Human CD28$^-$CD8$^+$ T Cells Contain Greatly Expanded Functional Virus-Specific Memory CTL Clones[1]

Michael P. Weekes, Andrew J. Carmichael,[2] Mark R. Wills, Kim Mynard, and J. G. Patrick Sissons

At birth, almost all human peripheral blood CD8$^+$ T cells express the costimulatory molecule CD28. With increasing age, the proportion of CD8$^+$ T cells that lack CD28 increases. Because the Ag specificity of CD28$^-$CD8$^+$ T cells has not previously been defined, we studied the contribution of CD28$^-$CD8$^+$ T cells to the memory CD8$^+$ CTL response against two human persistent viruses, human CMV (HCMV) and HIV. From PBMC of healthy virus carriers we generated multiple independent CTL clones specific for defined viral peptides and sequenced their TCR β-chains. We designed clonotypic oligonucleotides complementary to each β-chain hypervariable sequence and quantified the size of individual immunodominant CTL clones in PBMC. Some individual CTL clones were very large, comprising up to 3.1% of all CD8$^+$ T cells in PBMC, and were generally maintained at a stable level for months. Individual virus-specific CTL clones were consistently more abundant in purified CD28$^-$ cells than in the CD8$^+$ population as a whole. Because CD28$^-$CD8$^+$ cells as a population have been reported to proliferate poorly in response to mitogen, we studied the function of these virus-specific CD28$^-$ CTL clones by quantifying the frequency of peptide-specific CTL precursors using limiting dilution analysis. CD28$^-$CD8$^+$ T cells contained high frequencies of functional memory CTL precursors specific for peptides of HCMV or HIV, generally higher than in the CD8$^+$ T cell population as a whole. We conclude that in asymptomatic HCMV and HIV infection, human CD28$^-$CD8$^+$ T cells contain high frequencies of functional virus-specific memory CTL clones. ***The Journal of Immunology*****, 1999, 162: 7569–7577.**

The disulfide-linked homodimer CD28 on the surface of T cells binds to members of the B7 family on APCs and costimulates T cell activation (1). In humans, all thymocytes and the vast majority of cord blood CD8$^+$ T cells express CD28. The proportion of CD8$^+$ T cells that lack surface expression of CD28 increases with age, so that in adulthood, 25–50% of CD8$^+$ T cells are CD28$^-$ (2). In advancing HIV infection, >50% of CD8$^+$ T cells are CD28$^-$ (3–6). CD28$^-$CD8$^+$ T cells express the adhesion molecules CD11a (LFA-1), CD54 (ICAM-1), and CD58 (LFA-3), but do not express molecules associated with acute activation, such as CD25, CD69, or HLA-DR (2). In redirected cytotoxicity assays, freshly isolated CD28$^-$CD8$^+$ T cells are capable of TCR-mediated cytolysis, demonstrating that the TCR/CD3 complex is functional and that these cells possess cytotoxic activity (2). In healthy control donors and in HIV-infected subjects, several investigators have observed that CD28$^-$CD8$^+$ T cells show impaired proliferative responses to stimulation with mitogen in vitro (3, 5, 6), although it has been possible to generate CD28$^-$CD8$^+$ T cell clones (2). CD28$^-$CD8$^+$ T cells tend to have shortened telomeres (7). It has therefore been suggested that CD28$^-$CD8$^+$ T cells may have impaired proliferative potential and that some or all of these cells might be terminally differentiated. The same TCR sequence can be found in both CD8$^+$CD28$^+$ and CD8$^+$CD28$^-$ cells, indicating that CD28$^+$ and CD28$^-$ cells represent different states of differentiation of the same lineage (8), but as yet the Ag specificity of CD28$^-$CD8$^+$ T cells has not been determined.

To address the relationship between CD28$^-$CD8$^+$ T cells and Ag-specific T cell memory, we studied human virus-specific CD8$^+$ CTL, which play an important role in the control of human persistent virus infections (9). CD8$^+$ CTL recognize viral peptide Ag presented by MHC class I molecules on the surface of virus-infected cells via the TCR $\alpha\beta$ heterodimer that is generated during thymic development by rearrangement of variable (V), diversity (D), and joining (J) gene segments. Additional TCR diversity is generated by imperfect joining of these segments, exonucleotide nibbling at the joins, and addition of nongermline-encoded N-region nucleotides. The regions spanning the V-(D)-J joins constitute the hypervariable CDR3 regions that interact with the middle of the bound peptide and account for approximately 50% of TCR's interaction with peptide (10).

Following primary infection with the betaherpesvirus HCMV,[3] the virus persists lifelong in a latent state in cells of the myeloid lineage under the control of the immune system (11). In immunocompromised individuals, including patients with advanced HIV infection, HCMV reactivation often causes serious disease (12). We previously studied the HCMV-specific memory CTL response of healthy, long term HCMV carriers and quantified high frequencies of MHC-restricted HCMV-specific CTL precursors (CTLp) in peripheral blood, a large proportion of which recognized the viral tegument protein pp65. In most donors, the CTL response to this protein was highly focused on a small number of epitopes within pp65 (13). We analyzed the clonal composition of memory CTL in

Department of Medicine, University of Cambridge Clinical School, Cambridge, United Kingdom

Received for publication January 19, 1999. Accepted for publication April 1, 1999.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Program Grant G9202171 and Project Grant G9518990PB from the Medical Research Council. M.P.W. is a recipient of a Wellcome Trust Prize Studentship, and A.J.C. is a Lister Institute Research Fellow.

[2] Address correspondence and reprint requests to Dr. A. J. Carmichael, Department of Medicine, University of Cambridge Clinical School, Hills Road, Cambridge, UK CB2 2QQ. E-mail address: ac71@medschl.cam.ac.uk

[3] Abbreviations used in this paper: HCMV, human CMV; CTLp, CTL precursor; LDA, limiting dilution analysis; PerCP, peridinin chlorophyll protein.

