Rapid CD8$^+$ T Cell Repertoire Focusing and Selection of High-Affinity Clones into Memory Following Primary Infection with a Persistent Human Virus: Human Cytomegalovirus[1]

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To investigate the mechanism of selection of individual human CD8$^+$ T cell clones into long-term memory following primary infection with a persistent human virus (human CMV (HCMV)), we undertook a longitudinal analysis of the diversity of T cell clones directed toward an immunodominant viral epitope: we followed this longitudinally from early T cell expansion through the contraction phase and selection into the memory pool. We show that following initial HCMV infection, the early primary response against a defined epitope was composed of diverse clones possessing many different TCR Vβ segments. Longitudinal analysis showed that this usage rapidly focused predominantly on a single TCR Vβ segment within which dominant clones frequently had public TCR usage, in contrast to subdominant or contracted clones. Longitudinal clonotypic analysis showed evidence of disproportionate contraction of certain clones that were abundant in the primary response, and late expansion of clones that were subdominant in the primary response. All dominant clones selected into memory showed similar high functional avidity of their TCR, whereas two clones that greatly contracted showed substantially lower avidity. Expression of the IL-7R is required for survival of murine effector CD8$^+$ T cells into memory, but in primary HCMV infection IL-7R was not detected on circulating Ag-specific cells until memory had been established. Thus, the oligoclonal T cell repertoire against an immunodominant persistent viral epitope is established early in primary infection by the rapid selection of public clonotypes, rather than being a stochastic process. ***The Journal of Immunology,*** **2007, 179: 3203–3213.**

During a primary virus infection, naive virus-specific T cells encounter viral Ags and undergo extensive clonal expansion (which may exceed a 1000-fold). Following a decrease in the viral load (either because the virus is cleared as in an acute infection or because latency is established), expanded virus-specific T cells undergo a contraction phase in which >90% of the cells are lost and the remaining cells constitute a memory T cell pool. A number of factors have been suggested that may determine selection of T cells from the initial expanded population into the long-term memory pool, including expression of receptors for the IL-2 cytokine family (in particular the IL-7R), TNF family receptors, perforin, and IFN-γ, as well as the up-regulation of antiapoptotic factors (reviewed in Refs. 1 and 2).

The TCR usage of cells that survive the contraction phase and become established into the memory pool has been investigated in a number of model viral systems to determine whether particular TCR Vβ framework regions and CDR3 are preferentially selected into the memory pool (which would indicate that they confer a survival advantage). In the murine lymphocytic choriomeningitis virus model, a number of studies have concluded that whereas particular Vβ framework genes are favored during clonal expansion in the primary response, the primary response is nevertheless polyclonal, and in addition, the responding TCR repertoire seen in primary lymphocytic choriomeningitis virus infection is representatively selected into memory (3, 4).

Model infections with murine CMV also show extensive polyclonal T cell expansions following primary infection, and the same TCR repertoire is retained into memory (5, 6). Again, in an acute influenza model, the same TCR repertoire detected in the acute phase was also represented in the memory T cell pool (7).

It has recently been demonstrated in a number of murine models that a small subset of cells up-regulates expression of the IL-7Rα during the peak of the immune response, and these IL-7Rα^+ cells go on to enter the memory pool (8, 9). If IL-7Rα expression was ubiquitous in the primary responding population, this could explain why a representation of this initial repertoire is maintained into the memory pool.

In humans, a number of studies have analyzed the T cell repertoire in primary and memory phases following primary human virus infection. During primary EBV infection in humans, the acute phase of infection is associated with a large oligoclonal expansion of virus-specific T cells, followed by widespread apoptosis (10). In the acute phase, CD8$^+$ T cell clones that recognize lytic epitopes are up to 10 times more abundant than clones responding to latent viral proteins. However, in the contraction phase, these highly expanded clones specific for lytic epitopes are lost to a greater extent than the clones specific for latent epitopes (11–13). A subsequent analysis of the T cell response to a single defined lytic epitope confirmed that clones that dominated the primary response did not dominate the memory response. An analysis of T cell populations generated to an individual latent EBV peptide showed that the T cell response was heterogeneous and stably

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maintained over time, and concluded that the primary response largely determined the composition of the T cell memory pool (14).

In an important and ubiquitous human herpes virus infection, human CMV (HCMV)[3] infection, it is well established that in many healthy virus carriers the HCMV-specific memory CD8^{+} T cell response includes a high frequency of T cells specific for a lytic phase viral tegument protein pp65 (UL83) and the major immediate early protein IE1 (UL123) (15–18). It is uncertain whether HCMV expresses any proteins during latency. Recently, CD8^{+} T cells specific for epitopes within proteins from many other HCMV open reading frames have been described, although these studies also confirmed that pp65 (UL83) and IE1 (UL123) are among the most frequently recognized (19–21).

There is little information on the breadth of TCR usage and phenotype of the CD8^{+} T cell response to primary HCMV infection, and the factors in humans that influence selection of CD8^{+} T cells into long-term memory. We have previously shown that in long-term virus carriers, analysis of the clonal composition of the memory CD8^{+} T cells repertoire specific to four different pp65 epitopes revealed a high degree of clonal focusing. In a given long-term carrier, the majority of clones specific for a defined pp65 peptide used only one or two different TCRs. Among donors who had the same MHC class I allele, CD8^{+} T cell clones from different donors that recognized the same pp65 peptide-MHC complex often used the same TCR Vβ family. Thus, the large population of circulating pp65 peptide-specific memory CD8^{+} T cells was composed of only a few individual clones that have undergone extensive clonal expansion in vivo (22). The finding that the CD8^{+} T cell repertoire in long-term HCMV carriers is often focused onto a few Vβ TCR framework genes and shows selection of public CDR3 usage suggests that the primary T cell response might become focused with differential selection of certain clones into memory (in contrast to the stochastic, nonselective retention of the broad repertoire of the primary response reported in a number of murine systems). Primary infection by HCMV is a highly appropriate natural human infection in which to investigate whether selection of CD8^{+} T cells in the memory pool is stochastic, or is dependent on particular TCRs or on IL-7Rα expression.

In the present study, we have undertaken a longitudinal analysis of the clonal composition of the CD8^{+} T cell response in five subjects (three normal immunocompetent subjects and two renal transplant recipients) undergoing primary HCMV infection and followed for up to 5 years. We investigated whether focusing of the TCR repertoire occurs during the resolution of primary HCMV infection and whether TCR affinity is a determinant either of entry into the virus-specific memory pool or of the hierarchy of clonal dominance. In addition, we investigated whether expression of IL-7Rα during the course of primary HCMV infection in immunocompetent individuals is related to selection of T cells into the memory pool.

Materials and Methods

Donors

Immunocompetent subjects in the acute phase of HCMV infection attending Cambridge University Hospitals Trust were identified by clinical symptoms and abnormal liver function test results. Primary infection was confirmed by plasma HCMV DNAaemia quantitation and serological testing showing HCMV-specific IgM with subsequent isotype switching to HCMV-specific IgG (Health Protection Agency laboratory, Cambridge University Hospitals).

Long-term healthy HCMV carriers were identified by the detection of HCMV-specific IgG in serum. The MHC class I tissue type of each donor was determined by serological typing (Lymphocyte ABC-120; Biotest Diagnostics).

Cryopreserved PBMC from two HCMV-negative patients who had received a HCMV-positive renal transplant were obtained from I. ten Berge (Renal Transplant Unit) and R. Van Lier (Laboratory for Experimental Immunology, Academic Medical Centre, Amsterdam, The Netherlands). These patients were treated with cyclosporine, mycophenolate mofetil, and low dosage of prednisolone, and they had been longitudinally followed in time after transplantation.

