

# Development of Epstein-Barr Virus-specific Memory T Cell Receptor Clonotypes in Acute Infectious Mononucleosis

By Sharon L. Silins, Simone M. Cross, Suzanne L. Elliott,  
Stephanie J. Pye, Scott R. Burrows, Jacqueline M. Burrows,  
Denis J. Moss, Victor P. Argæet, and Ihor S. Misko

*From the Epstein-Barr Virus Unit, Queensland Institute of Medical Research, The Bancroft Centre, Brisbane, Australia 4029*

## Summary

The importance of cytotoxic T lymphocytes (CTLs) in the immunosurveillance of Epstein-Barr virus (EBV)-infected B cells is firmly established, and the viral antigens of CTL recognition in latent infection are well defined. The epitopes targeted by CTLs during primary infection have not been identified, however, and there is only limited information about T cell receptor (TCR) selection. In the present report, we have monitored the development of memory TCR- $\beta$  clonotypes selected in response to natural EBV infection in a longitudinal study of an HLA-B8<sup>+</sup> individual with acute infectious mononucleosis (IM). By stimulating peripheral blood lymphocytes with HLA-B8<sup>+</sup> EBV-transformed B lymphoblastoid cells, the primary virus-specific CTL response was shown to include specificities for two HLA-B8-restricted antigenic determinants, FLRGRAYGL and QAKWRLQTL, which are encoded within the latent EBV nuclear antigen EBNA-3. TCR- $\beta$  sequence analysis of CTL clones specific for each epitope showed polyclonal TCR- $\beta$  repertoire selection, with structural restrictions on recognition that indicated antigen-driven selection. Furthermore, longitudinal repertoire analysis revealed long-term preservation of a multiclonal effector response throughout convalescence, with the re-emergence of distinct memory T cell clonotypes sharing similar structural restrictions. Tracking the progression of specific TCR- $\beta$  clonotypes and antigen-specific TCR-V $\beta$  family gene expression in the peripheral repertoire *ex vivo* using semiquantitative PCR strongly suggested that selective TCR- $\beta$  expansions were present at the clonotype level, but not at the TCR-V $\beta$  family level. Overall, in this first analysis of antigen-specific TCR development in IM, a picture of polyclonal TCR stimulation is apparent. This diversity may be especially important in the establishment of an effective CTL control during acute EBV infection and in recovery from disease.

EBV is a gammaherpesvirus of humans that has potent B cell growth-transforming activity (1, 2). The virus persists as a latent infection of B cells under the control of class I-restricted CD8<sup>+</sup> CTLs that recognize the latent viral antigens, which include the EBV nuclear antigens (EBNAs)<sup>1</sup> 2-6 and the latent membrane proteins (3). Primary infection generally occurs asymptotically in early childhood; however, if infection is delayed until adolescence or adulthood, it can lead to the self-limiting lymphoproliferative disease acute infectious mononucleosis (IM) (4). A vigorous cellular immune reaction, dominated by activated and proliferating CD8<sup>+</sup> CTLs, is mounted during acute IM in re-

sponse to virus-infected B cells in the peripheral blood and lymphoid tissues (5). It is not yet clear from functional studies whether the majority of these expanded, activated T cells are specific for EBV antigens or alternatively result from nonspecific, antigen-independent "bystander" activation (6, 7). Only a limited number of studies have investigated the diversity of the expanded IM T cell repertoire; two studies have demonstrated selectively amplified subpopulations of CD8<sup>+</sup> T cells of undetermined antigenic specificity in the peripheral blood of acutely infected IM patients (8, 9), while another has found no evidence of dominant T cell expansions (10). Although the biological significance of these repertoire differences is unknown, they may reflect the variable clinical manifestations of the disease, ranging from mild symptomatic seroconversion to development of severe acute or chronic IM.

<sup>1</sup>Abbreviations used in this paper: EBNA, EBV nuclear antigen; IM, infectious mononucleosis; LCL, lymphoblastoid cell line.

CD8<sup>+</sup> CTLs recognize virus-infected cells via a surface  $\alpha\beta$  TCR heterodimer with specificity for peptide antigen presented by class I MHC molecules. The potential diversity of the T cell repertoire is enormous, generated through recombination of variable (TCRAV and TCRBV), diversity (TCRBD), and joining (TCRAJ and TCRBJ) gene elements during T cell ontogeny. Diversity is further enhanced by the imperfect joining of the receptor gene segments and by the addition of non-germline-encoded N region nucleotides to the coding ends of the V-(D)-J junctional regions (reviewed in reference 11). Molecular modeling of the TCR predicts that complementarity determining regions CDR1 and CDR2, encoded by TCRAV and TCRBV gene elements, interact with the MHC molecule while the hypervariable CDR3, spanning the V-(D)-J junctional regions, directly contacts the bound peptide (12). Although a broadly selected antigen-specific TCR repertoire may be of advantage to the host, several factors are thought to limit the diversity of the expressed repertoire for a particular epitope. These include positive and negative thymocyte selection, as well as structural constraints imposed by a given peptide/MHC combination (13). Lymphocyte competition with successive antigen encounters may also lead to selection of oligoclonal repertoires within the memory T cell pool. While a number of studies have investigated the TCR usage of CTLs specific for a variety of foreign antigens, only a limited number of studies have directly monitored the antigen-specific TCR repertoire development of memory T cells (14–16). In one such study, a focusing of specific TCR clonotypes selected during the course of an immune response was shown, providing support for preferential restimulation in shaping the peripheral antigen-specific T cell repertoire (14). In contrast, another study has shown no major change in repertoire selection in the primary and memory T cell responses (16). Despite these developments, much remains to be learned about T cell specificity selection and progressive evolution within the long-term memory T cell compartment.

