3.2. Cell sorting

Magnetic activated cell sorting (MACS) was performed on PB mononuclear cells (MNCs) using Vario-MACS columns (Miltenyi Biotec, Bergisch Gladbach, Germany) after staining with anti-CD3, CD4, CD8 and CD56 immunobeads (Miltenyi Biotec). Anti-fluorescein-isothiocyanate (FITC) and phycoerythrin (PE) (Miltenyi Biotec) immunobeads were used to collect αβT-cells and γδT-cells after staining with FITC-conjugated anti-T-cell receptor (TCR)Vδ2 and PE-conjugated TCRpanα/β monoclonal Abs (Immunotech; Beckman Coulter, Marseille, France), respectively. $CD56^+$NK-cells were collected using Lymphocyte Separation column, after depleting $CD3^+$cells by Lymphocyte Depleting column (Miltenyi Biotec). The procedures yielded >97% purity. $CD3^+$T-cells, γδT-cells, and αβT-cells were further purified (>99.9%) using the FACS Aria Flow Sorter (BD Biosciences, San Jose, CA, USA). $CD34^+$cells were enriched from BM cells by Indirect CD34 MicroBead Kit (Miltenyi Biotec).[13] To further purify $CD34^+$cells (>99.9%), lin^-CD34^+cells were sorted from BM by FACS Aria after staining with conjugated lineage mixtures of a) PE-Cy5-conjugated anti-CD3, CD4, CD8, CD10, CD20, CD11b, CD14, and CD235a (Immunotech), b) FITC-conjugated anti-CD3, CD4 and Vδ2 (Immunotech), c) PE-conjugated anti-TCRpanα/β, CD16 and CD56 (Immunotech), and d) allophycocyanin-conjugated anti-CD34 (BD Biosciences). Nonviable cells were excluded by propidium iodide staining (MBL, Nagoya, Japan). Isotype-matched control Abs determined backgrounds. Second sorting avoided contaminations. Flow-cytometric data were analyzed with FlowJo software (Tree Star, Inc., Ashland, OR, USA). More than 99.9% of purity was confirmed by re-analysis.

3.3. Real-time polymerase chain reaction (PCR) for EBV DNA

TaqMan real-time PCR for EBV DNA was performed as described previously.[5] Gene dosages were analyzed by ABI PRISM 7700 (Applied Biosystems, Foster City, CA, USA). DNA was mixed with TaqMan Universal PCR Master Mix (Applied 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. EBV-seropositive healthy persons show <200 copies EBV/ml and <40 copies EBV/μgDNA in PB and MNCs, respectively.

3.4. Southern blotting for EBV-TR or TCR/immunoglobulin (Ig) genes

Blotting analyses were performed by the established protocols.[14] Briefly, 5μg of high molecular weight DNA were digested with *Eco*RI and/or *Bam*HI, and were electrophoresed on 0.9% agarose gels. DNA was transferred to Byodine-B membranes (Pall Life Sciences, Ann Arbor, MI, USA) and hybridized with ^{32}P-labeled probe of a 5.2-kb *Bam*HI-*Eco*RI fragment containing the EBV-TR sequence, TCR genes (Cβ1, Jβ1, Jβ2, and Jγ) and IgH gene (JH). Normal control DNA was extracted from MNC of healthy EBV-seropositive adults.

3.5. Sequencing of TCRδ and TCRγ gene rearrangements

Genomic DNA was extracted from γδT-cells by 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 BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and 3130*xl* Genetic Analyzer (Life Technologies Corp., CA, USA).

3.6. Double staining for EBV and lymphocyte markers

Double staining of BM clot samples was performed by *in situ* hybridization for

EBER and immunostaining for lymphocyte marker to detect EBV-infected cells.[15] Briefly, sections were de-waxed, dehydrated and then treated with proteinase-K. The sections were hybridized with FITC-conjugated EBER probe (Dako, Copenhagen, Denmark). EBER positivity was detected by the combination with anti-FITC Abs, ChemMateENVISION (horseradish peroxidase-labeled polymer reagent, Dako), and diaminobenzidine substrate kit (Dako). The same slide was stained with either Abs against CD34 or each lymphocyte marker to search for $EBER^{+}CD34^{+}$BM cells.

