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Clonal Expansions in Acute EBV Infection Are Detectable in the CD8 and not the CD4 Subset and Persist with a Variable CD45 Phenotype[1]

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We have applied a sensitive global analysis of TCR heterogeneity to compare clonal dynamics of $CD4^+$ and $CD8^+$ T cells in acute infectious mononucleosis. Using this approach, we are able to identify a broad representation of the total virus-specific population without the bias of in vitro culture and then to track their phenotype and fate by their unique molecular footprint. We demonstrate a large number of Ag-driven clones using different TCRs in the acute phase, all $CD8^+$. The diverse large clones generated in the CD8 subset in response to this virus contrast with the complete lack of detectable clonal expansion in the CD4 compartment. Many of the same clones remain detectable in directly ex vivo $CD8^+$ T cells for at least a year after resolution of infectious mononucleosis, although the clone size is reduced. Thus, memory CD8 cells following EBV infection persist at relatively high circulating frequency and represent a subset of the large range of clonotypes comprising the acute effectors. Separation of samples into CD45RA (naive) and CD45RO (memory) fractions shows the accumulation of identical CDR3 region defined clonotypes in both CD45RO and CD45RA fractions and sequencing confirms that dominant long-lived monoclonal expansions can reside in the CD45RA pool. *The Journal of Immunology,* **2000, 165: 5729–5737.**

Epstein-Barr virus is a ubiquitous human γ herpes virus, worldwide in distribution, with 90–95% of most adult populations showing serological evidence of infection (1). The severe symptomatic nature of infectious mononucleosis (IM)[3] allows analysis of individuals with a defined onset of infection. Resolution of acute infection is followed by life-long asymptomatic persistent viral carriage in the vast majority of infected individuals. The cellular immune response is known to be primarily responsible for control of this virus and therefore provides a convenient model of a successful T cell response to a chronic viral infection. However, EBV is associated with a number of malignancies in cases where there is a failure of normal cellular immunity. Detailed characterization of the physiological T cell response capable of controlling EBV should contribute to the optimization of CTL immunotherapy, a therapeutic approach already under trial for EBV-associated malignancies (2).

Large $CD8^+$ clonal expansions have been described in IM (3), and there is some evidence that clones seen in the memory CTL response are selected during the acute phase and may persist at high circulating frequencies (4), contributing to the $CD8^+$ expansions seen in healthy adults (5). In contrast, $CD4^+$ clonal expansions are rarely detectable in healthy individuals, even with highly sensitive techniques (6–8), but it is unclear whether this is because large clones do not arise during primary antigenic challenges or whether they are less persistent than the CD8 clones (9). If large CD4 expansions fail to develop during acute antigenic activation of CD4 cells, this could be a result of a smaller overall CD4 Ag-specific response or one that is composed of many smaller clones. New techniques for analyzing Ag-specific responses directly ex vivo have allowed a re-evaluation of antiviral CD8 responses for EBV (10, 11) and other viruses (reviewed in Ref. 12) and have highlighted the large proportion of the response lost during in vitro culture and therefore not detected by conventional cytotoxic assays. Much less is known about the size of CD4 responses in viral infections (13), and these may have been similarly underestimated by limiting dilution analyses (14). Equivalent techniques for direct analysis of responding CD4 cells have only become available more recently and have not yet allowed parallel dissection of the CD4 and CD8 components of a human antiviral response.

TCR repertoire analysis with a sensitive, global method should allow a direct comparison of the clonotypic composition of CD4 and CD8 cells. This approach was chosen to avoid the need for in vitro selection of responding clones, which inevitably involves focusing on functionally delineated responses and can introduce bias into TCR usage (15). Studies of TCR usage, because of their laborious nature, have tended to concentrate on responses to one or two well-described or immunodominant epitopes, particularly the highly conserved EBV-specific response restricted by HLA-B8 (4, 16, 17), which may not be typical of EBV responses restricted by other alleles (18, 19). Although studies with HLA-peptide tetramers have highlighted the striking potential magnitude of responses specific for single EBV epitopes (10), other work has revealed that the numerically immunodominant response is not necessarily that

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[3] Abbreviations used in this paper: IM, infectious mononucleosis; CTLp, CTL precursor; BC, constant region of the β-chain; BV, variable region of the β-chain.

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which is most protective (20). In addition, accumulating data stress the role of a multispecific T cell response in maximizing viral control (21–23), all of which points to the importance of also analyzing smaller responses to subdominant epitopes. Epitopes recognized in class II-restricted responses have not been well defined in many viral infections such as EBV, so that a functionally directed analysis of virus-specific CD4 clonotypes has not been feasible.

Thus, the modified heteroduplex technique (7, 24) is used in this study to provide as broad coverage as possible of the in vivo repertoire of clones responding to acute infection with EBV (regardless of epitope) and to allow sensitive tracking of their fate following disease resolution. Analysis of the CD4 and CD8 fractions is undertaken in parallel to determine whether the well-recognized difference in oligoclonality of these subsets in steady state situations is also detectable during the challenge of an acute lymphoproliferative antiviral response. We combine this method with phenotypic separation of T cell subsets to investigate the CD45 phenotype of persistent EBV-specific clones in the light of increasing evidence that the differentially spliced isoforms of the CD45 molecule may not have a straightforward association with naive and memory pools.

Materials and Methods

Patients and volunteers

Eight patients (age range, 18–24 years) diagnosed with acute IM on the basis of characteristic clinical features, atypical lymphocytosis, and a positive monospot test were recruited through University College Hospital hematology department and local student general practitioner services. EBV infection was confirmed by serological tests for viral capsid Ag IgM Abs. Patients were first sampled within 10 days of symptom onset and underwent longitudinal follow-up for up to 1 year (Table I). Controls were age-matched healthy laboratory volunteers who had not had an illness suggestive of IM. Local ethical committee approval for the study was obtained, and all donors gave written informed consent.

Isolation of PBMC and T cell subsets

PBMC were isolated from heparinized blood samples by Ficoll-Hypaque density centrifugation, and the nonadherent fraction was recovered after a 1-h plastic adherence step at 37°C. CD4 and CD8 T cell subsets were isolated by positive selection using directly conjugated anti-CD4 or anti-CD8 MiniMacs magnetic beads (Miltenyi Biotech, Bisley, Surrey, U.K.). Separated fractions were always >95% pure as assessed by mAb staining and FACS analysis.

Purification of CD45RA and CD45RO subsets was achieved by incubation of nonadherent PBMC with saturating concentrations of a mixture of mAb supernatants (anti-CD14, anti-CD19, anti-CD11b, anti-CD4, and either UCHL1 or SN130 for CD45RA or CD45RO negative selection, respectively). Unwanted stained populations were then removed using goat anti-mouse Ig MiniMacs beads (Miltenyi Biotech). CD45RA populations were highly purified by repeating this process until the number of contaminating CD45RO cells was <1%.

