoxicity against WT1 peptide–pulsed LCLs. These results indicated that TCR gene–modified Tc1 cells exhibit cytotoxicity against peptide–loaded LCLs in a WT1 peptide–specific manner. TCR gene–transduced Tc1 cells from HLA-$A24^+$ donors could respond to both autologous and allogeneic WT1 peptide–pulsed HLA-$A24^+$ LCLs with similar efficacy. Moreover, HLA-$A24^+$ and HLA-$A24^-$ TCR gene–transduced Tc1 cells showed similar levels of cytotoxicity against HLA-$A24^+$ LCLs pulsed with WT1 peptide (data not shown), indicating that cytotoxicity was mediated by the transduced TCR but not by the endogenously expressed TCR.

In addition to Tc1 cells, Th1 cells also showed detectable cytotoxicity against HLA-$A24^+$ WT1 peptide–loaded LCLs when they were transduced with WT1-specific TCR genes (Figure 3B). Cytotoxic activity of TCR gene–transduced Th1 cells was also WT1 peptide specific and restricted to HLA-A24 because they were unable to respond to CMV peptides pulsed or unpulsed LCLs (Figure 3C-D) or HLA-$A24^-$ LCLs loaded with WT1 peptide (data not shown). HLA-A24–restricted cytotoxicity of TCR gene–modified Th1 and Tc1 cells was further confirmed by a blocking assay using HLA-A24–specific mAb (Figure 3E). Cytotoxic activity of TCR gene–transduced Th1 and Tc1 cells against WT1 peptide–pulsed HLA-$A24^+$ LCLs was measured in the presence or absence of anti–HLA-A24 mAb or control anti–HLA-A2 mAb. Cytotoxic activity of TCR gene–transduced Tc1 cells was inhibited by the addition of anti–HLA-A24 mAb but not with anti–HLA-A2 mAb (Figure 3E). Similarly, the cytotoxic activity of TCR gene–transduced Th1 cells was also blocked by anti–HLA-A24 mAb but not by anti–HLA-A2 mAb (Figure 3E).

Cytotoxic activity of TCR gene–transduced Th1 and Tc1 cells against naturally processed WT1 peptide was demonstrated using HLA-$A24^+$ LCLs retrovirally transduced with *WT1* gene as target

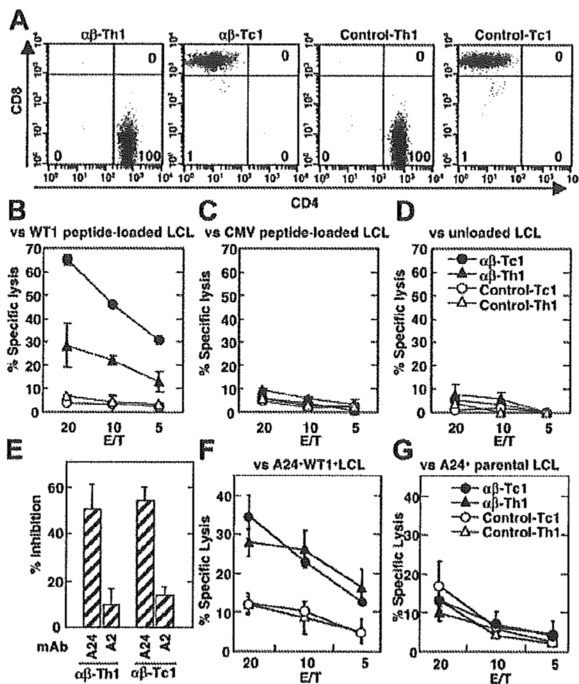

Figure 3. HLA-A24–restricted antitumor activity of TCR gene–transduced Th1 and Tc1 cells against WT1 peptide–loaded LCLs. (A) WT1-specific TCR α and β chain genes were lentivirally transduced to nonspecific Th1 and Tc1 cells obtained from an HLA-A24+ healthy donor as described in "Materials and methods." Ten days after infection, TCR gene–transduced and control T cells were stained with FITC-labeled anti-CD4 mAb and PE-labeled anti-CD8 mAb and sorted to CD4+ and CD8+ T cells by a FACSVantage instrument. After isolation, staining profile of Th1 and Tc1 cells was analyzed by FACSCalibur instrument and Cell Quest software. Percentage of cells in each quadrant is indicated in flow cytometer plots. (B-D) Cytotoxic activity of TCR gene–transduced and control Th1 and Tc1 cells against WT1 peptide–pulsed (B), CMV peptide–pulsed (C), or unloaded (D) LCLs (TAK-LCL, which are derived from an HLA-A24+ patient with leukemia) was evaluated by 4-hour ^{51}Cr-release assay. Nonspecific cytotoxicity of Th1 and Tc1 cells against HLA-A24− LCLs was less than 10%. (E) Blocking of cytotoxicity against WT1 peptide–pulsed HLA-A24+ LCLs by anti–HLA-A2 and anti–HLA-A24 mAbs was evaluated at an effector-to-target (E/T) ratio of 20 for Tc1 cells and E/T of 40 for Th1 cells. The percentage of inhibition was calculated by the following formula; % Inhibition = (% cytotoxicity with mAb) / (% cytotoxicity without mAb) × 100. Percentage of cytotoxicity of TCR gene–transduced Tc1 and Th1 against WT1 peptide–loaded LCL was 77% and 57%, respectively. Background cytotoxicity of Tc1 and Th1 against unloaded LCLs was 9% and 13%, respectively. (F-G) Cytotoxic activity of TCR gene–transduced and control Th1 and Tc1 cells against HLA-A24+ LCLs derived from healthy donors and retrovirally transduced with *WT1* gene (F) and their parental A24+ LCLs (G) was evaluated by 4-hour ^{51}Cr-release assay. Similar results were obtained using TCR gene–transduced Th1 and Tc1 cells derived from 2 other HLA-A24+ and 2 HLA-A24− healthy volunteers. Error bars indicate standard error (SE) in triplicate samples.

cells. Both TCR gene–transduced Th1 and Tc1 cells showed cytotoxic activity against *WT1* gene–transduced LCLs (Figure 3F) but not parental *WT1*-negative LCLs (Figure 3G). Control Th1 and Tc1 cells showed significant cytotoxicity against neither *WT1* gene–transduced LCLs nor their parental LCLs (Figure 3F-G). Thus, these results clearly demonstrate that the reactivity of the WT1-specific CD8+ Tc clone was successfully transferred into CD4+ Th1 and CD8+ Tc1 cells by transferring TCR genes.

Cytokine production by TCR gene–engineered Th1 and Tc1 cells in response to WT1 peptide–pulsed HLA-A24+ LCLs

Next, we investigated the antigen-specific cytokine production of TCR gene–transduced Th1 and Tc1 cells. When WT1-specific TCR gene–transduced Tc1 cells were cocultured with HLA-A24+ LCLs loaded with WT1 peptide, high levels of IFN-γ were detected in the culture supernatants (Figure 4A). The supernatants of TCR gene–transduced Tc1 cells cocultured with control CMV peptide–loaded or unloaded LCLs contained undetectable IFN-γ, showing that IFN-γ production from TCR gene–transduced Tc1 cells was WT1 peptide specific. Likewise, TCR gene–transduced Th1 cells produced high levels of IFN-γ in response to LCLs loaded with HLA-A24–binding WT1 peptide. Such IFN-γ production by TCR gene–modified Th1 cells was not induced by stimulation with unloaded LCLs (Figure 4A). Interestingly, in contrast to TCR gene–transduced Tc1 cells, TCR gene–transduced Th1 cells also produced high levels of IL-2 when cocultured with WT1 peptide–loaded LCLs (Figure 4B). In contrast, TCR gene–transduced Th1 and Tc1 cells produced little IL-2 in response to control CMV peptide–pulsed or peptide–unloaded HLA-A24+ LCLs (Figure 4B). IL-4 was not detected in the culture supernatants of TCR gene–transduced Th1 and Tc1 cells, which indicated that TCR gene–modified Th1 and Tc1 cells were successfully polarized into type-1 T cells in terms of cytokine production pattern (data not shown). TCR gene–transduced Th1 and Tc1 cells with similar reactivity were induced from both HLA-A24+ and HLA-A24− donors (data not shown). TCR gene–transduced Th1 and Tc1 cells prepared from HLA-A24+ donors produced similar level of cytokines against autologous and allogeneic WT1 peptide–loaded HLA-A24+ LCLs. Moreover, they produced cytokines in response to stimulation with WT1 peptide–pulsed HLA-A24+ LCLs but not with peptide–pulsed HLA-A24− LCLs (data not shown). Therefore, the capability of these cells to produce cytokines in response to WT1 peptide was mediated by the transduced genes, encoding the HLA-A24–restricted, WT1-specific TCRs.