4. Results

4.1. Target cells and clinical phenotype of patients

T-cell type Patient-1 had fever and skin lesions. $CD4^{-}CD8^{-}\gamma\delta$T-cells increased to 30% of PB-MNC, exclusively expressing Vδ2/Vγ9 **(Fig.1)**. PCR products amplified by Vδ2/Vγ9 primers indicated clonal proliferation of Vδ2/Vγ9 T-cells. Sequencing of Vγ9-JγP1 transcripts determined an N-region sequence **(Table 2)**. γδT-cells and αβT-cells had 4×10^{5} and 4×10^{3} copies EBV/μgDNA, respectively. Southern blotting using MNC showed rearranged TCRγ genes. EBV^{+}Vδ2/Vγ9 T-cells have been sustained in this patient for 6 years after diagnosis. Patient-2 suffered hydroa vacciniforme characterized by cutaneous disease often associated with photosensitivity and HMB. $CD3^{+}$T-cells, $CD8^{+}$T-cells and NK-cells carried 2×10^{5}, 5×10^{3} and 9×10^{3} copies EBV/μgDNA, respectively. It indirectly indicated a higher percentage of $EBV^{+}CD4^{+}$T-cells. She received a sibling donor SCT at 25 years of age for cutaneous lymphoma ($EBER^{+}CD4^{+}$T-cells and NK-cells). Patient-3 had fever and hepatitis. $CD4^{+}$T-cells, $CD8^{+}$T-cells and NK-cells had 3×10^{4}, 90 and <40 copies EBV/μgDNA, respectively. He received a sibling donor BMT.

NK-cell type Patient-4 showed skin and enteral infiltrations with EBV^+NK-cells. $CD4^+$T-cells, $CD8^+$T-cells and NK-cells carried 2×10^3, 1×10^3, and 4×10^5 copies EBV/μgDNA, respectively. She attained EBV-free remission after cord blood transplantation. Patient-5 had fever and HMB. T-cells and NK-cells carried 3×10^4 and 8×10^5 copies EBV/μgDNA, respectively. Low-dose prednisolone has controlled skin infiltrations with $EBER^+CD4^+$T-cells. Patient-6 showed fever and HMB. $CD4^+$T-cells, $CD8^+$T-cells and NK-cells had 8×10^3, 4×10^4, and 4×10^5 copies EBV/μgDNA, respectively.

4.2. Clonality of EBV-infected subsets

The clonotype of EBV in purified cells was assessed by the TR size. The blotting sensitivity was determined to be at least 5% EBV-infected cells as determined by add-back experiments. Blot hybridization analysis of EBV-TR sequences using MNC showed a single band in Patient-1. αβT-cells (>99.9%) showed an equal-sized TR to that of γδT-cells in Patient-1 in *Eco*RI or *Bam*HI digestion **(Fig.2)**. Patient-2 showed a single band in NK-cells, although $CD4^+$T-cells were the major target. A TR band in NK-cells of Patient-2 was the same size as one of biclonal bands in $CD3^+$T-cells. Patient-3 showed a single TR band in $CD4^+$T-cells but not $CD8^+$T-cells.

Clonal TR bands were detected in $CD3^+$T-cells (>99.5%) of all patients with NK-cell LPD. Blotting analysis of EBV-TR using MNC showed biclonal bands in Patient-4. TR(s) in $CD3^+$T-cells of Patient-4 (biclonal) and Patient-5 (monoclonal) were the same size as those in NK-cells of the same patients. On the other hand, $CD3^+$T-cells (>99.9%) from Patient-6 had a larger TR band than NK-cells (>99.9%) **(Fig.2)**, although the blotting using MNC showed the same sized band as NK-cells.

4.3. EBV load in BM $CD34^+$cells

Virus loads were re-quantified using highly purified subsets from PB and BM cells to further assess the origin of EBV-infection. EBV DNA was not detectable in BM-derived lin$^-$CD34$^+$cells of any 5 patients studied, nor in seropositive controls **(Fig.3)**. EBV was not detected in CD34$^+$cells by the double staining of EBER *in situ* hybridization and immunohistochemistry for BM samples **(Fig.4)**.