Phenotyping

Expression of different BV segments and activation markers by $CD4^+$ and $CD8^+$ subsets was assessed by flow cytometric analysis following triple staining of nonadherent PBMC with directly conjugated Abs to CD3, CD4, or CD8 and a panel of BV-specific mAbs (Immunotech, Marseilles, France) or CD45RO or HLA-DR (all from Sigma, Poole, U.K.) or CD28 (Becton Dickinson, San Jose, CA). Expression of these markers by BV expansions was analyzed by gating on $CD8^+$ T cells that had also been stained with the relevant BV-specific mAbs and CD45RO or HLA-DR or CD28.

Induction of EBV-specific CTL

PBMC were washed and resuspended in RPMI 1640 and 10% FCS with 1% human serum at a concentration of 1×10^6/ml in a 24-well plate. PBMC from acute IM samples stored in liquid nitrogen were thawed directly in IL-2-enriched medium (containing 30% (v/v) supernatant from the IL-2-secreting cell line MLA-144 (MLA-SN) and 100 U/ml of rIL-2) as described previously (25). The autologous B lymphoblastoid cell line was irradiated (10,000 cGy) and added to give a responder to stimulator ratio of 40:1. Restimulation was conducted 7 days later with the autologous lymphoblastoid cell line at a responder to stimulator ratio of 4:1 and subsequently weekly at a ratio of 10:1. After 2 wk the culture medium was IL-2 enriched as described above. Chromium release assays for EBV specificity and HLA restriction were conducted as described previously (25).

RNA extraction and cDNA synthesis

Total RNA was extracted from 2 to 4×10^6 PBMC or purified subsets of T cells by the guanidinium thiocyanate/acid phenol method using RNAzol B (Biogenesis, Bournemouth, U.K.). Approximately 5 μg of total RNA was used for first-strand cDNA synthesis using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Gaithersburg, MD) with RNasin (Promega, Madison, WI) and random hexamer primers.

Oligonucleotides

Oligonucleotides were synthesized and HPLC purified by the Imperial Cancer Research Fund (London, U.K.). Two BC primers were used: the consensus internal BC primer (5′-CACCCACGAGCTCAGCTCCACGTGGTC) and an external BC primer (5′-TGCTGA CCCCACTGTCGACCTCCTTCCCATT), which is 30 bp 3′ to the internal primer. The BV primers used were as previously described (24) for the heteroduplex PCRs. Amplification of the Cβ-chain to estimate the total amount of TCR β-chain mRNA in different samples was conducted using two primers for the C region of the β-chain (5′-TGGGAAGGAGGTCGACAGTG and 5′-TGG CCTTCCCTAGCAGGATCT) in conjunction with the PCRs below.

Heteroduplex analysis

Twenty-six PCRs were conducted for each analysis in a final volume of 50 μl and using one-fortieth of the cDNA each. Hot start PCR was conducted with initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min with a final extension period at 72°C for 10 min. Ten microliters of the PCR products was visualized on a 1% agarose gel. DNA carriers consisting of cDNAs encoding BV1–24 cloned from T cell clones or lines were as described previously and were provided by G. Casorati (24). These were amplified using the same TCRBV primers and reaction conditions as those for the sample cDNAs, but with the external BC primer. A 20-μl aliquot of each sample PCR product was mixed with 400 ng of the appropriate BV-matched carrier PCR product, denatured at 95°C for 5 min, and allowed to reanneal at 50°C for 1 h. The mixtures were kept on ice until loading on a 12% nondenaturing polyacrylamide gel run at 10 mA for 16 h at 4°C. Heteroduplex gels were stained with ethidium bromide for 30 min for initial visualization of DNA and then blotted onto nylon Hybond N^+ membranes in 20× SSC. The DNA was denatured and fixed to the membrane by a 20-min incubation in 0.4 M NaOH. The external BC probe was end labeled with [γ-^{32}P]ATP using polynucleotide kinase (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were prehybridized for 15 min in Rapid Hyb Buffer (Amersham Pharmacia Biotech) at 42°C, hybridized at 42°C for 1 h, and then washed in 5× SSC/0.1% SDS for 20 min at room temperature, followed by two washes of 15 min in 1× SSC/0.1% SDS at 42°C. Filters were exposed to film at −70°C for 6 h. For clonotypic probing, the filter was stripped by washing in 0.5% SDS at 80°C and rehybridized with the appropriate N region probe (5′-AGGATCCCCCAACGAGC), with a final wash with 0.2× SSC/0.1% SDS at 50°C.

Cloning and sequencing of BV PCR products

Cloning of PCR products was conducted using the TA cloning kit (Invitrogen, San Diego, CA). The ligation reaction was incubated at 14°C overnight and then used immediately for transformation of TOP10F′ One Shot competent *Escherichia coli* cells (Invitrogen). White colonies were distinguished from blue colonies not containing an insert and were further tested for inserts by plasmid Miniprep and restriction digestion with *Eco*RI (Amersham Pharmacia Biotech). Plasmid DNA was purified using Wizard Plus Minipreps (Promega) and sequenced using a modified version of the dideoxy chain termination DNA sequencing method with T7 DNA polymerase from the Sequenase version 2.0 kit (Amersham Pharmacia Biotech).

Results

In IM there is a diverse array of oligoclonal Ag-driven T cell expansions

Eight patients with acute infectious mononucleosis were recruited, six of them within 10 days of symptom onset, and their TCR repertoires were analyzed directly ex vivo. All six patients analyzed

very early in the course of the infection had lymphocytosis composed mainly of activated CD8 cells and had one or more large TCRBV-restricted expansions detectable by mAb staining (Table I) as previously noted in a larger cohort (3). However, molecular dissection of their T cell repertoire showed a much more diverse set of oligoclonal expansions than had been visualized with mAb staining alone. Available mAbs only cover half the repertoire, and because the percentage of cells staining with them can be highly variable in healthy individuals, only large expansions will be detectable. By contrast, TCR analysis by the modified heteroduplex technique has been shown to detect clonal expansions down to a frequency of 1 cell in 10,000, visualized as discrete bands with a unique, reproducible migration pattern (7, 24). The heteroduplex technique demonstrated not only oligoclonal expansions in those BV families that were expanded by mAb staining but also in many of those that appeared within the reference range with mAb. This is illustrated for donor 7, who had four TCRBV expansions detectable with a panel of 16 BV-specific mAbs (Table I) compared with at least one expansion demonstrable in 21 of 22 BV-specific PCR-heteroduplex tracks (Fig. 1*A*). Almost every BV PCR product from all patient PBMC samples was dominated by oligoclonal expansions, often with little background polyclonal smear remaining.

The pattern of heteroduplexes visualized in each patient was unique, consistent with a different set of Ag-driven clonal expansions occurring in each patient (shown for donors 7 and 3, Fig. 1, *A* and *B*). This was very different from the situation seen in healthy age-matched controls, exemplified in Fig. 1*C*, where fresh PBMC were polyclonal, as indicated by the smear pattern in every TCRBV track. Occasionally a few faint heteroduplex bands were visible in the healthy donors, which became more obvious on enriching for the $CD8^+$ fraction (7). Thus, most of the different TCR expansions seen in IM could be assumed to be disease related.

