

## **A functional link for major TCR expansions in healthy adults caused by persistent Epstein-Barr virus infection.**

S L Silins, ... , C W Schmidt, I S Misko

*J Clin Invest.* 1998;**102**(8):1551-1558. <https://doi.org/10.1172/JCI4225>.

### **Research Article**

Dramatic clonal expansions of unknown functional significance have been documented in the T cell receptor (TCR) alpha beta peripheral blood repertoires of apparently healthy adults. In this study, we provide evidence that persistent infection with the ubiquitous Epstein-Barr virus (EBV) causes major distortions within the memory repertoire of healthy virus carriers. Using complementarity determining region 3 (CDR3) length analysis to measure repertoire diversity, dominant expansions that dramatically skewed the entire TCRBV6 blood repertoire towards oligoclonality were enriched in the CD8(+)CD45RO+CD45RA- subset of HLA B8(+) healthy virus carriers. Evidence of phenotypic heterogeneity between individuals was also observed for these expansions based on their variable coexpression of CD45RO and CD45RA. TCR junctional region sequencing revealed that these expansions were clonal and that they represented commonly selected HLA B8-restricted memory cytotoxic T cells that recognize the immunodominant latent EBV epitope, FLRGRAYGL. Furthermore, the functional identity of these virus-specific CD8(+) T cells was confirmed by their FLRGRAYGL-specific cytotoxicity. Therefore, the functional significance of dramatic clonal expansions in healthy adults can be linked in some cases to virus-specific CD8(+) T cells that play an essential role in immunosurveillance. This first identified link for expansions in the circulation of healthy adults strongly implies that restricted-memory TCR responses to environmental antigens play a pivotal role in expansion development, which should have an important impact on studies interpreting [...]

**Find the latest version:**

<https://jci.me/4225/pdf>



# A Functional Link for Major TCR Expansions in Healthy Adults Caused by Persistent Epstein-Barr Virus Infection

Sharon L. Silins, Simone M. Cross, Kenia G. Krauer, Denis J. Moss, Christopher W. Schmidt, and Ihor S. Misko  
Epstein-Barr Virus Unit, Queensland Institute of Medical Research and University of Queensland Joint Oncology Program, Brisbane, Queensland, Australia 4029

## Abstract

Dramatic clonal expansions of unknown functional significance have been documented in the T cell receptor (TCR)  $\alpha\beta$  peripheral blood repertoires of apparently healthy adults. In this study, we provide evidence that persistent infection with the ubiquitous Epstein-Barr virus (EBV) causes major distortions within the memory repertoire of healthy virus carriers. Using complementarity determining region 3 (CDR3) length analysis to measure repertoire diversity, dominant expansions that dramatically skewed the entire TCRBV6 blood repertoire towards oligoclonality were enriched in the CD8<sup>+</sup>CD45RO<sup>+</sup>CD45RA<sup>-</sup> subset of HLA B8<sup>+</sup> healthy virus carriers. Evidence of phenotypic heterogeneity between individuals was also observed for these expansions based on their variable coexpression of CD45RO and CD45RA. TCR junctional region sequencing revealed that these expansions were clonal and that they represented commonly selected HLA B8-restricted memory cytotoxic T cells that recognize the immunodominant latent EBV epitope, FLRGRAYGL. Furthermore, the functional identity of these virus-specific CD8<sup>+</sup> T cells was confirmed by their FLRGRAYGL-specific cytotoxicity. Therefore, the functional significance of dramatic clonal expansions in healthy adults can be linked in some cases to virus-specific CD8<sup>+</sup> T cells that play an essential role in immunosurveillance. This first identified link for expansions in the circulation of healthy adults strongly implies that restricted-memory TCR responses to environmental antigens play a pivotal role in expansion development, which should have an important impact on studies interpreting TCR expansion patterns in health and disease. (*J. Clin. Invest.* 1998. 102:1551–1558.) Key words: CDR3 • T cell memory • CD8 • CD45RO • antigen-specific T cells

## Introduction

Unusually high expansions of  $\alpha\beta$  T cells within the human peripheral repertoire are known to occur in various blood-associated malignancies, autoimmune, inflammatory, and infectious

diseases (1–7). Clonality and conserved features of antigen specificity in many of these expansions have been linked to the pathogenesis of the disease. More recently, however, the significance of clonal dominance in disease has been questioned given that dramatic clonal expansions have also been reported, although at a lower frequency than in disease, within the circulating T cell repertoires of apparently healthy individuals (8–16). In healthy individuals,  $\alpha\beta$  T cell expansions can persist unaltered for years, predominantly occur within the CD8<sup>+</sup> T lymphocyte subset (8, 10, 14, 15), often express a CD45RO<sup>+</sup> memory phenotype (9, 11, 12), and appear to accumulate with age (8, 9). A key issue is what causes these major repertoire perturbations and what functional significance they have in cell-mediated immunity and in disease development. In particular, do expansions in the circulation arise as a normal consequence of repeated exposure of the immune system to environmental antigens and to persistent virus infections? Alternatively, are they autoreactive T cells, similar to B cells in benign monoclonal gammopathies, that are thought to be responding to premalignant conditions and age-related defects?

Diversity within the T cell repertoire can be measured by studying the distribution of T cell receptor (TCR)<sup>1</sup> rearrangements. The majority of T cells express an  $\alpha\beta$  TCR heterodimer that is generated through somatic recombination of variable (TCRAV and TCRBV), diversity (TCRBD), and joining (TCRAJ and TCRBJ) gene elements during T cell ontogeny (for review see reference 17). Repertoire diversity is further increased by the imprecise joining of the different gene segments and the quasi-random insertion or deletion of nucleotides at the V-(D)-J junctional regions that span the major antigen binding site or complementarity determining region 3 (CDR3) (18–20). Therefore, the CDR3 loop is highly variable in length and in codon usage between functionally distinct TCR clonotypes. Extreme clonotypic expansions do not necessarily elevate the frequencies of TCRBV gene families, as they have been detected within populations with normal TCRBV gene frequencies (9, 12).

Several features of the CD8<sup>+</sup> T cell memory response to EBV make it an ideal model system to address a possible link between persistent virus infection and the presence of large clonal expansions within the peripheral blood repertoires of healthy individuals. Firstly, we have shown previously that the class I-restricted memory response to the latent EBV epitope, FLRGRAYGL, is restricted by a single TCRBV6S2/BJ2S7 clonotype that is highly conserved in many HLA B8<sup>+</sup> individuals (21). (TCRBV6S2 replaces BV6S3\**a* under the new designation according to Arden et al. [22].) Secondly, functional assays have estimated that this public clonotype is highly fre-

Address correspondence to Dr. Sharon L. Silins, Queensland Institute of Medical Research, The Bancroft Centre, 300 Herston Rd., Brisbane, Queensland, Australia 4029. Phone: 61-7-3362-0342; FAX: 61-7-3362-0106; E-mail: sharonS@qimr.edu.au

Received for publication 8 June 1998 and accepted in revised form 21 August 1998.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.  
0021-9738/98/10/1551/08 \$2.00

Volume 102, Number 8, October 1998, 1551–1558  
<http://www.jci.org>

1. Abbreviations used in this paper: CDR, complementarity determining region; CTL, cytotoxic T cell; IM, infectious mononucleosis; TCR, T cell receptor.

quent within the peripheral blood repertoire (23) and that memory EBV-specific cytotoxic T cell (CTL) responses are stably maintained for life (24). The present study has explored the impact that selection of this EBV-specific clonotype has on the TCR  $\alpha\beta$  repertoire of healthy virus carriers. By examining the diversity of the TCRBV6 repertoire within different T cell populations of HLA B8<sup>+</sup> adults, we herein show that expansion of the EBV-specific clonotype in healthy virus-infected individuals dramatically skews the entire CD8<sup>+</sup> CD45RO<sup>+</sup> CD45RA<sup>-</sup> memory BV6 repertoire towards oligoclonality. This study identifies a basis for environmental antigens in the development of extreme T cell expansions in the blood of healthy adults.

## Methods

**Donors.** Peripheral blood samples were taken from two healthy, HLA B8<sup>+</sup>, long-term EBV-seropositive donors (viral capsid antigen IgG<sup>+</sup>, EBV nuclear antigen IgG<sup>+</sup>), D1 and D2, aged 51 and 37 yr, respectively. Peripheral blood was also taken from two HLA B8<sup>+</sup>, EBV-seronegative donors (viral capsid antigen IgG<sup>-</sup>, EBV nuclear antigen IgG<sup>-</sup>), D3 and D4, aged 56 and 55 yr, respectively. PBMCs were isolated from heparinized blood by centrifugation over Ficoll-Paque (Pharmacia Biotechnology, Melbourne, Australia). The HLA B8<sup>+</sup> status of each donor was initially determined by FACScan<sup>®</sup> analysis of PBMCs using anti-HLA B8 mAb (clone 59HA-1; One Lambda, Los Angeles, CA) and confirmed by serological typing of the donor's PBMCs.