0022-1767/99/$02.00

HCMV carriers by generating multiple independent peptide-specific CTL clones and sequencing the hypervariable VDJ region of the TCR β-chain of each clone. We found that the strong memory CTL response against a given viral peptide contains individual CTL clones that had undergone extensive expansion in vivo (14). In HIV infection there is a vigorous HIV-specific CD8$^+$ CTL response that is temporally associated with partial control of viral replication (15–17). During asymptomatic HIV infection, a high frequency of HIV-specific memory CTLp is maintained in peripheral blood for years, but in advancing HIV disease the HIV-specific CTL response is impaired (18).

To determine the contribution of CD28$^-$CD8$^+$ T cells to Ag-specific CTL memory, in asymptomatic carriers of HCMV and HIV we generated multiple independent CTL clones specific for defined peptides of HCMV and HIV and used the hypervariable TCR β-chain sequence of each immunodominant CTL clone to design a complementary oligonucleotide as a highly specific clonotypic probe. Using these probes we found large clone sizes of individual CTL clonotypes in the CD28$^-$ subpopulation of peripheral blood CD8$^+$ T cells. We complemented these molecular studies by demonstrating high frequencies of functional virus-specific memory CTLp within the CD28$^-$ subpopulation using limiting dilution analysis (LDA).

Materials and Methods

Donors

Two healthy HIV-seronegative laboratory donors and three asymptomatic HIV-infected subjects attending Addenbrooke's Hospital (Cambridge, U.K.) were studied. All donors were HCMV seropositive, as determined by an IgG ELISA (Captia HCMV IgG immunoassay, Centocor, Malvern, PA). MHC class I tissue types were determined by serological typing (Lymphotype ABC-120, Biotest, Dreieich, Germany). The tissue types, CD4$^+$ T cell counts, and plasma HIV RNA loads of the HIV-infected subjects were: H0009 (A2 A24 B62 B27), CD4 count 400/μl, viral load 3,000 copies/ml, no antiretroviral treatment; H0018 (A2 A3 B7 B44), CD4 count 390/μl, viral load 1,700 copies/ml, no antiretroviral treatment; and H0045 (A11 A29 B8 B44), CD4 count 250/μl, viral load 10,000/ml, started antiretroviral treatment 07/97 after which viral load was $<$50 copies/ml. The tissue types of the healthy HCMV carriers were: 005, A31 A33 B44 B35; and 011, A2 A23 B62 B44.

Viruses and cell lines

The recombinant vaccinia viruses used were vac pp65, vac gag, vac env, and vac T7 as a negative control as previously described (13, 18). Lymphoblastoid B cell lines (LCL) were generated from PBMC by EBV transformation (18).

Synthetic peptides

HCMV pp65 peptides were 17a (VFPTKDVAL, HLA-B35), 53 (DDVWTSGSDSDEELV, HLA-B35), 56 (TPRVTGGGAM, HLA-B7), 69 (NLVPMVATV, HLA-A2), and 72 (EFFWDANDIY, B12 (44)) (13). HIV env gp120 peptides were 740.18 (TQACPKVSFEPIPIHYCAPA), 740.19 (PIPIHYCAPAGFAILKCNNK, HLA-A2), and 7035.1 (AAEQLWVTVYY GVPVWKEAT, HLA-A11). HIV Gag peptides were 703.3 (LRPG GKKKYKLKHIV, HLA-A3) and 728 (KWILGLNKIVRMY, HLA-B27). The Medical Research Council AIDS Reagent Project, U.K., provided all HIV peptides.

Preparation of defined subsets of CD8$^+$ cells for LDA and oligonucleotide probing

PBMC were prepared from fresh heparinized venous blood samples by Ficoll-Hypaque (Lymphoprep, Nyegaard, Oslo, Norway) density gradient centrifugation. CD16$^+$ NK cells were depleted using anti-CD16 IgM (Leu 11b; Becton Dickinson, Oxford, U.K.) and complement (13). For healthy laboratory donors, CD4$^+$ cells were also depleted using anti-CD4-conjugated MACS microbeads according to the manufacturer's instructions (Miltenyi Biotech, Dreiech, Germany); enrichment for CD8$^+$ cells gave improved sensitivity for clonotype detection. An aliquot of CD16$^-$ (CD4$^-$) cells was further depleted of CD28$^+$ cells using FITC-conjugated anti-CD28 (Coulter, Luton, U.K.) and anti-FITC MACS microbeads.

Negatively selected cells were washed once in PBS and resuspended in RPMI 1640 supplemented with 10% human HCMV-seronegative AB serum (Blood Transfusion Service, Addenbrookes Hospital), 10% FCS (Myoclone, Life Technologies, Paisley, U.K.), 2 mM L-glutamine, 10^5 IU of penicillin/l, and 100 mg of streptomycin/l (referred to as RPMI-HuAB). Aliquots of these cells were used as responder cells in LDA (see below), for RNA extraction, and for flow cytometry.

mAbs and surface phenotyping

Vβ-chain usage was determined by three-color immunofluorescence using a panel of TCR-specific FITC-conjugated mAbs (the panel included Vβ 1, 2, 3, 5.1, 5.2, 6.7, 7, 8.1, 11, 12, 13.6, 14, 16, 17, 19, 20, 21.3, 22 (Coulter); and Vβ 13.1/13.3 and Vα2 (Serotec, Columbus, OH)) with PE-conjugated anti-CD8 and peridinin chlorophyll protein (PerCP)-conjugated CD3 (Becton Dickinson). Other antibodies used were FITC-conjugated anti-CD28 (Coulter) and anti-CD4 (Becton Dickinson). The proportion of Vβ^+ cells was determined by staining aliquots of cells with the relevant Vβ-specific mAb, PE-conjugated anti-CD28 and PerCP-conjugated anti-CD8 (Becton Dickinson). The purity of the CD28-depleted lymphocyte populations was high (median, 98.5%; range, 89.4–99.8%; $n = 21$).

