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Research Article

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

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Abstract

Dramatic clonal expansions of unknown functional significance have been documented in the T cell receptor (TCR) αβ 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 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 αβ 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, αβ T cell expansions can persist unaltered for years, predominantly occur within the $CD8^+$ T lymphocyte subset (8, 10, 14, 15), often express a $CD45RO^+$ 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)[1] rearrangements. The majority of T cells express an αβ 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 $CD8^+$ 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 $B8^+$ 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-

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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 αβ repertoire of healthy virus carriers. By examining the diversity of the TCRBV6 repertoire within different T cell populations of HLA $B8^+$ adults, we herein show that expansion of the EBV-specific clonotype in healthy virus-infected individuals dramatically skews the entire $CD8^+$ $CD45RO^+$ $CD45RA^-$ 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 $B8^+$, long-term EBV-seropositive donors (viral capsid antigen IgG^+, EBV nuclear antigen IgG^+), D1 and D2, aged 51 and 37 yr, respectively. Peripheral blood was also taken from two HLA $B8^+$, EBV-seronegative donors (viral capsid antigen IgG^-, EBV nuclear antigen IgG^-), 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 $B8^+$ status of each donor was initially determined by FACScan® 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 $CD8^+CD45RA^+CD45RO^-$, $CD8^+CD45RO^+CD45RA^-$, and $CD8^+CD45RO^+CD45RA^+$ T cells. 1–2 × 10^7 PBMCs were incubated with anti–human CD4 mAb-labeled magnetic beads (Dyna beads M-450; DYNAL, Melbourne, Australia) to enrich for $CD8^+$ lymphocytes according to the manufacturer's recommendation. The $CD8^+$-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 $CD8^+CD45RA^+CD45RO^-$, $CD8^+CD45RO^+CD45RA^-$, and $CD8^+CD45RO^+CD45RA^+$ population, using a Becton Dickinson FACS® Vantage Flow Cytometer. The purity of the recovered cells was > 98%.

RNA isolation and cDNA synthesis. Total RNA was extracted from 1–5 × 10^5 PBMCs or sorted $CD8^+CD45RA^+CD45RO^-$, $CD8^+CD45RO^+CD45RA^-$, and $CD8^+CD45RO^+CD45RA^+$ cells using Total RNA Isolation Reagent (Advanced Biotechnologies, London, UK). First strand cDNA was synthesized using an antisense TCRBC primer (C_{b1}) 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 (Vβ6) (25) and a nested 3′ TCRBC-specific primer (C_{b2}) (21). Amplifications were performed in 25-μl reaction volumes using first strand cDNA representing material from 1–5 × 10^4 cells, 10 pmol of each 5′ and 3′ primer, 200 μM dNTPs, 1.5 mM $MgCl_2$, 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 (CβP*: 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 Vβ6 and C_{b2} 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 $CD8^+CD45RA^+CD45RO^-$ and $CD8^+CD45RO^+CD45RA^-$ cells from seropositive donor D2 were activated weekly by stimulation with the γ-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 μg/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 μCi of ^{51}Cr at 37°C for 90 min, with and without peptide, washed twice by centrifugation, and used in standard 4-h ^{51}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 $B8^+$ 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 $B8^+$ 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-