**Isolation and enrichment of human CD8<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup>, CD8<sup>+</sup>CD45RO<sup>+</sup>CD45RA<sup>-</sup>, and CD8<sup>+</sup>CD45RO<sup>+</sup>CD45RA<sup>+</sup> T cells.** 1–2 × 10<sup>7</sup> PBMCs were incubated with anti-human CD4 mAb-labeled magnetic beads (Dyna beads M-450; DYNAL, Melbourne, Australia) to enrich for CD8<sup>+</sup> lymphocytes according to the manufacturer's recommendation. The CD8<sup>+</sup>-enriched population was centrifuged and the pelleted cells were stained for three-color, immunofluorescent flow cytometry using anti-human CD8 mAb conjugated with tricolor (clone 3B5; CALTAG, Sydney, Australia), anti-human CD45RA mAb conjugated with FITC (clone L48; Becton Dickinson, Sydney, Australia), and anti-human CD45RO mAb labeled with PE (clone UCHL-1; Becton Dickinson). Cells were sorted into three subsets, a CD8<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup>, CD8<sup>+</sup>CD45RO<sup>+</sup>CD45RA<sup>-</sup>, and CD8<sup>+</sup>CD45RO<sup>+</sup>CD45RA<sup>+</sup> population, using a Becton Dickinson FACS<sup>®</sup> Vantage Flow Cytometer. The purity of the recovered cells was > 98%.

**RNA isolation and cDNA synthesis.** Total RNA was extracted from 1–5 × 10<sup>5</sup> PBMCs or sorted CD8<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup>, CD8<sup>+</sup>CD45RO<sup>+</sup>CD45RA<sup>-</sup>, and CD8<sup>+</sup>CD45RO<sup>+</sup>CD45RA<sup>+</sup> cells using Total RNA Isolation Reagent (Advanced Biotechnologies, London, UK). First strand cDNA was synthesized using an antisense TCRBC primer (C<sub>b1</sub>) as described previously (21). For all RNA and cDNA reactions, a negative control was included where either cells or RNA were replaced with water.

**TCRBV6 PCR amplification.** TCRBV6 transcripts were amplified to saturation with a 5' TCRBV6 family-specific primer (V $\beta$ 6) (25) and a nested 3' TCRBC-specific primer (C<sub>b2</sub>) (21). Amplifications were performed in 25- $\mu$ l reaction volumes using first strand cDNA representing material from 1–5 × 10<sup>4</sup> cells, 10 pmol of each 5' and 3' primer, 200  $\mu$ M dNTPs, 1.5 mM MgCl<sub>2</sub>, 1.25 U of *Taq* polymerase (*AmpliTaq Gold*; Perkin-Elmer Corp., Norwalk, CT), and a GeneAmp PCR 9600 system (Perkin-Elmer Corp.). The PCR conditions consisted of denaturation at 95°C for 15 s, annealing at 55°C for 40 s, and extension at 72°C for 40 s for 35 cycles, followed by a 5-min final extension at 72°C. PCR products were separated on 2% (0.5× Tris-buffered EDTA) NuSieve GTG agarose gels (FMC BioProducts, Rockland, ME), excised, and purified using a QIAEX gel extraction kit (Qiagen, Chatsworth, CA).

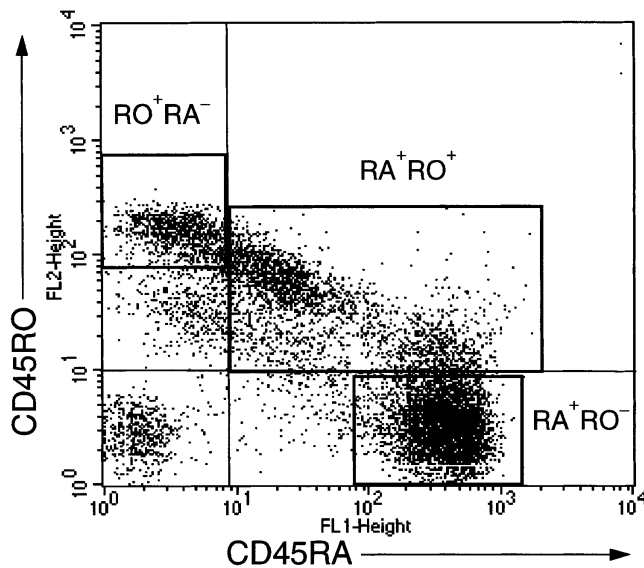
**Repertoire diversity analysis: CDR3 length determination and quantitation of CDR3 distribution.** The technique of CDR3 length determination and quantitation of CDR3 distribution to analyze TCR repertoire diversity is based on the methodology described by Pannetier et al. (26). QIAEX-purified TCRBV6 PCR products were labeled with a nested 3'-FAM fluorophore-labeled primer specific for the TCRBC gene (C $\beta$ P\*: 5'-FAM-TTCTGATGGCTCAAACAC-3'; Research Genetics Inc., Huntsville, AL) in a PCR run-off reaction. PCR conditions were identical to those described above, except that 2% of QIAEX-purified TCRBV6 PCR product was used as a template for five cycles of elongation (run-off) and a 5-min final extension at 72°C. The fluorescent PCR run-off products were heat-denatured at 95°C for 2 min and were separated on a 6% acrylamide gel together with size standards (GENESCAN-1000 ROX; Applied Biosystems, Brisbane, Australia) on an Applied Biosystems 373A DNA sequencer. Data were processed using the GENESCAN Analysis 2.1 Software (Applied Biosystems) which records the fluorescence intensity in each peak. Percent relative peak intensity is the specific peak area/total area of all peaks.

**Repertoire diversity analysis: TCR V-D-J junctional region sequencing.** QIAEX-purified TCRBV6 PCR products were ligated into the pGEM-T Vector System (Promega, Madison, WI) and then used to transform *Epicurian coli* SURE Competent Cells (Stratagene, La Jolla, CA) according to the respective manufacturers' instructions. Plasmid inserts were amplified by PCR and QIAEX-extracted according to the conditions described above, and then sequenced in both directions using the V $\beta$ 6 and C<sub>b2</sub> primers with a PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit and an ABI377 DNA sequencer (Applied Biosystems). Diversity within the BV6 gene family was measured by the frequency at which a particular cloned TCR V-D-J insert occurred. *n* signifies the number of identical TCR V-D-J junctional region sequences/total number of recombinant clones tested.

**Cytotoxicity assay.** Unsorted PBMCs and sorted populations of CD8<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup> and CD8<sup>+</sup>CD45RO<sup>+</sup>CD45RA<sup>-</sup> cells from seropositive donor D2 were activated weekly by stimulation with the  $\gamma$ -irradiated (80 Gy) LCL from an HLA A1/B8 homozygous individual, BM, at a stimulator/responder cell ratio of 1:200. After 32 d, these populations were used as effectors against HLA A1/B8 homozygous PHA T cell blasts, with and without adsorbed FLRGRAYGL peptide (100  $\mu$ g/ml, 0.5 ml). Peptides were purchased from Chiron Mimotopes (Melbourne, Australia), dissolved in DMSO, and diluted in serum-free RPMI 1640 medium for use in cytotoxicity assays. Target cells were incubated with 100  $\mu$ Ci of <sup>51</sup>Cr at 37°C for 90 min, with and without peptide, washed twice by centrifugation, and used in standard 4-h <sup>51</sup>Cr-release assays. The mean spontaneous lysis for targets in culture medium was < 20%, the mean maximum lysis in 0.5% SDS was > 90% of total uptake, and the variation about the mean specific lysis was < 5%.