We have recently reported that despite an enormous TCR repertoire potential in each individual, the CTL response to the immunodominant HLA-B8-restricted EBV epitope FLRGRAYGL consists of public TCRs that are highly conserved and that reproducibly emerge in the EBV-specific immune responses of different individuals (17, 18). The most restricted of these public responses is found in individuals who are HLA-B8<sup>+</sup>, where a single public TCR is found to dominate the response in a number of unrelated donors (17). This TCR is known to be cross-reactive with the alloantigen HLA-B44 (19). In HLA-B8<sup>+</sup>/B44<sup>+</sup> individuals, tolerance to this background MHC antigen has been shown to diversify the FLRGRAYGL-specific TCR repertoire, resulting in the development of a number of alternate types of public TCRs (18). A strong genetic bias in TCR gene recombination and preferential selection of public TCRs in the periphery in response to a persistent viral infection are thought to be important factors in the development of these exceptionally reproducible memory TCR responses.

In the present report, we have monitored the EBV-specific memory TCR- $\beta$  response during the course of primary EBV infection in an HLA-B8<sup>+</sup> individual with acute IM. This was achieved by determining the TCR- $\beta$  sequences of CTLs specific for the EBNA-3-encoded epitopes FLRGRAYGL or QAKWRLQTL after their isolation at various times after primary infection. The results show selection of TCR- $\beta$  diversity within the T cells responding to single viral epitope determinants, with evidence of antigen-driven clonal expansions and long-term maintenance of a multiclonal effector response selected early in the infection. Furthermore, a public TCR- $\beta$  chain was identified in the primary antiviral response, and PCR tracking of PBMCs *ex vivo* showed an expansion of this chain in the peripheral repertoire after clinical diagnosis.

## Materials and Methods

**Cell Donors.** Donor BT (HLA-A1, 31; -B8, 35) was diagnosed with acute IM based on clinical symptoms and serology. PBMCs were isolated from heparinized blood by centrifugation over Ficoll-Paque (Pharmacia Biotech, Melbourne, Australia) at the time of clinical diagnosis (day 1/sample referred to as BTI) and at different times thereafter (day 36/BTII, day 85/BTIII, and day 196/BTIV). Samples BTI and BTII were taken during the acute IM stage when the patient was IgM<sup>+</sup> for EBV viral capsid antigen and IgG<sup>-</sup> for EBNA. Samples BTIII and BTIV were taken during convalescence when the patient showed recovery from the disease and IgG EBNA antibodies developed. The HLA-B8<sup>+</sup> status of BT was initially determined by FACScan<sup>®</sup> analysis (Becton Dickinson & Co., Sydney, Australia) of PBMCs using an anti-HLA-B8 mAb (clone 59HA-1; One Lambda Inc., Los Angeles, CA), and confirmed by serological typing of the donor's PBMCs. The virus infecting BT was identified as a type A EBV isolate by PCR analysis using type-specific primers (20). Control PBMCs were isolated from healthy EBV-seronegative donors PP (HLA-A1, 24; -B8, 14) and WH (HLA-A1, -; -B8, 57) and seropositive donors JP (HLA-A2, 28; -B8, 62) and BM (HLA-A1, -; -B8, -).

**Establishment and Maintenance of Cell Lines.** Lymphoblastoid cell lines (LCLs) were established from donors BM (HLA-A1, -; -B8, -) and DJM (HLA-A24, 29; -B44, 47) by transformation of B cells with exogenous type A EBV (WIL and BL74 isolates, respectively) as described (21). PBMCs were stimulated with PHA as described (22). LCLs were routinely maintained in growth medium consisting of RPMI 1640 medium, 2 mM glutamine, 100 IU/ml penicillin, 100  $\mu$ g/ml streptomycin, and 10% (vol/vol) heat-inactivated fetal bovine serum. PHA blasts were maintained in continuous culture for 6–8 wk in growth medium containing 20 U/ml rIL-2 (23, 24).

**Selection and Synthesis of Peptides.** Two HLA-B8-restricted CTL epitopes, FLRGRAYGL and QAKWRLQTL, were selected to monitor the development of the TCR- $\beta$  repertoire in donor BT during acute IM and convalescence. Both of these epitopes are found in EBNA-3 in type A, but not in type B, EBV isolates (25). Peptides were purchased from Chiron Mimotopes (Melbourne, Australia), dissolved in DMSO, and diluted in serum-free RPMI 1640 medium for use in cytotoxicity assays.

**Agar Cloning of T Cells.** T cell clones were generated as described (26), except that PBMCs from the acute IM donor were exposed to growth medium containing 20 U/ml rIL-2 after their isolation over Ficoll-Paque to avoid apoptosis of T cells (27). Briefly, *ex vivo* PBMCs from donor BT were activated by stimu-

lation with the  $\gamma$ -irradiated (80 Gy) LCLs from the HLA-A1/B8 homozygous individual BM at a stimulator/responder cell ratio of 1:20. After 3 d, dispersed cells were seeded in 0.35% agarose (Sea-Plaque; FMC BioProducts, Rockland, ME) containing RPMI 1640 medium, 10% FBS, 25% (vol/vol) supernatant from MLA-144 cultures (TIB-201; American Type Culture Collection, Rockville, MD), and 30 U/ml rIL-2. Colonies were harvested after an additional 3 d, and were amplified in culture with biweekly restimulation with rIL-2 and specific LCLs. Colonies were routinely phenotyped using mAbs (all from Becton Dickinson) detecting the antigens CD4 (clones SK3 and SK4; anti-human Leu-3a,b-FITC), CD8 (clone SK2; anti-human Leu-2b-PE), and CD3 (clone SK7; anti-human Leu-4 PerCP). Flow cytometric analysis was performed on a FACScan<sup>®</sup> (Becton Dickinson).

**Cytotoxicity Assay.** Colonies from donor BT were initially screened using the rapid visual T cell-T cell killer assay (28) to identify FLRGRAYGL- and QAKWRLQTL-specific CD8<sup>+</sup> CTL clones. This procedure requires  $<10^3$  cells per assay. Epitope-specific CTL clones were further amplified in culture, and used as effectors against BM and DJM LCLs, and autologous PHA T cell blasts were pulsed with the appropriate peptide (100  $\mu$ g/ml). Target cells, with or without peptide, were incubated with 100  $\mu$ Ci of <sup>51</sup>Cr at 37°C for 90 min, 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  $<5\%$ .