All expansions detected are $CD8^+$, whereas the CD4 cells remain polyclonal

The $CD4^+$ helper response is known to be important in the immune control of acute viral infections, and an increase in total $CD4^+$ T cell numbers has been documented in IM despite the inversion in CD4/CD8 ratio resulting from the far larger increase in $CD8^+$ numbers. There was also evidence of some activation of $CD4^+$ T cells in IM compared with healthy controls (data not shown and demonstrated previously (26, 27)), although, again, this was far outweighed by the dramatic activation in the $CD8^+$ fraction. However, as shown in Table I, there were no expansions in the $CD4^+$ T cells detectable with V region-specific mAb staining in any of the IM donors. More surprisingly, there were no clonal expansions detected in the $CD4^+$ purified fraction of any of the IM patients, even by the highly sensitive heteroduplex method. This is demonstrated for one representative patient in Fig. 2, for whom IM

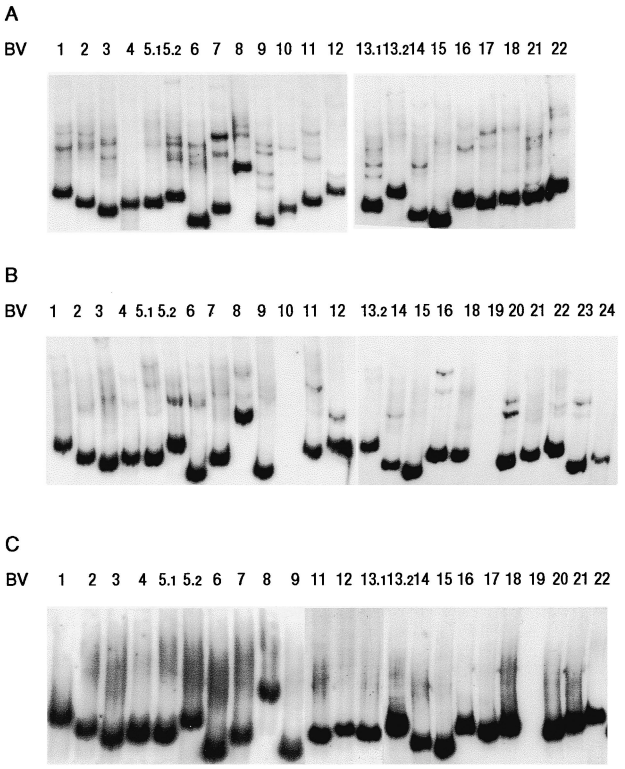

FIGURE 1. A comparison of overall clonality in PBMC from two IM donors and a healthy age-matched control. RNA was extracted from fresh PBMC and subjected to heteroduplex analysis of global TCR usage. Samples were electrophoresed through a polyacrylamide gel, and gels were blotted and hybridized with a TCRBC probe. *A*, The autoradiograph shows heteroduplex samples BV1–22 from IM donor 7 taken 3 days after symptom onset (shown with TCRBV primer and carrier used above each track). The heteroduplexes show a specific migration pattern above the dense carrier homoduplex in each track. *B*, Heteroduplex analysis of a different IM patient (donor 3) sampled within 5 days of disease onset, in whom fresh PBMC are shown to contain multiple heteroduplexes with a different pattern from donor 7 and with little background smear. *C*, Heteroduplex analysis of PBMC from a healthy donor who had never had IM, showing the typical polyclonal smear patterns above each carrier homoduplex.

Table I. *Patient sampling and detection of clonal expansions*

IM Donor	Lymphocyte Count at Recruitment	IM Sample (from acute symptom onset)	Follow-Up Samples (mo from onset)	TCRBV Expanded[a] by mAb Staining in		% of Heteroduplex Bands Segregating with	
				CD8 T cells	CD4 T cells	CD8 T cells	CD4 T cells
1		N/A	5	None	None	Not done	Not done
2	2.0×10^9/L	N/A	2, 11	None	None	100	0
3	5.1×10^9/L	5 days	1, 2, 3, 5, 6, 9	BV3, 5.1, 8, 12, 14, 16, 20, 23	None	100	0
4	8.8×10^9/L	6 days	1, 4	BV3, 14	None	100	0
5	10.6×10^9/L	7 days	1, 6	BV17, 21	None	100	0
6	4.7×10^9/L	9 days	1.5	BV16, 22	None	100	0
7	10.2×10^9/L	3 days	2	BV13.1, 13.6, 16, 22	None	100	0
8	7.0×10^9/L	10 days	2.5	BV3, 13.6	None	Not done	Not done

[a] Greater than mean + 3 SD for healthy age-matched controls.

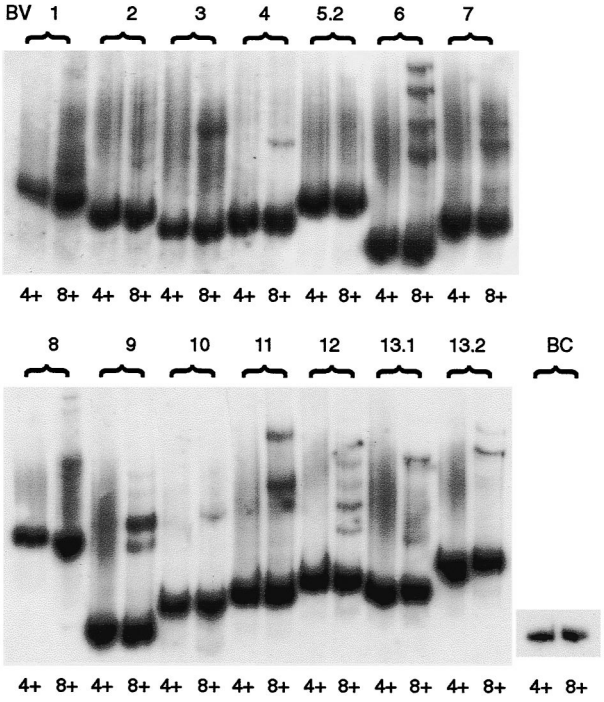

FIGURE 2. Segregation of all the IM heteroduplexes with the $CD8^+$ purified T cells. Fresh T cells from IM donor 4 were fractionated into $CD4^+$ and $CD8^+$ subsets using magnetic beads (both >95% purity on mAb staining). Heteroduplex analysis was performed on the two fractions in parallel, and samples were run in adjacent tracks for comparison. Samples for $CD4^+$ T cells (4+) and $CD8^+$ T cells (8+) are shown for BV1–13.2, demonstrating segregation of all heteroduplex bands with the $CD8^+$ subset. The last two tracks (BC) are the PCR product of the control amplification of cDNA with two β-chain constant region primers, hybridized with the same probe for the external portion of the C region, which hybridizes with the carrier in the BV samples. PCR on serial dilutions of cDNA suggested equivalent inputs of mRNA for $CD4^+$ and $CD8^+$ subset PCR.