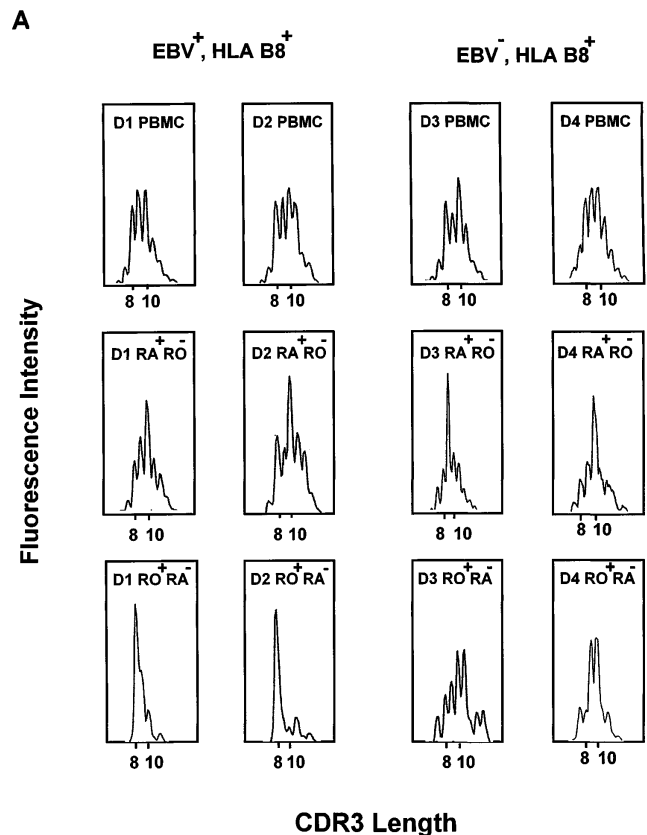
## Results

**EBV-infected healthy adults have dramatically distorted TCRBV6 memory repertoires.** Previously we have shown that the class I-restricted memory response to the latent EBV epitope, FLRGRAYGL, is dominated in many HLA B8<sup>+</sup> individuals by a single TCRBV6S2/BJ2S7 clonotype (21). To determine whether clonal expansions of this public EBV-specific clonotype can be linked to repertoire distortions within healthy individuals, we examined the extent of diversity within the peripheral TCRBV6 repertoire of two normal adult EBV-seropositive, HLA B8<sup>+</sup> donors, D1 and D2. Both donors have been found previously to have a highly restricted FLRGRAYGL-specific TCR memory response (21), with circulating CTL precursor frequencies of the EBV clonotype as high as 1:2,000 PBMCs reported for donor D1 (23). Repertoire diversity was mea-



**Figure 1.** Three-color immunofluorescence analysis of PBMCs of adult donor D2. CD8<sup>+</sup>-enriched cells were simultaneously stained with tricolor-conjugated CD8 in combination with FITC-conjugated CD45RA and PE-conjugated CD45RO mAbs. Cells were gated on size and tricolor fluorescence. Three distinct populations could be defined within CD8<sup>+</sup> T cells from adult PBMCs: CD45RO<sup>+</sup>CD45RA<sup>-</sup> (RO<sup>+</sup>RA<sup>-</sup>), CD45RO<sup>+</sup>CD45RA<sup>+</sup> (RO<sup>+</sup>RA<sup>+</sup>), and CD45RA<sup>+</sup>CD45RO<sup>-</sup> (RA<sup>+</sup>RO<sup>-</sup>) cells.

sured by a PCR-based approach that determines the distribution of CDR3 lengths within a given TCRBV6 family; gaussian-shaped peak profiles represent a diverse array of clonotypes of varying CDR3 lengths, whereas oligoclonal peak profiles have been shown to represent clonally expanded T cells (for review see reference 27). TCRBV6 family repertoire diversity was analyzed *ex vivo* in unsorted PBMCs and in sorted populations of either CD8<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup> (naive) or CD8<sup>+</sup>CD45RO<sup>+</sup>CD45RA<sup>-</sup> (memory) T cells that were > 98% pure. Fig. 1 shows the three-color immunofluorescence FACS<sup>®</sup> profile from individual D2. Consistent with the demarcation parameters of previous studies in defining memory and naive T cell populations, only the strongly staining single-positive CD45RO<sup>+</sup>CD45RA<sup>-</sup> cells and CD45RA<sup>+</sup>CD45RO<sup>-</sup> cells were collected, respectively. CDR3 length profiles obtained from three independent analyses of these different T cell populations were found to be highly reproducible (Fig. 2). An extreme predominance of a rearrangement with an 8-amino acid CDR3 length, corresponding exactly in size to the TCRB chain CDR3 region of the public EBV-specific clonotype, was recorded in the CD8<sup>+</sup>CD45RO<sup>+</sup>CD45RA<sup>-</sup> T cell subset of each EBV-seropositive donor (Fig. 2 A); relative peak intensities were 80 and 71% for donors D1 and D2, respectively (Fig. 2 B). This dramatic expansion was enriched within the CD45RO<sup>+</sup> memory subset since gaussian-like profiles of polyclonality were recorded in both unsorted PBMCs and in sorted CD8<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup> T cells of each EBV-seropositive donor (Fig. 2 A). The apparent clonal predominance of an 8-amino acid CDR3 rearrangement was found to be specific to EBV-infected donors, since TCRBV6 repertoire analysis of two HLA B8<sup>+</sup>, EBV-seronegative adults (D3 and D4) showed no evidence of repertoire focusing (Fig. 2, A and B).



**CDR3 Length**

**B**

CDR3 SIZE (amino acids)	RO <sup>+</sup> RA <sup>-</sup>			
	D1	D2	D3	D4
7	-	-	6%	11%
8	80%	71%	14%	9%
9	-	-	19%	25%
10	15%	8%	20%	18%
11	-	16%	25%	23%
12	5%	-	6%	13%
13	-	5%	10%	1%

**Figure 2.** CDR3 length distribution in TCRBV6 within different T cell populations of HLA B8<sup>+</sup>, EBV-seropositive (D1, D2), and EBV-seronegative (D3, D4) adults. (A) Profiles are displayed of fluorescence intensity (arbitrary units) as a function of CDR3 size (amino acids) for TCRBV6 of unsorted PBMCs and sorted populations of CD8<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup> (RA<sup>+</sup>RO<sup>-</sup>) and CD8<sup>+</sup>CD45RO<sup>+</sup>CD45RA<sup>-</sup> (RO<sup>+</sup>RA<sup>-</sup>) cells. CDR3 length, defined by Chothia et al. (43), is deduced from the fragment size. Each CDR3 peak is spaced three nucleotides or one amino acid apart. (B) Relative percent peak intensities derived from the CDR3 length profiles of the CD8<sup>+</sup>CD45RO<sup>+</sup>CD45RA<sup>-</sup> (RO<sup>+</sup>RA<sup>-</sup>) subset of each adult are reported. Relative peak intensities are calculated as described in Methods.

*EBV-specific TCR clonotypes account for the TCRBV6 expansions in the blood.* Repertoire diversity was next examined at the clonotype level by determining the TCR V-D-J junctional region sequences of recombinant clones that contained TCRBV6 inserts from the different T cell populations (Fig. 3, A-C). In the CD8<sup>+</sup>CD45RO<sup>+</sup>CD45RA<sup>-</sup> subset of both seropositive donors, a striking percentage of the total TCRBV6 repertoire was found to be dominated by a BV6S2/BJ2S7 rearrangement that was identical at the amino acid level

A

**EBV<sup>+</sup> Adult D1**

Clone	TCRBV	FW	CDR3	FW	TCRBJ	n
<b>PBMC</b>						
LC1	BV685	CAS	SPOQGETQ	YFG	BJ287	2/17
LC2	BV685	CAS	SRLKATEA	FFG	BJ181	1/17
LC3	BV681	NONFUNCTIONAL				1/17
LC4	BV682	CAS	RNRGRRENTEA	FFG	BJ181	1/17
LC5	BV681	NONFUNCTIONAL				1/17
LC6	BV685	CAS	SLAGTQETQ	YFG	BJ285	1/17
LC7	BV685	CAS	SADRLWG	NFG	BJ283	1/17
LC8	BV682	CAS	SFGGPGAL	HFG	BJ186	1/17
LC9	BV685	CAS	SLDRGVGY	TFG	BJ182	1/17
LC10	BV685	NONFUNCTIONAL				1/17
LC11	BV685	CAS	SLAQDYGY	TFG	BJ182	1/17
LC12	BV681	NONFUNCTIONAL				1/17
LC13	BV685	CAS	SGGKRVRYGY	TFG	BJ182	1/17
LC14	BV685	CAS	STQVGVGPPQ	HFG	BJ185	1/17
LC15	BV682	CAS	SPGLASLDTQ	YFG	BJ283	1/17
LC16	BV682	CAS	SLREGAEA	FFG	BJ181	1/17

**RA<sup>+</sup>RO<sup>-</sup>**

LC17	BV682	CAS	RDPGQDYGY	TFG	BJ182	3/17
LC18	BV681	CAS	SHGGRROPQ	HFG	BJ185	1/17
LC19	BV685	CAS	RPQTSGETQ	YFG	BJ285	1/17
LC20	BV681	CAS	SLGYGEEQ	YFG	BJ287	1/17
LC21	BV682	CAS	SLELAGYNEQ	FFG	BJ281	1/17
LC22	BV685	CAS	SLIAGSSYEQ	YFG	BJ287	1/17
LC23	BV682	CAS	SFGLTDTQ	YFG	BJ283	1/17
LC24	BV682	CAS	SLVKGGSPVVTYEQ	YFG	BJ287	1/17
LC25	BV688	CAS	SLALDRFPQETQ	YFG	BJ285	1/17
LC26	BV682	CAS	SLPGIHRGYEQ	YFG	BJ287	1/17
LC27	BV685	CAS	SRPSGLAGVREQ	YFG	BJ287	1/17
LC28	BV685	CAS	SLQEQETQ	YFG	BJ285	1/17
LC29	BV682	CAS	SLGRGRGLYNEQ	FFG	BJ281	1/17
LC30	BV681	CAS	SFQGSYGY	TFG	BJ182	1/17
LC31	BV685	CAS	SLFPFRTSGYEQ	YFG	BJ287	1/17