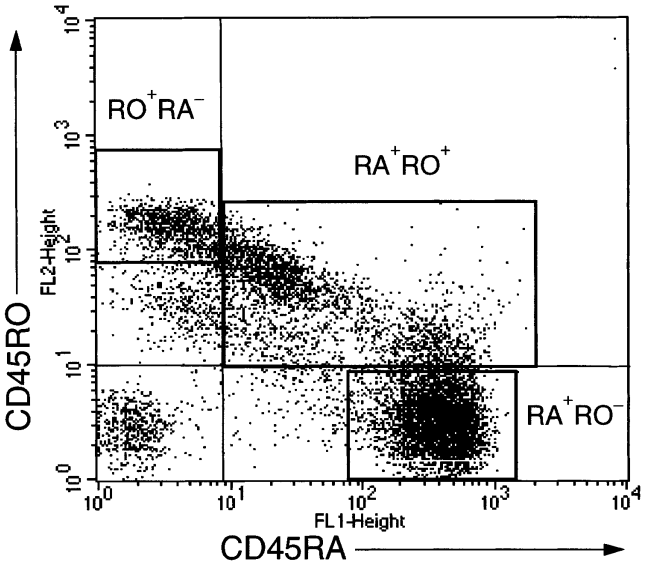
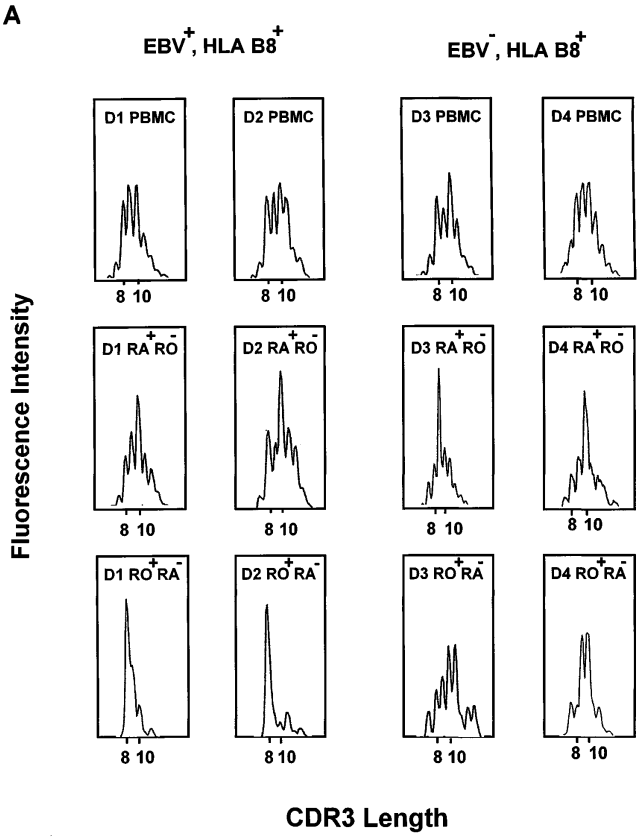


Figure 1. Three-color immunofluorescence analysis of PBMCs of adult donor D2. $CD8^+$-enriched cells were simultaneously stained with tricolor-conjugated CD8 in combination with FITC-conjugated CD45RA and PE-conjugated CD45RO mAbs. Cells were gated on size and tricolor fluorescence. Three distinct populations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



B

RELATIVE PEAK INTENSITIES

CDR3 SIZE (amino acids)	RO^+RA^-			
	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 $B8^+$, 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 $CD8^+CD45RA^+CD45RO^-$ (RA^+RO^-) and $CD8^+CD45RO^+$ $CD45RA^-$ (RO^+RA^-) 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 $CD8^+$ $CD45RO^+CD45RA^-$ (RO^+RA^-) subset of each adult are reported. Relative peak intensities are calculated as described in Methods.

sured by a PCR-based approach that determines the distribution of CDR3 lengths within a given TCRBV 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 $CD8^+CD45RA^+CD45RO^-$ (naive) or $CD8^+CD45RO^+$ $CD45RA^-$ (memory) T cells that were > 98% pure. Fig. 1 shows the three-color immunofluorescence FACS® 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 $CD45RO^+$ $CD45RA^-$ cells and $CD45RA^+CD45RO^-$ 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 $CD8^+CD45RO^+CD45RA^-$ 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 $CD45RO^+$ memory subset since gaussian-like profiles of polyclonality were recorded in both unsorted PBMCs and in sorted $CD8^+$ $CD45RA^+CD45RO^-$ 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 $B8^+$, EBV-seronegative adults (D3 and D4) showed no evidence of repertoire focusing (Fig. 2, *A* and *B*).