Generation of virus-specific CTL in LDA

The methodology of LDA used has previously been described in detail (18). Replicate microcultures ($n = 18$–27) of responder cells were set up over an appropriate range of dilutions. APCs were autologous irradiated peptide-pulsed PBMC in RPMI-HuAB medium supplemented with human rIL-2 (Medical Research Council AIDS Reagent Project; final concentration, 5 IU/ml). On day 14, the cells in each individual well were divided into five aliquots that were assayed for cytotoxicity against different radiolabeled target cells in 4-h ^{51}Cr release cytotoxicity assays. Target cells comprised autologous or MHC-mismatched LCL that had been infected for 18 h with vac pp65, vac gag, vac env, or vac T7 (multiplicity of infection = 10) and radiolabeled with ^{51}Cr (Amersham, Aylesbury, U.K.) for 45 min at 37°C. Peptide-pulsed target cells were prepared by first labeling LCL with ^{51}Cr and then pulsing with 50 μl of peptide (40 μg/ml) for a further 30 min. Target cells were washed three times and counted. The method of analysis has been described previously (13, 18).

Derivation of formal single-cell clones and expansion of clonally derived CTL microcultures

From selected LDA microcultures, formal single-cell clones were generated by subculture at 0.5 cells/well (14). Multiple independent clonally derived CTL cultures were generated by mitogen restimulation of residual cells from selected LDA microcultures at or below the dilution where $<$50% of replicate microcultures showed peptide-specific killing (14); at these low dilutions, individual LDA microcultures contained single peptide-specific clones (our own unpublished observations). mRNA was extracted, and cDNA was synthesized as previously described (14).

TCR β-chain PCR amplification and sequencing

To determine TCR β-chain V region usage of CTL lines or clones, PCR amplification and sequencing were performed using a panel of TCR Vβ primers and a Cβ primer as previously described (14). The hypervariable CDR3 region was defined by Kabat et al., starting at position 95 of Vβ and finishing at the phenylalanine of the motif FGXG within Jβ (19). Sequence data presented in Table I are available from EMBL/GenBank/DDBJ under accession numbers AJ011545–AJ011556 and AJ010895.

Quantitative clonotypic analysis

To quantify an individual peptide-specific CTL clone in PBMC, we designed a complementary oligonucleotide probe 15–20 nucleotides long based on the TCR β-chain hypervariable NDN region and the ends of the adjoining V and J regions. Such probes are highly specific for individual CTL clonotypes, as reflected by very low binding of individual clonotypic probes to amplified negative control cDNA (20) (Fig. 1*A*). mRNA was extracted from CD16-depleted or CD16- and CD28-depleted cells, reverse transcribed into cDNA, and amplified in duplicate using a Vβ-specific primer (14) and a Cβ-specific primer. A positive control sample from the formal single cell CTL clone and a negative control sample from the pooled PBMC of four HCMV- and HIV-seronegative donors was also amplified at the same time in duplicate using the same primers. Forty microliters of each PCR product was separated on a 1.3% agarose gel. PCR products were transferred overnight to a Zeta-Probe nylon filter (Bio-Rad, Hercules, CA) using standard techniques (21). Blotting buffer was 0.6 M NaOH/0.2 M NaCl. The filter was washed in 2$\times$ SSC and baked at 80°C

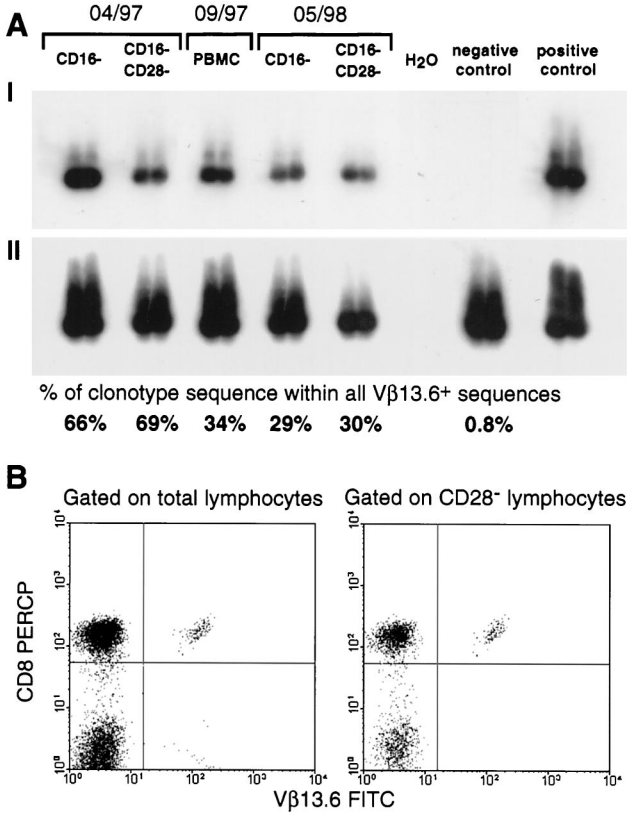

FIGURE 1. Quantitation of the in vivo clone size of donor H0009's env peptide 740.19-specific CTL clone 9A by clonotype probing. *A*, Probing was conducted as described in *Materials and Methods*. mRNA was extracted from CD16-depleted or CD16- and CD28-depleted PBMC, reverse transcribed into cDNA, and amplified using a Vβ13.6-specific primer and a Cβ-specific primer. PCR products were blotted onto a filter, which was first probed using the labeled clonotypic probe (I). By stripping the filter and reprobing with a conserved constant region-specific probe that detects all TCRs (II), the relative abundance of clonotype sequence compared with the total sequence within Vβ13.6 was calculated. In the CD16$^-$ population on 20/05/98, the percentage of clonotype sequence within all Vβ13.6 sequences was 29%. *B*, The percentages of Vβ13.6$^+$ cells were determined by flow cytometry by staining an aliquot of CD16-depleted PBMC with FITC-conjugated Vβ13.6-specific mAb, anti-CD28-PE, and anti-CD8-PerCP. In this example, on 20/05/98 in donor H0009's CD16-depleted PBMC, 77% of the Vβ13.6$^+$ lymphocytes were CD8$^+$; 3.4% of the total CD8$^+$ T cells were Vβ13.6$^+$. By gating on CD28$^-$ cells, 100% of the CD28$^-$ Vβ13.6$^+$ lymphocytes were CD8$^+$; 6.3% of CD28$^-$ CD8$^+$ T cells were Vβ13.6$^+$. Therefore in the CD16-depleted population the clone size was $0.29 \times 1/(0.77) \times 0.034 \times 10^6 = 13{,}000$ cells of the clone/10^6 CD8$^+$ lymphocytes. In the CD16-CD28-depleted population the corresponding clone size was $0.30 \times 1/(1.0) \times 0.063 \times 10^6 = 19{,}000$ cells of the clone/10^6 CD8$^+$ lymphocytes (calculation explained in *Materials and Methods*).