Cytotoxicity of, and cytokine production by, TCR gene–transduced Th1 and Tc1 cells in response to leukemia cells

Finally, we examined whether TCR gene–transduced Th1 and Tc1 cells exhibit antitumor activity against freshly isolated leukemia cells that present naturally processed WT1 peptide on MHC molecules. Freshly isolated leukemia cells were examined for their WT1 expression by the real-time PCR method, and all leukemia cells used in this experiment were found to show a strong WT1 mRNA expression (see the legend for Figure 5B-G), as reported previously.[21] The FACS-sorted TCR gene–transduced Th1 and Tc1 cells were significantly expanded by WT1 peptide–loaded LCLs without changing their purity (Figure 5A). On the other hand,

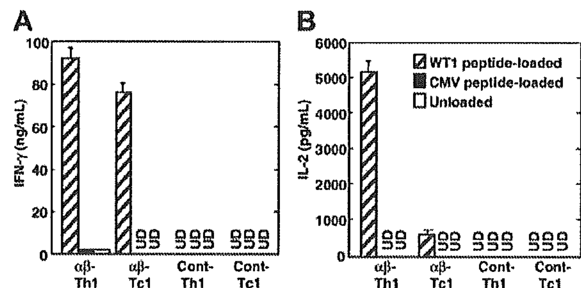

Figure 4. WT1 peptide–specific cytokine production by TCR gene–transduced Th1 and Tc1 cells. TCR gene–transduced Th1 and Tc1 cells (1 × 10^5), obtained from nonspecific Th1 and Tc1 cells of the same healthy donor of T cells used in the experiments of Figure 3, were cocultured with WT1 peptide–pulsed, CMV peptide–pulsed, or unloaded HLA-A24+ LCLs (TAK-LCL) (5 × 10^4). After 20 hours, supernatants were harvested from culture, and their IFN-γ (A) and IL-2 levels (B) were determined by ELISA. UD represents undetected. Similar results were obtained using TCR gene–transduced Th1 and Tc1 cells derived from 2 other HLA-A24+ and 2 HLA-A24− healthy volunteers. Error bars indicate standard error (SE) in triplicate samples.

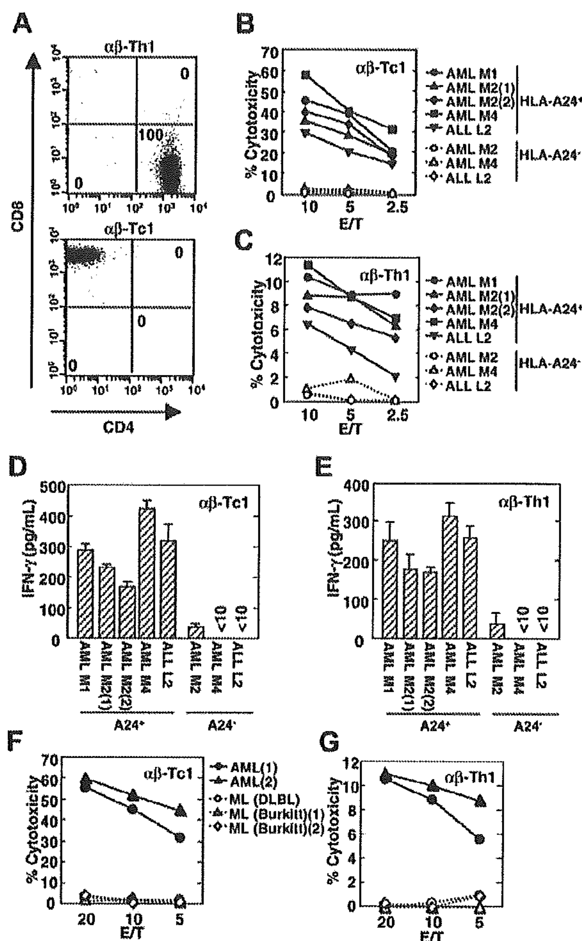

Figure 5. HLA-A24–restricted cytotoxicity of, and IFN-γ production by, TCR gene–transduced Tc1 and Th1 cells against freshly isolated leukemia cells. TCR gene–transduced CD8+ Tc1 and CD4+ Th1 cells were isolated by a FACSVantage instrument restimulated with WT1 peptide (CMTWNQMNL)–pulsed HLA-A24+ LCLs and expanded in the presence of IL-2. (A) After expansion, TCR gene–transduced Th1 and Tc1 cells were stained with FITC-labeled anti-CD4 mAb and PE-labeled anti-CD8 mAb, and the staining profile of Th1 and Tc1 cells was analyzed by a FACSCalibur instrument and Cell Quest software. Percentage of cells in each quadrant is indicated in flow cytometer plots. (B-C) Cytotoxic activity of TCR gene–transduced Tc1 (B) and Th1 cells (C) against freshly isolated leukemia cells was evaluated by 4-hour ^{51}Cr release assay. (D-E) TCR gene–transduced Tc1 (D) and Th1 cells (E) were cocultured with leukemia cells. After 20 hours, culture supernatant was harvested, and IFN-γ levels in the supernatant were measured by ELISA. WT1 expression level of leukemia cells used in the experiments of Figure 5B-E was determined by quantitative real-time PCR to be HLA-A24+ AML M1 (3.5×10^{-1}), AML M2[1] (2.7×10^{-1}), AML M2[2] (8.6×10^{-2}), AML M4 (8.8×10^{-1}), ALL L2 (5.3×10^{-1}), HLA-A24- AML M2 (1.5×10^{0}), AML M4 (2.5×10^{-1}), and ALL L2 (5.8×10^{-1}). Expression levels were expressed as relative values against K562 cells which strongly express WT1. Error bars indicate standard error (SE) in triplicate samples. (F-G) Cytotoxic activity of TCR gene–transduced Tc1 (F) and Th1 cells (G) against HLA-A24+ WT1+ freshly isolated leukemia cells and HLA-A24+ WT1- malignant lymphoma (ML) cells was evaluated by 4-hour ^{51}Cr release assay. WT1 expression level of leukemia and lymphoma cells used in the experiments of Figure 5F-G was determined to be AML[1] (1.5×10^{0}), AML[2] (7.2×10^{-1}), ML (DLBL) (7.3×10^{-5}), ML (Burkitt)[1] (3.5×10^{-4}), and ML (Burkitt)[2] (7.8×10^{-5}). AML indicates acute myeloid leukemia; ALL, acute lymphoid leukemia; DLBL, diffuse large B cell lymphoma; and Burkitt, Burkitt lymphoma.

control Th1 and Tc1 cells could not be expanded by stimulation with WT1 peptide–loaded LCLs (data not shown). Th1 and Tc1 cells, transduced with WT1-specific TCR genes and stimulated with WT1 peptide–loaded allogeneic LCLs, showed no significant cytotoxicity against HLA-A24+ LCLs used as stimulator cells (data not shown). Thus, alloreactive T cells were not generated by stimulation with WT1 peptide–pulsed allogeneic LCLs. Tc1 cells, transduced with WT1-specific TCR genes and stimulated with antigenic peptide, exhibited strong cytotoxicity against HLA-A24+ but not HLA-A24− leukemia cells (Figure 5B). Consistent with the cytotoxic activity of TCR gene–transduced Tc1 cells, they also produced significant levels of IFN-γ in response to HLA-A24+ freshly isolated leukemia cells (Figure 5D). Such strong IFN-γ production was not induced by coculture with HLA-A24− leukemia cells.