**mRNA Isolation and cDNA Synthesis.** Poly A<sup>+</sup> RNA was extracted from  $1-5 \times 10^6$  CTLs or PBMCs using a QuickPrep Micro mRNA purification kit (Pharmacia Biotech.). An antisense TCRBC primer (C<sub>b1</sub>) was used to generate first-strand cDNA from 0.5–2  $\mu$ g poly A<sup>+</sup> RNA, and this was followed by RNA hydrolysis and the removal of excess primer as described previously (17).

**Amplification and Sequencing of Rearranged TCR  $\beta$  Sequences.** TCR- $\beta$  rearranged sequences were amplified with five sets of 5' TCRBV family-specific oligonucleotides (V $\beta$ 1-5.1, V $\beta$ 5.2-9, V $\beta$ 10-14, V $\beta$ 15-19, and V $\beta$ 20-24) and a 3' TCRBC (C $\beta$ ) constant primer. The oligonucleotides V $\beta$ 1-20/C $\beta$  and V $\beta$ 21-24 were synthesized according to the methods of Panzara et al. (29) and Kalams et al. (15), respectively. Amplifications were performed in 25- $\mu$ l reaction volumes using 0.5  $\mu$ l cDNA, 10 pmol of each V $\beta$  and C $\beta$  primer, 200 mM dNTPs, 20 mM MgCl<sub>2</sub>, 1.25 U of Taq polymerase (Ampli-Taq), and a GeneAmp PCR 9600 system (Perkin-Elmer Cetus Corp., Norwalk, CT). The PCR conditions consisted of denaturation at 95°C for 15 s, annealing at 60°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.  $\beta$ -Actin was also amplified as a control for cDNA integrity (30). PCR products were excised from 2.5% (0.5 $\times$  Tris-buffered EDTA) NuSieve GTG agarose gels (FMC BioProducts) and purified using a QIAEX gel extraction kit (QIAGEN Inc., Chatsworth, CA). Recovered PCR products were sequenced in both directions with a PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit and a 373A DNA sequencer (Applied Biosystems, Inc., Foster City, CA). In the event that nucleotide sequence analysis of a PCR product revealed more than one specific sequence, the product was subsequently ligated into the pGEM-T vector system (Promega, Madison, WI). The nucleotide sequence of six clones was determined for each ligation.

**Semiquantitative PCR Analysis of TCR V $\beta$  Gene Expression in PBMCs.** TCRBV families 3, 4, 5.1, and 6 were amplified using a 5' sense V $\beta$  3, 4, 5.1, and 6 primer in combination with a 3'

antisense TCRBC constant primer (C<sub>b2</sub>; 5'-ATTCACCCAC-CAGCTCAGCTCCACG-3'), respectively. Total TCRBC was amplified using a 5' sense TCRBC primer (Cb5'; 5'-CGTGT-TCCCACCCGAGGTCCG-3') and C<sub>b2</sub>. Amplifications were performed in 25- $\mu$ l reaction volumes using 0.5  $\mu$ l cDNA, 10 pmol of each V $\beta$  and C $\beta$  primer, 200 mM dNTPs, 20 mM MgCl<sub>2</sub>, and 1.25 U Taq polymerase. For semiquantification of V $\beta$  expression, the exponential phase of the PCR was determined for each cDNA preparation and primer pair. The amplification schedule was 95°C for 15 s, 60°C for 40 s, and 72°C for 40 s. The amplified products were resolved on 2.5% agarose gels, stained with ethidium bromide, and for some experiments, transferred to nylon membranes (Hybond N; Amersham International, Little Chalfont, UK), and then hybridized with a digoxigenin-11-dUTP (DIG)-labeled TCRBC probe that was detected using the DIG luminescent detection system according to the protocols described by the manufacturer (Boehringer Mannheim Australia, Castle Hill, Australia). Relative quantification of the amplified products was performed using a computing densitometer and ImageQuant software version 3.3 (Molecular Dynamics, Inc., Sunnyvale, CA). The expression of TCRBV families was calculated as the percent of total TCR- $\beta$ .

**Semiquantitative PCR Analysis of Public FLRGRAYGL-specific TCR- $\beta$  Gene Expression in PBMCs.** The public BV6/BJ2S7 FLRGRAYGL-specific rearrangement has previously been quantitated by PCR within the TCRBV6 family (17). For analysis of the public chain, first-strand cDNA (200 ng) was used as a template for PCR amplification using 10 pmol each of a 5' sense TCRBV6 family-specific primer (V $\beta$ 6) and a 3' antisense TCRBC primer (C<sub>b2</sub>) according to the above-specified PCR conditions. The amplified transcripts were resolved on 2% Nusieve GTG agarose gels, purified, and 1  $\mu$ l of this material (diluted 1/100) was used for a second round of PCR using 10 pmol each of V $\beta$ 6 and either a degenerate primer complementary to the CDR3 region of the FLRGRAYGL-specific public  $\beta$  chain ( $\beta_{CDR31}$ ; 5'-GTACTGCTC(G,A)TA(A,G,C,T)GC(C,T)TG-3') or C<sub>b2</sub> for use as a standard for calculation of the relative abundance of the product obtained with the TCRBV6- $\beta_{CDR31}$  primer set. For semiquantification of TCRBV expression, the exponential phase of the PCR was determined for each cDNA preparation and primer pair. The amplified products were resolved on 2.5% agarose gels, stained with ethidium bromide, and quantified densitometrically as described above.