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 $CD8^+CD45RO^+CD45RA^-$ 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$^+$ Adult D1**

Clone	TCRBV	FW	CDR3	FW	TCRBJ	n
PBMC						
LC1	BV6S5	CAS	SPGQGETQ	YFG	BJ2S7	2/17
LC2	BV6S5	CAS	SRLKATEA	FFG	BJ1S1	1/17
LC3	BV6S1	NONFUNCTIONAL				1/17
LC4	BV6S2	CAS	RNRGRRENTEA	FFG	BJ1S1	1/17
LC5	BV6S1	NONFUNCTIONAL				1/17
LC6	BV6S5	CAS	SLAGTQETQ	YFG	BJ2S5	1/17
LC7	BV6S5	CAS	SADRLWG	NFG	BJ2S3	1/17
LC8	BV6S2	CAS	SFGGPGAL	HFG	BJ1S6	1/17
LC9	BV6S5	CAS	SLDRGVGY	TFG	BJ1S2	1/17
LC10	BV6S5	NONFUNCTIONAL				1/17
LC11	BV6S5	CAS	SLAQDYGY	TFG	BJ1S2	1/17
LC12	BV6S1	NONFUNCTIONAL				1/17
LC13	BV6S5	CAS	SGGGKVRYGY	TFG	BJ1S2	1/17
LC14	BV6S5	CAS	STQVGVGQPQ	HFG	BJ1S5	1/17
LC15	BV6S2	CAS	SPGLASTDTQ	YFG	BJ2S3	1/17
LC16	BV6S2	CAS	SLREGAEA	FFG	BJ1S1	1/17
RA$^+$RO$^-$						
LC17	BV6S2	CAS	RDPGQDYGY	TFG	BJ1S2	3/17
LC18	BV6S1	CAS	SHGGRRQPQ	HFG	BJ1S5	1/17
LC19	BV6S5	CAS	RPGTSGETQ	YFG	BJ2S5	1/17
LC20	BV6S1	CAS	SLGYGEEQ	YFG	BJ2S7	1/17
LC21	BV6S2	CAS	SLELAGYNEQ	FFG	BJ2S1	1/17
LC22	BV6S5	CAS	SLYAGSSYEQ	YFG	BJ2S7	1/17
LC23	BV6S2	CAS	SFGLDTDTQ	YFG	BJ2S3	1/17
LC24	BV6S2	CAS	SLVKGQGPSVVTYEQ	YFG	BJ2S7	1/17
LC25	BV6S8	CAS	SLALDREPQETQ	YFG	BJ2S5	1/17
LC26	BV6S2	CAS	SLPGIGHRGYEQ	YFG	BJ2S7	1/17
LC27	BV6S5	CAS	SRPSGLAGVREQ	YFG	BJ2S7	1/17
LC28	BV6S5	CAS	SLGQETQ	YFG	BJ2S5	1/17
LC29	BV6S2	CAS	SLGRGRGLYNEQ	FFG	BJ2S1	1/17
LC30	BV6S1	CAS	SFGGSYGY	TFG	BJ1S2	1/17
LC31	BV6S5	CAS	SLFPRTSGYEQ	YFG	BJ2S7	1/17
RO$^+$RA$^-$						
LC32	**BV6S2**	**CAS**	**SLGQAYEQ**	**YFG**	**BJ2S7**	**8/13 (61%)**
LC33	BV6S1	CAS	RDPGQDYGY	TFG	BJ1S2	2/13
LC34	BV6S1	CAS	SLGYGEEQ	YFG	BJ2S7	1/13
LC35	BV6S1	NONFUNCTIONAL				1/13
LC36	BV6S1	CAS	SLGASGALYNEQ	FFG	BJ2S1	1/13
PUBLIC FLRGRAYGL CLONOTYPE						
BV6S2		CAS	SLGQAYEQ	YFG	BJ2S7	