As compared with TCR gene–transduced Tc1 cells, TCR gene–transduced Th1 cells showed weak but significant cytotoxicity against freshly isolated leukemia cells in an HLA-A24–restricted manner (Figure 5C). The cytotoxicity of Th1 cells was apparently lower than that of Tc1 cells. Lower cytotoxicity of Th1 cells is considered to be due to an inherent property of CD4+ Th cells. As shown in Figure 5E, TCR gene–transduced Th1 cells produced similar levels of IFN-γ as Tc1 cells in response to freshly isolated HLA-A24+ but not HLA-A24− leukemia cells. Thus, HLA class I–restricted Th1 cells engineered with TCR genes efficiently recognize antigen peptide on leukemia cells. WT1-specific cytotoxicity and IFN-γ production was considered to be mediated by transduced WT1-specific, HLA-A24–restricted TCRs because we succeeded in preparing HLA-A24–restricted WT1-specific T cells from naive T cells from HLA-A24–negative donors. In addition, control nontransduced T cells could not be expanded by stimulation with WT1 peptide–loaded LCLs.

To demonstrate whether TCR gene–transduced Th1 and Tc1 cells surely exert WT1-specific antileukemia activity, we next examined their antitumor activity against WT1− malignant lymphoma cells as control target cells. As shown above (Figure 5B-C), both TCR gene–modified Th1 and Tc1 cells showed a significant cytotoxicity against WT1 expressing freshly isolated AML cells (Figure 5F-G). In contrast, they showed no detectable cytotoxicity against HLA-A24+ malignant lymphoma cells (Figure 5F-G). Consistently, IFN-γ was not detected in the supernatant of TCR gene–modified Th1 and Tc1 cells cocultured with HLA-A24+ lymphoma cells (data not shown). Thus, antitumor activity by TCR gene–transduced Th1 and Tc1 cells against leukemia cells is demonstrated to be WT1 specific. In summary, we demonstrated that HLA-A24–restricted tumor-specific responsiveness of a Tc clone can be successfully transferred into both nonspecifically activated Th1 and Tc1 cells by TCR gene transduction.

Discussion

In the present paper, we have tested an alternative strategy for preparation of tumor-specific Th1 and Tc1 cells by transferring MHC class I–restricted tumor antigen–specific TCR genes. We have previously demonstrated a critical role of both tumor-specific Th1 cells and tumor-specific Tc1 cells for complete rejection of an established tumor mass in animal models.[2,3,19,27] It is now generally accepted that tumor-specific CD4+ Th cells, in addition to CD8+ Tc cells, play an important role in tumor rejection in both animal models and humans.[2-6,27-30] However, vaccination with both HLA class I and class II binding tumor-specific peptides to patients with cancer was ineffective for inducing simultaneous activation of antitumor Th and Tc cells.[31] This is possibly due to strong immunosuppression and the existence of CD4+CD25+ regulatory T cells in tumor-bearing hosts.[32-35] Therefore, it is important to develop a more efficient vaccination protocol to activate both tumor-specific Th and Tc cells for effective induction of antitumor immunity in tumor patients.

Adoptive transfer of tumor antigen–specific Th and Tc cells expanded ex vivo represents a promising strategy to treat patients with

cancer.[36,37] However, generation of tumor-specific Th cells from patients with tumors is very difficult because few tumor-specific peptides that bind with HLA class II have been identified so far.[38,39] To develop a novel method to generate both tumor antigen–specific Th1 and Tc1 cells, we investigated the antitumor activity of gene–modified Th1 and Tc1 cells transduced with HLA class I–restricted TCR genes. By using a highly efficient lentiviral gene transfer system, TCR gene transduction to nonspecifically activated Th1 and Tc1 cells resulted in marked surface expression of the transduced TCR (Figures 1-2). These TCR gene–transduced Th1 and Tc1 cells responded to WT1 peptide antigen restricted by the same HLA molecule of the original Tc clone (Figures 3-4). Moreover, TCR gene–modified tumor-specific Th1 and Tc1 cells demonstrated antitumor activity in response to *WT1* gene–transduced LCLs or HLA-A24$^+$ leukemia cells, which were expressing naturally processed WT1 peptide on MHC molecules (Figure 3F-G and Figure 5B-G). The recognition of leukemia cells by TCR gene–transduced Th1 and Tc1 cells was found to be WT1 specific because WT1-negative HLA-A24$^+$ lymphoma cells failed to stimulate TCR gene–modified T cells (Figure 5F-G). It is interesting to investigate whether the efficacy of recognition by TCR gene–transduced Th1 and Tc1 cells correlates with WT1 expression level of leukemia cells, because the level of WT1 mRNA expression of lung cancer cell lines was found to correlate with their susceptibility against WT1-specific CTL-mediated cytotoxicity.[22] However, we could not conclude it in this study because freshly isolated leukemia cells always express strong WT1 without exception, and the number of available leukemia cells is limited.

The most important finding in the present experiments is that MHC class I–restricted TCR genes obtained from tumor-specific Tc clone were successfully transferred to Th1 cells and that these engineered Th1 cells can respond to antigenic peptide in an MHC class I–restricted manner. MHC class I–restricted tumor-specific Th1 cells but not Tc1 cells produced high levels of IL-2 in response to the WT1 peptide (Figure 4). Therefore, TCR gene–transduced Th1 cells are beneficial for enhancing antitumor cellular immunity mediated by cotransferred Tc1 cells and/or host-derived antitumor effector cells. The generation of MHC class I–restricted Th cells by TCR gene transfer has been reported by Clay et al[12] and Morgan et al,[20] but these investigators demonstrated only that gene–modified Th cells produced IFN-γ in response to stimulation with antigen peptide–pulsed T2 cells but not with naturally presented antigen bound by MHC molecules.[12,20] However, we have clearly demonstrated that (1) CD4$^+$ Th1 cells that were transduced with HLA-A24–restricted TCR genes produced IL-2 in addition to IFN-γ (Figure 4) and (2) TCR gene–modified Th1 cells showed substantial cytotoxicity and IFN-γ production in response to leukemia cells expressing naturally processed tumor antigen peptide on MHC molecules (Figure 5). Our gene–modified class I–restricted Th1 cells possibly exerted strong antitumor activities because we prepared Th1 cells under type-1 T-cell–inducing conditions. We showed that Th1 cells that were transduced with HLA class I–restricted TCR genes showed a significant antitumor activity against freshly isolated leukemia cells (Figure 5) that express HLA class II. However, they showed low levels of antitumor activity against HLA-A24$^+$ WT1$^+$ leukemia cell lines that express no HLA class II molecules, while TCR gene–modified Tc1 cells exhibited similar antitumor activity against both leukemia cell lines and freshly isolated leukemia cells. TCR gene–transduced Th1 cells exhibit both cytotoxicity (cytotoxicity at an E/T ratio of 10 is 9% on average; Figure 5B) and cytokine production (235 pg/mL IFN-γ production on average; Figure 5D) by coculture with freshly isolated HLA class II–expressing leukemia cells but not with HLA class II–negative leukemia cell lines (cytotoxicity at an E/T ratio of 10 is 3% and 30 pg/mL IFN-γ production; data not shown). This indicates that, in addition to the recognition of HLA class I–binding antigenic peptide on leukemia cells by class I–restricted TCR complex, ligation of CD4 with HLA class II appeared to be required for full activation in response to naturally processed WT1 antigen peptide. This is consistent with previous observations that the CD4/MHC class II or CD8/MHC class I interactions are required for the reactivity of naturally occurring class I–restricted Th cells or class II–restricted Tc cells, respectively.[40-43] However, some naturally occurring class I–restricted Th cells were also reported to exert their function in the absence of CD4/class II ligation.[44] In addition, it was reported that some Tc cells recognized antigen in a CD8-independent way.[43,45,46] This discrepancy may be explained by different strength of the interaction between the class I–restricted TCR of different class I–restricted Th cells and antigen peptide presented on MHC molecule. Thus, it might be possible to prepare TCR gene–modified Th1 cells that can respond to MHC class II$^-$ target cells if nonspecific Th cells are transferred with class I–restricted TCR genes that have a high binding avidity with MHC-bound antigen. In general, most freshly isolated leukemia cells express high levels of MHC class II molecules.[47] Therefore, TCR gene–transduced Th1 cells will be useful for most patients with leukemia irrespective of the strength of the interaction between TCR and HLA-bound antigenic peptide. Even in the case of nonhematopoietic tumor cells, most of which express no HLA class II molecules, class I–restricted TCR gene–transduced Th1 cells should exert antitumor activity by recognizing HLA class I–binding tumor antigen peptide cross-primed by professional APCs such as DCs. WT1, which is widely expressed in leukemia cells, is a potential target antigen for immunotherapy of patients with leukemia.[48,49] In addition to leukemia cells, WT1 has been found to be expressed on various types of other tumors, including lung, colon, or breast cancer.[50-52] Thus, our present method to transduce class I–restricted WT1-specific TCR genes may be applied to patients with various types of tumors.