Peptides

The following peptides of HCMV pp65 were used: TPRVTGGGAM (aa 417–426), restricted through HLA-B7; NLVPMVATV (aa 495–503), restricted through HLA-A2; and VLEETSVML (aa 316–324), restricted through HLA-A2 (supplied by Proimmune). Peptides were diluted in RPMI 1640 (Invitrogen Life Technologies) and used at a final concentration of 40 μg/ml, unless otherwise stated.

mAbs and tetramers

PBMC were isolated from fresh heparinized blood by Ficoll-Hypaque (Nycomed) density gradient centrifugation. mAbs used were specific for CD3, CD4, CD8, CD45RA, CD45RO, CD28, CD27 (Caltag Laboratories), Vβ1, Vβ2, Vβ3, Vβ5.1, Vβ5.2, Vβ6, Vβ7, Vβ8, Vβ11, Vβ12, Vβ13, Vβ14, Vβ16, Vβ17, Vβ20, Vβ21.3, Vβ22 (Beckman Coulter), and IL7-Rα (R&D Systems). Abs were conjugated to FITC, PE, TriColor, or allophycocyanin. Peptide MHC class I tetramers of HLA-B7 and HLA-A2 containing the HCMV pp65 peptides TPRVTGGGAM and NLVPMVATV (Proimmune), respectively, were also used conjugated to PE or allophycocyanin. Data were collected on a FACSCalibur flow cytometer (BD Biosciences) and analyzed using WinMDI software (J. Trotter, Scripps, http://facs.scripps.edu).

Purification of cell populations

For PCR amplification of TCR Vβ and Vα regions and for limiting dilution analysis culture, PBMC were stained with PE-conjugated peptide MHC class I tetramer (as described above) washed in MACS buffer (1$\times$ PBS, 2 mM EDTA, 0.5% FCS) and stained with anti-PE microbeads (Miltenyi Biotec). Tetramer-binding cells were isolated on a LS MACS column (Miltenyi Biotec), according to manufacturer's instructions. The purity of the tetramer-positive fraction was analyzed by flow cytometry by staining pre- and postsorted samples with mAbs for CD3 and CD8. For quantitative clonotypic analysis of purified CD8^{+} T cells, PBMC were stained with CD8 microbeads (Miltenyi Biotec) and isolated by LS MACS column (Miltenyi Biotec), according to manufacturer's instructions.

Direct ex vivo cytotoxicity assays

Unmanipulated PBMC were isolated from whole blood and were used in direct ex vivo assays at E:T ratios of 5:1, 10:1, and 20:1 in triplicate by plating cells out in 50 μl of RPMI 10 medium. Target cells used comprised autologous or HLA-mismatched lymphoblastoid cell lines (LCLs). A total of 10^6 LCLs was washed in PBS and 10 μl of $Na_2Cr^{57}O_4$ (Amersham) added to the cell pellet, and incubated at 37°C for 45 min. A total of 50 μl of 40 μg/ml peptide was then added and incubated for an additional 45 min at 37°C. One autologous LCL sample was left without peptide to act as an unpulsed control. Target cells were then washed three times in acidified RPMI 10 medium and added to the assay plate (containing effectors, medium-only wells, and wells containing Nonidet P-40, as described above) at 2000 cells/well in 120 μl of medium. Plates were incubated at 37°C for 4 h, after which 70 μl of supernatant was harvested from each well and used to quantify radioactive emission. Percent specific lysis was calculated using the following formula: percent specific lysis = 100 $\times$ (target release – spontaneous release)/(maximum release – spontaneous release).

Generation of HCMV-specific CD8^{+} T cell clones in limiting dilution assays and functional avidity assays

At multiple time points for each subject, epitope-specific T cell clones were generated from single-cell cultures by limiting dilution analysis, followed by recloning, as previously described (22). On day 14, using split-well analysis, cells were assayed for cytotoxicity against radiolabeled target cells in 4-h ^{51}Cr release assays, as previously described (16). T cell clones were assayed in ^{51}Cr release assays against target LCLs pulsed with a range of dilutions of peptide. Functional avidity was calculated by plotting the

[3] Abbreviations used in this paper: HCMV, human CMV; LCL, lymphoblastoid cell line; PCC, pigeon cytochrome C.

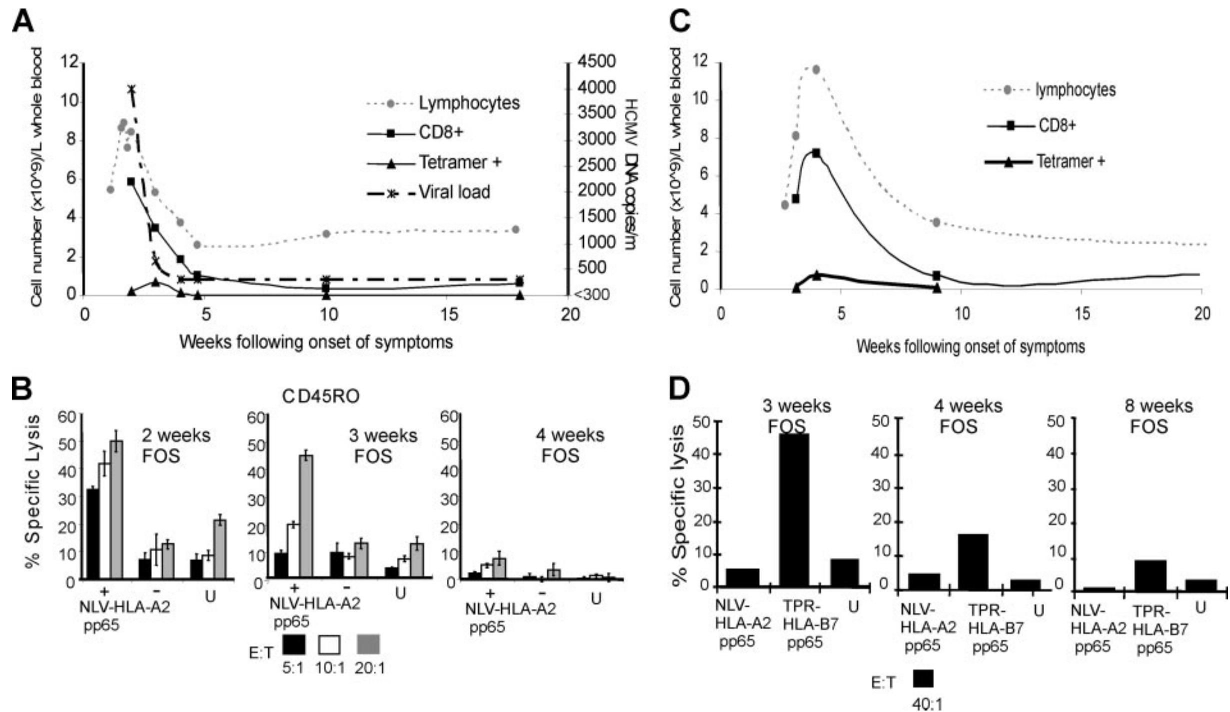

FIGURE 1. Lymphocytosis and $CD8^+$ T cell responses in two immunocompetent donors undergoing primary HCMV infection. Peripheral blood total lymphocyte counts, total $CD8^+$ T cell, and pp65-peptide $tetramer^+$ cell frequencies were determined in two donors (donor 106 in *A* and *B*; donor 104 in *C* and *D*). The HCMV viral load was also determined for one of the patients studied (*A*). Unstimulated PBMC were used as effector cells in chromium release assays to determine direct ex vivo cytotoxicity against target cells matched for HLA-A2 or B7 and loaded with either HCMV pp65-NLV or TPR peptides or unloaded control (U). In both donors, the ex vivo cytotoxic activity per tetramer + cell was highest at the earlier time point and decreased with time.

normalized percent specific lysis against log [peptide] M and determining the concentration of peptide required for 50% maximum specific lysis.

PCR amplification of TCR Vβ and Vα regions

Total RNA was extracted from purified $CD8^+$ cells or whole PBMC using RNeasy extraction kits (Qiagen). First-strand cDNA synthesis was conducted using the Reverse Transcriptase A3500 System kit (Promega). PCR was performed, as previously described (23), using a panel of 39 Vβ family primers and a Cβ chain primer (22) or a panel of 34 Vα primers and a Cα chain primer (24) (synthesized by Sigma Genosys). Sequencing of TCR β-chain or α-chain PCR products was performed by Medical Research Council Geneserve (Genome Campus).