B **EBV$^+$ Adult D2**

Clone	TCRBV	FW	CDR3	FW	TCRBJ	n
PBMC						
AS1	BV6S1	CAS	SSNRGNEQ	FFG	BJ2S1	1/12
AS2	BV6S2	CAS	SLLAGEL	FFG	BJ2S2	1/12
AS3	BV6S2	CAS	SARDGSYEQ	YFG	BJ2S7	1/12
AS4	BV6S3	NONFUNCTIONAL				1/12
AS5	BV6S3	CAS	SLGGSGPSSYNEQ	FFG	BJ2S1	1/12
AS6	BV6S6	CAS	SFSGEETQ	YFG	BJ2S5	1/12
AS7	BV6S1	NONFUNCTIONAL				1/12
AS8	BV6S2	CAS	SLAPGQHSYEQ	YFG	BJ2S7	1/12
AS9	BV6S2	CAI	GGGGTDTQ	YFG	BJ2S3	1/12
AS10	BV6S1	CAS	SLNREETQ	YFG	BJ2S5	1/12
AS11	BV6S1	CAS	SPNRAVGQGVNGY	TFG	BJ1S2	1/12
AS12	BV6S1	CAS	SLVGPDEQ	YFG	BJ2S5	1/12
RA$^+$RO$^-$						
AS13	BV6S1	NONFUNCTIONAL				1/13
AS14	BV6S1	CAS	SFSGLAYNEQ	FFG	BJ2S1	1/13
AS15	BV6S2	CAS	SSTSGGYTGEL	FFG	BJ2S2	1/13
AS16	BV6S1	NONFUNCTIONAL				1/13
AS17	BV6S1	CAS	SWTGSTDTQ	YFG	BJ2S3	1/13
AS18	BV6S2	CAS	SLAGEETGEL	FFG	BJ2S2	1/13
AS19	BV6S5	CAS	SSVPSTDTQ	YFG	BJ2S3	1/13
AS20	**BV6S2**	**CAS**	**SLGQAYEQ**	**YFG**	**BJ2S7**	**1/13**
AS21	BV6S1	NONFUNCTIONAL				1/13
AS22	BV6S2	CAS	SYSASGILVNEQ	FFG	BJ2S1	1/13
AS23	BV6S6	CAS	SPGQGVGEL	FFG	BJ2S2	1/13
AS24	BV6S2	CAS	SSPRAGTLGDTQ	YFG	BJ2S3	1/13
AS25	BV6S2	CAS	SFMTSGSSYNEQ	FFG	BJ2S1	1/13
RO$^+$RA$^-$						
AS26	**BV6S2**	**CAS**	**SLGQAYEQ**	**YFG**	**BJ2S7**	**15/18(83%)**
AS27	**BV6S2**	**CAS**	**SSGQAYEQ**	**YFG**	**BJ2S7**	**1/18 (6%)**
AS28	BV6S1	CAS	SPNRAVGQGVNGY	TFG	BJ1S2	1/18
AS29	BV6S6	CAS	SPGPNYEQ	YFG	BJ2S7	1/18
PUBLIC FLRGRAYGL CLONOTYPE						
BV6S2		CAS	SLGQAYEQ	YFG	BJ2S7	