Recently, we have proposed adoptive immunotherapy using tumor antigen–specific Th1 cells as a useful strategy for tumor immunotherapy (Th1 cell therapy).[2,3,19,27] Indeed, combining Th cells with Tc cells is a powerful method to treat patients with cancer.[37] As shown in the present experiments, class I–restricted tumor-specific TCR genes can successfully transfer tumor specificity to Th1 and Tc1 cells. Because these genetically engineered tumor-specific Th1 cells are obtained from polyclonally activated Th1 cells, it is possible to prepare a large number of tumor-specific Th1 cells from a relatively small number of PBMCs in a short culture period. In contrast to class II–binding tumor-specific peptides, hundreds of class I–binding peptides have been identified. These peptides should enable us to generate a database of tumor-specific TCR genes from a variety of tumor-specific Tc clones expressing various HLA genotypes. Therefore, we believe that our established TCR gene–modified tumor-specific Th1 cells will be applicable to many patients with cancer and may become a powerful tool for developing a novel tailor-made Th1 cell therapy.

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MAJOR ARTICLE

Differences between T Cell–Type and Natural Killer Cell–Type Chronic Active Epstein-Barr Virus Infection

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Infections of T cells and natural killer (NK) cells play a central role in the pathogenesis of chronic active Epstein-Barr virus (CAEBV) infection. To characterize the virologic and cytokine profiles of T cell–type and NK cell–type infection, 39 patients with CAEBV infection were analyzed. Patients with T cell–type infection had higher titers of immunoglobulin G against early and late EBV antigens, suggesting lytic cycle infection. However, the pattern of EBV gene expression was latency type II; *BZLF1*, which is a hallmark of lytic cycle infection, could not be detected in any patients, regardless of infection type. Patients with CAEBV infection had high concentrations of proinflammatory, T helper cell type 1, and anti-inflammatory cytokines. The cytokine profile in patients with NK cell–type infection was similar to that in patients with T cell–type infection, but the concentration of IL-13 was high in patients with NK cell–type infection. These findings should help to clarify the pathogenesis of CAEBV infection and facilitate the development of more-effective treatments.

Epstein-Barr virus (EBV) is a ubiquitous virus that infects most individuals by early adulthood. Primary EBV infection is usually asymptomatic but sometimes progresses to infectious mononucleosis, which resolves spontaneously after the emergence of EBV-specific immunity [1, 2]. EBV infection can be chronic in apparently immunocompetent hosts [3, 4]. Chronic active EBV (CAEBV) infection is characterized by chronic or recurrent mononucleosis-like infectious symptoms, such as fever, persistent hepatitis, extensive lymphadenopathy, hepatosplenomegaly, pancytopenia, uveitis, interstitial pneumonia, hydroa vacciniforme, and hypersensitivity to mosquito bites [3–5]. Patients with CAEBV infection have an unusual pattern of EBV-related antibodies and high viral loads in peripheral blood [3–7]. CAEBV infection is associated with high mortality and morbidity.

Recent studies have indicated that the clonal expansion of EBV-infected T cells and natural killer (NK) cells plays a central role in the pathogenesis of CAEBV infection [5, 8–11]. In a previous study, we found that patients with CAEBV infection fall into 2 clinically distinct groups, on the basis of whether the infected cells in their peripheral blood were mainly T cells or NK cells [5]. T cell–type infection is characterized by fever and high titers of EBV-related antibodies, whereas NK cell–type infection is characterized by hypersensitivity to mosquito bites and high titers of IgE. Furthermore, patients with T cell–type infection have significantly poorer outcomes [5, 12]. EBV-infected T cells might become activated and release inflammatory cytokines, such as interferon (IFN)–γ or tumor necrosis factor (TNF)–α, resulting in severe inflammation and fever [13, 14]. However, it is still not known why these 2 manifestations of the disease have different symptoms and courses.

The purpose of the present study was to gain a better

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understanding the pathogenesis of CAEBV infection by characterizing the virologic profiles of T cell–type and NK cell–type infection and identifying the differences between them. We analyzed 20 patients with T cell–type infection and 19 patients with NK cell–type infection. The 2 types of CAEBV infection were compared in both virologic and immunologic analyses, including an analysis of cytokine profiles.

PATIENTS, MATERIALS, AND METHODS

Patients. Thirty-nine patients with CAEBV infection were enrolled in the present study. Informed consent was obtained from all of the patients or their parents. Of the 39 patients, 24 had been included in our previous study of the clinical characteristics of CAEBV infection [5]. All of the patients met the following diagnostic criteria [5]: (1) they had EBV-related illness or symptoms for >6 months (including fever, persistent hepatitis, extensive lymphadenopathy, hepatosplenomegaly, pancytopenia, uveitis, interstitial pneumonia, hydroa vacciniforme, or hypersensitivity to mosquito bites); (2) they had increased quantities of EBV in either affected tissue or peripheral blood (the quantity of EBV was defined as increased when $\geqslant 1$ of the following criteria was met: EBV DNA was detected in either affected tissue or peripheral blood by Southern-blot hybridization, EBV-encoded small RNA 1 [EBER-1]–positive cells were detected in either affected tissue or peripheral blood [15], or $>10^{2.5}$ copies/μg DNA was detected in peripheral-blood mononuclear cells [PBMCs] [15]); and (3) they did not manifest evidence of any previous immunologic abnormalities or of any other recent infection that might explain the condition (all of the patients examined were negative for antibody against HIV).

Cells. For the EBV gene-expression experiment, a lymphoblastoid cell line (LCL) that was transformed with B95-8 virus was used as a positive control, and BJAB, an EBV-negative B cell line, was used as a negative control.

Samples. Samples were collected at the time of diagnosis or before the administration of immunosuppressive therapy, such as chemotherapy or hematopoietic stem-cell transplantation. EDTA-treated peripheral blood collected from patients was centrifuged and separated into plasma and cell fractions; the cell fractions were separated into PBMCs on Ficoll-Paque density gradients (Pharmacia Biotech).