Bacterial cloning and sequencing of TCR Vβ PCR products

Cloning of TCR Vβ PCR products was conducted using the Topo TA blunt end cloning kit (Invitrogen Life Technologies), according to manufacturer's instructions. Briefly, PCR products were purified by gel electrophoresis and ligated into the TOPO-TA vector. Chemically competent DH5-α *Escherichia coli* were transformed and plasmid DNA was extracted following selection and growth of individual colonies. Sequencing of vectors containing inserts of the appropriate size was performed by Medical Research Council Geneserve (Genome Campus).

Quantitative clonotypic analysis

Clonotypic analysis was performed, as previously described (23). Briefly, for each biological HCMV-specific clone, a complementary oligonucleotide was designed based on the TCR hypervariable region of the clone to be quantified (referred to as the clonotypic probe). A conserved TCR C region probe was also designed. RT-PCR was conducted from RNA extracted from PBMC-purified $CD8^+$ T cells in duplicate with a Vβ family-specific primer and a Cβ-specific primer on total $CD8^+$ cDNA. PCR products were also generated from cDNA from a defined biological CTL clone (positive control) and from a mixed population of PBMC derived from five HCMV-negative donors (negative control). Duplicate PCR products were separated on 1.5% agarose and transferred overnight onto ζ probe nylon filter in blotting buffer (0.6 M NaOH, 0.2 M NaCl). A total of 15 pmol of each oligonucleotide probe was end labeled with [γ-^{32}P]dATP (Amersham) using T4 polynucleotide kinase (Promega), according to manufacturer's instructions. Unincorporated material was removed using a G-25 Sephadex spin column (Amersham). Filters were hybridized overnight at 10–15°C below the melting temperature of the clonotype probe in 15 ml of hybridization buffer (7% SDS, 0.25 M Na_2HPO_4, 10% polyethylene 8000) containing the γ-^{32}P end-labeled probe. Filters were then washed three times in wash buffer (5% SDS, 0.25 M Na_2HPO_4), and radioactive signal was quantified using an Instant Imager (Beckman Coulter). Filters were stripped in 0.4 M NaOH and rehybridized with either further clonotype probes or the TCR β-chain C region probe. The proportion of the clonotypic sequence within the amplified sequence was calculated as follows: 100 × ((test sample cpm probed with clonotypic probe)/(test sample cpm probed with constant probe))/((positive control cpm probed with clonotypic probe)/(positive control cpm probed with constant probe)).

Results

Primary HCMV infection in immunocompetent donors is characterized by a large burst of $CD8^+$ T cells that are directly cytotoxic ex vivo

We studied five subjects (three normal immunocompetent subjects and two kidney transplant recipients). In two of these normal subjects, we were able to perform a detailed kinetic, phenotypic, and cytotoxicity analysis of the primary $CD8^+$ T cell response.

A previous study from our laboratory found a peak lymphocytosis of 4.9×10^9 $CD8^+$ T cells/L blood (normal range 1.5–3.5 × 10^9/L) in one immunocompetent donor (donor 104) undergoing primary HCMV infection (23), suggesting the actual magnitude of the response is much larger than in immunosuppressed patients. In the present study, the magnitude of $CD8^+$ responses was determined at several time points in primary HCMV infection in a further subject (donor 106) (Fig. 1*A*), in which a peak response of 5.85×10^9 $CD8^+$ T cells/L was seen at 11 days following onset. These primary donors generated $CD8^+$ T cells specific for either TPR-HLA-B7 (donor 104) or NLV-HLA-A2 (donor 106). Both