C **EBV$^-$ Adult D3**

Clone	TCRBV	FW	CDR3	FW	TCRBJ	n
PBMC						
JS1	BV6S1	CAS	SLLPANTGEL	FFG	BJ2S2	1/15
JS2	BV6S3	CAS	SPSPTDL	TFG	BJ2S6	1/15
JS3	BV6S5	CAS	SLGQGSEKL	FFG	BJ1S4	1/15
JS4	BV6S3	CAS	SLASVSAGEL	FFG	BJ2S2	1/15
JS5	BV6S6	CAS	SLDATYNEQ	FFG	BJ2S1	1/15
JS6	BV6S1	CAS	SPTGGVAYEQ	YFG	BJ2S7	1/15
JS7	BV6S2	CAS	SLEQVDYGY	TFG	BJ1S2	1/15
JS8	BV6S1	CAS	SLGRGSSYEQ	YFG	BJ2S7	1/15
JS9	BV6S5	CAS	SLQGGDTEA	FFG	BJ1S1	1/15
JS10	BV6S2	CAS	STRRAGEL	FFG	BJ2S2	1/15
JS11	BV6S2	CAS	SEDLGVGQPQ	HFG	BJ1S5	1/15
JS12	BV6S2	CAS	SLPGGQGTYEQ	YFG	BJ2S7	1/15
JS13	BV6S3	CAS	SLAGRSSYEQ	YFG	BJ2S7	1/15
JS14	BV6S2	CAS	LLGQQETQ	YFG	BJ2S5	1/15
JS15	BV6S6	CAS	SLNEGTEQ	YFG	BJ2S7	1/15
RA$^+$RO$^-$						
JS16	BV6S5	CAS	SPKWRHYGY	TFG	BJ1S2	3/23
JS17	BV6S2	CAS	ERDPSWGTDTQ	YFG	BJ2S3	2/23
JS18	BV6S2	CAG	GVAGEEETQ	YFG	BJ2S5	1/23
JS19	BV6S5	CAS	SFGWTAPYGY	TFG	BJ1S2	1/23
JS20	BV6S3	CAS	SWDTTLRTDTQ	YFG	BJ2S3	1/23
JS21	BV6S2	CAS	SIRGASSYNEQ	FFG	BJ2S1	1/23
JS22	BV6S3	CAS	SIGTAYGY	TFG	BJ1S2	1/23
JS23	BV6S2	CAS	SSGSYEQ	YFG	BJ2S7	1/23
JS24	BV6S1	CAS	SLTSGRPYEQ	YFG	BJ2S7	1/23
JS25	BV6S3	CAS	AGLDRVYQETQ	YFG	BJ2S5	1/23
JS26	BV6S2	CAS	SLATGTSGGWLPSEQ	FFG	BJ2S1	1/23
JS27	BV6S1	NONFUNCTIONAL				1/23
JS28	BV6S2	CAS	SLFASEGPGGETQ	YFG	BJ2S5	1/23
JS29	BV6S2	CAS	RSVWGTDTQ	YFG	BJ2S3	1/23
JS30	BV6S6	CAS	SLNEGTEQ	YFG	BJ2S7	1/23
JS31	BV6S3	CAS	SLLGHEQ	YFG	BJ2S7	1/23
JS32	BV6S2	CAS	HAQANGNTI	YFG	BJ1S3	1/23
JS33	BV6S1	CAS	SLYYNAQGLDTGEL	FFG	BJ2S2	1/23
JS34	BV6S3	CAS	SKYRGQNSPL	HFG	BJ1S6	1/23
JS35	BV6S3	CAS	RGGGGHEQ	FFG	BJ2S1	1/23
RO$^+$RA$^-$						
JS36	BV6S2	CAS	ERDPSWGTDTQ	YFG	BJ2S3	4/19
JS37	BV6S3	CAS	SPQAIDEQ	FFG	BJ2S1	1/19
JS38	BV6S3	CAS	SGTGGLAGDYNEQ	FFG	BJ2S1	1/19
JS39	BV6S3	CAS	SLNEGTEQ	YFG	BJ2S7	1/19
JS40	BV6S2	CAS	SLETRAYGY	TFG	BJ1S2	1/19
JS41	BV6S3	CAS	RPADSNEQ	FFG	BJ2S1	1/19
JS42	BV6S1	NONFUNCTIONAL				1/19
JS43	BV6S1	NONFUNCTIONAL				1/19
JS44	BV6S2	CAS	SILRSGSYNEQ	FFG	BJ2S1	1/19
JS45	BV6S3	CAS	SLTADNYEQ	YFG	BJ2S7	1/19
JS46	BV6S1	CAS	SLYRTGYMADTQ	YFG	BJ2S3	1/19
JS47	BV6S2	CAS	SSGGGY	TFG	BJ1S2	1/19
JS48	BV6S2	CAS	SLRQMNTEA	FFG	BJ1S1	2/19
JS49	BV6S3	CAS	SLGQIYEQ	YFG	BJ2S7	1/19
JS50	BV6S3	CAS	SQFLARTDTQ	YFG	BJ2S3	1/19