for 30 min. It was then incubated in 15 ml of prehybridization buffer (7% SDS and 0.25 M Na_2HPO_4) for 1 h at 10–15°C below the melting temperature of the clonotypic probe. Fifteen picomoles of clonotypic probe was end labeled with [γ-^{32}P]dATP using T4 polynucleotide kinase (Promega) as described by the manufacturer. The probe was separated from unincorporated label using a Sephadex G-25 spin column (Amersham). Hybridization was conducted overnight at 10–15°C below the melting temperature of the clonotypic probe in hybridization buffer (7% SDS, 0.25 M Na_2HPO_4, and 10% polyethylene glycol 8000). The filter was washed for 15 min three times in wash buffer (5% SDS and 20 mM Na_2HPO_4). The amount of probe that had bound to each sample on the filter was quantitated using an Instant Imager (Beckman, Palo Alto, CA). The filter was exposed to x-ray film, stripped by soaking in 0.4 M NaOH for 30 min, and washed in 2× SSC. It was then rehybridized with a TCR β-chain constant region probe (AGATCTCTGCTTCTGATG) following the same protocol as that described above. In each experiment it was possible to correct for differences in radioactive labeling of the clonotypic and constant region probes by measuring the radioactivity of each probe bound to the positive control sample as a reference standard (TCR β-chain sequences amplified from the biologically derived CTL clone contain the unique hypervariable NDN region and constant region in equal amounts). The proportion of clonotype sequence within the total amplified TCR Vβ sequence for a given population of CD8$^+$ cells was calculated as follows: % of clonotype within Vβ-expressing cells = 100 × ([cpm clonotypic probe/cpm TCR constant probe for the T cell population of interest]/[cpm clonotypic probe/cpm TCR constant probe for the positive control clone]).

We quantified cells that were Vβ^+ in the CD8$^+$ and CD4$^+$ populations by flow cytometry using Vβ-specific mAbs. Because the TCR mRNA from CD4$^+$ cells has the effect of diluting the clonotype in the total TCR mRNA preparation, we corrected for the proportion of Vβ^+ cells that were CD8$^+$. The clone size within total CD8$^+$ lymphocytes could then be estimated from the relative abundance of clonotype sequence within all sequences encoding the same Vβ segment multiplied by the proportion of the Vβ^+ CD8$^+$ cells within the total CD8$^+$ T cell population. For the example in Fig. 1, the clone size per 10^6 CD8$^+$ T cells was therefore: % of clonotype sequence within all Vβ13.6$^+$ sequences × 1/(% of Vβ13.6$^+$ cells that are CD8$^+$) × % of Vβ13.6$^+$CD8$^+$ cells in total CD8$^+$ cells × 10^6.

Results

Identification of MHC-restricted HIV and HCMV peptides

We identified five CTL epitopes in HIV env or gag and determined their MHC restriction (22). We identified five CTL epitopes restricted by four different HLA alleles from HCMV pp65 and determined their MHC restriction (13, 14).

The clonal composition of memory CTL specific for HIV gag and env epitopes is highly focused

To determine how many different TCR Vβ gene segments were used by CTL specific to a given peptide, unstimulated PBMC and peptide-specific bulk CTL lines (obtained by pooling the residual cells from LDA microcultures that showed strong peptide-specific killing) were studied by two-color immunofluorescence using mAbs specific for defined Vβ gene segments. In unstimulated PBMC of donor H0009, 2.6% of CD8$^+$ T cells were Vβ13.6$^+$, and 3.6% were Vβ22$^+$. After stimulation with peptide 740.19, Vβ13.6$^+$ cells expanded to 84.6% of the CD8$^+$ population; only 0.1% were Vβ22$^+$. After stimulation with peptide 728, 2.1% of responder CD8$^+$ cells were Vβ13.6$^+$, and 29.8% were Vβ22$^+$.

We next analyzed the clonal composition of the peptide-specific CTL response to identify how many different CTL clones contribute to the peptide 740.19-specific Vβ13.6$^+$ CTL population. We generated formal single cell clones and multiple independent, clonally derived CTL cultures specific for HIV env and gag peptides, and sequenced each TCR β-chain (Table I). In each of the three HIV-infected subjects studied, the clonal composition of the memory CTL responses to gag and env peptides was highly focused; for each donor, one predominant TCR β-chain nucleotide sequence was detected in multiple, independently derived formal CTL clones and clonally derived CTL cultures specific for a given peptide. In donor H0009, six of six independent peptide 740.19-specific CTL clones and clonal microcultures derived at two time points had the same Vβ13.6$^+$ TCR β-chain nucleotide sequence, demonstrating that this CTL clone accounted for the large majority of the peptide 740.19-specific memory CTLp in this donor. In the same donor, gag peptide 728-specific CTL were also focused, since both a CTL clone and the Vβ22$^+$ cells within a peptide-specific bulk CTL line had the same β-chain sequence. Only 30% of CD8$^+$ lymphocytes within the peptide 728-specific bulk CTL line were however Vβ22$^+$, indicating that other clones expressing different Vβ segments may be involved in the recognition of this