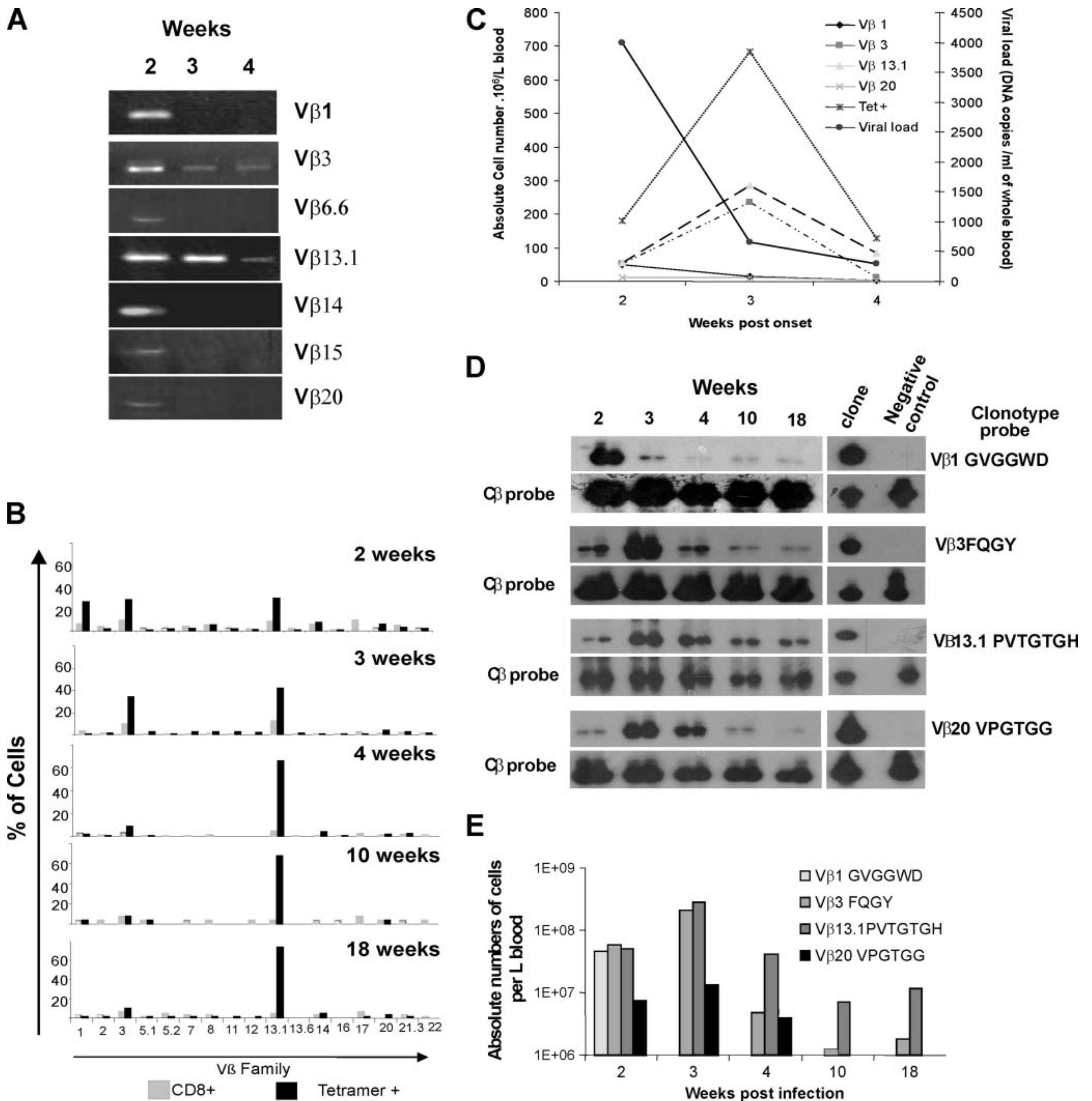

FIGURE 2. Early focusing of TCR Vβ family usage of NLV-pp65-specific CD8$^+$ T cells following the onset of primary HCMV infection in immunocompetent donor 106. NLV-pp65 tetramer$^+$ cells were separated from PBMC by MACS magnetic bead sorting, and mRNA was extracted from these Ag-specific cells and used in RT-PCR with a panel of 25 TCR Vβ family primers (*A*). At 2 wk after onset, Vβ1, Vβ3, Vβ6.6, Vβ13.1, Vβ14, Vβ15, and Vβ20 TCR were identified; by 4 wk only Vβ3- and Vβ13.1-positive cells were detectable. *B*, PBMC from donor 106 were stained with CD8 and HLA-A2 pp65 tetramer in conjunction with mAbs specific for a given TCR Vβ family (*C*, ▨ = percentage of cells in the whole CD8$^+$ population; ■ = percentage of cells in the pp65 peptide-specific population). *C*, The absolute number of tetramer-positive T cells in each TCR Vβ family at 2, 3, and 4 wk following onset of symptoms in comparison with viral DNA load at the same time points. *D*, Quantitative clonotypic analysis of four different NLV-pp65-specific CD8$^+$ T cell clones from donor 106 following primary HCMV infection and selection of T cells into long-term memory. At successive time points, CD8$^+$ T cells were purified from whole PBMC and amplified by RT-PCR with Vβ primers specific for Vβ1, Vβ3, Vβ13.1, and Vβ20. Duplicate PCR products from each time point were transferred to membranes and probed with the CDR3 clonotypic specific probe specific for each of the four immunodominant clones, followed by a C region probe. *E*, The contribution of each clonotype to the CD8 T cell pool at each time point following onset of symptoms.

donors demonstrated a similar expansion of tetramer-positive CD8$^+$ cells reaching a peak of Ag-specific cells a few weeks after onset that rapidly resolved over the next few weeks (Fig 1, *A* and *C*). In donor 106, plasma HCMV DNAemia when first measured was 4000 DNA copies/ml at the time of peak lymphocytosis and fell sharply to below the limit of detection (<300 DNA copies/ml) at 4 wk after onset.

In donor 106, 2 wk following the onset of symptoms (defined as first onset), 3% of circulating CD8$^+$ T cells were specific for the NLV-HLA-A2 pp65 peptide; 1 wk later this increased 7-fold to

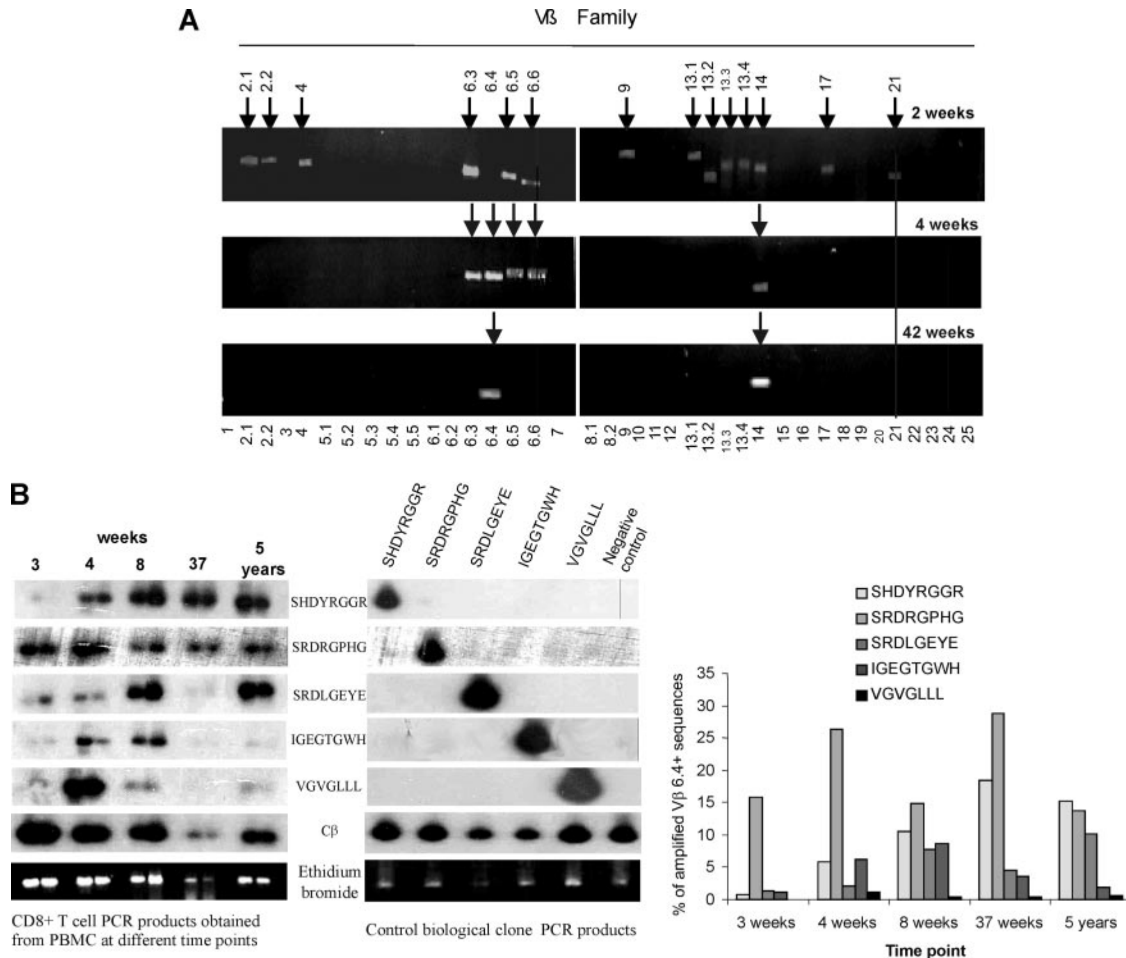

FIGURE 3. Early clonal focusing of the pp65 peptide-specific CD8$^+$ T cell response following primary HCMV infection in a renal transplant recipient 6017009. *A*, Tetramer$^+$ cells were enriched from whole PBMC to high purity by MACS sorting following staining with MHC-I tetramers. mRNA was extracted and used in RT-PCR with a panel of 25 Vβ family primers. Time is weeks following first detection of HCMV DNAemia. Quantitative clonotypic analysis of five different TPR-pp65-specific CD8$^+$ T cell clones from donor 104 following primary HCMV infection and selection of T cells into long-term memory. *B*, At successive time points, CD8$^+$ T cells were purified from whole PBMC and amplified by RT-PCR with Vβ primers specific for Vβ6.4. Duplicate PCR products from each time point were transferred to membranes and probed sequentially with a CDR3 clonotypic probe specific for each of the five clonotypes, followed by a C region probe. The percentage of contribution of each clone to its Vβ family was calculated.

20% of all circulating CD8$^+$ T cells being specific for this single HCMV epitope. Similarly, in donor 104, the pp65 TPR-HLA-B7 response increased from 3 to 4 wk following first onset to 11.7% of all circulating CD8$^+$ T cells. The frequency of virus-specific T cells fell rapidly in both donors over the next few weeks. PBMC from donor 106 were also stained with VLE-HLA-A2 I-E-specific tetramer at the same time points following first onset: a small 0.8% population was detectable at 2 wk, but this failed to expand further, and later contracted to an undetectable level (data not shown).

At both 2 and 3 wk following first onset of symptoms, direct ex vivo Ag-specific cytotoxicity against peptide-pulsed target cells was demonstrable (23). This same phenomenon was also observed in donor 106: strong direct ex vivo cytotoxicity was evident that was lost after the resolution of the lymphocytosis (Fig. 1, *B* and *D*).

The TCR usage of HCMV-specific CD8$^+$ T cells is diverse early in primary HCMV infection and rapidly focuses during resolution

TCR usage by peptide-specific cells was analyzed by RT-PCR of purified tetramer-positive cells (using a panel of primers that can amplify all TCR Vβ families and subfamilies) and by flow cytometry using a panel of Vβ-specific mAbs.

In donor 106, at 2 wk after onset, seven different TCR Vβ families (Vβ1, Vβ3, Vβ6.6, Vβ13.1, Vβ14, and Vβ20) were detectable in tetramer-positive cells using RT-PCR (Fig. 2*A*). This rapidly changed at the 3-wk time point, at which four of these TCR Vβ families (Vβ1, Vβ6.6, Vβ14, Vβ15, and Vβ20) were no longer detectable within the peptide-specific repertoire, but Vβ3 and Vβ13.1 were detected at all time points investigated up to and including 18 wk.