**RO<sup>+</sup>RA<sup>-</sup>**

<b>LC32</b>	<b>BV682</b>	<b>CAS</b>	<b>SLGQAYEQ</b>	<b>YFG</b>	<b>BJ287</b>	<b>8/13 (61%)</b>
LC33	BV681	CAS	RDPGQDYGY	TFG	BJ182	2/13
LC34	BV681	CAS	SLGYGEEQ	YFG	BJ287	1/13
LC35	BV681	NONFUNCTIONAL				1/13
LC36	BV681	CAS	SLGASGALYNEQ	FFG	BJ281	1/13

**PUBLIC FLRGRAYGL CLONOTYPE**

BV682	CAS	SLGQAYEQ	YFG	BJ287
-------	-----	----------	-----	-------

B

**EBV<sup>+</sup> Adult D2**

Clone	TCRBV	FW	CDR3	FW	TCRBJ	n
<b>PBMC</b>						
AS1	BV681	CAS	SSNRGNEQ	FFG	BJ281	1/12
AS2	BV682	CAS	SLIAGEL	FFG	BJ282	1/12
AS3	BV682	CAS	SARDGSYEQ	YFG	BJ287	1/12
AS4	BV683	NONFUNCTIONAL				1/12
AS5	BV683	CAS	SLGGSSPSSYNEQ	FFG	BJ281	1/12
AS6	BV686	CAS	SFSGBETQ	YFG	BJ285	1/12
AS7	BV681	NONFUNCTIONAL				1/12
AS8	BV682	CAS	SLAPQSHSYEQ	YFG	BJ287	1/12
AS9	BV682	CAS	GGGTTDTQ	YFG	BJ283	1/12
AS10	BV681	CAS	SLNRBETQ	YFG	BJ285	1/12
AS11	BV681	CAS	SPNRRAVQQGVNGY	TFG	BJ182	1/12
AS12	BV681	CAS	SLVGPDDQ	YFG	BJ285	1/12

**RA<sup>+</sup>RO<sup>-</sup>**

AS13	BV681	NONFUNCTIONAL				1/13
AS14	BV681	CAS	SFSGLAYNEQ	FFG	BJ281	1/13
AS15	BV682	CAS	SGTGGGLADYNEQ	FFG	BJ282	1/13
AS16	BV681	NONFUNCTIONAL				1/13
AS17	BV681	CAS	SWTGETDTQ	YFG	BJ283	1/13
AS18	BV682	CAS	SLAGEETGEL	FFG	BJ282	1/13
AS19	BV685	CAS	SSVFTDTQ	YFG	BJ283	1/13
<b>AS20</b>	<b>BV682</b>	<b>CAS</b>	<b>SLGQAYEQ</b>	<b>YFG</b>	<b>BJ287</b>	<b>1/13</b>
AS21	BV681	NONFUNCTIONAL				1/13
AS22	BV682	CAS	SYASAGLLVNEQ	FFG	BJ281	1/13
AS23	BV686	CAS	SPQQGVGEL	FFG	BJ282	1/13
AS24	BV682	CAS	SSPRAGTLGDTQ	YFG	BJ283	1/13
AS25	BV682	CAS	SPMTGSSSYNEQ	FFG	BJ281	1/13

**RO<sup>+</sup>RA<sup>-</sup>**

<b>AS26</b>	<b>BV682</b>	<b>CAS</b>	<b>SLGQAYEQ</b>	<b>YFG</b>	<b>BJ287</b>	<b>15/18 (83%)</b>
<b>AS27</b>	<b>BV682</b>	<b>CAS</b>	<b>SSGQAYEQ</b>	<b>YFG</b>	<b>BJ287</b>	<b>1/18 (6%)</b>
AS28	BV681	CAS	SPNRRAVQQGVNGY	TFG	BJ182	1/18
AS29	BV686	CAS	SPGPNYEQ	YFG	BJ287	1/18

**PUBLIC FLRGRAYGL CLONOTYPE**

BV682	CAS	SLGQAYEQ	YFG	BJ287
-------	-----	----------	-----	-------

C

**EBV<sup>-</sup> Adult D3**

Clone	TCRBV	FW	CDR3	FW	TCRBJ	n
<b>PBMC</b>						
JS1	BV681	CAS	SLLPANTGEL	FFG	BJ282	1/15
JS2	BV683	CAS	SPSPDTL	TFG	BJ286	1/15
JS3	BV685	CAS	SLGQGSRKL	FFG	BJ184	1/15
JS4	BV683	CAS	SLASVSAGEL	FFG	BJ282	1/15
JS5	BV686	CAS	SLDATYNEQ	FFG	BJ281	1/15
JS6	BV681	CAS	SPTGGVAYEQ	YFG	BJ287	1/15
JS7	BV682	CAS	SLEQVDYGY	TFG	BJ182	1/15
JS8	BV681	CAS	SLGRGSSYEQ	YFG	BJ287	1/15
JS9	BV685	CAS	SLQGGDEA	FFG	BJ181	1/15
JS10	BV682	CAS	STRRAGEL	FFG	BJ282	1/15
JS11	BV682	CAS	SEDLGVGPPQ	HFG	BJ185	1/15
JS12	BV682	CAS	SLPGGGTYEQ	YFG	BJ287	1/15
JS13	BV683	CAS	SLAGRSSYEQ	YFG	BJ287	1/15
JS14	BV682	CAS	LLGQQETQ	YFG	BJ285	1/15
JS15	BV686	CAS	SLNEGTEQ	YFG	BJ287	1/15

**RA<sup>+</sup>RO<sup>-</sup>**

JS16	BV685	CAS	SPKWRHYGY	TFG	BJ182	3/23
JS17	BV682	CAS	ERDPSWGTDTQ	YFG	BJ283	2/23
JS18	BV682	CAG	GVAGEETQ	YFG	BJ285	1/23
JS19	BV685	CAS	SFGWTAPYGY	TFG	BJ182	1/23
JS20	BV683	CAS	SWTTLRLTDTQ	YFG	BJ283	1/23
JS21	BV682	CAS	SIRGASSYNEQ	FFG	BJ281	1/23
JS22	BV683	CAS	SIGTAYGY	TFG	BJ182	1/23
JS23	BV682	CAS	SSGSYEQ	YFG	BJ287	1/23
JS24	BV681	CAS	SLTSGRPYEQ	YFG	BJ287	1/23
JS25	BV683	CAS	AGLDRVYQETQ	YFG	BJ285	1/23
JS26	BV682	CAS	SLATGTSGGWLPSEQ	FFG	BJ281	1/23
JS27	BV681	NONFUNCTIONAL				1/23
JS28	BV682	CAS	SLFASGPGGETQ	YFG	BJ285	1/23
JS29	BV682	CAS	RSVWGTDTQ	YFG	BJ283	1/23
JS30	BV686	CAS	SLNEGTEQ	YFG	BJ287	1/23
JS31	BV683	CAS	SLLGHEQ	YFG	BJ287	1/23
JS32	BV682	CAS	HAQANGNTI	YFG	BJ183	1/23
JS33	BV681	CAS	SLYNAOGLDTGEL	FFG	BJ282	1/23
JS34	BV683	CAS	SKYRQNSPL	HFG	BJ186	1/23
JS35	BV683	CAS	ROGGGHEQ	FFG	BJ281	1/23

**RO<sup>+</sup>RA<sup>-</sup>**

JS36	BV682	CAS	ERDPSWGTDTQ	YFG	BJ283	4/19
JS37	BV683	CAS	SPQAIDEQ	FFG	BJ281	1/19
JS38	BV683	CAS	SGTGGLAGDYNEQ	FFG	BJ281	1/19
JS39	BV683	CAS	SLNEGTEQ	YFG	BJ287	1/19
JS40	BV682	CAS	SLETRAYGY	TFG	BJ182	1/19
JS41	BV683	CAS	RPADSNEQ	FFG	BJ281	1/19
JS42	BV681	NONFUNCTIONAL				1/19
JS43	BV681	NONFUNCTIONAL				1/19
JS44	BV682	CAS	SILRSGSYNEQ	FFG	BJ281	1/19
JS45	BV683	CAS	SLTADNYEQ	YFG	BJ287	1/19
JS46	BV681	CAS	SLYRTGYMADTQ	YFG	BJ283	1/19
JS47	BV682	CAS	SSGGY	TFG	BJ182	1/19
JS48	BV682	CAS	SLRQMNTEA	FFG	BJ181	2/19
JS49	BV683	CAS	SLGQYEQ	YFG	BJ287	1/19
JS50	BV683	CAS	SQFLARLDTQ	YFG	BJ283	1/19