## Results

**Isolation of FLRGRAYGL- and QAKWRLQTL-specific CTL Clones during the Course of Primary EBV Infection.** In the present study, two well-defined HLA-B8-restricted CTL epitopes, FLRGRAYGL and QAKWRLQTL, were selected to monitor the development of the TCR- $\beta$  repertoire in donor BT during acute IM and convalescence. Multiple FLRGRAYGL- and QAKWRLQTL-specific CTL colonies were isolated from BT PBMCs at each of the time points (BTI, BTII, BTIII, and BTIV) in a longitudinal study (Table 1). These time points spanned the period from acute IM (BTI and BTII), when the donor was IgM<sup>+</sup> and EBNA IgG<sup>-</sup>, to convalescence (BTIII and BTIV), when the donor had recovered clinically from the disease and EBNA IgG antibodies had developed. The use of the HLA-B8 homozygous BM LCL provided the essential re-

**Table 1.** Isolation of Codominant FLRGRAYGL- and QAKWRLQTL-specific CTL Colonies Generated In Vitro from Donor BT during Acute IM and Convalescence

Donor	Proportion of epitope-specific CD8 <sup>+</sup> CTL colonies screened using the rapid visual T cell-T cell killer assay	
	FLRGRAYGL-specific	QAKWRLQTL-specific
BTI*	7/27	10/27
BTII	9/66	19/66
BTIII	9/55	13/55
BTIV	9/75	10/75

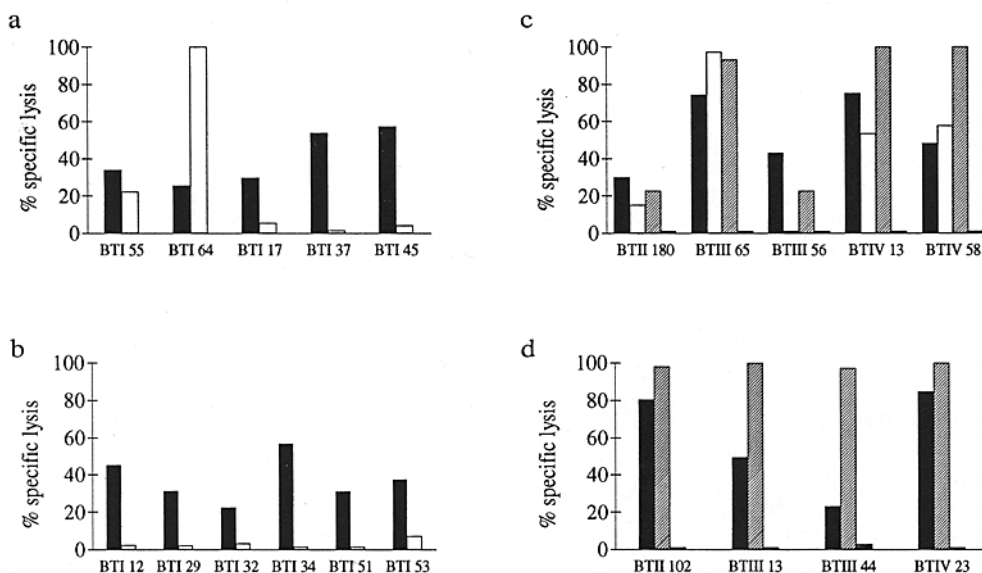
\*IM patient BTI was tested on four separate occasions after diagnosis. BTI at day 1, BTII at day 36, BTIII at day 85, and BTIV at day 196.

stimulation population and also potentiated the likelihood of generating epitope-specific CTL clones that were restricted through HLA-B8.

Cytotoxicity data in Fig. 1 *a* clearly show that the FLRGRAYGL-specific CTL clones isolated at day 1 (BTI) included two clones (BTI 55 and BTI 64) that cross-reacted with the alloantigen HLA-B44 on DJM LCL targets, suggesting that these clones expressed the public TCR rearrangement BV6/BJ2S7 (18). Three other clones (BTI 17, BTI 37, and BTI 45) showed no such cross-reactivity, and were categorized putatively as expressing distinct TCR rearrangements. As expected, the six QAKWRLQTL-specific CTL clones did not cross-react with the DJM LCL targets (Fig. 1 *b*). Unfortunately, none of the BTI epitope-specific clones grew sufficiently well for TCR analysis. CTL clones generated from subsequent bleeds, however, were

easier to establish in culture, even though donor BT was still with acute disease at the BTII bleed (IgM<sup>+</sup>; EBNA IgG<sup>-</sup>). Cytotoxicity profiles of representative clones that were available for TCR analysis are featured in Fig. 1, *c* and *d*. Four FLRGRAYGL-specific clones (BTIII 180, BTIII 65, BTIV 13, and BTIV 58) cross-reacted with the DJM LCL target (Fig. 1 *c*), and were subsequently shown (Fig. 2) to express the public BV6/BJ2S7 rearrangement. By contrast, clone BTIII 56 (Fig. 1 *c*) did not cross-react with the DJM LCL target and was shown (Fig. 2) to express a distinct TCR rearrangement. Every FLRGRAYGL- and QAKWRLQTL-specific clone lysed autologous PHA blasts pulsed with the exogenous peptide FLRGRAYGL or QAKWRLQTL, respectively (Fig. 1, *c* and *d*), thus confirming their epitope specificity initially designated on the basis of T cell-T cell killing assays.

**Selection of a Public FLRGRAYGL-reactive  $\beta$  Chain in Primary Infection.** We have previously shown that a single public TCR dominates the FLRGRAYGL CTL response of a number of healthy, EBV-seropositive HLA-B8<sup>+</sup> individuals (17). To confirm the cytotoxicity data indicating selection of the public rearrangement during the primary antiviral response, the TCRB (V-D-J-C) segments expressed by FLRGRAYGL-reactive CTL clones from patient BT at day 36 (BTII), still during the acute IM stage, were identified using TCRBV family-specific PCR followed by direct sequencing (Fig. 2 *a*). The TCR- $\alpha$  chains were not determined, since in-frame rearrangements of both  $\alpha$  alleles have been detected in T cell clones, whereas the control of  $\beta$  expression by allelic exclusion results in only a single in-frame rearrangement in each T cell clone (34). Of the two clones available for TCR analysis, BTII 180 and BTII 36 both expressed a single in-frame BV6/BJ2S7 rearrangement that was identical at the amino acid level to the public  $\beta$  chain common to other HLA-B8<sup>+</sup> donors. Variation in codon usage within the N-D-N re-



**Figure 1.** Specific lysis by CTL clones isolated from IM donor BT during acute IM (BTI and BTII) and convalescence (BTIII and BTIV). CTLs analyzed by T cell-T cell killing to be FLRGRAYGL- (*a* and *c*) or QAKWRLQTL- (*b* and *d*) specific were tested against BM (HLA-A1, -; B8, -) and DJM (HLA-A24, 29; B44, 47) LCLs and autologous PHA-blasts pulsed with peptide FLRGRAYGL (*c*) or QAKWRLQTL (*d*). E:T ratio = 2:1. Clones isolated at day 1 (BTI), day 36 (BTII), day 85 (BTIII), and day 196 (BTIV) are shown. ■, BM LCL; □, DJM LCL; ▨, BT PHA-blasts + peptide; ▩, BT PHA-blasts.