Table I. *Multiple independent peptide-specific CTL clones or microcultures had the same TCR β-chain nucleotide sequence*

Donor	Peptide	Clono-type	Date of Sample	CTL Clone/ Culture	% Specific Lysis		Vβ	Cβ
					+ Peptide	Control		
H0009	env 740.19	9A	09/96	Clone 3.2	54	0	Vβ13.6 CAS SYTLDNQPQH	FG Jβ1 Cβ1
			04/97	Culture 1	54	0	Vβ13.6 CAS SYTLDNQPQH	FG Jβ1 Cβ1
				Culture 2	51	12	Vβ13.6 CAS SYTLDNQPQH	FG Jβ1 Cβ1
				Culture 3	52	0	Vβ13.6 CAS SYTLDNQPQH	FG Jβ1 Cβ1
				Culture 4	46	0	Vβ13.6 CAS SYTLDNQPQH	FG Jβ1 Cβ1
				Culture 5	42	4	Vβ13.6 CAS SYTLDNQPQH	FG Jβ1 Cβ1
	gag 728	9B	02/96	Clone 5.6	78	3	Vβ22.1 CAS SLGSYEQY	FG Jβ2 Cβ2
			09/96	Bulk CTL line			Vβ22.1 CAS SLGSYEQY	FG Jβ2 Cβ2
H0018	gag 703.3		06/96	Clone 4.6	70	0	Vβ5.2 CAS TSGWGSYEQY	FG Jβ2 Cβ2
		18A	01/98	Culture 2	32	0	Vβ13.1 CAS NLAPYEQY	FG Jβ2 Cβ2
				Culture 3	42	3	Vβ13.1 CAS NLAPYEQY	FG Jβ2 Cβ2
				Culture 4	50	2	Vβ13.1 CAS NLAPYEQY	FG Jβ2 Cβ2
			04/98	Culture 3	72	10	Vβ13.1 CAS NLAPYEQY	FG Jβ2 Cβ2
				Culture 4	47	0	Vβ13.1 CAS NLAPYEQY	FG Jβ2 Cβ2
H0045	env 740.18	45A	01/97	Clone 1.1	62	2	Vβ13.1 CAS RQGAPNQPQH	FG Jβ1 Cβ2
			04/97	Culture 2	36	0	Vβ13.1 CAS RQGAPNQPQH	FG Jβ1 Cβ2
				Culture 5	32	0	Vβ13.1 CAS RQGAPNQPQH	FG Jβ1 Cβ2
		45B		Culture 1	34	0	Vβ13.1 CAS SDPLNEAF	FG Jβ1 Cβ1
				Culture 3	34	0	Vβ7	
	env 7035.1		01/97	Clone 1.1	55	1	Vβ6 CAS SLGGLF	FG Jβ2 Cβ2
			04/97	Culture 4	32	0	Vβ6 CAS GMTQY	FG Jβ2 Cβ2
		45C		Culture 1	32	0	Vβ6 CAS SFGELF	FG Jβ2 Cβ2
				Culture 2	45	15	Vβ6 CAS SFGELF	FG Jβ2 Cβ2
			04/98	Culture 6	40	0	Vβ6 CAS SFGELF	FG Jβ2 Cβ2
				Culture 8	57	2	Vβ6 CAS SFGELF	FG Jβ2 Cβ2
H0018	pp65 56	18B	01/98	Clone 1B[a]	74	1	Vβ6.4 CAS SHDRQGASSPLH	FG Jβ1 Cβ1
		18C		Clone 2K	78	2	Vβ6.4 CAS SHDREGGNSPLH	FG Jβ1 Cβ1
		18D		Clone 1K	75	7	Vβ6.4 CAS SERTGGSYEQY	FG Jβ2 Cβ2
H0045	pp65 72	45D	01/97	Clone 2	74	0	Vβ8 CAS SPIQGYYEQY	FG Jβ2 Cβ2

[a] The TCR β-chain sequence of this clone has been reported elsewhere (14) and is shown here for comparison.

peptide. In donors H0018 and H0045, CTL specific for three different HIV peptides were also highly focused. These results closely resemble our previous observation of clonal focusing of CTL specific for peptides of pp65 in healthy HCMV carriers (14). In all donors in the current study we also identified the clonotypes of immunodominant CTL specific for given pp65 peptides (Table I) (14).

Large clone sizes of HIV and HCMV peptide-specific CTL clones in CD28$^-$CD8$^+$ cells demonstrated using clonotype-specific oligonucleotide probes

For each CTL clone, we designed a complementary oligonucleotide based on the hypervariable region of the TCR β-chain. To quantify each clone within the CD16-depleted or CD16- and CD28-depleted cells, cDNA was synthesized, amplified using the relevant Vβ-specific primer, and blotted onto a filter that was probed using the labeled clonotypic probe. By stripping the filter and reprobing with a conserved internal probe that detects all TCRs, the relative abundance of clonotype sequence compared with the total sequence within that Vβ family could be calculated. From the relative abundance of clonotype sequence within the Vβ family, and the proportion of Vβ$^+$ cells in the CD8$^+$ population (determined by flow cytometry), the clone size within the CD16-depleted or CD16- and CD28-depleted T cell populations could be estimated (Fig. 1).

We found very large clone sizes of individual CTL clones specific for pp65 peptides in both healthy HCMV carriers (Table II). A single CTL clone comprised up to 1.4% of all CD8$^+$ T cells in PBMC. The size of pp65-specific CTL clones generally remained stable over time. HCMV-specific CTL clone sizes were similar or increased in CD28-depleted CD8$^+$ cells compared with those in nondepleted cells. In donor 011, the size of Vβ17$^+$ pp65 peptide 69-specific CTL clone 11A was 1.5- to 3-fold greater in the CD16$^-$CD4$^-$CD28$^-$ population than in the CD16$^-$CD4$^-$ population when examined at four different time points (Table II). In donor 005, the size of Vβ20$^+$ pp65 peptide 17/18-specific CTL clone 5A was also generally greater in CD28$^-$ cells, in this case because of an increased percentage of Vβ20$^+$ cells within the CD28$^-$CD8$^+$ subpopulation; during the period of study, 14–20% of all CD8$^+$ cells were Vβ20$^+$, whereas within the CD28$^-$CD8$^+$ subpopulation, 28–38% of cells were Vβ20$^+$.