Titers of anti–EBV nuclear antigen (EBNA) antibodies were measured by use of an anticomplement immunofluorescence method. Titers of anti–viral capsid antigen (VCA) and anti–early antigen–diffuse restricted (EA-DR) IgG were measured by use of an immunofluorescence method. These titers were measured in all patients.

DNA was extracted from either 2×10^6 PBMCs or 200 μL of plasma by use of a QIAamp blood kit (Qiagen). To differentiate between free EBV DNA molecules and virions or nucleocapsids, selected plasma samples were examined for EBV content by digestion with deoxyribonuclease (RQ1 RNase-free DNase; Promega) for 30 min at 37°C [16, 17]. As controls, pGEM-BALF5, a control plasmid DNA containing an EBV target gene, and the supernatants of LCL and BJAB cultures were used. LCL was treated with *n*-butyrate and phorbol 12-myristate 13-asetate, to induce lytic cycle infection, and the culture supernatants were used as a control that contained enveloped virions.

For patients from whom fresh samples were available ($n = 19$), RNA was extracted from 2×10^6 PBMCs by use of an RNA extraction kit (QIAamp RNA Blood Mini Kit; Qiagen). cDNA was synthesized by use of Superscript reverse transcriptase II (Gibco Life Technology), as described elsewhere [18].

Determination of EBV-infected cells. To determine which cells were infected with EBV, PBMCs were fractionated into $CD3^+$, $CD4^+$, $CD8^+$, $CD16^+$, $CD19^+$, and $CD56^+$ cells by use of an immunobead method (DynaBeads; Dynal A/S) [5]. For some patients with T cell–type infection from whom a sufficient quantity of PBMCs was obtained, fractionation into $CD4^+$ and $CD8^+$ cells was also performed. The fractionated cells were analyzed by either quantitative polymerase chain reaction (PCR) or in situ hybridization with the EBER-1 probe [15]. Patients were defined as having T cell–type infection when $CD3^+$ cells either were the main cells giving a positive hybridization signal with EBER-1 or contained more EBV DNA than other cells in the blood sample [5]. Patients were defined as having NK cell–type infection when $CD16^+$ or $CD56^+$ cells were the main ones containing EBV [5]. Repeated tests were performed for some patients, and similar results were obtained; in the present study, representative results are shown.

For some patients, infected cells were identified in biopsied or autopsied tissues, such as lymph nodes, liver, and spleen. Double labeling by use of in situ hybridization with the EBER-1 probe and immunostaining with surface marker antibody were performed as described elsewhere [19].

Clonality of EBV. The clonality of EBV was determined by Southern blotting with a terminal-repeat probe, as described elsewhere [20, 21]. PBMC-extracted genomic DNA was digested with *Bam*HI, subjected to gel electrophoresis, transferred to a nylon membrane, hybridized with a ^{32}P-labeled *Xho*I fragment from the terminal region of EBV, and visualized by autoradiography.

Quantification of EBV DNA. Both PBMCs and plasma from all of the patients were assayed for viral load. A real-time quantitative PCR assay with a fluorogenic probe was performed as described elsewhere [15, 22]. As a positive standard for quantification, pGEM-BALF5 was used [15]. The quantity of EBV DNA was calculated as the number of copies per microgram or per milliliter of plasma.

Amplification of EBV-specific RNA transcripts by PCR. To detect latent gene expression (*EBNA1*, *EBNA2*, and *LMP1* [latent member protein]), nested PCR was performed essentially as described elsewhere [23]. For *EBNA1*, 3 different primer

sets were used, to determine promoter usage. Approximately 50 ng of total RNA (converted to cDNA) was used as template. The amplified products were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized by UV light. To detect *BZLF1*, which encodes a transactivator protein and is expressed in lytic cycle infection, nested PCR was performed with the primers described by Prang et al. [24]. To detect the gene for gp350/220, which is also expressed in lytic cycle infection, nested PCR was performed with the outer primers described by Kelleher et al. [25] and newly selected inner primers (5′-CATCACCGGTGACACCAAGT-3′ and 5′-TGCTGGCGAACTGGTGGACA-3′). To detect a housekeeping gene, the human glyceraldehyde-3-phosphate dehydrogenase gene was amplified by single PCR; the sequences of the primer pair were 5′-GAAGGTGAAGGTCGGAGT-3′ and 5′-GAAGATGGTGATGGGATTTC-3′. All of the primer pairs used in the present study were designed to span introns, to avoid amplification of genomic DNA.

Quantification of cytokine gene transcription by use of a real-time PCR assay. Cytokine gene transcription was quantified by use of a real-time PCR assay, as described elsewhere [18]. Transcription of the genes for IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12p35, IL15, IFN-γ, and TNF-α was assessed by use of a TaqMan Cytokine Gene Expression Plate 1 (Applied Biosystems). A predeveloped primer/probe (Assays-On-Demand Gene Expression Products; Applied Biosystems) was used to measure IL-13 separately. Each well contained TaqMan primers and probes for assaying human cytokine mRNA and rRNA as an endogenous control. Approximately 25 ng of total RNA (converted to cDNA) was used to quantify the expression of each cytokine gene. All of the assays were conducted in duplicate. To calculate the relative expression of a cytokine gene in cells, the value for the expression of a cytokine gene was divided by that for the internal control rRNA, by use of the comparative threshold cycle method described by the manufacturer (P/N 4306744; Applied Biosystems) [18, 26].

Determination of plasma cytokine concentration by use of ELISA. Plasma cytokine concentrations were determined in all of the patients. Plasma concentrations of IL-1β, IL-4, IL-10, IL-12, IL-13, and IFN-γ were determined by use of sandwich-type ELISA kits (R&D Systems); these assays were conducted in accordance with the manufacturer's instructions [18, 27]. Sample values were determined from a standard curve. The minimum detectable concentrations of IL-1β, IL-4, IL-10, IL-12, IL-13, and IFN-γ were 1, 10, 3.9, 5, 32, and 8 pg/mL, respectively.

Statistical analyses. Statistical analyses were conducted by use of StatView software (version 5.0; SAS Institute). Fisher's exact test or the χ^2 test was used to compare differences in clinical measurements. The Mann-Whitney *U* test was used for statistical comparisons of laboratory data, viral load, cytokine gene expression, and plasma cytokine concentrations. $P<.05$ was considered to be statistically significant.

RESULTS

Characteristics of T cell–type and NK cell–type CAEBV infection. Of the 39 patients with CAEBV infection, 20 had infections that were defined as T cell type, and 19 had infections that were defined as NK cell type. The differences in clinical and laboratory measurements between the 2 types of CAEBV infections are shown in table 1. The patients with T cell–type infection had a poorer prognosis (death rate, 60%); 12 of them died, with the causes of death including hepatic failure ($n=4$), malignant lymphoma ($n=2$), and cardiac complications ($n=2$) (for death by other causes, $n=4$). Three patients received hematopoietic stem-cell transplantation; 2 are still alive, and 1 relapsed and died shortly afterward. T cell–type infection was strongly characterized by high fever and anemia; other characteristics of T cell–type infection were hepatomegaly and lymphadenopathy, although the statistical significance was marginal. In contrast, the patients with NK cell–type infection had a better prognosis (death rate, 26%); 5 of them died, with the causes of death including complications related to hematopoietic stem-cell transplantation ($n=3$), sepsis ($n=1$), and interstitial pneumonia ($n=1$). Seven patients received hematopoietic stem-cell transplantation; 4 are still alive, and 3 died of transplantation-related complications. NK cell–type infection was characterized by large granular lymphocytosis, hypersensitivity to mosquito bites, and a high IgE concentration. These observed differences between the 2 types of CAEBV infection are in agreement with the results of our previous study [5].