5. Discussion

The current study found that EBV$^+$T/NK-cells clonally proliferated in various subsets of patients. EBV clonotype, as determined by EBV TR size, in the minor subsets notably coincided with that in the major subsets of infection. On the other hand, BM CD34$^+$cells carried no EBV. These data suggest that EBV can infect lymphocytes having differentiation ability, that then clonally distribute to T/NK-cells, but not involve infection of CD34$^+$hematopoietic stem cells (HSCs).

It remains controversial in regard to whether CAEBV is an infection or clonal disease. EBER$^+$T-cells and NK-cells can be found in the tonsils of acute IM patients.[16] EBV-shedding T/NK-cell lines were established from CAEBV patients.[17,18] Excessive activation of lytic virus may raise the chance of ectopic infection. While cytotoxic CD8$^+$T-cells clonally expand in IM patients,[19] non-CD8$^+$T-cells were infected and clonally proliferated in our patients. PB γδT-cells consist of polyclonal Vδ2/Vγ9$^+$T-cells in healthy persons. This subset was clonally infected with EBV in Patient-1. Toyobe *et al.*[20] reported that a CAEBV patient with biclonal expansion of Vβ7$^+$ and Vβ9$^+$ cells in PB CD4$^+$T-cells. Both clones were infected with EBV strain that was indistinguishable from each other. Endo *et al.*[21] analyzed EBV clonotype in 2 CAEBV patients having biphenotypic expansions of CD4$^+$ and CD8$^+$T-cells. Both

subsets carried a TR band with the same size. Germinal center EBV^+B-cells could evolve LPD.[22] Diverse T/NK-cell infection in this line might not represent spreading lytic viruses, but differentiation and expansion of T/NK-cell clones with latent episome. An alternative explanation is integration of the EBV gene into the host genome.[23] EBV latent genes are expressed in heterogeneous and restricted patterns in CAEBV patients.[24] Specific integration of EBV gene rarely occurs in transformed cells. Taken together, EBV could infect T/NK-cells at differentiating stages, and these cells proliferate and enter the circulation. Multiple EBV strains affect EBV-associated tumors in a single host.[25] Site specific clonality should be investigated to understand EBV^+T/NK-cell LPD.

The major concern is how clonal EBV^+T/NK-cells are allowed to proliferate in patients. EBV^+B-cell LPD arises in X-linked LPD patients. A Caucasian patient with B-cell type CAEBV had defective cytotoxicity due to *PRF1* mutations.[26] Although cytotoxic defects are implicated in CAEBV patients,[27] EBV-infected T/NK-cells may have altered cytotoxicity. Neither a genetic defect nor familial occurrence has been identified in T/NK-cell type CAEBV or EBV-HLH.[28,29] The end-to-end length of fused TR itself may drive the clonal selection of EBV-infected cells as an oncogenic component during primary infection.[30]

This study demonstrated that BM $CD34^+$cells were not a niche of EBV infection. Human immunodeficiency virus (HIV)-infected $CD34^+$HSCs were detected in HIV-positive patients.[31] HTLV-1 or HHV8 infects $CD34^+$HSCs.[32] Primitive HSCs are resistant to HIV infection, although these cells become increasingly susceptible once they begin to differentiate into committed HSCs.[33] EBV might not infect BM, but infect peripheral or thymic $CD34^+CD4^-CD8^-$T-cell precursors (DN1/2) prior to the

differentiation into αβT-cells and γδT-cells. Dormant $CD34^+$cells may be protected from infection or eliminated after infection. The curative SCT for clonal disease of EBV^+T/NK-cell progenitors could be explained by the eradication of EBV-infected hematopoietic cells and the reset of virus-specific immunity. Further study on the origin of EBV^+T/NK-cells may therefore shed some light on the pathogenesis and specific therapy of EBV-associated T/NK-cell LPD/lymphoma.