CTL	TCRBV	FW	CDR3	FW	TCRBJ
<b>a</b>					
BTII 180	BV6	C A S tgt gcc agc	S L G Q A Y E Q agc ttg gga <u>cag gcc tac gag cag</u>	Y F G tac ttc ggg	BJ2S7
BTII 36	BV6	C A S tgt gcc agc	S L G Q A Y E Q agc tta ggt <u>cag gcc tac gag cag</u>	Y F G tac ttc ggg	BJ2S7
<b>b</b>					
BTIII 65	BV6	C A S tgt gcc agc	S S G Q A Y E Q agc tcc gga <u>cag gct tac gag cag</u>	Y F G tac ttc ggg	BJ2S7
BTIII 56	BV5	C A S tgt gcc agc	S P M D R P N Q P Q agc cct atg <u>gac aga</u> cca <u>aat cag ccc cag</u>	H F G cat ttt ggt	BJ1S5
BTIII 60	BV12	C A I tgt gcc atc	S A A G E T T E A agt gcc <u>gca ggg gag</u> acc <u>act gaa gct</u>	F F G ttc ttt gga	BJ1S1
<b>c</b>					
BTIV 13	BV6	C A S tgt gcc agc	S L G Q A Y E Q agc ttg gga <u>cag gcc tac gag cag</u>	Y F G tac ttc ggg	BJ2S7
BTIV 58	BV6	C A S tgt gcc agc	S L G Q A Y E Q agc tta ggg <u>cag gcc tac gag cag</u>	Y F G tac ttc ggg	BJ2S7
LC13	BV6	C A S tgt gcc agc	S L G Q A Y E Q agc tta ggg <u>cag gcc tac gag cag</u>	Y F G tac ttc ggg	BJ2S7

**Figure 2.** TCR- $\beta$  junctional region sequences from FLRGRAYGL-reactive CTL clones isolated at different time points after primary EBV infection in patient BT. The V-D-J region sequences of clones isolated at (a) day 36/BTII, (b) day 85/BTIII, and (c) day 196/BTIV after clinical diagnosis are shown. A translated amino acid sequence is shown above each corresponding nucleotide sequence. The actual sequence obtained for each TCR- $\beta$  chain extended at least 80 nucleotides further 5' of the sequence shown. For comparison, the TCR- $\beta$  junctional region of a representative public FLRGRAYGL-specific clone from an HLA-B8<sup>+</sup> donor LC (LC13) is also shown. Designations for TCRBV and TCRBJ gene elements follow that of Arden et al. (31) and Toyonaga et al. (32), respectively. For each clone, the deduced amino acid sequence of the CDR3-equivalent loop, defined according to Chothia et al. (33), is shown putatively supported by two frame work branches (FW). TCRBJ germline sequences are underlined and in normal print. TCRBD germline sequences are italicized and underlined. A single in-frame TCR- $\beta$  rearrangement was detected for each clone, and an out-of-frame rearrangement was also detected in BTIII 56. The TCR- $\beta$  sequences of clones BTII 180, BTII 36, BTIII 65, BTIV 13, and BTIV 58 were reconfirmed by independent analyses starting from new RNA isolations from each of these clones. These sequences are available from EMBL/GenBank/DBJ under accession numbers Z73085–Z73090.

regions of these clones showed that they were generated by different rearrangement events, indicating strong selection for this public TCR component given the potential diversity of FLRGRAYGL-specific TCR- $\beta$  chains (18).

Development of the FLRGRAYGL-specific memory TCR response was monitored longitudinally by sequencing the TCRB genes of the FLRGRAYGL-specific CTLs that were isolated at further time points after diagnosis. At day 85 (BTIII), a highly homologous public  $\beta$  chain, with a single amino acid substitution in the CDR3 region, was identified for clone BTIII 65 (Fig. 2 b). In addition, a number of distinct  $\beta$  chains were expressed by clones BTIII 56 and BTIII 60 (Fig. 2 b). Comparison of these chains with previously reported public FLRGRAYGL-specific  $\beta$  rearrangements showed no structural homology between these chains. At day 196 (BTIV), the public  $\beta$  response was found to persist, with clones BTIV 13 and BTIV 58 expressing the public BV6/BJ2S7 rearrangement (Fig. 2 c). Notably, clones BTIV 13 and BTII 180, which were isolated 160 d apart, had identical rearrangements, suggesting that they arose by antigen-driven expansion from a single progenitor clone. Although two other FLRGRAYGL-specific CTL lines isolated at day 196 were found to be non-clonal since they contained several in-frame  $\beta$  chains, the BV6/BJ2S7 public chain was confirmed by PCR screening not to be present in these lines (data not shown). Overall, a polyclonal memory CTL response containing a public

TCR- $\beta$  component was selected and maintained long-term in response to FLRGRAYGL after a primary symptomatic EBV infection in patient BT.