Figure 3. Diversity of TCRBV6 junctional region sequences within different T cell repertoires of HLA B8<sup>+</sup>, EBV-seropositive, and EBV-seronegative adults. The V-D-J junctional region amino acid sequences of recombinant BV6 clones are shown for unsorted PBMCs and sorted populations of CD8<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup> (RA<sup>+</sup>RO<sup>-</sup>) and CD8<sup>+</sup>CD45RO<sup>+</sup>CD45RA<sup>-</sup> (RO<sup>+</sup>RA<sup>-</sup>) cells of EBV seropositive adults D1 (A) and D2 (B) and the seronegative adult D3 (C). The frequency (n) of each clone was determined as detailed in Methods. For comparison, the V-D-J junctional region of the public FLRGRAYGL-specific clonotype is also shown at the bottom of A and B. Rearrangements corresponding to the public FLRGRAYGL-specific clonotype are highlighted in bold type. In donor D2 (B) a variant of the public clonotype (clone AS27), involving a single amino acid substitution (L to S) in the CDR3 region, was identified that is known to be FLRGRAYGL specific (33). For each clone, the deduced amino acid sequence of the CDR3 loop, defined according to Chothia et al. (43), is shown putatively supported by two frame work branches (FW). Out-of-frame rearrangements are designated nonfunctional. Designations for TCRBJ gene elements follow that of Toyonaga et al. (44) and the TCRBV6 subtypes were assigned according to Arden et al. (22). The nucleotide sequences of each of these clones are available from EMBL/GenBank/ DDBJ under accession numbers AJ224204–AJ224305.

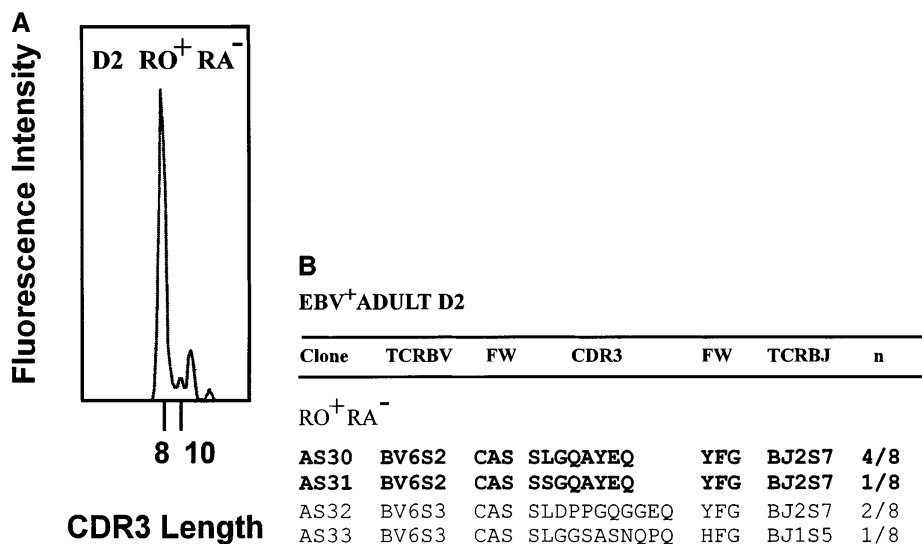
to the public EBV-specific TCRB chain (Fig. 3, A and B); the impact of this clonotype was 61% dominance (8/13 clones; LC32) in the case of donor D1 and an even higher 83% dominance (15/18 clones; AS26) in donor D2. This expansion did not result from preferential amplification of either the BV6S2 subfamily or of the SLGQAYEQ CDR3 sequence within the BV6S2 subset since neither of these were found to be overrepresented within the PBMCs and naive populations of either seropositive donor (Fig. 3, A and B) and of the HLA B8<sup>+</sup> EBV-seronegative donor, D3 (Fig. 3 C). Further confirmation that this TCRB chain's functional identity was the public EBV-specific clonotype was that for each individual, it displayed a unique codon usage within the V-D-J junctional region that corresponded precisely to the TCRB chain sequence of FLRGRAYGL-specific CTLs that previously had been functionally characterized from donors D1 and D2 (data not shown; available from accession numbers AJ224278, AJ224302, and AJ224303) (21) and that it was specific to the two HLA B8<sup>+</sup> EBV-infected individuals and was not found in any T cell population within the uninfected HLA B8<sup>+</sup> donor, D3 (Fig. 3 C). Overall, junctional region sequencing showed that the dominant peaks obtained from CDR3 length analysis were clonal and that they corresponded precisely to the public TCRB chain rearrangement that recognizes the EBV-encoded epitope, FLRGRAYGL. The PBMCs and naive CD8<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup> repertoires of both virus carriers expressed a polyclonal distribution, showing no signs of dramatic clonal predominance (Fig. 3, A and B). Analysis of data from the seronegative donor, D3, also showed a high degree of clonotype diversity within the PBMCs and CD8<sup>+</sup>CD45RO<sup>+</sup>CD45RA<sup>-</sup> and CD8<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup> T cell subsets (Fig. 3 C); the only evidence of repertoire perturbation within the memory compartment was a 21% (4/19 clones) expansion of a functionally undefined TCRBV6S2/BJ2S3 rearrangement (clone JS36). Interestingly, the EBV-specific BV6S2/BJ2S7 rearrangement was detected, albeit at low levels, within the naive compartment of seropositive donor D2 (Fig. 3 B).

Repeated repertoire analysis on seropositive donor D2 after 7 mo showed that the EBV-specific clonotype's impressive restriction on TCRBV6 diversity was persistent. Fig. 4 shows an identical CD8<sup>+</sup>CD45RO<sup>+</sup>CD45RA<sup>-</sup> repertoire profile to

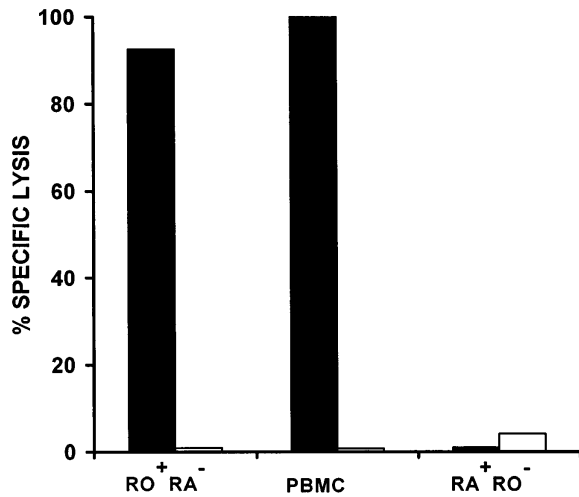
that obtained 7 mo earlier (Fig. 2 A), with > 70% of clonotypes being dominated by an 8-amino acid CDR3 length expansion (Fig. 4 A) that was identified by sequencing as the BV6S2/BJ2S7 EBV-specific clonotype (Fig. 4 B).

To fully confirm that the clonal expansions within the memory repertoire represent the public EBV-specific clonotype, the FLRGRAYGL-specific cytotoxicity of the memory and naive CD8<sup>+</sup> populations from seropositive adult D2 was assessed (Fig. 5). After several weeks of in vitro stimulation, unsorted PBMCs and the CD8<sup>+</sup>CD45RO<sup>+</sup>CD45RA<sup>-</sup> subset were found to efficiently lyse target cells sensitized with FLRGRAYGL peptide, whereas the CD8<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup> subset showed no detectable cytotoxicity (Fig. 5). These results correlated with the clonotype data that showed a predominance of the EBV-specific BV6S2/BJ2S7 clonotype within the CD8<sup>+</sup>CD45RO<sup>+</sup>CD45RA<sup>-</sup> subset. In contrast, low lying pools of virus-specific T cells such as those that were detected in the naive subset of this donor (Fig. 3 B) are likely to go undetected since they fall below the sensitivity levels of the cytotoxicity assay. Overall, these studies confirmed a functional link for the monoclonally expanded T cells responsible for major repertoire distortions in healthy HLA B8<sup>+</sup> adults with EBV specificity.