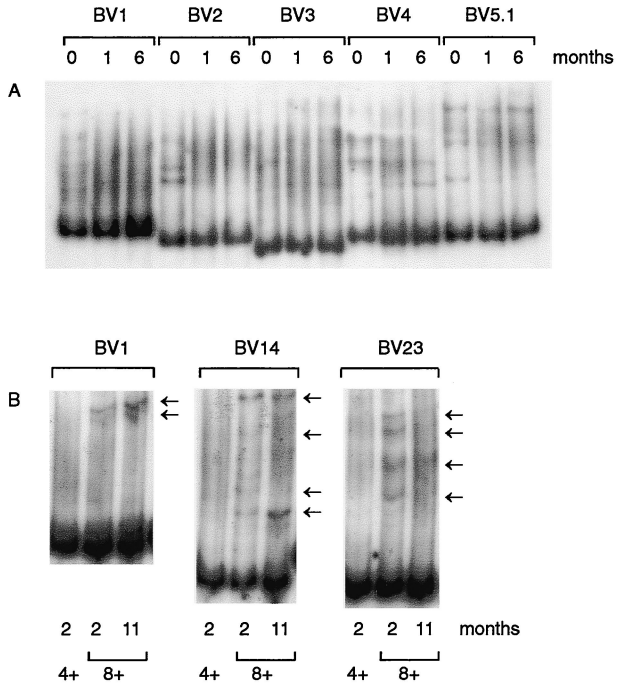

FIGURE 3. Longitudinal heteroduplex tracking of clones following IM. *A*, Samples from donor 3 were analyzed by heteroduplex on a total of six further occasions following the IM sample. Heteroduplex analysis of five representative families (BV1–5.1) from the acute sample (0) and two follow-up samples (1 and 6 mo) are shown. The analysis was conducted on total fresh PBMC, stored as cDNA and analyzed in parallel. Representative TCRBV (BV4 and BV5.1) illustrate maintenance of acute clonotypes, as evidenced by preservation of their heteroduplex footprint on follow-up, whereas other clonotypes (BV2) become undetectable by 1 mo. *B*, Fresh PBMC were taken from donor 2 at 2 mo, and $CD4^+$ (4+) and $CD8^+$ (8+) T cells were purified using magnetic bead separation (purity >95%). $CD8^+$ T cells were also separated from PBMC 11 mo following disease onset. Stored cDNA samples from each sample were subjected to heteroduplex analysis in parallel. Representative TCRBV are shown, illustrating polyclonal patterns in $CD4^+$ T cells and preservation of $CD8^+$ heteroduplexes except in BV23.

T cells have been fractionated using magnetic beads into $CD4^+$ and $CD8^+$ fractions (purity >97%) and analyzed in parallel, with similar inputs of total TCR β-chain mRNA, as suggested by the TCRBC PCR control. All the visible clones are in the $CD8^+$ T cells, with polyclonal smear patterns throughout the $CD4^+$ T cells. Parallel molecular TCR analysis of highly purified CD4 and CD8 subsets in five other IM patients showed the same segregation of all clones detectable by heteroduplex analysis with the CD8, not CD4, cells (Table I).

Persistence of clonotypes at detectable levels in unstimulated memory pool

Longitudinal follow-up of donors with the heteroduplex technique showed that many of the TCR expansions seen in the acute response persisted at a level where they could be detected directly ex vivo despite full symptom resolution. A large proportion (~50%) of the bands seen in the first sample taken at the height of the illness remained visible for the duration of follow-up (up to 11 mo; Fig. 3). Longitudinal tracking was also performed in purified $CD8^+$ T cells to remove the variability attributable to the decrease in the percentage of circulating $CD8^+$ T cells with time after IM (Fig. 3*B*). A number of clones became undetectable in the PBMC compartment over time and were only visible in purified CD8 cells, indicating a decrease in clone size, while other clones contracted to below the threshold of detection even in purified CD8 cells (Fig. 3*B*). A marked reduction in clone size was also evidenced by the quantitation of large TCRBV expansions by mAb staining, showing contraction of most expansions to within the reference ranges by 3 wk after symptom onset (data not shown). Thus, the circulating memory pool of CD8 cells in healthy carriers was composed of a large subset of the effectors present in acute infection. No new clones appeared on recovery that had not been visualized in IM (Fig. 3).

The data shown in Fig. 3*B* highlight the polyclonality of the $CD4^+$ T cell compartment, visualized by the uniform smear pattern. This lack of detectable clonal expansion in the CD4 cells persisted throughout follow-up (shown for 2 mo after IM, Fig. 3*B*).

EBV-specific CTL comprise many of the same clonotypes seen in fresh ex vivo samples

To investigate the functional specificity of the clones visualized, EBV-specific CTL lines were generated from two of the donors using restimulation with the autologous B lymphoblastoid cell line to gain as broad a representation as possible of the physiological in vivo repertoire of EBV-CTL. In the case of donor 3, the T cell line was generated from PBMC taken 6 mo after acute disease; for donor 4 both acute and memory (4 mo after IM) PBMC were restimulated in parallel. All T cell lines generated exhibited a high percentage (35–75%) of EBV-specific lysis of the autologous B

lymphoblastoid cell line with which they had been stimulated, detectable at low E:T cell ratios and in repeated CTL assays over a number of weeks.

The $CD8^+$ cells from the T cell lines were purified and used for heteroduplex analysis. Samples from fresh $CD8^+$ T cells and the T cell line generated from the same PBMC were run in adjacent tracks for comparison (representative examples in Fig. 4). The T cell lines were enriched for many of the same clonotypes seen in the acute ex vivo sample, some of which had subsequently become barely detectable or undetectable, even in the $CD8^+$-enriched fraction (Fig. 4*A*). This provided strong supporting evidence that the clonotypes of the effectors expanded in acute disease overlapped with the clonotypes involved in the memory CTL response when these individuals were rechallenged with EBV in vitro. Clonotypic identity between the fresh and CTL samples was further confirmed by sequencing (see below). The results of the selective in vitro expansion of EBV-specific CTL suggested that some of the clones that became undetectable in fresh circulating $CD8^+$ T cells had not undergone clonal exhaustion and were capable of re-expansion.

The comparison of EBV-specific CD8 cells capable of proliferating in vitro in acute infection and in the memory phase from donor 4 revealed very similar patterns of EBV-specific CTL lysis in terms of HLA restriction (data not shown). Parallel visualization of the fresh CD8 cells and those from the T cell lines from both IM and memory (4 mo) samples revealed some clonotypic identity (BV6, Fig. 4*B*), whereas other clones were either preferentially expanded in the IM or memory T cell lines (BV9, Fig. 4*B*). Thus, global tracking of clonotypes by their molecular footprint showed that the memory population of EBV CTL circulating after disease resolution and capable of expanding in response to virus challenge in vitro was derived from the pool of disease-related CD8 cells found in acute infection.