**Selection of TCR- $\beta$  Diversity within the QAKWRLQTL Response.** TCR- $\beta$  selection and development in the QAKWRLQTL-specific memory response was monitored in parallel with the FLRGRAYGL response in patient BT. Five distinct  $\beta$  chain sequences were identified for the six clones isolated at day 36 (BTII; Fig. 3 a). Despite this overall diversity, three of the clones (BTII: 158, 68, and 141) preferentially used the TCRBV gene BV3, and five clones (BTII: 102, 176, 175, 158, and 68) expressed a common TCRBJ element, BJ2S5. The CDR3 region, which interacts directly with the peptide epitope (35), was also restricted in length to 8–11 amino acids in all of the clones. Conservation of a non-germline-encoded proline residue in the hypervariable CDR3 region of clones BTII 102, BTII 176, and BTII 175 was also observed. Further, in the response at day 85 (BTIII), all the QAKWRLQTL-specific clones expressed proline in the second position of the CDR3 region (Fig. 3 b), a finding that is likely to reflect selection by antigen. Several  $\beta$  chains identified earlier at day 36 (BTII: 102, 176, and 175) were found to recur at this later time point in clones BTIII 1, BTIII 13, and BTIII 44. These same chains, as well as the BV4/BJ1S2 rearrangement of clone BTIII 185, were found to reemerge at day 196 (Fig. 3 c). Overall, a diverse QAKWRLQTL-specific

CTL	TCRBV	FW	CDR3	FW	TCRBJ
<b>a</b>					
BTII 102	BV5.1	C A S tgc gcc agc	S P E V G E T Q agc ccc <u>gag atg</u> ggg <u>gag acc cag</u>	Y F G tac ttc ggg	BJ2S5
BTII 176	BV5.1	C A S tgc gcc agc	S P E V G E T Q agc ccc <u>gag atg</u> ggg <u>gag acc cag</u>	Y F G tac ttc ggg	BJ2S5
BTII 175	BV21	C A S tgt gcc agc	S P G S A R S G E T Q agc ccc ggc <u>agc gca</u> cgg agt ggg <u>gag acc cag</u>	Y F G tac ttc ggg	BJ2S5
BTII 158	BV3	C A S tgt gcc agc	S L L Y Q G G E T Q agt tta ctc tat <u>cag gga gga</u> <u>gag acc cag</u>	Y F G tac ttc ggg	BJ2S5
BTII 68	BV3	C A S tgt gcc agc	S F L P L R A Q E T Q agt ttc ctc ccc <u>ctg cag gcc</u> <u>caa gag acc cag</u>	Y F G tac ttc ggg	BJ2S5
BTII 141	BV3	C A S tgt gcc agc	S G S G G A N T E A agt ggc tgc <u>ggg gag gca</u> <u>aac act</u> <u>gaa gcl</u>	F F G tte ttt gga	BJ1S1
<b>b</b>					
BTIII 1	BV5.1	C A S tgc gcc agc	S P E V G E T Q agc ccc <u>gag atg</u> ggg <u>gag acc cag</u>	Y F G tac ttc ggg	BJ2S5
BTIII 13	BV5.1	C A S tgc gcc agc	S P E V G E T Q agc ccc <u>gag atg</u> ggg <u>gag acc cag</u>	Y F G tac ttc ggg	BJ2S5
BTIII 44	BV21	C A S tgt gcc agc	S P G S A R S G E T Q agc ccc ggc <u>agc gca</u> cgg agt ggg <u>gag acc cag</u>	Y F G tac ttc ggg	BJ2S5
BTIII 53	BV3	C A S tgt gcc agc	S P L P G T G A Y S N Q P Q agt cca cta ccc <u>gag acc gga</u> <u>gct</u> tat <u>gag aat cag ccc cag</u>	H F G cat ttt ggt	BJ1S5
BTIII 85	BV4	C S A tgc agc gca	I P Q G A A G Y atc <u>cca cag gga gcc</u> <u>gct ggc tac</u>	T F G acc ttc ggt	BJ1S2
<b>c</b>					
BTIV 32	BV21	C A S tgt gcc agc	S P G S A R S G E T Q agc ccc ggc <u>agc gca</u> cgg agt ggg <u>gag acc cag</u>	Y F G tac ttc ggg	BJ2S5
BTIV 54	BV5.1	C A S tgc gcc agc	S P E V G E T Q agc ccc <u>gag atg</u> ggg <u>gag acc cag</u>	Y F G tac ttc ggg	BJ2S5
BTIV 47	BV4	C S A tgc agc gca	I P Q G A A G Y atc <u>cca cag gga gcc</u> <u>gct ggc tac</u>	T F G acc ttc ggt	BJ1S2

**Figure 3.** TCR- $\beta$  junctional region sequences from QAKWRLQTL-reactive CTL clones isolated at different time points after primary EBV infection in patient BT. The V-D-J region sequences of clones isolated at (a) day 36/BTII, (b) day 85/BTIII, and (c) day 196/BTIV after clinical diagnosis are shown. The actual sequence obtained for each chain extended at least 80 nucleotides further 5' of the sequence shown. A single in-frame TCR- $\beta$  rearrangement was detected for each clone. TCRB V, J gene segments, and CDR3 region loops are presented and assigned as outlined in the legend of Fig 2. The conserved CDR3 proline residue is highlighted in bold type. These sequences are available from EMBL/GenBank/DDJB under accession numbers Z73091–Z73097.

response was found in patient BT that showed features of antigen-driven selection and long-term persistence of expanded populations of distinct CTL clonotypes.

*V $\beta$  Families Associated with FLRGRAYGL and QAKWRLQTL Recognition Are Not Selectively Expanded in the PBMC.* Identification of several V $\beta$  families involved in the specific immune recognition of EBV during IM next prompted us to determine whether, by selective amplification, any of these gene families represent major expansions in the peripheral T cell repertoire during the course of the disease. Using a semiquantitative PCR approach with primers specific for TCRBV families 3, 4, 5.1, and 6, the relative expression of each TCRBV gene was initially assessed in the healthy EBV-seronegative control donors PP and BM, and a third EBV-seropositive donor JP was also analyzed longitudinally during a 10-mo period (Table 2). This approach established a normal range of relative TCRBV expression and confirmed the validity of this technique since interindividual variation was observed and found to be reproducible between independent analyses. V $\beta$  repertoire analysis was next performed on sequential PBMC

samples from donor BT (Table 2). The results showed no apparent change in TCRBV 3, 4, 5.1, or 6 expression during the course of primary EBV infection. In addition, no selective perturbation in TCRBV 3, 4, 5.1, or 6 expression was identified when these results were compared to the normal control range (Table 2). These results imply that relative to the total TCR- $\beta$  repertoire, several V $\beta$  families known to be specifically associated with EB viral recognition were not preferentially expanded in response to a primary EBV infection in this individual.