Finally, to measure the full extent of the public EBV-specific clonotype's impact on TCRBV6 repertoire diversity we analyzed the intermediate stained, triple-positive CD8<sup>+</sup>CD45RO<sup>+</sup>CD45RA<sup>+</sup> T cells (Fig. 1) from both HLA B8<sup>+</sup> seropositive donors for CDR3 length and clonotype distribution (Fig. 6). At present it is unclear whether these T cells represent a transitional phenotype between the naive and memory subsets, or whether they contain memory cells that have reverted to a naive phenotype (28–30). In seropositive donor, D1, a skewed CDR3 profile was obtained (Fig. 6 A), but these expansions did not correspond in length (Fig. 6 A) nor in rearrangement sequence (Fig. 6 B) to the public EBV clonotype. Interestingly, one of these functionally unidentified expansions (clone LC38) was found only within the dual-positive CD45RO<sup>+</sup>CD45RA<sup>+</sup> subset (Fig. 6 B) and was not detected in either the naive or memory subsets (Fig. 3 A). The CD8<sup>+</sup>CD45RO<sup>+</sup>CD45RA<sup>+</sup> repertoire of seropositive donor D2 also displayed evidence of repertoire distortion (Fig. 6 A), but in this donor



**Figure 4.** Repeated analysis of TCRBV6 repertoire diversity within CD8<sup>+</sup>CD45RO<sup>+</sup>CD45RA<sup>-</sup> (RO<sup>+</sup>RA<sup>-</sup>) cells of HLA B8<sup>+</sup>, EBV-seropositive adult D2 isolated after a 7-mo period. (A) CDR3 length profiles are displayed of fluorescence intensity (arbitrary units) as a function of CDR3 size (amino acids) for TCRBV6. (B) The V-D-J junctional region amino acid sequences of recombinant BV6 clones are shown. TCRBV and J gene segments and CDR3 region loops are presented and assigned as outlined in the legend to Fig. 3. The frequency (*n*) of each clone was determined as detailed in Methods. The nucleotide sequences of each of these clones are available from EMBL/GenBank/DBJ under accession numbers AJ009914–AJ009929.



**Figure 5.** EBV-specific cytotoxicity of different T cell populations of HLA B8<sup>+</sup>, EBV-seropositive adult D2. Specific lysis by unsorted PBMCs and sorted CD8<sup>+</sup>CD45RO<sup>+</sup>CD45RA<sup>-</sup> (RO<sup>+</sup>RA<sup>-</sup>) and CD8<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup> (RA<sup>+</sup>RO<sup>-</sup>) cells of HLA B8<sup>+</sup> PHA blasts, with (filled bars) and without (open bars) adsorbed peptide, FLRGRAYGL. Effectors were tested in standard 4-h <sup>51</sup>Cr-release assays. Effector/target ratio: 10:1.

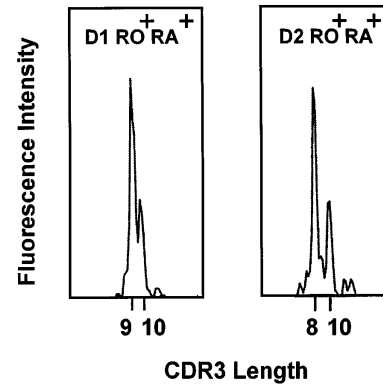
one of the expansions proved to be the EBV-specific clonotype (Fig. 6 B). Overall, these results show that not only do EBV-specific memory CTLs dominate the CD45RO<sup>+</sup> compartment of CD8<sup>+</sup> T cells, they can also impact on repertoire diversity within the intermediate CD45RO<sup>+</sup>CD45RA<sup>+</sup> subset of T cells.

## Discussion

This study uses a novel approach in monitoring antigen-specific T cells within the peripheral blood that provides evidence of a functional link between persistent EBV infection and T cell expansions in healthy adults. A public BV6S2/BJ2S7 clonotype that recognizes the EBV-encoded class I-restricted epitope, FLRGRAYGL, was found to dominate the TCRBV6 repertoire in the CD8<sup>+</sup>CD45RO<sup>+</sup>CD45RA<sup>-</sup> memory compartment of normal, EBV-seropositive individuals. So dramatic was the impact of selection of this EBV-specific clonotype that it occupied as much as 80% of the entire TCRBV6 family message. Dominant expansion of a single clonotype within TCRBV6 is significant given that this is one of the most prevalent of the known 25 functional BV families expressed in the normal human repertoire (31, 32), containing a higher than average number of seven functional subfamilies (22). Therefore, selection of the EBV-specific clonotype dramatically distorts a large and diverse BV population. Whether expansion of this clonotype increases the percentage of CD8<sup>+</sup> T cells expressing BV6 is at present unknown and is difficult to determine given the lack of antibodies specific for the BV6S2 subfamily that is predominantly rearranged to BJ2S7 within the public EBV-specific clonotype. However, from a previous study that monitored the relative expression of the BV6 family in healthy, HLA B8<sup>+</sup> EBV-seronegative and EBV-seropositive adults, no selective increase in TCRBV6 frequency was found to be associated with long-term exposure to EBV (33).

The identification of EBV-specific TCR clonotypes within

**A**



**B**

### EBV<sup>+</sup> ADULT D1

Clone	TCRBV	FW	CDR3	FW	TCRBJ	n
<b>RO<sup>+</sup>RA<sup>+</sup></b>						
LC37	BV6S1	CAS	RDPGQDYGY	TFG	BJ1S2	12/20
LC38	BV6S4	CAS	RYRDDSYNEQ	FFG	BJ2S1	6/20
LC39	BV6S5	CAS	SLDLAGGLYNEQ	FFG	BJ2S1	1/20
LC40	BV6S5	CAS	SQWREVNEQ	FFG	BJ2S1	1/20

### EBV<sup>+</sup> ADULT D2

Clone	TCRBV	FW	CDR3	FW	TCRBJ	n
<b>RO<sup>+</sup>RA<sup>+</sup></b>						
AS34	BV6S2	CAS	SLGQAYEQ	YFG	BJ2S7	11/26
AS35	BV6S2	CAS	SSGQAYEQ	YFG	BJ2S7	2/26
AS36	BV6S1	CAS	SPNRAVGQGVNGY	TFG	BJ1S2	3/26
AS37	BV6S1		NONFUNCTIONAL			3/26
AS38	BV6S1	CAS	SLILLSNTEA	FFG	BJ1S1	3/26
AS39	BV6S3	CAS	SLALATEQ	YFG	BJ2S7	1/26
AS40	BV6S1	CAS	SAWVSSYNEQ	FFG	BJ2S1	1/26
AS41	BV6S3	CAS	SQAPPGGAYEQ	YFG	BJ2S7	1/26
AS43	BV6S3	CAS	SQAPPRGAYEQ	YFG	BJ2S7	1/26

**Figure 6.** Diversity of the TCRBV6 repertoire within the intermediate stained CD8<sup>+</sup>CD45RO<sup>+</sup>CD45RA<sup>+</sup> (RO<sup>+</sup>RA<sup>+</sup>) repertoires of HLA B8<sup>+</sup>, EBV-seropositive donors D1 and D2. (A) CDR3 length profiles are displayed of fluorescence intensity (arbitrary units) as a function of CDR3 size (amino acids) for TCRBV6. (B) The V-D-J junctional region amino acid sequences of recombinant BV6 clones are shown. TCRBV and J gene segments and CDR3 region loops are presented and assigned as outlined in the legend to Fig. 3. The frequency (n) of each clone was determined as detailed in Methods. The nucleotide sequences of each of these clones are available from EMBL/GenBank/DDBJ under accession numbers AJ009914–AJ009929.

the CD8<sup>+</sup> T cell memory subset is consistent with earlier studies that functionally identified EBV-specific CTLs predominantly within the CD8<sup>+</sup>CD45RO<sup>+</sup>CD45RA<sup>-</sup> compartment (34). There was also phenotypic heterogeneity between different individuals in detecting the public EBV-specific clonotype within the dual-positive, CD45RO<sup>+</sup>CD45RA<sup>+</sup> cells. The significance of this phenotypic heterogeneity is at present unknown, but it may reflect differential levels of antigen-specific T cell activation in vivo that alter the turnover and/or recycling of antigen-specific cells between the naive and memory pools. Indeed, recent studies by Bunce and Bell (35) suggest that antigen-specific, memory CD4<sup>+</sup> T cells are divided between two

different compartments, a CD45RA<sup>+</sup> and a CD45RO<sup>+</sup> subset. In this model antigen restimulation is required for stable maintenance of these memory cells within the CD45RO<sup>+</sup> pool. Variable expression of CD45RO and CD45RA may also reflect functional differences among clonotypically identical T cells such as effector and long-term memory functions. A similar observation has been made recently by Callan et al. (36) using soluble tetrameric MHC for detecting virus-specific T cells in primary EBV-infected donors clinically diagnosed with infectious mononucleosis (IM). In these studies they found that in individuals several years after IM, a subset of virus-specific T cells expressing CD45RA could be detected. This was unlike the situation in IM where the majority of antigen-specific cells expressed CD45RO. Whether, as in our study, these cells co-express CD45RA and CD45RO was not determined, but this observation could prove to be important in understanding the maintenance and stability of clonotypic expansions within the circulation.