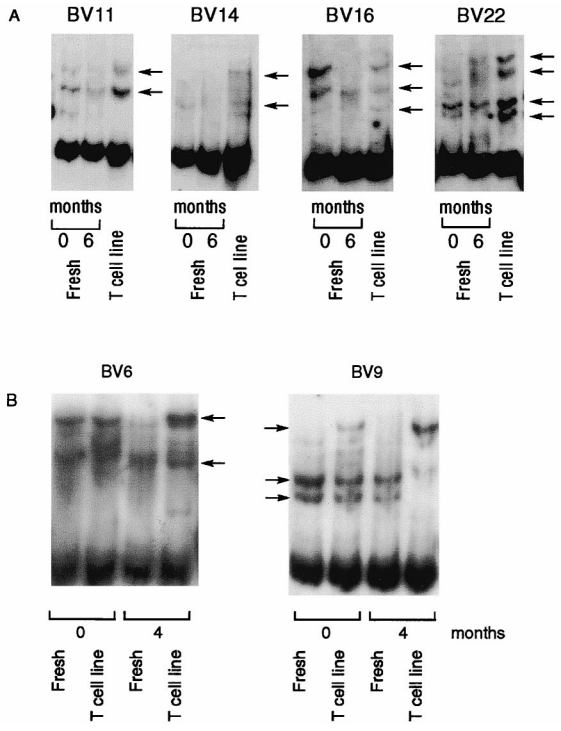

FIGURE 4. Comparison of heteroduplex footprints between ex vivo IM $CD8^+$ T cells and EBV-specific CTL. *A*, Fresh $CD8^+$ T cells from IM (0 mo) and 6 mo follow-up (donor 3) were analyzed by heteroduplex in parallel with $CD8^+$ T cells purified from the CTL generated from the 6 mo PBMC sample. The T cell line demonstrated 70% specific lysis of the autologous lymphoblastoid cell line at the time cells were harvested for RNA extraction. Many of the heteroduplexes that were prominent in the ex vivo IM $CD8^+$ T cells and barely detectable at 6 mo were enriched in the CTL population (arrowed). *B*, Heteroduplex was performed simultaneously on stored cDNA from fresh $CD8^+$ T cells (0 and 4 mo) and the EBV-specific T cell lines derived from them (donor 4). Selected TCRBV run in adjacent tracks of the polyacrylamide gels are shown. Arrows indicate conserved clonal patterns, except the upper arrow in BV9, which indicates a clone visible only after in vitro restimulation.

There is a disproportionate loss of the $CD45RO^+$ phenotype within oligoclonally expanded $CD8^+$ T cells following IM

At the onset of acute infectious mononucleosis, there was a striking $CD8^+$ lymphocytosis of HLA-DR^+ $CD45RO^+$ T lymphocytes. Oligoclonal expansions detectable by TCRBV mAb staining were also enriched for the HLA-DR^+ phenotype, as noted in a previous study (3). This enrichment persisted on follow-up, even when the percentage of $CD8^+$ T cells within a TCRBV family had contracted to within reference ranges, suggesting ongoing EBV-related activation. This is illustrated for one donor in Fig. 5*A*, for whom a BV22 expansion accounting for 10.5% of $CD8^+$ T cells is shown to have a higher fraction positive for HLA-DR than the rest of the $CD8^+$ T cells both at the onset of symptoms (time 0) and on full recovery (2 mo).

By contrast, the percentage of $CD45RO^+$ $CD8^+$ T cells was usually extremely high in acute disease, but fell much more rapidly and disproportionately more so in expansions (mirrored by an increase in CD45RA staining). Thus, in the same donor (Fig. 5*B*), 90% of circulating $CD8^+$ T cells were $CD45RO^+$ at time 0, but by 2 mo 39% were $CD45RO^+$, and only 28% of the BV22 $CD8^+$ T

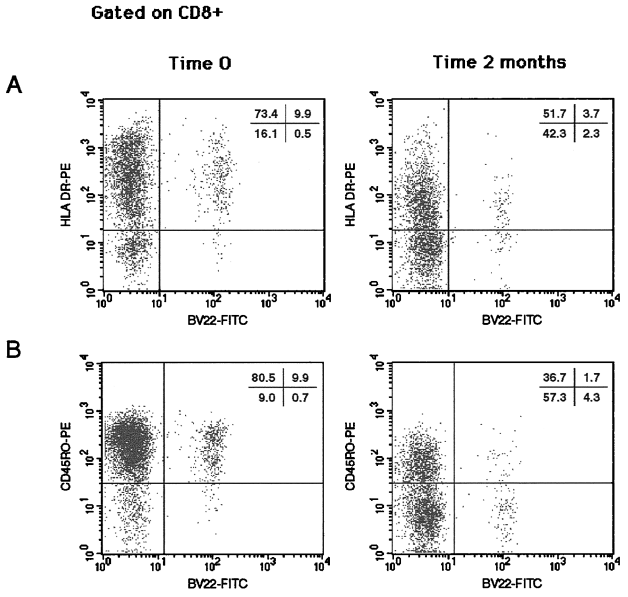

FIGURE 5. Triple-color staining and FACS analysis of T cells during and after IM to examine the phenotype of TCRBV expansions. PBMC were analyzed by flow cytometry using mAb to CD8, HLA-DR, or CD45RO and a TCRBV (in this example, BV22, donor 7). Twelve thousand events were acquired within the live gate set on size and granularity, and data were then gated on $CD8^+$ T cells during analysis. *Left panels*, Staining patterns in IM; *right panels*, Staining patterns at 2 mo when the disease had fully resolved. *A*, The percentage of $BV22^+$ $CD8^+$ T cells compared with remaining $CD8^+$ T cells staining positive for HLA-DR was 95 vs 82% at time 0 and 61 vs 55% at 2 mo, respectively. *B*, The percentages of $BV22^+$ $CD8^+$ T cells and remaining $CD8^+$ T cells staining positive for CD45RO were 93 and 90% at time 0 and 28 and 39% at 2 mo, respectively.

cells were still CD45RO$^+$. There was therefore a discrepancy between staining of CD8$^+$ T cells with CD45 isoform Abs and other markers of activation such as HLA-DR, particularly following resolution of the initial large Ag-driven lymphocytosis. This suggested that activated CD8 cells generated after the acute phase in this viral response could have the CD45RO$^-$CD45RA$^+$ phenotype previously associated with a naive resting status.

Molecular TCR analysis reveals an accumulation of clonal expansions in the CD45RA$^+$ pool following IM resolution

The phenotypic data showing accumulation of activated, Ag-experienced CD8 cells with a CD45RA phenotype after IM suggested that there may be reversion of individual clones from a CD45RO to an RA phenotype. The CD45 phenotype of clonal expansions in the response to EBV was therefore further investigated by probing TCR usage at the molecular level. T cells taken from two individuals during the acute stage of IM were fractionated into CD45RA and CD45RO subsets. The CD45RA fractions were highly purified by negative selection, such that the level of contaminating CD45RO$^+$ or CD45RA$^+$RO$^+$ double-positive T cells was $<$1%. Heteroduplex analysis was conducted simultaneously for the two fractions to allow the phenotype of multiple different T cell clonotypes to be identified directly ex vivo. For this purpose, RT-PCR was conducted on the purified fractions in parallel, and TCR BV-matched samples were run in adjacent tracks for comparison. Initially, most of the clonal expansions segregated with the CD45RO$^+$ fraction, with only a few of the clones also faintly detectable in the CD45RA$^+$ T cells (Fig. 6, *A* and *C*). If this were a result of small amounts of contaminating CD45RO$^+$ T cells being picked up by the highly sensitive PCR analysis of the CD45RA$^+$ T cells, carryover would be expected to be most obvious for the most intense bands, rather than to occur with this selective pattern.