Using flow cytometry in donor 106, at 2 wk after onset, tetramer-positive cells were distributed in three Vβ families, Vβ1, Vβ3, and Vβ13.1, each of which accounted for ~30% of tetramer-binding cells, with the remaining 10% being shared between Vβ8, Vβ14, and Vβ20 (Fig. 2*B*) (Abs for Vβ6.4, Vβ13.4, and Vβ15 were not available). One week later, this position had changed markedly in that no Vβ1 cells were detectable among the tetramer-positive population, although there were still large Ag-specific expansions in the Vβ3 and Vβ13.1 families, with Vβ13.1 beginning to dominate the response, accounting for over 40% of pp65 peptide-specific cells. At the 4-wk time point, the dominance of Vβ13.1 population was even more marked, with over 60% of peptide-specific cells now within this family, whereas the contribution of Vβ3 cells decreased to <10% of the overall tetramer-binding

TABLE I. *TcR amino acid sequences of pp65 peptide-specific clones from two immunocompetent donor patients with primary HCMV infection*[a]

Donor	Vβ		CDR3 β		Jβ	Vα		CDR3 α		Jα
104	6.4	**CAS**	SSRDRGPHGQ	**YFG**	2.7	12	**CAT**	EIRMDSSYKL	**IFG**	12
	6.4	**CAS**	SSRDLGEYEQ	**YFG**	2.7	3	**CAT**	EGRMDSSYKL	**IFG**	12
	6.4	**CAS**	SHDYRGRRSPL	**HFG**	1.6	12	**CAT**	RPPGAGSYQL	**TFG**	28
	6.4	**CAS**	SLIGEGTGWHQ	**YFG**	2.7	3	**CAT**	VSRMDSSYKL	**IFG**	12
	6.4	**CAS**	SLVGVGLLLNEQ	**YFG**	2.7	3	**CAT**	EGRMDSSYKL	**IFG**	12
106	1	**CAS**	SGVGGWDEQ	**YFG**	2.7	nd		nd		nd
	3	**CAS**	SFQGYTEA	**FFG**	1.1	16	**CAD**	YYGQNF	**VFG**	26
	13.1	**CAS**	SPVTGTGHYGY	**TFG**	1.2	18	**CAS**	NTGNQF	**YFG**	49
	20	**CAW**	VPTGTGGTEA	**FFG**	1.1	nd		nd		nd

[a] Donor 104 is specific for HLA-B7 restricted TPR and donor 106 specific for HLA-A2 restricted NLV.

population, a pattern that was maintained at 10 and 18 wk following the onset of symptoms. These results are internally consistent with the RT-PCR analysis.

In donor 104, at the 3-wk time point, there were expansions in four different Vβ families, but only one of these, namely Vβ6.4, was still detectable late in long-term memory. At the 5-year time point, an additional band was detected in the TCR Vβ6.6 subfamily that may be due to the emergence of a new clone that was not detected during the primary immune response (data not shown).

We identified an additional HLA-B7-positive immunocompetent donor (107) with a very recent primary infection that we followed over a 3-wk period: a large TPR-pp65-specific tetramer-positive population (4.8% of CD8$^+$ T cells) was observed on initial diagnosis that contracted to 2.5% over a 3-wk period. TCR Vβ analysis showed that these T cells were composed of multiple TCR Vβ families, Vβ1, Vβ6.2, Vβ6.4, Vβ6.6, and Vβ14, and small Vβ18 and Vβ24 bands. FACS analysis of tetramer-positive cells confirmed Vβ1 and Vβ14 and showed the loss of Vβ1 over time, with an increase in Vβ14 cells to ~one-third of the tetramer-positive population after 3 wk (data not shown). This is in good agreement with our results from other HLA-B7 donors (donors 104 and 6017009).

The Vβ repertoire of Ag-specific cells was also investigated in subjects with primary HCMV infection following renal transplantation from HCMV-seropositive donors to seronegative recipients. In patient 6017009, following the first detection of HCMV DNAemia, the TPR-HLA-B7 peptide-specific CD8$^+$ T cell repertoire was very broad with expansions in 14 different families comprising Vβ2, Vβ4, Vβ9, Vβ14, Vβ17, and Vβ21, as well as subfamilies of Vβ6 and Vβ13. Two weeks later, however, only expansions within Vβ14 and subfamilies of Vβ6 remained. At a later time point, 42 wk following primary infection, Vβ14 was again present, but only the Vβ6.4 subfamily was detected (Fig. 3*A*). In a second patient, 3574666, five different Ag-specific expansions were seen at 2 wk following the first detection of HCMV DNAemia, one of which had contracted below the limit of detection 1 year following primary infection (data not shown).

Thus, in both immunocompetent and immunosuppressed individuals, the pp65 peptide-specific response in primary HCMV infection was broad, including CD8$^+$ T cell clones using multiple TCR Vβ families. Focusing of the peptide-specific repertoire occurred rapidly, as follows: within the first few weeks of infection the contribution of many Vβ families to the peptide-specific repertoire contracted below the limit of detection, resulting in the dominance of clones within an individual Vβ family during long-term memory.

Focusing of the pp65-specific immune response in primary HCMV infection occurs by the selection of clones with public TCR usage and contraction of private clonotypes

Limiting dilution assays were used to generate biological T cell clones from which TCR Vα and Vβ chains were sequenced to identify CDR3 usage (Table I). In parallel, cDNA prepared from sorted tetramer-positive cells in PBMC was PCR amplified using TCR Vβ-specific primers, the PCR product was cloned into bacteria, and multiple bacterial clones were sequenced. The bacterial cloning of TCR Vβ PCR products derived from specific TCR Vβ families followed by sequencing allowed us to compare the actual TCR diversity directly ex vivo with the sequence of those T cell clones that were able to proliferate as a clone in vitro.

In analyzing the peptide-specific response of donor 104, we derived five different Vβ6.4$^+$ biological clones that used either Vα3

TABLE II. *Comparison of TcR β−chain amino acid sequences from HLA-B7 TPR-pp65-specific clones and HLA-A2 NLV-pp65-specific clones from unrelated HCMV carriers*

Donor	HLA	Vβ		CDR3 β		Jβ
HOO18	B7	6.4	**CAS**	SSHDRQGASSPL	**HFG**	1.6
HOO18	B7	6.4	**CAS**	SSHDREGGSSPL	**HFG**	1.6
002	B7	6.4	**CAS**	SSHDTGGYNSPL	**HFG**	1.6
025	B7	6.4	**CAS**	STHDIQGLGSPL	**HFG**	1.6
104	B7	6.4	**CAS**	SSHDYRGRRSPL	**HFG**	1.6
002	B7	6.4	**CAS**	SFRDYGNYEQ	**YFG**	2.7
018	B7	6.4	**CAS**	SNRDRGDYEQ	**YFG**	2.7
104	B7	6.4	**CAS**	SSRDRGPHGQ	**YFG**	2.7
104	B7	6.4	**CAS**	SSRDLGEYEQ	**YFG**	2.7
3574666	B7	6.4	**CAS**	SLRDRGSYEQ	**YFG**	2.7
6017009	B7	6.4	**CAS**	SSRDRGSYEQ	**YFG**	2.7
008	A2	13.1	**CAS**	SPTTGTGHYGY	**TFG**	1.2
004	A2	13.1	**CAS**	SYQTGTGVYGY	**TFG**	1.2
001	A2	13.1	**CAS**	SYQTGTGNYGY	**TFG**	1.2
106	A2	13.1	**CAS**	SPVTGTGHYGY	**TFG**	1.2