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FIGURE LEGENDS

Figure 1. A representative flow-cytometric analysis of lymphocytes in Patient-1. TCRγδ cells increased to ~30% of peripheral blood lymphocytes, exclusively expressing Vγ9/Vδ2, but with only a negligible expression of HLA-DR. $V\gamma4^+$ or $V\delta3^+$ cells were <1% of lymphocytes. More than 99% of $V\delta2^+$ 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 EBV-terminal repeated (TR) sequence. TCRαβ+cells and Vδ2+cells in Patient (Pt)-1, and $CD3^+$cells and $CD56^+$cells in Pt-6 showed >99.9% of purity. *Eco*RI (E) or *Bam*HI (B)-digested DNA was separated by electrophoresis (2~5μg/lane) and hybridized with a ^{32}P-labeled 5.2-kb *Bam*HI-*Eco*RI fragment containing the TR repeat sequences. The blotting sensitivity was determined to be at least 0.5% contamination of EBV-infected cells. Arrow, a clonal band. * non-specific band in each digestion. NC: normal control of EBV-seropositive healthy adult lymphocytes, PC: positive control of Raji cell lines.

Figure 3. Quantification of EBV DNA in highly purified cell fractions (>99.9%) obtained from 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*). EBV-seropositive healthy carriers or

patients with immune thrombocytopenic purpura were used as controls.

Figure 4. Immunohistochemical staining of bone marrow (BM) cells obtained from Patient-5 (a) and Patient-6 (b). 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 EBER using diaminobenzidine as a chromogen of peroxidase (positive: brown nucleus, *closed arrow*). There were no double positive cells for CD34 and EBER in BM cells.

Table 1. Clinical profiles of patients studied

Patient	1	2	3	4	5	6
Sex	f	f	m	f	m	m
Age (yrs) at onset, at the study	6, 7	8, 25	2, 5	4, 4	1, 10	3, 8
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 DNA[1] PB/MNC	100/106	300/104	600/na	3000/na	300/105	2000/na
Major targeted subsets	γδT	CD4	CD4	CD56	CD56	CD56
CD3/CD19/CD56 (%)	70/28/2	75/18/7	69/24/7	82/5/13	47/17/36	58/15/27
$CD3^+HLA\text{-}DR^+$ (%)	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
Clonality[2] EBV-TR	M	B	M	B	M	M
TCR	R	R	na	G	G	G
Outcome	AOD	Death post-SCT	ADF post-SCT	ADF post-SCT	AOD	AOD

1) Each value means the copy number of EBV DNA (PB: /ml, MNC: /μg DNA).
2) Clonality was screened by Southern blotting for PB-MNC derived DNA probed with EBV-TR, TCR and IgH genes. There was no evidence of clonally proliferating B cells. EBV: Epstein-Barr virus, CAL: coronary artery lesion, CNS: central nervous system, HMB: hypersensitivity to mosquito bites, 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, B: biclonal, 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-JγP1Junctional Transcripts Expressed by γδT-Cells

	Vγ9	N region	JγP1
Germline	ATT CCG TCA GCC ****TAC TGT GCC TTG TGG GAG GTG		ACC ACT GGT TGG ATC ← *JγT3 primer*
	Vγ9 primer →		
Pt	TGT GCC TTG TGG GAG GTG	CAG	ACC ACT GGT TGG TTC AAG ATA TTT GCT GAA GGG ACT AAG
	C A L W E V	Q	T T G W