T cells taken from the same donors 6 or 9 mo after the acute infection were also analyzed by heteroduplex according to CD45 phenotype and run in adjacent tracks. Following disease resolution, many of the clones that predominantly segregated with the CD45RO$^+$ phenotype were also identified in the CD45RA$^+$ fraction by the presence of the same heteroduplex band (indicated with arrows in Fig. 6, *B* and *C*). We therefore went on to investigate the cytolytic potential of CD45RA and CD45RO clonotypes.

EBV-specific CTL precursors with proliferative potential in vitro were derived from the CD45RO clonotypes

EBV-specific CTL precursors (CTLp) were likely to reside in the CD45RO fraction in IM, since almost all circulating CD8 cells and detectable clonotypes had a CD45RO$^+$ phenotype at this time point (Fig. 6*A*). However, in the memory phase, many of the same clonotypes could also be visualized in the CD45RA pool (Fig. 6*B*), making it difficult to be certain that the CTLp were still derived from the CD45RO fraction.

The differential segregation of two clones according to CD45 isoform was therefore used as an opportunity to investigate their contribution to the memory CTL response. In donor 3, two distinct clones were visualized in BV23, one segregating with CD45RA, and one segregating with CD45RO (Fig. 7*A*, first two tracks). To validate this unexpected finding of a prominent heteroduplex unique to the CD45RA fraction, the BV23 CD45RA$^+$ T cell PCR amplification products were cloned and sequenced. Twelve of 13 inserts sequenced from the PCR product were identical across the VDJ junction, confirming the presence of a large monoclonal CD45RA$^+$ expansion (with the junctional region sequence shown in Fig. 7*C*). The BV23 expansion represented 9.3% of circulating CD8$^+$ T cells and 13% of circulating CD8$^+$ CD45RA$^+$ T cells by

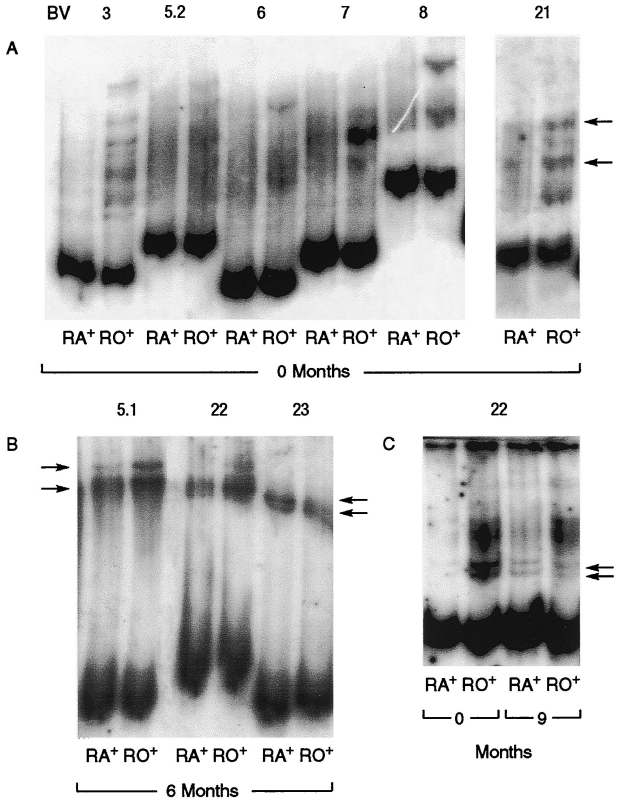

FIGURE 6. Visualization of clonal segregation according to CD45 isoform expression in IM and memory samples. *A*, PBMC from IM donor 5 at time 0 were fractionated into CD45RA$^+$ (RA$^+$) and CD45RO$^+$ (RO$^+$) T cell subsets with mAbs and magnetic beads. Heteroduplex analysis was performed on the two subsets in parallel and run in adjacent tracks. The TCRBV shown here demonstrate predominant segregation of heteroduplexes with CD45RO$^+$ T cells at this time point, although in some cases (TCRBV21) there is already evidence of selective overlap of clones between both isoforms (arrowed). *B*, Heteroduplex analysis of CD45RA$^+$ and CD45RO$^+$ fractionated T cells from donor 5 at 6 mo after IM, demonstrating the presence of the same clonotypes in both fractions (arrowed). *C*, PBMC from donor 3 at 0 and 9 mo after IM onset were separated according to expression of CD45 isoform, and fractionated subsets from both time points were analyzed in adjacent tracks to facilitate comparison of clonal segregation over time. Heteroduplexes that predominantly segregate with the CD45RO fraction in IM become more prominent in the CD45RA fraction by 9 mo, as illustrated for BV22 (arrowed).

mAb staining. Thus, this single clone accounted for an estimated 12% of the total CD8$^+$ naive (CD45RA$^+$) repertoire and had an approximate clone size of 5×10^7 cells/l in the periphery alone. Tracking of this large clone by its heteroduplex footprint and by clonotypic probing (data not shown) confirmed that it persisted at high frequency in the periphery for at least 9 mo, demonstrating that long-lived monoclonal expansions can occur in the CD45RA pool. Cloning and sequencing of the BV23 CD45RO$^+$ T cell PCR product from the same donor showed the presence of a different expansion, accounting for 4 of 10 inserts (Fig. 7*C*).

The BV23 heteroduplex product from the CTL line raised in this donor was analyzed alongside the total PBMC and CD45RA and RO fractions from the same time point before stimulation (Fig. 7*A*). T cells expressing BV23 were known to be an important component of the CTL line, since they accounted for the largest expansion within it by mAb staining (12% of the CTL line; data not shown). The predominant heteroduplex pattern in the in vitro expanded CTL sample mirrored that seen in the CD45RO$^+$ fraction, together with a new band not seen in any of the fresh samples.