Determination of EBV-infected cells. For 28 patients, fractionation of PBMCs followed by quantitative PCR was used to determine the cell types that were infected (table 2). Using this method, we determined that 15 patients had T cell–type infection, because $CD3^+$ cells contained more EBV DNA than did the other cell populations. In 3 of these patients, mainly $CD4^+$ T cells were infected; in 2 of these patients, mainly $CD8^+$ T cells were infected. In contrast, mainly $CD16^+$ cells (not $CD3^+$ cells) were infected in 13 patients, who were therefore determined to have NK cell–type infection (table 2). In some patients, both $CD16^+$ and $CD19^+$ cells contained more EBV DNA than did unfractionated cells, suggesting that both NK and B cells were infected with EBV.

For the remaining 11 patients, in situ hybridization was used to determine which cell types were infected. For 3 patients with NK cell–type infection, infected cells were identified by fractionating PBMCs and then performing EBER-1 in situ hybridization. EBER-1 was detected in 15%, 25%, and 60% of the $CD56^+$ cells in these 3 patients, indicating that most of the infected cells were NK cells (as described elsewhere [28]). For the other 8 patients, tissue samples were used to identify in-

Table 1. Differences in clinical and laboratory measurements between T cell–type and NK cell–type chronic active Epstein-Barr virus (EBV) infection.

	T cell–type infection (n = 20)	NK cell–type infection (n = 19)	P
Sex, M:F, no.	10:10	11:8	.43
Age at onset, mean ± SD, years	12.6 ± 9.5	8.0 ± 4.9	.19
Death rate, %	**60**	26	**.03**
Time to death from onset, mean ± SD, years	2.9 ± 1.3	5.4 ± 3.8	.27
Symptoms			
Fever >1 day/week, %	**81**	38	**.01**
Hepatomegaly, %	79	53	.09
Lymphadenopathy, %	58	32	.096
Large granular lymphocytosis, %	11	**58**	**.003**
Hypersensitivity to mosquito bites, %	11	**68**	**.0002**
Laboratory data			
WBC count, mean ± SD, cells/μL	5200 ± 5500	6100 ± 3200	.11
Hemoglobin concentration, mean ± SD, g/dL	**10.7 ± 1.2**	12.1 ± 1.9	**.03**
Platelet count, mean ± SD, 10^4 cells/μL	21.4 ± 7.6	19.2 ± 8.5	.52
IgG level, mean ± SD, mg/dL	1990 ± 1440	1590 ± 450	.36
IgE level, mean ± SD, mg/dL	190 ± 220	**5650 ± 6470**	**.002**
EBV-related antibody			
Anti-VCA IgG level, GMT	**2010**	310	**.001**
Anti–EA-DR IgG level, GMT	**610**	70	**.007**
Anti-EBNA level, GMT	27	45	.12
Viral load			
PBMCs, mean ± SD, copies/μg	$10^{3.9 \pm 0.7}$	$\mathbf{10^{4.4 \pm 0.6}}$	**.03**
Plasma, mean ± SD, copies/mL	$10^{2.8 \pm 1.1}$	$10^{2.5 \pm 2.0}$	.87

NOTE. Values in boldface indicate statistically significant results. Either Fisher's exact test or the χ^2 test was used to compare symptoms; the Mann-Whitney U test was used to compare laboratory data and viral load. EA-DR, early antigen–diffuse restricted; EBNA, EBV nuclear antigens; F, female; GMT, geometric mean titer; M, male; PBMCs, peripheral-blood mononuclear cells; VCA, viral capsid antigen; WBC, white blood cell.

fected cells. Double labeling with EBER-1 and surface markers showed that, in 5 patients, most EBER-1–positive cells were $CD3^+$, indicating that they had T cell–type infection. In 3 patients, most EBER-1–positive cells were $CD16^+$ (not $CD3^+$), indicating that they had NK cell–type infection. Some of these results have been described elsewhere [29].

Virologic analyses. EBV-related antibody titers were compared between the 2 types of CAEBV infection. The patients with T cell–type infection had significantly higher titers of anti-VCA and anti–EA-DR IgG (table 1). The anti-EBNA antibody titers were comparable between the 2 types of CAEBV infection. The higher titers of antibody against early and late EBV antigens (i.e., anti–EA-DR IgG and anti-VCA IgG) but not against latent antigen (i.e., anti-EBNA antibody) suggested the possibility of lytic cycle infection in T cells.

To examine whether lytic cycle infection existed in EBV-infected T or NK cells, for 19 patients from whom fresh samples were available (for T cell–type infection, $n = 11$; for NK cell–type infection, $n = 8$), reverse-transcription PCR was used to examine EBV gene expression in PBMCs. *BZLF1*, which encodes a transactivator protein and is a hallmark of lytic cycle infection [1], was not detected in any of the 19 patients (table 3); another lytic gene, for gp350/220, was also not detected. *EBNA1, LMP1,* and *LMP2A* were detected in PBMCs from nearly one-half of the 19 patients, indicating that they had the latency type II pattern. The Qp promoter, but not the Cp/Wp promoter, was used for *EBNA1* transcription. Representative results are shown in figure 1. Because it was possible that lytic cycle infection was present at sites other than PBMCs, EBV gene expression was examined in autopsy or biopsy samples from 2 patients with T cell–type infection. The *BZLF1* and gp350/220 genes were not detected in the livers, lymph nodes, or spleens of these patients (data not shown). The pattern of EBV gene expression in these tissue samples was also latency type II.

Next, the viral load in peripheral blood was investigated by use of real-time PCR. The viral load in PBMCs was higher in patients with NK cell–type infection (table 1). Interestingly, the viral load in plasma was similar between the patients with each type. To examine the viral load in plasma, plasma samples from selected patients were digested with deoxyribonuclease before extraction of DNA. As a preliminary experiment, pGEM-BALF5, a control plasmid DNA containing an EBV target gene, and the supernatant of an LCL culture containing enveloped virions were tested. The control plasmid DNA was sensitive to deoxy-

Table 2. Determination of Epstein-Barr virus (EBV)–infected cells in peripheral-blood mononuclear cells.

Type of chronic active EBV infection, patient	EBV DNA, copies/μg						Mainly infected cells	Clonality of cells
	Fractionated cells					Unfractionated cells		
	$CD3^+$	$CD4^+$	$CD8^+$	$CD16^+$	$CD19^+$			
T cell								
1	**220,000**	760	**230,000**	100,000	760	210,000	T ($CD8^+$)	Monoclonal
2	**90,000**	ND	ND	17,000	15,000	24,000	T	ND
3	**43,000**	**21,000**	1500	**12,500**	2400	8600	T ($CD4^+$)	Monoclonal
4	**23,000**	ND	ND	1100	1600	14,000	T	Monoclonal
5	**18,000**	ND	ND	6200	0	7300	T	ND
6	**17,000**	ND	ND	4300	5200	16,000	T	ND
7	**12,000**	ND	ND	5700	2700	11,000	T	ND
8[a]	12,000	4500	2500	6500	5000	51,000	T	Monoclonal
9	**11,600**	ND	ND	2700	2000	6100	T	Monoclonal
10	10,000	27,000	4900	1900	1200	42,000	T ($CD4^+$)	Oligoclonal
11	**7600**	ND	ND	80	260	6600	T	ND
12	**6600**	ND	ND	0	900	3900	T	Polyclonal
13	**3600**	ND	ND	840	950	3200	T	ND
14	**400**	**720**	90	250	20	340	T ($CD4^+$)	Monoclonal
15	160	10	**220**	0	20	200	T ($CD8^+$)	Polyclonal
NK cell								
1	120,000	ND	ND	**400,000**	**470,000**	210,000	NK, B	Monoclonal
2	21,000	ND	ND	**170,000**	3200	110,000	NK	Monoclonal
3	7400	ND	ND	**89,000**	17,000	78,000	NK	ND
4	11,000	ND	ND	**86,000**	18,000	75,000	NK	Monoclonal
5	10,000	ND	ND	**54,000**	23,000	36,000	NK	Monoclonal
6	3300	ND	ND	**35,000**	1900	20,000	NK	Monoclonal
7	7600	ND	ND	25,000	1700	28,000	NK	ND
8[a]	1800	ND	ND	**16,000**	**9200**	7000	NK, B	Oligoclonal
9	300	ND	ND	**15,000**	0	2000	NK	ND
10	200	ND	ND	15,000	1800	31,000	NK	ND
11	1600	ND	ND	4500	570	8100	NK	Monoclonal
12	50	ND	ND	**4300**	110	1600	NK	Oligoclonal
13	0	ND	ND	**2700**	**1300**	820	NK, B	ND

NOTE. Values in boldface indicate that EBV DNA was concentrated after fractionation. ND, not done.