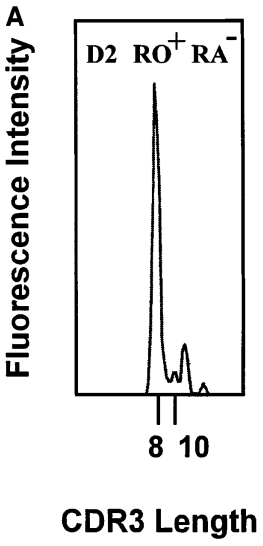
Figure 3. Diversity of TCRBV6 junctional region sequences within different T cell repertoires of HLA B8$^+$, EBV-seropositive, and EBV-seronegative adults. The V-D-J junctional region amino acid sequences of recombinant BV6 clones are shown for unsorted PBMCs and sorted populations of CD8$^+$CD45RA$^+$CD45RO$^-$ (RA$^+$RO$^-$) and CD8$^+$ CD45RO$^+$CD45RA$^-$ (RO$^+$RA$^-$) 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 $B8^+$ 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 $B8^+$ EBV-infected individuals and was not found in any T cell population within the uninfected HLA $B8^+$ 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 $CD8^+CD45RA^+CD45RO^-$ 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 $CD8^+CD45RO^+CD45RA^-$ and $CD8^+CD45RA^+CD45RO^-$ 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 $CD8^+CD45RO^+CD45RA^-$ 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 $CD8^+$ populations from seropositive adult D2 was assessed (Fig. 5). After several weeks of in vitro stimulation, unsorted PBMCs and the $CD8^+CD45RO^+CD45RA^-$ subset were found to efficiently lyse target cells sensitized with FLRGRAYGL peptide, whereas the $CD8^+CD45RA^+CD45RO^-$ 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 $CD8^+CD45RO^+CD45RA^-$ 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 $B8^+$ 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 $CD8^+CD45RO^+CD45RA^+$ T cells (Fig. 1) from both HLA $B8^+$ 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 $CD45RO^+CD45RA^+$ subset (Fig. 6 *B*) and was not detected in either the naive or memory subsets (Fig. 3 *A*). The $CD8^+CD45RO^+CD45RA^+$ repertoire of seropositive donor D2 also displayed evidence of repertoire distortion (Fig. 6 *A*), but in this donor



B

EBV^+ADULT D2

Clone	TCRBV	FW	CDR3	FW	TCRBJ	n
RO^+RA^-						
AS30	**BV6S2**	**CAS**	**SLGQAYEQ**	**YFG**	**BJ2S7**	**4/8**
AS31	**BV6S2**	**CAS**	**SSGQAYEQ**	**YFG**	**BJ2S7**	**1/8**
AS32	BV6S3	CAS	SLDPPGQGGEQ	YFG	BJ2S7	2/8
AS33	BV6S3	CAS	SLGGSASNQPQ	HFG	BJ1S5	1/8

Figure 4. Repeated analysis of TCRBV6 repertoire diversity within $CD8^+CD45RO^+CD45RA^-$ (RO^+RA^-) cells of HLA $B8^+$, 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/DDBJ under accession numbers AJ009914–AJ009929.