In the HIV-infected subjects, the clone sizes of HIV-specific and HCMV-specific CTL clonotypes were also generally stable over time (Table III). In donor H0018, we identified three different pp65 peptide 56-specific CTL clonotypes, each of which used Vβ6.4. cDNA samples from different time points were amplified using the Vβ6.4-specific primer, and the PCR products were analyzed with probes specific for each of three clonotypes (Fig. 2). The proportion of clonotype 18D within Vβ6.4-expressing cells was higher than that of clonotype 18C, and both remained stable over time. The proportion of clonotype 18B within Vβ6.4-expressing cells decreased significantly between 01/98 and 04/98, but remained stable thereafter. Importantly, a very similar decrease in the relative size of this clonotype was observed within the CD28-depleted population analyzed in parallel (Fig. 2). Clonotypes 18C and 18D act as internal controls for this decrease because they have the same Vβ segment as clonotype 18B. In the HIV-infected subjects, for both HIV-specific and HCMV-specific CTL clonotypes the clone sizes were similar or increased in the CD28-depleted populations compared with those in undepleted cells (Table III).

Table II. *Simultaneous comparison of clone size and peptide-specific CTLp frequency for healthy HCMV carriers*

					$CD16^-CD4^-$ Cells					$CD16^-CD4^-CD28^-$ Cells					
Donor	Peptide	Clono-type	Vβ	Date	Clonotype wells/wells analyzed[a]	% of clone in Vβ	% of $CD8^+$ cells that are $V\beta^+$	Clonal size[b]	CTLp frequency[c]	CTLp: clone size ratio[e] (%)	Clonotype wells/wells analyzed[a]	% of clone in Vβ	% of $CD8^+$ cells that are $V\beta^+$	Clonal size[b]	CTLp frequency[c]	CTLp: clone size ratio[d] (%)
011	pp65 69	11A	17	04/97	1/1	20	6.5	14,000	ND		4/4	54	4.3	25,000	ND	
				10/97		9	7.0	6,400	ND			35	4.0	15,000	ND	
				01/98		8	6.5	5,100	1,100	22		30	5.0	16,000	5,300	33
				04/98		9	7.6	7,400	11,000	149		26	4.8	13,000	23,000	177
				07/98		ND	ND	ND	860			ND	ND	ND	3,500	
	pp65 72	ND	ND	01/98		ND	ND	ND	56			ND	ND	ND	1,600	
				04/98					1,000						1,500	
				07/98					59						1,000	
005	pp65 17a	5A	20	05/97	1/1	3	13.5	4,600			ND	1	34.2	3,800		
				10/97		4	15.8	6,600				5	34.0	18,000		
				01/98		2	20.4	4,500	770	17		1	37.6	5,400	1,500	28
				07/98		6	15.0	10,000	53	0.5		4	27.6	12,000	45	0.4
	pp65 53	ND	ND	01/98					240						240	
				07/98					210						390	

[a] The proportion of responding peptide-specific wells that contained the CTL clonotype indicates the contribution of particular immunodominant clonotypes to the functional response in each population.
[b] Clonal size per 10^6 $CD8^+CD16^-CD4^-$ T cells or per 10^6 $CD8^+CD16^-CD4^-CD28^-$ T cells, respectively. The magnitude of nonspecific binding of the clonotype-specific probe to amplified negative control cDNA was always <0.8%.
[c] Peptide-specific CTLp frequency per 10^6 $CD8^+$ T cells measured by limiting dilution analysis. CTLp frequencies against unpulsed target cells were generally <5% of the peptide-specific CTLp frequency.
[d] Ratio of functional CTLp frequency (determined by LDA): clone size (determined by clonotype probing).

$CD28^-$ cells from asymptomatic HIV-infected subjects contain high frequencies of functional CTLp specific for HCMV and HIV peptides

We measured the frequency of memory CTLp specific for gag, env, and pp65 peptides in LDA, using as responder cells the CD16 (CD4-)-depleted PBMC or CD16 (CD4-)-CD28-depleted PBMC that were used for simultaneous clonotype probing (Tables II and III). The magnitude of MHC-restricted peptide-specific killing was very similar in individual microcultures derived from either CD16-depleted or CD16- and CD28-depleted responder cells (data not shown). We confirmed that CTL generated by stimulation with individual HIV or HCMV peptides could kill target cells infected with recombinant vaccinia viruses that expressed the relevant HCMV or HIV protein (data not shown) (22).

In healthy HCMV carriers, the frequencies of memory CTLp specific to pp65 peptides were high and showed some variability over time (Table II). In donor 011, the frequencies of CTLp specific for pp65 peptide 69 and for pp65 peptide 72 were significantly higher in $CD16^-CD4^-CD28^-$ cells than in $CD16^-CD4^-$ cells. In donor 005, the frequencies of CTLp specific for pp65 peptide 17a and for pp65 peptide 53 were similar in both responder cell populations.

For all three HIV-infected subjects, the LDA frequencies of memory CTLp specific for gag, env, and pp65 peptides were maintained at a stable level over a period of up to 13 mo (Table III). We found that the frequency of gag, env, or pp65 peptide-specific CTLp in the CD28-depleted responder cells was generally similar to or higher than the frequency of CTLp specific to the same peptide in the undepleted population. In HIV-infected subject H0009, $CD16^-CD28^-$ cells also contained higher frequencies of CTLp specific to pp65 peptide 69 and gag peptide 728 than $CD16^-$ cells. There were similar frequencies of peptide-specific CTLp in both CD28-depleted and undepleted cells in subjects H0018 and H0045.