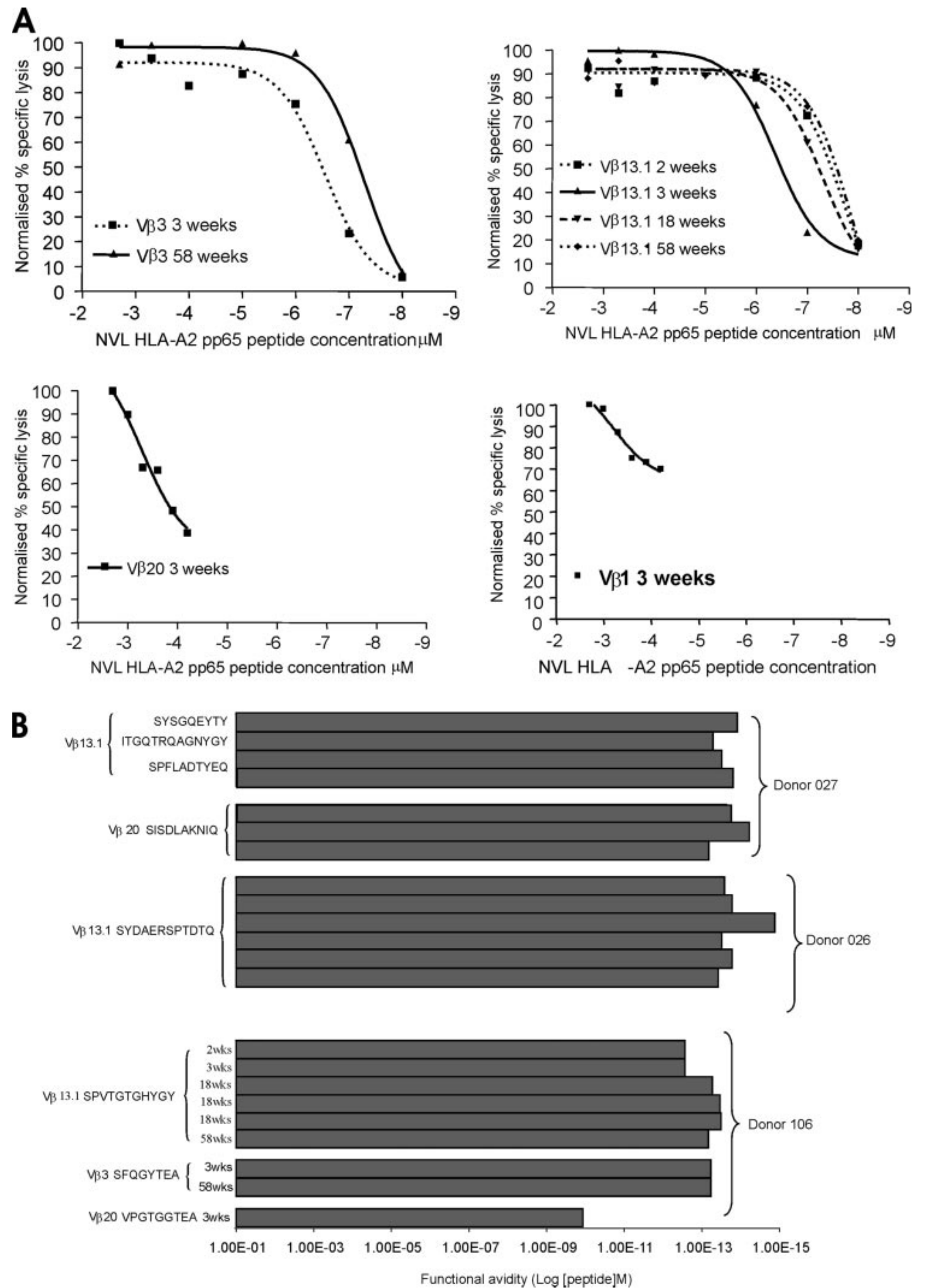

FIGURE 4. High functional avidity of NLV-pp65-specific CD8$^+$ T cell clones from donor 106 with primary HCMV and two unrelated long-term HCMV carriers (026, 027). *A*, T cell clones independently isolated from PBMC obtained at different time points were used in chromium release assays with target cells pulsed with a range of peptide concentrations (*x*-axis). Specific lysis was normalized so that the maximum value equates to 100%. Functional avidity was calculated by plotting the normalized percent specific lysis against log [peptide] M and determining the concentration of peptide required for 50% maximum specific lysis. The Vβ1 and Vβ20 clones obtained from donor 106 at 3 wk after onset had significantly lower functional avidity; this clone became contracted below the level of detection in long-term memory. *B*, Comparision of the functional avidities of Vβ13.1 and Vβ20 clones isolated from two unrelated long-term virus carriers.

or Vα12 chains. The same TCR Vα3 Jα12 amino acid sequence was detected in two different clones that had Vβ6.4 Jβ2.7 chains with different hypervariable sequences. From sorted tetramer-positive cells obtained at 3 wk following onset, 10 of 11 bacterial clones of the Vβ6.4 PCR product were of a single clonotype (SSRDRGPHGQ) and one of another (SSHDYRGGRSPL). From sorted tetramer-positive cells at 5 years after infection, bacterial clones of the Vβ6.4 PCR product yielded the same two clonotypes as well as an additional sequence, SSRDLGEYEQ. The bacterial cloning did not reveal any clonotype sequences that were not derived by biological cloning and sequencing (Table I).

In donor 106 at 2 and 3 wk after onset, all of seven bacterial Vβ1 clones from sorted tetramer-positive cells had the same sequence, and this was identical with the only Vβ1 biological clone isolated; all of 10 bacterial Vβ3 clones and all of 10 bacterial Vβ13.1 clones had the same sequence as the biological clone, and all of 6 bacterial Vβ20 clones had the same sequence as the only biological Vβ20 clone.

Thus, in summary, both techniques detected the same clonotypic TCR Vβ sequences, demonstrating CDR3 diversity is not underrepresented in those clones able to proliferate in vitro.

Quantitative clonotypic analysis was used to determine the frequency of individual Ag-specific clones during primary infection (Fig. 2, *D* and *E*). In donor 106, at 2 wk after onset, the GVGGWD (Vβ1), FQGY (Vβ3), and PVTGTGH (Vβ13.1) clones were codominant, each at a frequency of 5×10^7 cells/L (1% of CD8$^+$ T cells); however, the GVGGWD clone contracted significantly by 3 wk and remained rare thereafter. From the 3-wk time point onward, the PVTGTGH clone dominated the response, although the absolute numbers of cells of the clone decreased as primary

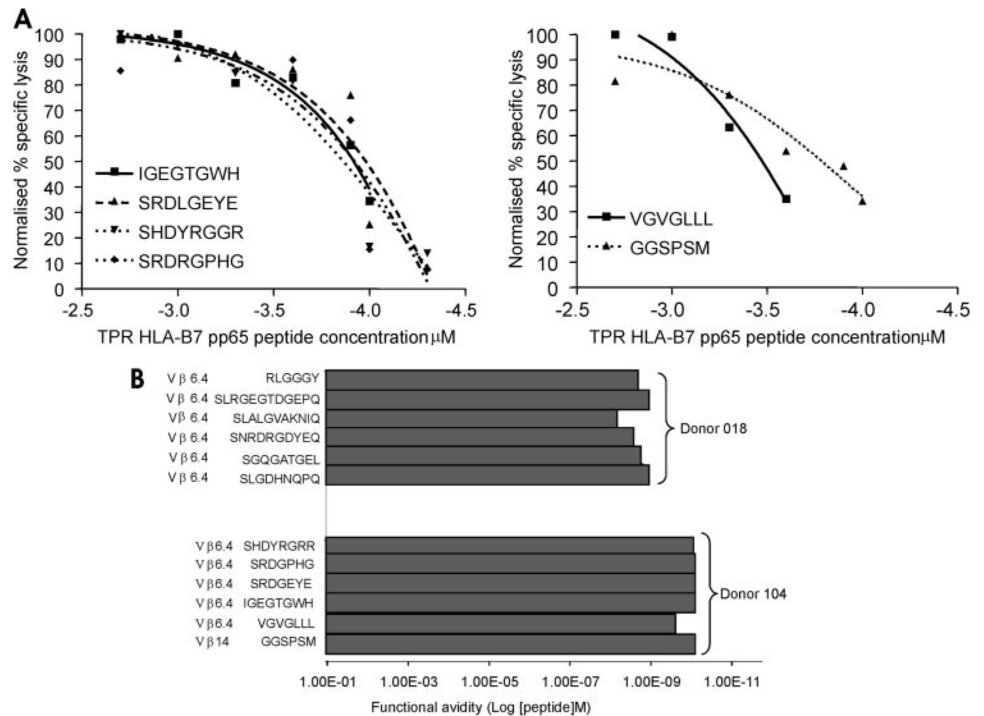

FIGURE 5. Analysis of the functional avidity of TPR-pp65-specific CD8$^+$ T cell clones from donor 104 with primary HCMV and an unrelated long-term HCMV carrier 018. T cell clones were used in chromium release assays with target cells pulsed with a range of peptide concentrations. Specific lysis was normalized so that the maximum value equates to 100%. Functional avidity was calculated by plotting the normalized percent specific lysis against log [peptide] M and determining the concentration of peptide required for 50% maximum specific lysis. *A*, Functional avidity of six different clonotypes in donor 104 (*B*) comparison of different clonotype functional avidities between donors 104 and 018.