[a] Infected cells were confirmed by double labeling of tissue samples.

ribonuclease (percentage of reduction of EBV DNA after digestion, 99.9%), but the LCL supernatant was resistant to the enzyme (percentage of reduction of EBV DNA after digestion, 55.7%). Five plasma samples from each group of patients were tested. After deoxyribonuclease digestion, the percentages of reduction of EBV DNA were 100%, 92.8%, 96.8%, 97.3%, and 100% in the samples from the patients with T cell–type infection and 99.8%, 98.3%, 100%, 100%, and 100% in the samples from the patients with NK cell–type infection. Thus, plasma from both groups of patients was sensitive to deoxyribonuclease, indicating that most of the EBV DNA in plasma, rather than consisting of enveloped virions, consisted of free EBV DNA molecules, which were likely derived from dead or damaged cells.

The clonality of EBV was analyzed by use of Southern blotting. The majority of both T cell–type and NK cell–type infections were monoclonal (table 2). There was a trend in that those patients with polyclonal or oligoclonal proliferation had lower viral loads, although there was no difference between the 2 types of CAEBV infection.

Cytokine profiles. Differences in the symptoms or immunologic responses between the 2 types of CAEBV infection might be due to the differences in the cytokine production profiles of either EBV-infected cells themselves or inflammatory cells. The plasma concentrations of cytokines (IL-1β, IL-4, IL-10, IL-12, IL-13, and IFN-γ) were estimated and compared between the 2 types of CAEBV infection. IL-1β, IL-10, and IFN-γ, none of which are detected in healthy individuals, were detected in plasma from many patients with CAEBV infection (figure 2); there were no significant differences between the T cell–type and NK cell–type infections. IL-13, which also is not detected in healthy individuals, was frequently detected in the patients with NK cell–type infection, and the concentration was higher than that in the patients with T cell–type infection. The patients who developed

Table 3. Summary of Epstein-Barr virus (EBV) gene expression in peripheral-blood mononuclear cells.

Type of chronic active EBV infection	*EBNA1*[a] Common	*EBNA1*[a] Qp	*EBNA1*[a] Cp/Wp	*EBNA2*	*LMP1*	*LMP2A*	*BZLF1*	gp350/220	*GAPDH*
T cell (n = 11)	4	1	0	0	4	3	0	0	11
NK cell (n = 8)	4	3	0	0	4	4	0	0	8

NOTE. Data are no. of samples positive for the indicated gene. EBNA, EBV nuclear antigens; GAPDH, human glyceraldehyde-3-phosphate dehydrogenase; LMP, latent membrane protein.

[a] For *EBNA1*, 3 different primer sets were used to determine promoter usage.

hemophagocytic syndrome had high concentrations of inflammatory cytokines, such as IL-1β and IFN-γ.

Next, the transcription of cytokine genes in PBMCs was investigated in the 19 patients from whom fresh samples were available. Assays were performed for IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12p35, IL-13, IL-15, IFN-γ, and TNF-α. Transcription of the genes for IL-1α, IL-1β, IL-10, IL-13, IL-12p35, IL-15, TNF-α, and IFN-γ was high in the patients with CAEBV infection, whereas transcription of the genes for IL-4 and IL-5 was undetectable in most patients. There were no statistical differences in the transcription of these cytokine genes between the 2 types of CAEBV infection, including for IL-13.

DISCUSSION

It has been shown that infections of T and NK cells play a central role in the pathogenesis of CAEBV infection. A recent national survey in Japan showed that the majority of patients with CAEBV infection had infections that belonged to either the T cell type or the NK cell type; only 2 of 82 patients had an infection that mainly involved B cells [12]. EBV-infected T cell or NK cell lines have been established from patients with CAEBV infection [20, 21]. In the present study, we identified 20 patients with CAEBV infection in whom mainly T cells were infected and 19 patients with CAEBV infection in whom mainly NK cells were infected. Recently, a small-scale study reported that, during acute EBV-associated hemophagocytic lymphohistiocytosis, EBV infection was predominant in $CD8^+$ cells, but that, in patients with CAEBV infection, EBV infection was predominant in non-$CD8^+$ cells [30]. However, the results of the present study indicated that, in some of our patients with CAEBV infection, EBV infection was predominant in $CD8^+$ T cells.

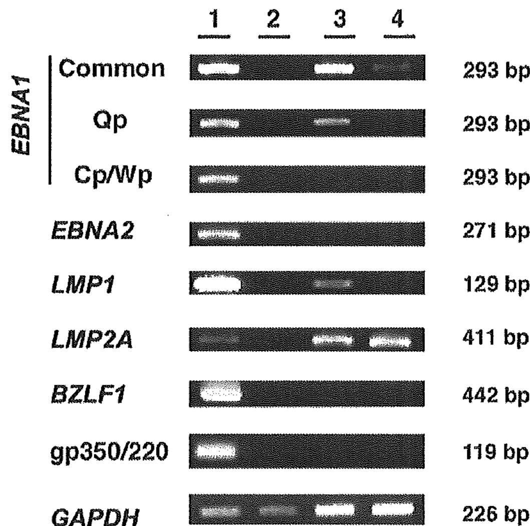

Figure 1. Expression of Epstein-Barr virus (EBV) genes in peripheral blood. *Lane 1:* EBV-positive lymphoblastoid cell line. *Lane 2:* BJAB, an EBV-negative B cell line. *Lane 3:* Peripheral blood from a patient with T cell–type chronic active EBV (CAEBV) infection. *Lane 4:* Peripheral blood from a patient with NK cell–type CAEBV infection. EBNA, EBV nuclear antigens; GAPDH, human glyceraldehyde-3-phosphate dehydrogenase; LMP, latent membrane protein.

To determine which cells were infected with EBV, we used immunomagnetic cell isolation to fractionate PBMCs, followed by quantitative PCR. This method is very rapid and convenient, but its disadvantage is the relatively poor purity of the selected cells. EBV-infected cells can contaminate uninfected cell fractions. Furthermore, the real-time PCR assay is so sensitive that the contaminating EBV genome can be detected; therefore, this method can determine only the cell population that is mainly infected. The low levels of EBV DNA seen in other cell populations do not always mean that they are infected with EBV; however, the results of the present study showed that, in some of our patients, both NK cells and B cells were infected with EBV (table 2). Perhaps >1 cell lineage harbors EBV in some patients with CAEBV infection. Electric cell sorting followed by EBER-1 in situ hybridization is a more accurate method for determining EBV-infected cell lineages. Using this method, Kasahara et al. found that, in some patients with CAEBV infection, different cell lineages were infected [30].