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

Figure 1

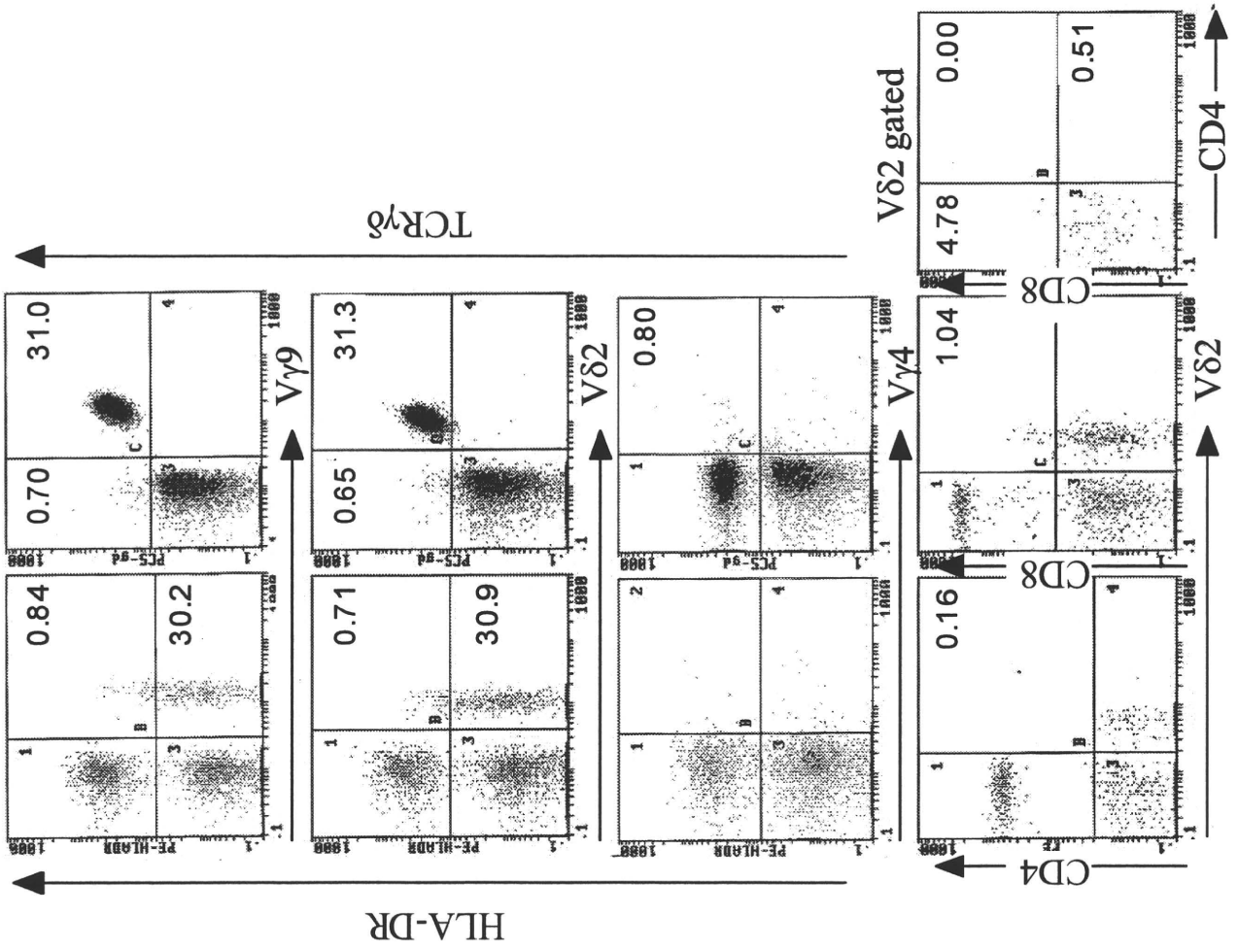
TCRγδ
HLA-DR
Vγ9
Vδ2
Vγ4
Vδ2
0.84
30.2
0.70
31.0
0.71
30.9
0.65
31.3
0.80
0.16
1.04
4.78
0.00
0.51
Vδ2 gated
CD8
CD4
CD4