Simultaneous comparison of clone size of defined CTL clonotypes and peptide-specific CTLp frequency

Tables II and III compare peptide-specific clone sizes and LDA frequencies of peptide-specific CTLp at the same time point in the same donor. The peptide-specific CTLp frequency measured in LDA reflects an aggregate of the individual contributions of different CTL clonotypes that are capable of responding to a given viral peptide in vitro. The contributions of particular immunodominant clonotypes to the functional response in vitro can be estimated from the proportion of responding peptide-specific microcultures that contained the CTL clonotype. For example, pp65 peptide 69-specific clonotype 9C from donor H0009 was identified in four independent peptide-specific microcultures on 04/97; similar observations were made for other clonotypes in other donors (Table III). Because this clonotype accounts for a large proportion of the total peptide 69-specific CTL response in this donor, we calculated the ratio of functional CTLp frequency: clone size as an estimate of the cloning efficiency of the specific CTL clonotype. In each cell population, these two values are, in fact, very similar; on 20/5/98, in the $CD16^-$ population, the size of clonotype 9C was 3800 clones/10^6 $CD8^+$ T cells, and the frequency of peptide-specific CTLp was 4300/10^6 $CD8^+$ T cells (Table III). Similarly, within the corresponding $CD16^-CD28^-$ subpopulation, the size of the clonotype was 14,000 clones/10^6 $CD8^+$ T cells, which was comparable to the frequency of peptide 69-specific CTLp in LDA, namely 12,000 CTLp/10^6 $CD8^+$ T cells. Where analyzed, for each donor the same immunodominant clone made up a similar proportion of all the peptide-specific clones in both the CD28-depleted and undepleted responder $CD8^+$ cells (Tables II and III).

Table III. *Simultaneous comparison of clone size and peptide-specific CTLp frequency for HIV-infected subjects*

					CD16$^-$ Cells						CD16$^-$CD28$^-$ Cells					
Donor	Peptide	Clono-type	Vβ	Date	Clonotype wells/wells analyzed[a]	% of clone in Vβ	% of CD8$^+$ cells that are Vβ$^+$	Clonal size[b]	CTLp frequency[c]	CTLp: clone size ratio[a] (%)	Clonotype wells/wells analyzed[a]	% of clone in Vβ	% of CD8$^+$ cells that are Vβ$^+$	Clonal size[b]	CTLp frequency[c]	CTLp: clone size ratio[d] (%)
H0009	env 740.19	9A	13.6	04/97	5/5	66	3.1	31,000	730	2	ND	69	5.4	37,000	ND	
				05/98		29	3.4	13,000	870	7		30	6.3	19,000	ND	
	gag 728	9B	22	04/97	1/1	2	3.0	1,500	8,400	560	ND	7	3.1	2,400	22,000	917
				05/98		3	2.9	1,700	3,100	182		1	3.0	500	6,500	1,300
	pp65 69	9C	13.1	04/97	4/4	2	ND	2,100	3,800	181	ND	8	ND	6,700	22,000	328
				05/98		4	5.9	3,800	4,300	113		18	7.3	14,000	12,000	86
H0018	gag 703.3	18A	13.1	01/98	1/1	11	ND	5,900	680	12	2/2	22	ND	11,000	380	3
				04/98	2/2	4	ND	2,400	640	27	ND	20	ND	9,500	530	6
				08/98		10	3.3	5,000	ND			47	2.8	23,000	ND	
	pp65 56	18B, C, D		01/98	Table I	Fig.2			680			Fig. 2			ND	
				04/98					640						5,400	
	pp65 69			01/98		ND	ND	ND	500						330	
				04/98					800						750	
H0045	env 740.18	45A	13.1	17/04/97		ND	4.5		ND			2	3.8	720	ND	
				25/04/97	2/4	3	ND	3,100	380	12	ND	2	ND	730	ND	
				04/98		0.3	6.4	410	290	71		1	5.3	390	370	95
	env 7035.1	45C		25/04/97	2/3	ND	ND	ND	100		ND	ND	ND	ND	ND	
				04/98	1/1				200		1 /1				290	
	pp65 72	45D	8	17/04/97		ND	0.9		ND			0	0.4	0	ND	
				25/04/97	1/1	0.04	ND	10	250	2,500	ND	0.1	ND	10	ND	
				04/98		0.06	4.2	50	530	1,060		0.32	2.3	100	1,300	1,300

Note: Footnotes *a*, *c*, and *d* are the same as in Table II.

[b] Clonal size per 10^6 CD8$^+$CD16$^-$ T cells or per 10^6 CD8$^+$CD16$^-$CD28$^-$ T cells, respectively. The magnitude of nonspecific binding of the clonotype-specific probe to amplified negative control cDNA was always <1.0%.

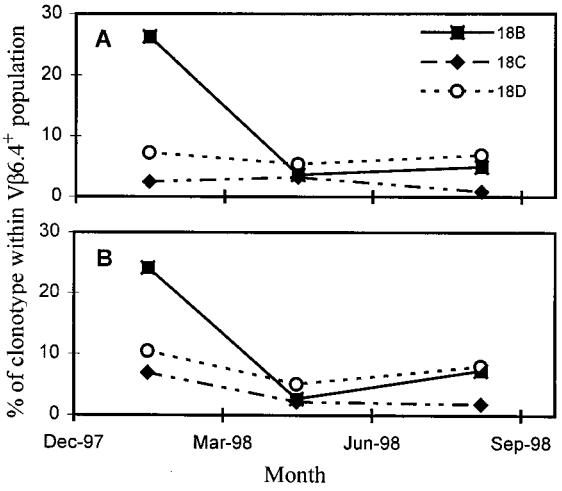

FIGURE 2. Quantitation of three CTL clonotypes specific for the same pp65 peptide in a single donor. In donor H0018 we identified three CTL clonotypes specific for peptide 56, all of which used Vβ6.4 but had different hypervariable sequences (see Table I). We quantified the proportion of each clonotype within Vβ6.4$^+$ cells at three time points: *A*, in CD16$^-$ PBMC; and *B*, in CD16$^-$CD28$^-$ PBMC. Because no Vβ6.4-specific mAb is currently available, we were not able to calculate the absolute clonal sizes.