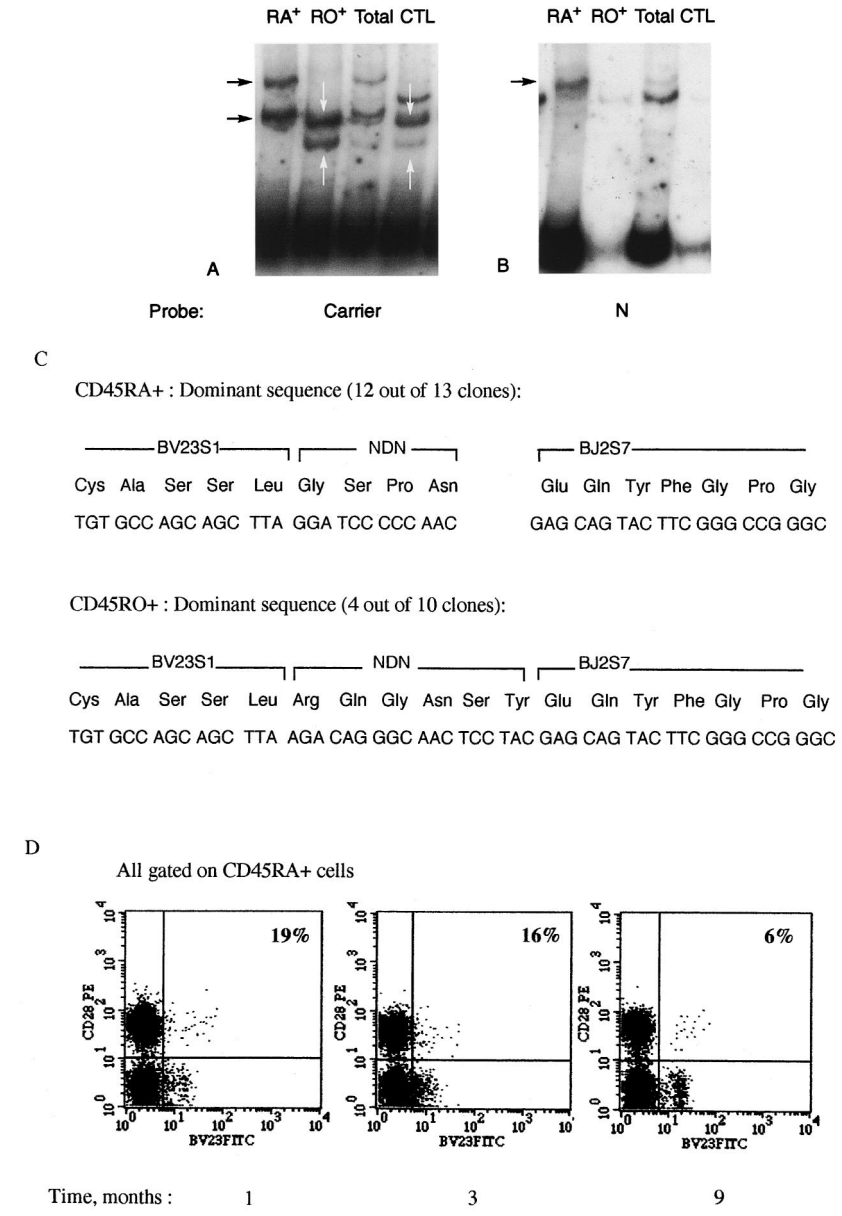

FIGURE 7. EBV-specific CTLp segregate with the CD45RO$^+$ fraction, whereas the CD45RA pool contains a large monoclonal expansion with an effector phenotype. *A*, There are distinct BV23 heteroduplexes in the CD45RA$^+$ (horizontal arrows) and CD45RO$^+$ (vertical arrows) T cells from IM donor 3. Both sets are visible in BV23 PBMC (total track), but only the RO$^+$ clone (and not the RA$^+$ clone) expands in the BV23 product of the T cell line derived from PBMC from the same 6 mo point (CTL track, vertical arrows). *B*, Clonotypic probing with an N region probe specific for the BV23 CD45RA clone (derived from the sequence in *C*) highlights the RA$^+$ clone forming a heteroduplex with the carrier in RA$^+$ and total tracks (arrow) and as a heteroduplex with the RO$^+$ clone (slightly lower pattern of migration) in the RO$^+$ and total tracks. The CD45RA clonotypic probe does not hybridize with the heteroduplexes in the T cell line track (CTL), confirming that the CTL are not derived from the CD45RA clone. *C*, Sequencing across the CDR3 region confirms the monoclonality of the BV23$^+$ CD45RA$^+$ expansion. CD8 T cells from the 9-mo follow-up sample from donor 3 were fractionated into CD45RA$^+$ and CD45RO subsets, and the RT-PCR product for BV23 was cloned and sequenced. Nucleotide and predicted protein sequences are shown, with the junctional (NDN) regions marked. Both dominant sequences used BJ2S7, but the CD45RO clone had an NDN region longer by 2 aa. *D*, The BV23 monoclonal expansion persists within the CD45RA pool and develops a CD28$^-$ phenotype following IM. CD8$^+$ T cells were stained for CD45RA, CD28, and BV23 and analyzed by flow cytometry; dot plots are shown after gating on CD45RA$^+$ cells from samples from donor 3 taken 1, 3, and 9 mo after IM. Percentages indicate the proportion of CD45RA$^+$BV23$^+$ cells staining positively for CD28$^+$ at each time point.

The molecular footprint formed by the CD45RA$^+$ clone was not detectable in the CTL sample (Fig. 7*A*). This was confirmed by clonotypic probing with a probe specific for the N region of the CD45RA clonotype (derived from the N region sequence; Fig. 7*C*). This clonotypic probe hybridized with the CD45RA and total PBMC tracks, but did not hybridize with any of the heteroduplices in the T cell line track (Fig. 7*B*). These results indicated that the predominant clone in the T cell line was that derived from the CD45RO$^+$ CD8$^+$ T cells (confirmed by sequencing of the T cell line BV23 product; data not shown). The data suggest that, at least in the case of some clones, EBV-specific CTLp capable of expansion in vitro may selectively reside in CD45RO$^+$ CD8$^+$ T cells, whereas clonally expanded CD45RA$^+$ CD8$^+$ cells may constitute effectors (prone to apoptose in culture).

To investigate this further, we extended the phenotypic analysis of the BV23CD45RA$^+$ cells, since recent studies in healthy donors have described a CD45RA$^+$CD28$^-$CD27$^-$ subset within the CD45RA pool that exhibits direct cytolytic, but not CTL precursor, activity (28). The percentage of BV23 cells staining for CD45RA increased following IM, consistent with the development of the large monoclonal expansion described above. Costaining with CD28 and CD45RA of the BV23 expansion (Fig. 7*D*) revealed that the BV23 CD45RA$^+$ subset contained a high proportion of CD28$^-$ cells by 1 mo after IM onset, which increased further over time after disease resolution. The proportion of CD27$^-$ cells in the CD45RA$^+$ subset increased over time in parallel with that of CD28$^-$ cells (data not shown). Thus we documented the development of monoclonal expansions in the CD45RA subset following acute EBV infection with a phenotype consistent with that described for CD8 effectors.

Discussion

Global analysis of the direct ex vivo clonal composition of the dramatic CD8$^+$ lymphocytosis at the onset of IM revealed extensive CDR3 region defined TCR expansions, with no restriction on V region usage. The presence of heteroduplex bands representing clonal expansions in almost every TCRBV examined, and the dramatic reduction in the polyclonal background smear usually seen, implied that most of the CD8$^+$ proliferation was Ag driven. Accumulating data from mouse and human models acquired by a

combination of highly sensitive techniques are consistent with this finding, indicating that there can be a large Ag-specific component to acute virus-driven CD8 proliferation (10, 29, 30). Although the magnitude of the EBV-specific CD8 expansion in IM has been demonstrated (10), the breadth of clones involved in the acute response was not previously recognized, since analysis after screening with TCRBV mAbs had only identified some of the largest expansions (3).