*Tracking the Development of a Public FLRGRAYGL-specific  $\beta$  Chain in PBMCs Ex Vivo.* Having demonstrated the presence of a public FLRGRAYGL-reactive compartment of CTL clones from the acute to the convalescent stage of the infection, we were interested in directly monitoring the progressive evolution of this T cell population ex vivo. Using a double-step semiquantitative PCR procedure that we had developed previously (17), the public  $\beta$  rearrangement was tracked relative to TCRBV6 expression in peripheral blood preparations BTI, BTII, BTIII, and BTIV (Fig. 4). Two HLA-B8<sup>+</sup> EBV seronegative donors, PP and WH,

**Table 2.** Relative Expression of TCRBV3, 4, 5.1, and 6 in PBMCs

Donors	Percent of TCR- $\beta$ *			
	BV3	BV4	BV5.1	BV6
<b>Controls</b>				
PP	11	14	16	21
BM	8	12	11	10
JP (1/95) <sup>‡</sup>	7	7	7	16
JP (10/95)	11	10	7	8
JP (11/95)	7	6	8	11
Normal range	7–11	6–14	7–16	8–21
<b>IM Donor</b>				
BTI <sup>§</sup>	9	6	4	7
BTII	10	10	5	7
BTIII	10	9	6	11
BTIV	9	10	7	10

\*PBMCs were analyzed by semiquantitative PCR for relative expression of TCRBV3, 4, 5.1, and 6, as described in Materials and Methods. Data are expressed as the percent of total TCR- $\beta$ .

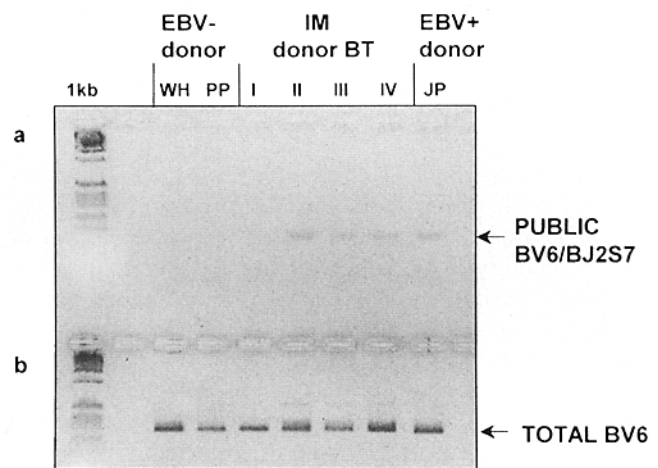
<sup>‡</sup>Donor JP was tested on three separate occasions during a 10-mo period.

<sup>§</sup>IM donor BT was tested on four separate occasions after diagnosis: BTI at day 1, BTII at day 36, BTIII at day 85, and BTIV at day 196.

and a seropositive HLA-B8<sup>+</sup> donor, JP, were also assessed (Fig. 4). The analysis showed that from the time of diagnosis (BTI) to day 36 (BTII) a threefold increase in expression of the public  $\beta$  chain had occurred. This expansion was also shown to be stably maintained during days 85 (BTIII) and 196 (BTIV). Furthermore, this upregulated level of the public  $\beta$  chain was found to be similar to that observed in the healthy virus carrier JP, who is known to have a public FLRGRAYGL-specific CTL component (our unpublished observations). The identity of each amplified product was confirmed by direct sequencing, which in the case of samples BTI, BTII, BTIII, BTIV, and JP resulted in a single clearly readable BV6/BJ2S7 public sequence. In contrast, the low abundant products obtained in samples PP and WH were shown to be mixed and unreadable in sequence (data not shown). These data indicate that the public memory CTL component of the FLRGRAYGL response was expanded during the early immune reaction to EBV infection and subsequently maintained at stable levels during convalescence.

## Discussion

In this first analysis of EBV-specific TCR selection and development in a natural primary infection, several important findings have been made concerning the specificity, emergence, diversity, and longevity of the T lymphocyte



**Figure 4.** Semiquantitative detection of the public TCR- $\beta$  chain in PBMC ex vivo. Semiquantitative PCR amplification of (a) public BV6/BJ2S7 TCR- $\beta$  sequences and (b) total TCRBV6 sequences from two EBV-seronegative donors (PP and WH), the IM donor BT at different times after diagnosis (BTI/day 1; BTII/day 36; BTIII/day 85; BTIV/day 196), and the EBV-seropositive donor JP. - denotes control reactions excluding template.

clonotypes comprising the memory CTL response. First, a component of the primary virus-specific CTL response in IM was shown to be specific for two antigenic determinants, FLRGRAYGL and QAKWRLQTL, that are encoded within the latent viral antigen EBNA-3. Second, TCR- $\beta$  repertoire selection was found to be polyclonal for both these epitopes, although structural restrictions on recognition were documented that provide evidence for antigen-driven selection. Third, longitudinal repertoire analyses revealed that a multiclonotypic CTL response was maintained during the course of primary infection with the reemergence of distinct memory T cell clonotypes sharing similar structural restrictions.

While it is well known that an HLA class I-restricted, virus-specific CTL component is activated in IM (7, 36), the target epitopes of this response have not been identified previously. This study is, therefore, important in demonstrating that EBNA-3-specific CTL precursors can be isolated from the peripheral blood during a primary symptomatic EBV infection. Interestingly, in healthy virus-immune donors, a hierarchy of epitope immunodominance has been found in which the HLA-B8-restricted EBNA-3 response is typically dominated by FLRGRAYGL-reactive CTLs (25). Yet during primary infection in donor BT, the CTL response to FLRGRAYGL and QAKWRLQTL was codominant in that specific clones were reactivated at similar frequencies after LCL stimulation in vitro. Although a number of hierarchical mechanisms are thought to control epitope immunogenicity, including selective generation of peptides by antigen processing, differential binding and competition of peptides for MHC molecules, and TCR repertoire availability (37), the codominance of these responses during the acute infectious stage possibly reflects immune stimulation

under conditions of high viral antigen load. Indeed, there is an increased prevalence of virus-infected B cells in the peripheral blood of IM donors compared to healthy virus carriers (38, 39). Recent evidence suggests that these cells express the full spectrum of latent viral genes *in vivo*, and that they disappear from the circulation during convalescence (40). CTLs targeted to latent viral determinants may, therefore, play a crucial role in limiting EBV's colonization of the lymphoid system during acute infection.