Originally, CAEBV infection was characterized by an unusual pattern of EBV-related antibodies, such as high titers of anti-VCA and anti–EA-DR IgG or the absence of anti-EBNA antibody [3, 4, 6, 31], although recent observations indicate that high titers of these EBV-related antibodies are not necessary for CAEBV infection to be diagnosed [5]. In patients with CAEBV infection—and especially in patients with T cell–type infection who have high titers of EBV-related antibody—high

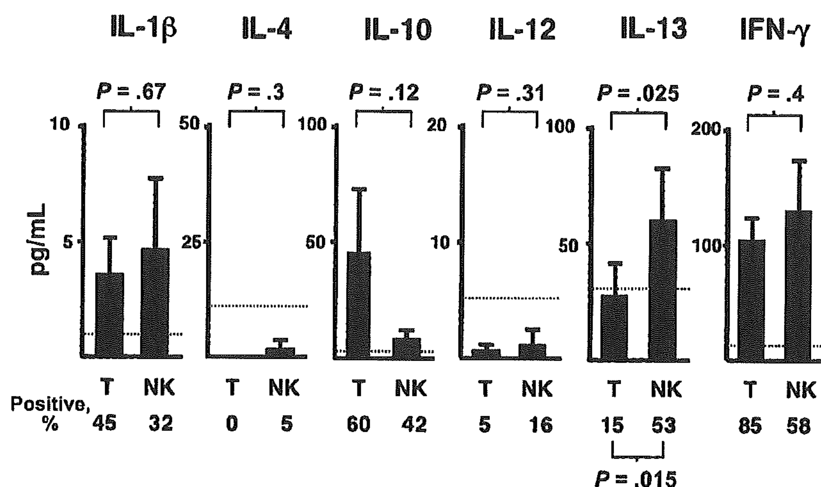

Figure 2. Comparison of plasma cytokine concentrations. Concentrations were estimated by use of an immunoassay and compared between patients with T cell–type ($n = 20$) and NK cell–type ($n = 19$) chronic active Epstein-Barr virus infection. Boxes and error bars indicate means and SEs, respectively; the dotted lines indicate the upper limits of healthy individuals. The Mann-Whitney *U* test was used to compare plasma cytokine concentrations, and Fisher's exact test was used to compare positivity rates. IFN, interferon; IL, interleukin.

titers of antibodies against the early and late EBV antigens and the existence of cell-free EBV DNA in plasma suggest the possibility of lytic cycle infection [6, 32, 33]. In the present study, a deoxyribonuclease-digestion experiment showed that the presence of EBV DNA in plasma was attributable to free nucleic acids that were likely released from dead or damaged cells. Furthermore, the pattern of EBV gene expression in PBMCs was latency type II, which supports the absence of lytic cycle replication in the PBMCs, at least, of patients with CAEBV infection. Lytic cycle infection may occur in tissue, although our results for tissue samples, while limited, showed no sign of a lytic cycle. It is also possible that we did not detect the occurrence of lytic cycle replication if lytic cycle infection occurred in <1% of EBV-infected cells. There is no definite proof of lytic cycle replication in tissues from patients with CAEBV infection. Some investigators have reported the expression of early or late EBV gene transcripts, such as those for BZLF1 or viral IL-10 [30, 34, 35], whereas other investigators have reported the absence of expression of these transcripts in tissue samples [36].

Our observation here of the absence of lytic cycle infection is particularly important with regard to selection of the treatment strategy for patients with CAEBV infection. Antiviral drugs that suppress viral DNA polymerase and lytic cycle replication may not be suitable for the treatment of CAEBV infection; however, therapies that reduce or eliminate EBV-infected T cells or NK cells may be suitable choices. Chemotherapy or hematopoietic stem-cell transplantation are suitable in this regard—the successful treatment of CAEBV infection by hematopoietic stem-cell transplantation has been reported [37, 38]. Alternatively, EBV-related antigens expressed in T cells or NK cells may be the targets of treatment. Cytotoxic T cells that were generated from LCL and targeted to latency type III antigens have been administered to patients with CAEBV infection [39, 40]. On the basis of the present result that the pattern of EBV gene expression was latency type II, cytotoxic T cells specific for latency type II antigens, such as LMP1 or LMP2A, would be more favorable for the control and eradication EBV-infected cells if they are inducible [41, 42].

In the present study, patients with CAEBV infection had high concentrations of proinflammatory (IL-1β), Th1-type (IFN-γ), and anti-inflammatory (IL-10) cytokines. Transcription of the genes for these cytokines was also high in PBMCs. The upregulation of various cytokine genes has also been reported in patients with CAEBV infection in other studies [43–45]. These cytokines are thought to be produced either by EBV-infected T cells or NK cells or by reacting inflammatory cells. On the one hand, it has been shown that EBV-infected T cells produce proinflammatory (IL-6 and TNF-α), Th1-type (IL-2 and IFN-γ), and anti-inflammatory (transforming growth factor β1) cytokines [14, 46]. Shen et al. reported that, during EBV-infected nasal NK/T cell lymphoma, human IL-10, an anti-inflammatory cytokine that suppresses cytotoxicity against EBV-infected cells, was expressed [47]. On the other hand, reacting inflammatory cells, such as macrophages, can produce most of the cytokines seen in the present study. Unfortunately, because of our study design, it is impossible to determine whether EBV-infected or reacting cells were the main sources of these cytokines. However, the high concentrations of and the elevated

transcription of genes for various cytokines must contribute to the diverse symptoms seen in patients with CAEBV infection.

One of the purposes of the present study was to find virologic differences between the T cell–type and the NK cell–type infection. One main difference was that we found frequent detection and a high concentration of IL-13 in the patients with NK cell–type infection. IL-13 is a Th2-type cytokine that induces the differentiation of B cells, the production of antigen-specific antibody, and a class switch to IgE and that also suppresses the cytotoxic functions of monocytes and macrophages [48]. IL-13 is primarily produced by activated T cells and is not usually detected in plasma from healthy individuals [49]. The cytokine is produced by Reed-Sternberg cells during Hodgkin disease, which is associated with EBV infection [50]. Although the reason why IL-13 was produced in the patients with NK cell–type infection is unclear, the high concentration of IL-13 may explain the high serum IgE levels and the hypersensitivity to mosquito bites, both of which are frequently seen in patients with NK cell–type infection [5].

The other difference between the 2 types of CAEBV infection is that the patients with NK cell–type infection had a higher viral load in PBMCs. This is particularly interesting, because NK cell–type infection is usually milder and progresses slowly [5, 12]. In contrast, the viral load in plasma was similar between the 2 types of CAEBV infection. These results suggest that sources of EBV DNA other than PBMCs exist in patients with T cell–type disease, the more severe, rapid type of CAEBV infection. In patients with T cell–type infection, the cell-free EBV DNA may come from tissue, such as lymph nodes or the spleen, where EBV-infected T cells infiltrate and proliferate. Indeed, patients with T cell–type infection have a higher incidence of hepatomegaly and lymphadenopathy, as was shown in the present study. The higher viral load in plasma could also be explained by the naturally high rate of apoptosis in activated T cells. It is still unclear why T cell–type infection is severe and progresses rapidly. The distribution of infected cells, determined by the differences in homing receptors among cells, may determine the symptoms and prognosis. A recent animal model showed that activated T cells are selectively trapped in the liver, primarily by intracellular adhesion molecule 1, which is constitutively expressed on sinusoidal endothelial cells and Kupffer cells [51]. We previously reported a patient with primary EBV infection who had severe hepatitis and whose liver was infiltrated with EBV-infected $CD8^+$ cells. In patients with T cell–type CAEBV infection, EBV-infected, presumably activated T cells might accumulate in the liver and cause hepatitis. Although further studies are necessary, our findings should help to clarify the pathogenesis of CAEBV infection and facilitate the development of more-effective treatments.

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