A number of studies have shown that there is a much greater propensity for detectable clonal expansions to develop in the CD8 than the CD4 compartment over time in healthy donors, and some of these have now been clearly linked to ongoing responses to persistent viral infections such as EBV (5). Lack of detectable, persistent CD4 clones could result from the smaller overall CD4 responses to antigenic challenges encountered and the smaller individual CD4 clonal burst sizes. Recent data using methods for direct ex vivo analysis suggest that the size of the total Ag-specific CD4 response is at least 35-fold smaller than that of the CD8 response in acute and memory phases of lymphocytic choriomeningitis virus (31). Virus-specific CD4 frequencies decreased substantially following resolution of acute murine γ herpes infection (32), and study of a human class II-restricted response using HLA-peptide tetramers necessitated in vitro expansion to detect CD4 cells specific for an influenza epitope in chronic carriers (33). Thus it was of interest to compare the clonality of the CD4 and CD8 subsets in IM, since no previous studies have examined either the size of the CD4 Ag-specific response or CD4 clonality in this infection. It was striking that no $CD4^+$ T cell expansions were detectable, even in the early stages of acute infection with EBV in this study and despite the high sensitivity of the heteroduplex technique. The other study in which CD4 and CD8 TCR clonality were analyzed simultaneously in an acute immune response found a few transient $CD4^+$ clonal expansions in the two individuals examined, with a brief undefined flu-like illness (34). However, the $CD4^+$ T cell expansions documented by single-stranded conformation polymorphism were far fewer than those in the $CD8^+$ fraction and disappeared more rapidly (by 21 days after infection), consistent with murine data indicating a shorter duration of CD4 than CD8 responses (13). Thus, it is possible that transient $CD4^+$ T cell clonal expansions occurred in the relatively long incubation period of IM and had already resolved by the time of symptom onset and recruitment. The CD4 T cell response contributes to the maintenance of effective CTL responses (35, 36), as illustrated by the association of EBV-related lymphoproliferative disease with $CD4^+$ T cell depletion in AIDS. However, far fewer $CD4^+$ T cells of a given clonotype may be required, since their effects are amplified through the actions of secreted cytokines. This difference in clonal dynamics observed in an immune response where both $CD4^+$ and $CD8^+$ T cells are known to participate suggests a much tighter control on CD4 clone size.

Termination of the acute response to EBV infection resulted in a rapid reduction of CD8 clone size in most cases, presumably due to the massive apoptosis known to occur (37). However on longitudinal repertoire analysis, a large proportion of the acute clonotypes remained detectable in fresh ex vivo $CD8^+$ T cells after the full resolution of IM, and no new clonal expansions arose. Thus, the initial EBV memory repertoire (followed up to 1 year) represented a large subset of the primary array of clonal expansions. This requires clones to undergo numerous divisions to generate the initial expansion of effectors and yet to retain the capacity to enter the memory pool and divide further following rechallenge with the virus; such maintenance of replicative reserve may be mediated by up-regulation of telomerase (38). The evolution of the total EBV-specific CD8 response is compatible with the findings of a study focusing on TCR usage in the CTL response to two HLA-B8-restricted epitopes within EBNA-3 during IM and convalescence (17). By analyzing this functionally defined component (following in vitro expansion of CTL clones), maintenance of multiple responding TCR was demonstrated for 6 mo after IM despite the highly focused public TCR response reported to one of these epitopes in the long term memory response (16). A similar approach showed preservation of diverse TCR usage in the CTL response to an epitope within the lytic *trans*-activator protein BZLF1 during primary and persistent infection (19). Maintenance of the broad repertoire of clones visualized by heteroduplex analysis, which is likely to represent responses to a number of different epitopes, may be a crucial immunoprotective feature of the antiviral response, particularly in the case of less genetically stable viruses.

The CD45 phenotype of the clonal expansions documented in this work is in line with accumulating data that CD45RA does not reliably identify only naive $CD8^+$ T cells (39–42). The correlation between clonotypes identified in the $CD45RO^+$ fraction and those capable of re-expansion in EBV-CTL cultures is congruent with the original data showing segregation of most EBV-CTLp with this subset (43). It is now clear that EBV-specific CTL effectors might not have been detected by these limiting dilution analyses, since they would have been prone to apoptose in vitro. Some $CD8^+$ CTL effectors have been suggested to have a $CD45RA^+CCR7^-$ or $CD45RA^+CD27^-CD28^-$ phenotype outside the setting of an acute infection (28, 44), consistent with the accumulation of clonal expansions in the latter subset following acute EBV infection. The analysis of CD45 isoform expression of tetramer-positive $CD8^+$ T cells following the resolution of IM also showed a partial switch to CD45RA expression (10). The heteroduplex data presented here reveal that cells from the same clones (and therefore using the same TCRs) can express both CD45 phenotypes, as has been demonstrated for a single CMV-specific clonotype (45). This could be attributed to CD45RO to CD45RA reversion following an acute response or could indicate that some cells within a dividing clone are capable of maintaining expression of the CD45RA isoform. However, the very high initial proportion of $CD45RO^+$ CD8 cells and the progressive increase in the number of clonotypes in the CD45RA fraction over time following the acute antigenic challenge support the former interpretation. The functional specificity of the CD45RA clones could be explored for defined epitopes by techniques such as enzyme-linked immunospot or intracytoplasmic staining for IFN-γ, which do not require in vitro expansion. This would enable assessment of whether there was also functional reversion to a naive state or whether these EBV-related CD45RA clones actually have direct ex vivo effector function, as suggested by their phenotypic resemblance to those described in healthy individuals (28, 44). The demonstration of a large, long-lived CD45RA clone underscores the fact that Ag-driven $CD8^+$ T cells can maintain this phenotype for prolonged periods, accounting for a significant proportion of the circulating naive CD8 pool.

This is the first analysis of CD4 clonality in EBV infection and reveals profound differences in the heterogeneity of the responding population compared with that of the CD8 compartment. The clonal composition of $CD4^+$ and $CD8^+$ fractions needs to be compared simultaneously in other acute immune responses to support the assertion that the $CD4^+$ T cell response to Ag is composed of smaller clones. This would be congruent with their differing effector functions, with larger $CD8^+$ clones being required to achieve efficient direct CTL killing, whereas $CD4^+$ effects are mediated via cytokines. Most of the extensive repertoire of CD8 clones responding to primary symptomatic EBV infection persists at surprisingly high circulating frequencies following disease resolution. The data indicate that a relatively heterogeneous repertoire

of CTL can be maintained at least initially under the pressure of a persistent, genetically stable, virus infection. The assessment of clonality by mAb staining and heteroduplex analysis confirm that most acutely Ag-activated $CD8^+$ T cells express the low molecular weight isoform CD45RO. CD45RA is not a clear-cut naive marker for CD8s, in that large Ag-driven clones are found in this fraction. Thus, the ability to identify persistent clones constituting the ongoing cellular response, not only those capable of expansion in vitro, has allowed reassessment of a highly effective antiviral T cell response.

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