infection resolved. A fourth clone, PVGTGG, accounted for nearly 50% of the amplified Vβ20 sequences early in infection, but because the contribution of this family to the total CD8$^+$ population was small, this clone represented a quantitatively minor part of the overall NLV-pp65-specific response. In donor 104, the SRDRGPHG (Vβ6.4) clone dominated the TPR-pp65-specific response throughout primary infection. At five years following infection, however, there was codominance among the SRDRGPHG, SRDLGEYE, and SHDYRGGR clones (Fig. 3*B*). When the TCR β amino acid chain sequences of the immunodominant Vβ13.1 clone from donor 106 were compared with TCR sequences derived from unrelated long-term HCMV carriers who were HLA-A2, we observed striking conservation of Vβ and Jβ usage, and CDR3 amino acid sequences in these clones (Table II), indicating public TCR usage. We also identified two novel public TCRs in a large set of HLA-B7-restricted TPR-peptide-specific clones from unrelated HCMV carriers (Table II). Clones from other primary and long-term HLA-B7 HCMV carriers were found to have immunodominant Vβ6.4/Vα3 TCRs and show conserved CDR3 Vβ motifs, namely SXHDXXGXXSPL in association with Jβ1.6, and SXRDXGXXEQ motifs in association with Jβ2.7. Clones with these TCRs were selected from a broader repertoire during the resolution of primary infection in both immunocompetent and immunosuppressed individuals, whereas private clonotypes were heavily contracted, indicating a selection advantage conferred by the conserved TCR amino acid sequence.

Determination of TCR avidity of HCMV-specific clones in functional cytotoxicity assays

Cytotoxicity assays were used to determine the TCR avidities of HCMV-specific clones from long-term virus carriers to determine whether a relationship exists between cellular function and clonal dominance. Lysis of EBV-transformed B cell lines pulsed with decreasing concentrations of peptide produced dilution curves that were similar for all T cell clones specific for a given peptide that were selected into long-term memory, irrespective of donor origin, clonal dominance, or their derivation from either the CD45RO or CD45RA T cell memory pools. Differences were observed between clones specific for different peptides, with HLA-A2-NVL-restricted clones typically having functional avidity values of 1×10^{-8} μM, whereas HLA-B7-TPR-restricted clones had values of 1×10^{-4} μM (Figs. 4 and 5).

In primary infection, both dominant PVTGTGH (Vβ13.1) clones and subdominant FGQY (Vβ3) clones derived at different times during the resolution of primary infection in donor 106 showed similar dilution curves, both to one another and to HLA-A2-restricted clones from two unrelated long-term HCMV carriers (Fig. 4). A partial analysis of the functional properties of the GVGGWD (Vβ1) clone from donor 106 estimates the avidity at $5.5.10^{-5}$ υm: further analysis and a more accurate determination of avidity was not possible because this clone failed to survive long-term in vitro. However, the functional avidity of a Vβ20 (VPGTGGTEA) clone $1.2.10^{-5}$ υm that was only isolated early in infection and was not present in long-term memory was between 2 and 3 logs lower than either the dominant PVTGTGH (Vβ13.1) clones or the subdominant FGQY (Vβ3) clones that did survive into long-term memory. Similar TCR avidity was observed between all clones generated from donor 104, although the smallest clonotype within Vβ6.4 (VGVGLLL) did have a lower avidity than the codominant clonotypes (Fig. 5). TCR avidity was high in clones in long-term memory.

IL-7R was not expressed on circulating HCMV-specific cells during selection into memory

We investigated IL-7Rα expression by Ag-specific cells in primary HCMV infection in immunocompetent hosts. In donor 104, at both 3 and 8 wk after onset, Ag-specific cells were exclusively IL-7Rα negative (Fig. 6*A*). Similarly, in donor 106, during the first 10 wk after onset, all pp65-specific cells lacked the IL-7Rα, but at the 18-wk time point, a subset of cells up-regulated IL-7Rα, producing a bimodal expression pattern (Fig. 6*B*). Ag-specific cells at this time were distributed among both the CD45RA^{high} and CD45RO^{high} populations, and both CD45 subsets showed a similar

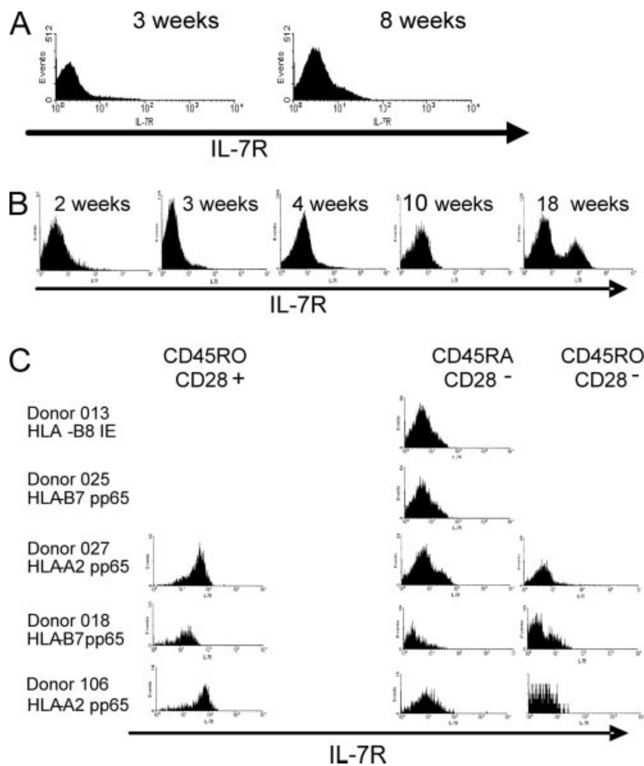

FIGURE 6. IL-7Rα expression on pp65-peptide-specific CD8$^+$ T cells in primary and long-term HCMV infection. PBMC were stained with mAbs for CD45RA, CD28, and IL-7Rα in conjunction with pp65 peptide tetramer. Plots are gated on Ag-specific cells, and unfilled histograms represent isotype controls. *A*, Donor 104; *B*, donor 106; *C*, long-term HCMV carriers.

pattern of bimodal expression of IL-7Rα (data not shown). Consequently, during the peak of infection, all circulating activated cells down-regulated IL-7Rα; but, in contrast to murine models, no subset of IL-7Rα^{high} cells was apparent in acute infection. Furthermore, several weeks after the onset of symptoms, there were still significant circulating populations of Ag-specific cells that remained IL-7Rα^-.

In the mouse, IL-7Rα expression is a requirement for the selection of memory CD8$^+$ T cells as follows: we investigated the IL-7Rα expression pattern by HCMV-specific CD8$^+$ cells in four healthy HCMV carriers and subject 106 in relation to other phenotypic markers (Fig. 6). HCMV-specific CD8$^+$ cells showed bimodal expression of IL-7Rα, with significant proportions of memory cells from all donors lacking the receptor. On peptide-specific cells, expression of IL-7Rα was closely associated with CD28 expression; CD28$^-$ cells lacked IL-7Rα, whereas some, but not all CD28$^+$ cells were also IL-7Rα^+. Bimodal expression of IL-7Rα was observed in clones specific for different peptides of either the I-E or pp65 proteins.