Figure 2

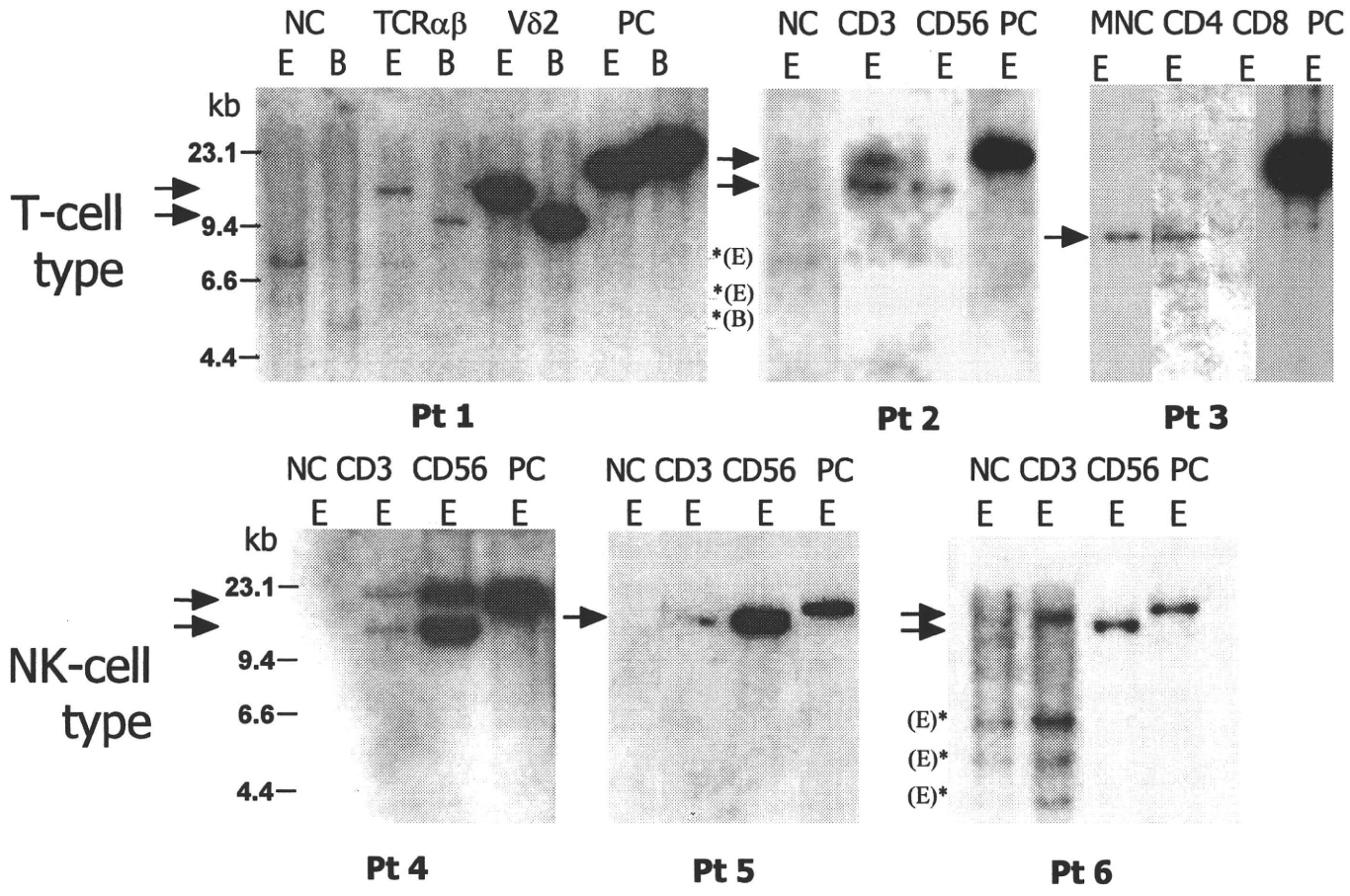
NC TCRαβ Vδ2 PC
E B E B E B E B
kb
23.1
9.4
6.6
4.4
T-cell type
*(E)
*(E)
*(B)
NC CD3 CD56 PC
E E E E
MNC CD4 CD8 PC
E E E E
Pt 1
Pt 2
Pt 3
NC CD3 CD56 PC
E E E E
NC CD3 CD56 PC
E E E E
NC CD3 CD56 PC
E E E E
kb
23.1
9.4
6.6
4.4
NK-cell type
(E)*
(E)*
(E)*
Pt 4
Pt 5
Pt 6