It has been postulated that life-long exposure to repeated or persistent subclinical virus infection may play a key role in the genesis of dramatically expanded T cell populations within healthy individuals (8, 9, 12, 37). Indeed, several features of CD8<sup>+</sup> T cell expansions, including their predominance and persistence within the activated/memory repertoire, are consistent with their development through environmental exposure. We provide evidence in this study that class I-restricted memory CTLs with specificity for EBV contribute to the major expansions that have been documented in the peripheral blood repertoires of healthy individuals. The maintenance of high levels of EBV-specific memory CTLs in healthy individuals throughout life (24) reflects their essential immunosurveillance role in controlling virus infection, a finding that is also characteristic of the long-term persistence of expanded clonotypes in healthy individuals. Indeed, the impressive ability of EBV-specific CTLs to cause complete regression of virus-induced lymphoproliferative lesions in immunosuppressed patients (38, 39) strongly suggests that their prevalence and dramatic impact on repertoire diversity are important for maintaining the life-long asymptomatic virus–host balance in normal health. Overall, EBV immunity is likely to have a major impact on repertoire diversity within a large proportion of the human population given the ubiquitous nature of this herpesvirus infection and the unprecedented level of TCR restriction that appears to be associated with the long-term memory CTL response to EBV. Aside from the HLA B8-restricted FLRGRAYGL response, we have identified recently several other highly restricted EBV-specific TCR responses that are presented through a range of HLA alleles. Studies are underway to assess the impact on repertoire diversity of these CD8<sup>+</sup> T cell memory responses.

Using the elegant MHC class I-peptide tetramer technology, massive expansions of CD45RO<sup>+</sup>, EBV-specific CD8<sup>+</sup> T cells have been documented recently within individuals afflicted by IM (36). Resolution of the disease and transition to the long-term virus carrier state are accompanied by a fall in virus-specific T cell precursors; the tetrameric complexes estimated the frequency of EBV-specific T cells of a defined epitope specificity as < 0.5% CD8<sup>+</sup> T cells in healthy virus carriers. Despite the culling of large numbers of virus-specific T cells after the peak of lymphoproliferation in IM, it is clear from the findings in this study that the resulting frequencies in healthy donors long term after primary infection can significantly

alter the  $\alpha\beta$  memory repertoire. This most likely is linked with the unusually high degree of TCR restriction that exists within the T cell response for the EBV-encoded epitope, FLRGRAYGL (21). Given the limitation of tetramer technology in only detecting antigen-specific T cells at > 0.1% to 0.5% of CD8<sup>+</sup> T cells (40), our approach at present provides a reliable and sensitive method of tracking virus-specific CD8<sup>+</sup> T cells in long-term memory responses.

The identified origin of these stably expanded clonotypes and their role in maintaining a balanced host–virus relationship in normal health is extremely important to studies interpreting patterns of clonal dominance in disease. Indeed, distinct patterns of clonal restriction and structural evidence of antigen specificity within the TCR V, J, and CDR3 regions have often been interpreted as evidence for a role of these T cells in disease pathogenesis. However, our findings emphasize the importance of HLA restriction and EBV immunostatus on the outcome of studies comparing peripheral blood repertoire diversity in health and disease. We would also predict that other highly focused, immunodominant memory T cell responses are likely to dramatically skew TCR repertoire distribution in healthy individuals. For example, dominant public TCR responses have also been reported in influenza virus infection (41). Ultimately, knowledge of T cell specificity is needed to address the issue of an immunoregulatory role versus stimulation by unrelated factors of expanded T cells in disease. Interestingly, this study also shows that the CD8<sup>+</sup> T cells with an intermediate CD45RO<sup>+</sup>CD45RA<sup>+</sup> phenotype may also harbor major clonotype expansions that are precluded from standard analyses that define strongly positive CD45RA<sup>+</sup> and CD45RO<sup>+</sup> phenotypes as naive and memory cells, respectively. More recent studies that have used CD45RA and CD27 expression to separate naive, memory, and effector human CD8<sup>+</sup> T cells within the circulation (42) might also prove useful in monitoring expansion development and in unraveling their functional significance. Overall, this first identified link for CD8<sup>+</sup> T cell expansions provides the basis for future studies monitoring the development of extreme clonal dominance within the normal repertoire and the effects of aging and disease on repertoire progression.

## Acknowledgments

We thank Dr. Xiaohua Chen and Alana Goldman for their helpful assistance in using the Applied Biosystems 373A DNA sequencer and the GENESCAN Analysis 2.1 Software, Grace Chojnowski and Paula Hall for their expert technical assistance with FACS<sup>®</sup> sorting, Drs. Scott Burrows and Rajiv Khanna for critically reviewing the manuscript, and Dr. John Whitson for performing the blood collections.

This work was supported by the National Health and Medical Research Council of Australia.

## References

1. Kluin-Nelemans, J.C., M.G.D. Kester, J.J. Melenhorst, J.E. Landegent, L. van de Corput, R. Willemze, and J.H.F. Falkenburg. 1996. Persistent clonal excess and skewed T cell repertoire in T cells from patients with hairy cell leukemia. *Blood*. 87:3795–3802.
2. Moss, P., G. Gillespie, P. Frodsham, J. Bell, and H. Reyburn. 1996. Clonal populations of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in patients with multiple myeloma and paraproteinemia. *Blood*. 87:3297–3306.
3. Choi, Y., J.A. Lafferty, J.R. Clements, J.K. Todd, E.W. Gelfand, J. Kappler, P. Marrack, and B.L. Kotzin. 1990. Selective expansion of T cells expressing V $\beta$ 2 in toxic shock syndrome. *J. Exp. Med.* 172:981–984.