Discussion

We have shown that following primary HCMV infection, the repertoire of CD8$^+$ T cells responding to a given viral peptide (as measured by TCR Vβ and Vα usage) is initially diverse, but rapidly becomes more focused as certain families of responding T cells contract below the level of detection. The cells that are maintained into the longer-term memory pool are a subset of the initial responding CD8$^+$ T cell population. It is of note that this focusing onto particular TCR usage occurs during the resolution of primary infection and then is maintained stably thereafter. The dominant virus-specific clonotypes selected into long-term memory often show conservation of amino acid motifs in their TCR hypervariable regions between unrelated donors (public clonotypes), whereas private clonotypes were generally subdominant or contracted below the limit of detection. The TCRs of CD8$^+$ clones derived from different donors, but specific to the same viral peptide, were shown to have remarkably similar functional avidities, whereas two clones that contracted below the level of detection had 2–3 logs lower functional avidity. IL-7Rα was not expressed on circulating Ag-specific CD8$^+$ T cells during the period of clonal focusing; IL-7Rα expression thus does not appear to be required for selection of human CD8$^+$ T cells into memory.

We have presented clear evidence that the very early TCR repertoire following primary infection becomes focused by differential contraction of some virus-specific clones, and this focusing is consistent with selection of particular TCR Vβ framework regions and conservation within the hypervariable CDR3 between unrelated HCMV carriers (public TCR usage). In particular, dominant Vβ13.1/Vα18 clones were found in response to NLV-HLA-A2 peptide in multiple donors, in agreement with a previous report in HCMV carriers of public TCR usage by CD8$^+$ T cells specific for the same peptide (25). We also note that this public TCR usage may not be confined to dominant clones because the TCR β-chain hypervariable amino acid sequence of a subdominant Vβ3 NLV-HLA-A2 peptide-specific clone in this study and a Vβ8 subdominant clone we previously identified (26) have now also been identified in a recent study by Trautmann et al. (25). The CDR3β amino acid sequences were identical between these different donors, but because each study found these clones independently, they were not until now recognized as being public TCRs.

Selection of public TCR usage to a particular peptide HLA restriction element is not unique to the NLV-HLA-A2 peptide. We have compared the TCR usage specific for TPR-HLA-B7 from a patient with primary HCMV with the TCR usage found in a number of long-term HCMV carriers, and have identified two novel public TCR motifs in the dominant clones using Vβ6.4/Vα3 and Vβ6.4/Vα12 TCRs.

The frequent selection and dominance of pp65-specific clones that have public TCR usage suggest that a functional advantage is conferred by the particular amino acid N-D-N motifs that make up the CDR3. What is the functional advantage that this TCR arrangement provides to these virus-specific T cell clones? The simplest hypothesis is that the TCRs selected have a much greater avidity for the peptide MHC complex. Evidence from influenza-specific memory responses has shown that a dominant public TCR Vβ17 amino acid sequence found in HLA-A2 patients was up to 10,000-fold more avid than subdominant clones (27).

In addition to showing that some of the initial T cell clones that respond to primary infection are not represented in memory, we have also shown that the clones that are selected into memory have a dominant and subdominant hierarchy. It may be that functional avidity both dictates selection into memory and determines position in the hierarchy. In a single donor in which we were able to isolate functional T cell clones very early following onset, we have shown that an initial clone using Vβ1 contracted below the level of detection, whereas a clone using Vβ20 contracted to the threshold of detection as follows: both of these clones had a 2- to 3-log lower functional avidity when compared with Vβ13.1 dominant clones. However, we observed similar T cell avidity between the Vβ13.1 dominant and Vβ3 subdominant clones in this same donor. These observations are similar to a detailed study of selection of pigeon cytochrome C (PCC)-specific CD4$^+$ T cells, in which the PCC-specific preimmune T cell repertoire in the 2B4β mouse model used was composed of five different clonotypes: in this latter study,

following exposure to Ag, the repertoire at day 7 was very different from the preimmune repertoire, and the two lowest affinity clonotypes were missing from the PCC-specific repertoire (28).

Similarly, in a second donor in whom the response focused onto TCR Vβ6.4 containing five clonotypes, the functional avidity of these clones was found to be similar to each other and to Vβ6.4 clones derived from an unrelated donor. Thus, functional avidity below a certain threshold may be sufficient for transient initial expansion, but not for sustained maintenance into memory, whereas dominant and subdominant clones that are selected into the memory pool can have very similar high functional avidities. It is interesting to note that in the PCC 2B4β mouse model, the study also demonstrated that clones that were above a certain affinity threshold were selected in the response, but beyond this higher affinity level there was no further skewing toward higher affinity clones (28).

Because IL-7Rα expression is needed for entry into the memory pool in murine models of infection (8, 9), the expression of IL-7Rα on Ag-specific cells during the resolution of primary HCMV infection was investigated in the present study. Low IL-7Rα expression on circulating cells did not prevent HCMV-specific cells from entering the memory pool in immunocompetent hosts, consistent with the findings of van Leeuwen et al. (29) in renal transplant recipients. Emerging evidence suggests that expression of IL-7Rα is a feature of memory T cells specific for acute, cleared infections in humans such as influenza and respiratory syncytial virus; it is interesting to note that in another persistent human herpes virus infection, EBV, IL-7Rα is also not present on memory CD8$^+$ T cells.

The individuals participating in this study had symptomatic primary infection that, it could be argued, might be characterized by a greater magnitude of viremia or a more vigorous CD8$^+$ T cell response than that in asymptomatic primary infection. This might affect the speed at which clonal focusing occurred or the timing of accumulation of pp65-specific memory cells within phenotypically distinct subsets. However, when the memory response of these donors is compared with that of long-term HCMV carriers used in this study, the frequencies, phenotype, and clonotypes of virus-specific cells are all internally consistent, as was the case with the memory response in renal transplant recipients; this argues against patients with symptomatic primary infection being an unrepresentative group.

The data generated by this study suggest a model of HCMV pp65-specific CD8$^+$ T cell selection following primary HCMV infection that is based on affinity driving survival and favoring selection into the T cell memory pool. Early infection generates a diverse virus-specific TCR repertoire, comprising clones with a range of affinities, including cells with public TCR usage. Determination of the viral load at the same time as either the absolute number of tetramer-positive Vβ-positive cells in peripheral blood or the absolute clone size at each time point following the onset of symptoms (Figs. 1, 2, and 4) demonstrates that some T cell clones are contracting at a time when the viral load is reducing, and these are the clones with lower functional affinities. At the same time, other clones are expanding in number: these clones undergo contraction later, after the viral load has already substantially decreased, and establish a dominant/subdominant hierarchy, but nevertheless have very similar functional avidities.

High plasma HCMV DNA loads are detected during primary HCMV infection: these viral DNA loads are reduced to background levels within 4–5 wk following onset of symptoms. During long-term carriage of the virus, periodic reactivation most likely occurs, and provides secondary stimulation to the HCMV-specific memory T cells. Our study clearly shows that the clones initially established in primary infection can be very long-lived, with some still being detectable 5 years after primary infection. However, there is also evidence that subdominant clones can expand and become more dominant (Fig. 3) (30), and that novel HCMV-specific T cell clonotypes may also arise months after hemopoietic stem cell transplantation (31).

There continues to be debate concerning the mechanisms by which T cells are selected into long-term memory (1, 2). The data presented in this study from our analysis of a human persistent virus infection provide evidence favoring an affinity-driven selection hypothesis. However, analysis of a larger panel of contracted clones is required to substantiate this message and to definitively determine the mechanistic basis of clonal focusing during primary HCMV infection.

Disclosures

The authors have no financial conflict of interest.

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