The availability of an HLA-B8<sup>+</sup> donor undergoing a primary symptomatic infection provided us with the unique opportunity to monitor antigen-specific TCR repertoire maturation from the acute to the convalescent stage of the disease. Of particular interest was the TCR response to the EBNA-3-encoded, HLA-B8-restricted epitope, FLR-GRAYGL, which is known to be highly conserved in healthy virus carriers despite a diverse repertoire of TCRs that are capable of reacting with this antigenic determinant (18). To account for this unprecedented level of TCR conservation, a model incorporating a strong genetic bias in TCR gene recombination and selective T cell maturation and expansion in the periphery in response to persistent immune stimulation was proposed (17). One prediction of this hypothesis is that public FLRGRAYGL-specific T cell precursors are likely to be present among the broad range of TCR affinities/avidities that are stimulated during primary infection. In the present study, a number of TCR- $\beta$  receptor chains other than the public rearrangement were identified in the responding IM memory T cell pool. There was, however, evidence of strong preferential selection of the public chain, since four out of five CTL clones arose from independent T cell precursors that were unique in codon usage within the CDR3 region. Tracking public TCR- $\beta$  clonotypes in the peripheral repertoire *ex vivo* over the course of the disease also provided evidence that shortly after the acute IM phase, a public compartment of circulating memory T cells were selectively expanded and preserved throughout the recovery phase. These latter findings are consistent with limiting dilution analyses that have shown high levels of EBV-specific memory CTL precursors in healthy seropositive donors that are maintained throughout life (41).

Although a polyclonal FLRGRAYGL- and QAKWRLQTL-specific CD8<sup>+</sup> effector response was selected in IM, restrictions on TCR recognition that appeared to be selectively maintained in the long-term memory response were apparent soon after virus challenge. A multiclonal precursor population of CTLs expressing highly conserved public chains was identified in the FLRGRAYGL response, while the QAKWRLQTL-specific CTLs showed a bias in TCRV and J gene usage with an amino acid conservation in the CDR3 region. Both of these observations are consistent with antigen playing an important role in shaping the selected repertoire. Moreover, the recurrent clonotypes of each epitope response shared distinct structural restrictions, implying their selective preservation over the course of the infection. Similar structural patterns of TCR restriction

have previously been reported for viral infections (42, 43). Progressive changes to the primary TCR repertoire have also been documented in CD4<sup>+</sup> helper cells of mice after challenge with antigen *in vivo* (14), as well as in the natural CD8-restricted immune response to HIV (44), where in both of these studies, CDR3 length restrictions and sequence conservation were reported.

The long-term consequences of primary infection with virus that persists are unknown in terms of TCR repertoire evolution. Although in our study, the TCR- $\beta$  responses for two separate antigenic determinants remained polyclonal up to 6 mo after infection, showing no dramatic signs of focusing as is known to occur in the FLR-GRAYGL responses of healthy virus carriers, the competition for restimulation between different lymphocytes may have been minimal if antigen load and resource availability were not limiting at this stage during convalescence. In this regard, clonal exhaustion from prolonged overstimulation by high doses of antigen (45) may play an important role in some of the more extreme complications of IM.

Preferential expansions of selected TCR-V $\beta$  subpopulations have been demonstrated during the acute phase of infection with simian immunodeficiency virus (SIV) (46), HIV (44), and EBV (8, 9). In the case of SIV and HIV, virus-specific cytotoxic function has been associated with amplified V $\beta$  families, suggestive of a role of these T cells in clearing or limiting the spread of virus *in vivo*. We used a novel approach to analyze the virus-induced TCR repertoire response, first by determining the TCR- $\beta$  chains of CTL clones specifically stimulated by viral antigens during natural primary infection, and then by monitoring antigen-specific TCRBV family expression in the peripheral repertoire *ex vivo*. This approach avoids several of the problems associated with direct repertoire analysis, namely the presence of clonal expansions in memory T cell populations in normal healthy individuals (47, 48), as well as the likely possibility of TCR-independent, "bystander" activation of memory CTLs induced by the cytokine-rich milieu that is produced during the course of acute viral infection (49). Based on our analyses, several V $\beta$  families found to be specifically associated with EBV recognition in IM were not found to be preferentially expanded in the peripheral repertoire during the course of the disease. This was despite longitudinal TCR sequencing data, which provided evidence of clonal expansions of several TCR- $\beta$  clonotypes expressing these gene families. Taken together, these findings emphasize the highly polyclonal nature of the TCR repertoire responding to EBV in IM.

A broadly selected, epitope-specific TCR repertoire is likely to be of advantage to the host against persistent viruses that use a variety of mechanisms to evade immune recognition. Indeed, it has been suggested that the limited TCR repertoire of HIV-1 envelope-specific CTLs may facilitate the immune escape of virus variants by restricting TCR recognition (15). Epitope loss variants that can escape CTL destruction have been identified in natural EBV isolates (50), although there appears to be minimal CTL pres-



sure for selection of these virus mutants (51). This lack of pressure may be a consequence of a multispecific antiviral T cell response, both at the level of CTL recognition and

TCR specificity selection. Overall, selection of TCR diversity is likely to be important in the establishment and maintenance of a balanced host-virus coexistence.

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Address correspondence to Dr. Sharon L. Silins, Queensland Institute of Medical Research, The Bancroft Centre, EBV Unit, 300 Herston Road, Brisbane, Australia 4029.

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