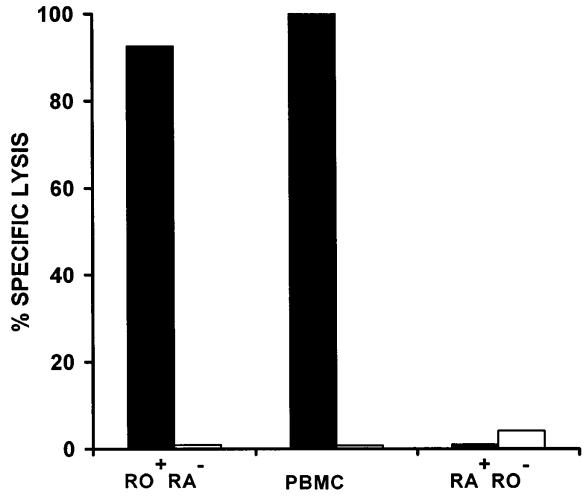


Figure 5. EBV-specific cytotoxicity of different T cell populations of HLA B8$^+$, EBV-seropositive adult D2. Specific lysis by unsorted PBMCs and sorted CD8$^+$CD45RO$^+$CD45RA$^-$ (RO$^+$RA$^-$) and CD8$^+$CD45RA$^+$CD45RO$^-$ (RA$^+$RO$^-$) cells of HLA B8$^+$ PHA blasts, with (*filled bars*) and without (*open bars*) adsorbed peptide, FLRGRAYGL. Effectors were tested in standard 4-h ^{51}Cr-release assays. Effector/target ratio: 10.1.

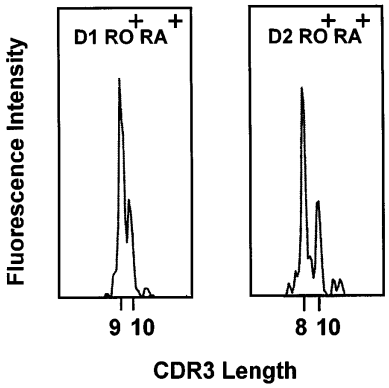
one of the expansions proved to be the EBV-specific clonotype (Fig. 6 *B*). Overall, these results show that not only do EBV-specific memory CTLs dominate the CD45RO$^+$ compartment of CD8$^+$ T cells, they can also impact on repertoire diversity within the intermediate CD45RO$^+$CD45RA$^+$ subset of 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$^+$CD45RO$^+$CD45RA$^-$ 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$^+$ 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$^+$ 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$^+$ADULT D1

Clone	TCRBV	FW	CDR3	FW	TCRBJ	n
RO$^+$RA$^+$						
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$^+$ADULT D2

Clone	TCRBV	FW	CDR3	FW	TCRBJ	n
RO$^+$RA$^+$						
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$^+$CD45RO$^+$CD45RA$^+$ (RO$^+$RA$^+$) repertoires of HLA B8$^+$, 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$^+$ T cell memory subset is consistent with earlier studies that functionally identified EBV-specific CTLs predominantly within the CD8$^+$CD45RO$^+$CD45RA$^-$ compartment (34). There was also phenotypic heterogeneity between different individuals in detecting the public EBV-specific clonotype within the dual-positive, CD45RO$^+$CD45RA$^+$ 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$^+$ T cells are divided between two

different compartments, a $CD45RA^+$ and a $CD45RO^+$ subset. In this model antigen restimulation is required for stable maintenance of these memory cells within the $CD45RO^+$ 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 coexpress 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 $CD8^+$ 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 $CD8^+$ T cell memory responses.

Using the elegant MHC class I–peptide tetramer technology, massive expansions of $CD45RO^+$, EBV-specific $CD8^+$ 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 $< 0.5\%$ $CD8^+$ 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 αβ 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 $CD8^+$ T cells (40), our approach at present provides a reliable and sensitive method of tracking virus-specific $CD8^+$ 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 $CD8^+$ T cells with an intermediate $CD45RO^+CD45RA^+$ phenotype may also harbor major clonotype expansions that are precluded from standard analyses that define strongly positive $CD45RA^+$ and $CD45RO^+$ phenotypes as naive and memory cells, respectively. More recent studies that have used CD45RA and CD27 expression to separate naive, memory, and effector human $CD8^+$ 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 $CD8^+$ 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.

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