Figure 3

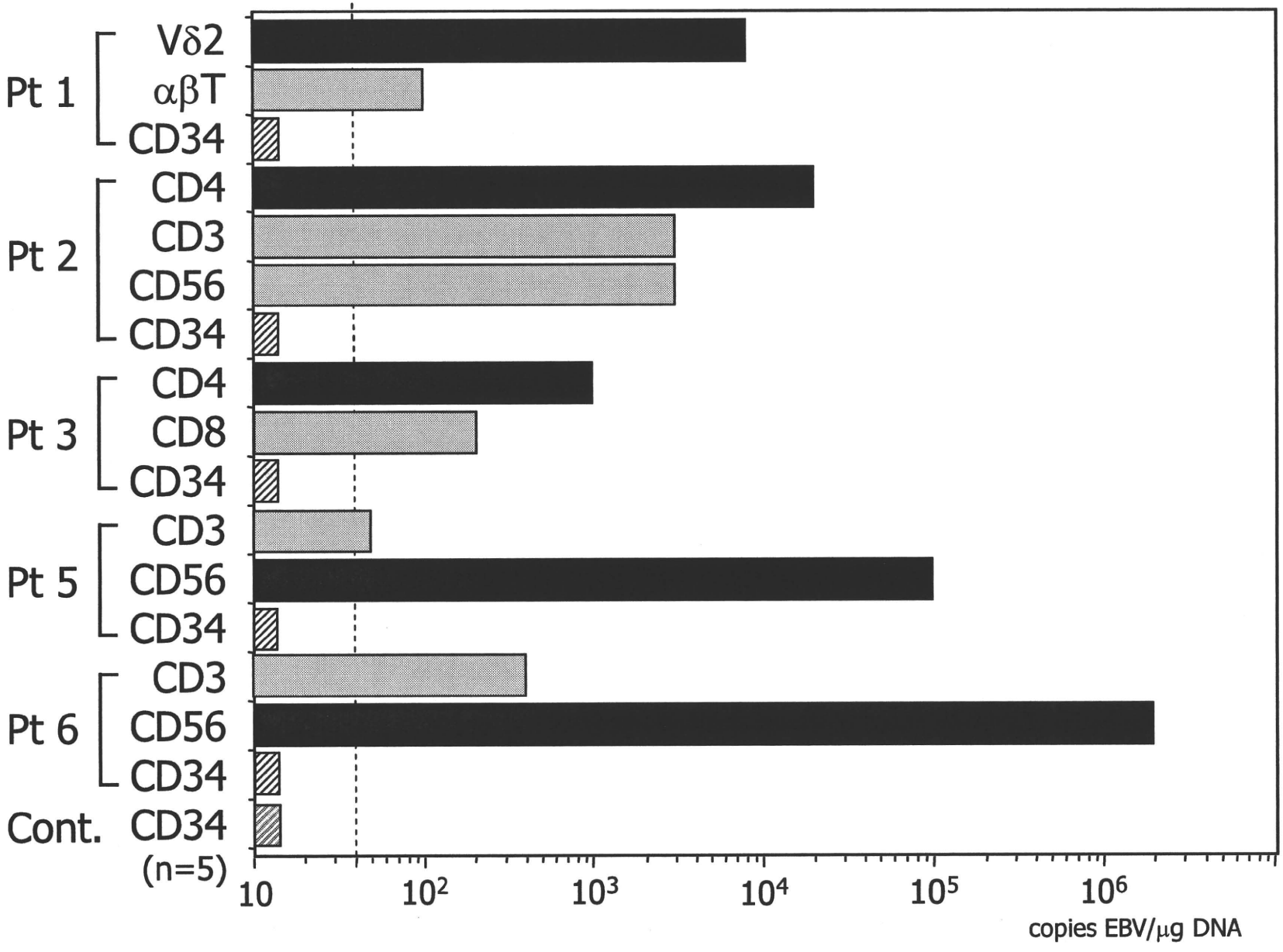
Vδ2
αβT
CD34
CD4
CD3
CD56
CD34
CD4
CD8
CD34
CD3
CD56
CD34
CD3
CD56
CD34
CD34
Pt 1
Pt 2
Pt 3
Pt 5
Pt 6
Cont.
(n=5)
10
10²
10³
10⁴
10⁵
10⁶
copies EBV/μg DNA

Figure 4