4. Paliard, X., S.G. West, J.A. Lafferty, J.R. Clements, J.W. Kappler, and P. Marrack. 1991. Evidence for the effects of a superantigen in rheumatoid arthritis. *Science*. 253:325–329.
5. Wucherpfennig, K.W., and D.A. Hafler. 1995. A review of T cell receptors in multiple sclerosis: clonal expansion and persistence of human T cells specific for an immunodominant myelin basic protein peptide. *Ann. NY Acad. Sci.* 756:241–258.
6. Pantaleo, G., J.F. Demarest, H. Soudeyans, C. Graziosi, F. Denis, J.W. Adelsberger, P. Borrow, M.S. Saag, G.M. Shaw, R.P. Sekaly, and A.S. Fauci. 1994. Major expansion of CD8<sup>+</sup> T cells with a predominant  $\nu$  beta usage during the primary immune response to HIV. *Nature*. 370:463–467.
7. Callan, M.F.C., N. Steven, P. Krausa, J.D.K. Wilson, P.A.H. Moss, G.M. Gillespie, J.I. Bell, A.B. Rickinson, and A.J. McMichael. 1996. Large clonal expansions of CD8<sup>+</sup> T cells in acute infectious mononucleosis. *Nat. Med.* 2:906–911.
8. Posnett, D.N., R. Sinha, S. Kabak, and C. Russo. 1994. Clonal populations of T cells in normal elderly humans: the T cell equivalent to “benign monoclonal gammopathy.” *J. Exp. Med.* 179:609–618.
9. Schwab, R., P. Szabo, J.S. Manavalan, M.E. Weksler, D.N. Posnett, C. Pannetier, P. Kourilsky, and J. Even. 1997. Expanded CD4<sup>+</sup> and CD8<sup>+</sup> T cell clones in elderly humans. *J. Immunol.* 158:4493–4499.
10. Wang, E.C.Y., P.A.H. Moss, P. Frodsham, P.J. Lehner, J.I. Bell, and L.K. Borysiewicz. 1995. CD8 high CD57<sup>+</sup> T lymphocytes in normal, healthy individuals are oligoclonal and respond to human cytomegalovirus. *J. Immunol.* 155:5046–5056.
11. Bonfert, V., J. Cihak, U. Losch, and H.W.L. Ziegler-Heitbrock. 1995. Preferential expression of V beta gene families in CD8 memory cells of apparently healthy donors. *Cell. Immunol.* 166:165–171.
12. Hingorani, R., I.H. Choi, P. Akolkar, B. Gulwani Akolkar, R. Pergolizzi, J. Silver, and P.K. Gregersen. 1993. Clonal predominance of T cell receptors within the CD8<sup>+</sup> CD45RO<sup>+</sup> subset in normal human subjects. *J. Immunol.* 151:5762–5769.
13. Grunewald, J., M. Jeddi-Tehrani, H. DerSimonian, R. Andersson, and H. Wigzell. 1992. A persistent T cell expansion in the peripheral blood of a normal adult male: a new clinical entity? *Clin. Exp. Immunol.* 89:279–284.
14. Fitzgerald, J.E., N.S. Ricalton, A. Meyer, S.G. West, H. Kaplan, C. Behrendt, and B.L. Kotzin. 1995. Analysis of clonal CD8<sup>+</sup> T cell expansions in normal individuals and patients with rheumatoid arthritis. *J. Immunol.* 154:3538–3547.
15. Morley, J.K., F.M. Batiwalla, R. Hingorani, and P.K. Gregersen. 1995. Oligoclonal CD8<sup>+</sup> T cells are preferentially expanded in the CD57<sup>+</sup> subset. *J. Immunol.* 154:6182–6190.
16. Lantelme, E., L. Granziero, L. Angman, and C. Giachino. 1997. Clonal predominance, but preservation of a polyclonal reservoir, in normal alpha beta T cell repertoire. *Hum. Immunol.* 53:49–56.
17. Hedrick, S.M., and F.J. Eidelman. 1993. T lymphocyte antigen receptor. In *Fundamental Immunology*. W.E. Paul, editor. Raven Press, New York. 383–420.
18. Jorgensen, J.L., U. Esser, B. Fazekas de St. Groth, P.A. Reay, and M.M. Davis. 1992. Mapping TCR-peptide contacts by variant peptide immunization of single-chain transgenics. *Nature*. 355:224–230.
19. Garcia, K.C., M. Degano, R.L. Stanfield, A. Brunmark, M.R. Jackson, P.A. Peterson, L. Teyton, and I.A. Wilson. 1996. An alpha-beta T cell receptor structure at 2.5 angstrom and its orientation in the TCR-MHC complex. *Science*. 274:209–219.
20. Garboczi, D.N., P. Ghosh, U. Utz, Q.R. Fan, W.E. Biddison, and D.C. Wiley. 1996. Structure of the complex between human T cell receptor, viral peptide and HLA-A2. *Nature*. 384:134–141.
21. Argat, V.P., C.W. Schmidt, S.R. Burrows, S.L. Silins, M.G. Kurilla, D.L. Doolan, A. Suhrbier, D.J. Moss, E. Kieff, T.B. Sculley, and I.S. Misko. 1994. Dominant selection of an invariant T cell antigen receptor in response to persistent infection by Epstein-Barr virus. *J. Exp. Med.* 180:2335–2340.
22. Arden, B., S.P. Clark, D. Kabelitz, and T.W. Mak. 1995. Human T cell receptor variable gene segment families. *Immunogenetics*. 42:455–500.
23. Silins, S.L., S.R. Burrows, D.J. Moss, R. Khanna, I.S. Misko, and V.P. Argat. 1995. T cell receptor repertoire for a viral epitope in humans is diversified by tolerance to a background major histocompatibility complex antigen. *J. Exp. Med.* 182:1–13.
24. Bourgault, I., A. Gomez, E. Gomard, and J.P. Levy. 1991. Limiting-dilution analysis of the HLA restriction of anti-Epstein-Barr virus-specific cytotoxic T lymphocytes. *Clin. Exp. Immunol.* 84:501.
25. Panzara, M.A., E. Gussoni, L. Steinman, and J.R. Oksenberg. 1992. Analysis of the T cell repertoire using the PCR and specific oligonucleotide primers. *Biotechniques*. 12:728–735.
26. Pannetier, C., M. Cochet, S. Darce, A. Casrouge, M. Zöller, and P. Kourilsky. 1993. The sizes of the CDR3 hypervariable regions of the murine T cell receptor beta chains vary as a function of the recombined germ-line. *Proc. Natl. Acad. Sci. USA*. 90:4319–4323.
27. Pannetier, C., J. Even, and P. Kourilsky. 1995. T cell repertoire diversity and clonal expansions in normal and clinical samples. *Immunol. Today*. 16:176–181.
28. Bell, E.B., and S.M. Sparshott. 1990. Interconversion of CD45R subsets of CD4 T cells in vivo. *Nature*. 348:163–166.
29. Rocha, B., C. Penit, C. Baron, F. Vasseur, N. Dautigny, and A.A. Freitas. 1990. Accumulation of bromodeoxyuridine-labeled cells in central and peripheral organs: minimal estimates of production and turnover rates of mature lymphocytes. *Eur. J. Immunol.* 20:1697.
30. Sprent, J., and D.F. Tough. 1994. Lymphocyte life-span and memory. *Science*. 265:1395–1400.
31. Rosenberg, W.M., P.A. Moss, and J.I. Bell. 1992. Variation in human T cell receptor V beta and J beta repertoire: analysis using anchor polymerase chain reaction. *Eur. J. Immunol.* 22:541–549.
32. Loveridge, J.A., W.M. Rosenberg, T.B. Kirkwood, and J.I. Bell. 1991. The genetic contribution to human T cell receptor repertoire. *Immunology*. 74:246–250.
33. Silins, S.L., S.M. Cross, S.L. Elliott, S.J. Pye, S.R. Burrows, J.M. Burrows, D.J. Moss, V.P. Argat, and I.S. Misko. 1996. Development of Epstein-Barr virus-specific memory T cell receptor clonotypes in acute infectious mononucleosis. *J. Exp. Med.* 184:1815–1824.
34. Merckenschlager, M., and P.C.L. Beverley. 1989. Evidence for differential expression of CD45 isoforms by precursors for memory-dependent and independent cytotoxic responses: human CD8 memory CTLp selectively express CD45RO (UCHL1). *Int. Immunol.* 1:450–459.
35. Bunce, C., and E.B. Bell. 1997. CD45RC isoforms define two types of CD4 memory T cells, one of which depends on persisting antigen. *J. Exp. Med.* 185:767–776.
36. Callan, M.F.C., L. Tan, N. Annels, G.S. Ogg, J.D.K. Wilson, C.A. O’Callaghan, N. Steven, A.J. McMichael, and A.B. Rickinson. 1998. Direct visualization of antigen-specific CD8<sup>+</sup> T cells during the primary immune response to Epstein-Barr virus in vivo. *J. Exp. Med.* 187:1395–1402.
37. Grunewald, J., and H. Wigzell. 1996. T cell expansions in healthy individuals. *The Immunologist*. 4:99–103.
38. Papadopoulos, E.B., M. Ladanyi, D. Emanuel, S. Mackinnon, F. Boudad, M.H. Carabasi, H. Castro-Melaspina, B.H. Childs, A.P. Gillio, T.N. Small, et al. 1994. Infusions of donor leukocytes to treat Epstein-Barr virus-associated lymphoproliferative disorders after allogeneic bone marrow transplantation. *N. Engl. J. Med.* 330:1185–1191.
39. Rooney, C.M., C.A. Smith, C.Y.C. Ng, S. Loftin, C. Li, R.A. Krance, M.K. Brenner, and H.E. Heslop. 1995. Use of gene-modified virus-specific T lymphocytes to control Epstein-Barr virus-related lymphoproliferation. *Lancet*. 345:9–13.
40. Kuroda, M.J., J.E. Schmitz, D.H. Barouch, A. Craiu, T.M. Allen, A. Sette, D.I. Watkins, M.A. Forman, and N.L. Letvin. 1998. Analysis of Gag-specific cytotoxic T lymphocytes in simian immunodeficiency virus-infected rhesus monkeys by cell complex class I-peptide complex. *J. Exp. Med.* 187:1373–1381.
41. Lehner, P.J., E.C.Y. Wang, P.A.H. Moss, S. Williams, K. Platt, S.M. Friedman, J.I. Bell, and L.K. Borysiewicz. 1995. Human HLA-A0201-restricted cytotoxic T lymphocyte recognition of influenza A is dominated by T cells bearing the V $\beta$ 17 gene segment. *J. Exp. Med.* 181:79–91.
42. Hamann, D., P.A. Baars, M.H.G. Rep, B. Hooibrink, S.R. Kerkhof-Garde, M.R. Klein, and R.A.W. van Lier. 1997. Phenotypic and functional separation of memory and effector human CD8<sup>+</sup> T cells. *J. Exp. Med.* 186:1407–1418.
43. Chothia, C., D.R. Boswell, and A.M. Lesk. 1988. The outline structure of the T cell alpha beta receptor. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:3745–3755.
44. Toyonaga, B., Y. Yoshikai, V. Vadasz, B. Chin, and T.W. Mak. 1985. Organization and sequences of the diversity, joining, and constant region genes of the human T cell receptor beta chain. *Proc. Natl. Acad. Sci. USA*. 82:8624–8628.