Other CTL clones (e.g., subdominant clonotype 45B identified in one of four peptide-specific Vβ13.1$^+$ microcultures; Table I), whose sizes we have not quantified, also contribute to the functional response observed in LDA. This is confirmed by the fact that in donor H0009 the frequency of gag peptide 728-specific CTLp is consistently greater than the clone size of 9B, and in donor H0045 the frequency of pp65 peptide 72-specific CTLp is greater then the clone size of 45D (Table III), resulting in a CTLp:clone size ratio in excess of 100%.

In general, the CTLp:clone size ratio was between 12–90%. In the case of the highly focused peptide 740.19-specific CTL response of donor H0009 (Table IA), the functional LDA frequency was substantially lower than the clone size (CTLp:clone size ratio, $<$7%). It is possible that this large clone genuinely had impaired function or that in vitro culture was suboptimal, possibly because the sequence of the env peptide 740.19 used to stimulate CTL in vitro may have differed from the endogenous HIV viral sequence(s) in H0009 that stimulated this clonal expansion in vivo.

Importantly, at each time point in each donor, the CTLp:clone size ratio of CD28-depleted cells was very similar to that of the undepleted CD8$^+$ T cell population as a whole, indicating that the large virus-specific clones in the CD28$^-$ population maintain their ability to function in vitro. The only exception was donor H0018, in whom the CTLp:clone size ratio of gag peptide 703.3-specific CTL was reduced in the CD28$^-$ population compared with that in the undepleted population.

Discussion

To examine the contribution of CD28$^-$CD8$^+$ T cells to Ag-specific T cell memory, we characterized the strong memory CTL responses against HCMV and HIV at the clonal level. We showed that the sizes of individual virus-specific CTL clones can be very large and that they are generally maintained at a stable level for months. Individual peptide-specific CTL clones were consistently more abundant in the purified CD28$^-$ cells. We have shown that these expanded CD28$^-$ clones were functional in quantitative LDA.

We previously found that the CTL response to four different HCMV pp65 peptides was highly focused; in general, the majority of CTL clones specific for a defined pp65 peptide from any one virus carrier used only one or two different TCR at the level of nucleotide sequence (14). Here we found a high degree of clonal focusing among memory CTL specific for a given HIV gag or env peptide/MHC complex within each of three HIV-infected subjects. Previous studies that have examined the clonal composition of the CTL response to other persistent human virus infections within individuals have observed a comparable degree of clonal focusing (23). In a single HIV-infected subject, multiple independent CTL clones specific for an HLA-B14-restricted env peptide derived at different time points over the course of 36 mo used the same TCR β-chain (24). Similarly, multiple independent CTL clones specific to an HLA-B8-restricted EBV peptide derived from one virus carrier at one time point all used the same TCR (25). The CTL responses to different HTLV-1 peptides have also been observed to be oligoclonal within individual donors (26).

In healthy HCMV carriers we previously measured very high frequencies of CTLp specific for individual pp65 peptides by LDA. Because the peptide-specific CTL response is highly focused at the clonal level, this indicated that the large population of circulating memory CTL against a given HCMV peptide contains individual CTL clones that had undergone extensive clonal expansion in vivo. Here, using the clonotype probing technique, we have produced direct evidence confirming that individual HIV- and HCMV-specific CTL clones can indeed be very large, sufficient to permit reproducible quantitation of the size of individual CTL clonotypes directly ex vivo independently of function. In this study individual peptide-specific CTL clones generally accounted for 2–66% of all CD8$^+$ T cells that expressed the same Vβ segment as the clone; this corresponded to a clone size of between 0.15–3.1% of all CD8$^+$ T cells. The clonotyping results also indicated that individual CTL clone sizes were maintained at a relatively constant level over time. In a healthy HCMV carrier, we found that the size of a Vβ17$^+$ pp65 peptide 69-specific CTL clonotype was between 5,100–14,000 cells/10^6 CD8$^+$ T cells when measured on four separate occasions over a 15-mo period (Table II). The sizes of individual CTL clones derived from other donors were also generally maintained with no more than a 4-fold change in clonal size over the period of observation. In an HIV-infected subject, one CTL clonotype showed a 7-fold reduction in size within Vβ6.4$^+$ cells, while the size of two other Vβ6.4$^+$ clonotypes specific for the same peptide remained stable (Fig. 2). Because both HIV and HCMV are persistent viruses characterized by sustained or intermittent viral reactivation and Ag expression, repeated exposure of virus-specific memory CTL to viral Ag in vivo over time may lead to selective expansion and maintenance of large memory CTL clones, possibly those that express certain high affinity TCR (27).

By clonotype probing of CD28-depleted cell populations, we found large clone sizes of individual HIV-specific and HCMV-specific CTL clones in CD28$^-$ cells. The clone size of HIV- and HCMV-specific CTL clonotypes was similar or increased within the CD28$^-$ population compared with that in undepleted CD8$^+$ cells in the HIV-infected subjects and in healthy HCMV carriers. In normal adult donors, oligoclonal expansions of unknown Ag specificity have previously been observed in CD8$^+$ T cells and in particular within the CD28$^-$CD8$^+$ T cell subpopulation (28–36). Our demonstration of large memory CD8$^+$ CTL clones that are specific for HCMV and HIV within the CD28$^-$CD8$^+$ subpopulation suggests that memory CTL specific for persistent viruses may account for many of these hitherto unexplained oligoclonal expansions.

In all three HIV-infected subjects, the LDA frequencies of functional memory CTLp specific for gag, env, and pp65 peptides were

maintained at stable levels over a period of up to 13 mo. In healthy HCMV carriers, the frequencies of memory CTLp specific to pp65 peptides were more variable. As shown in Table II, in some instances the clone size of an individual peptide-specific CTL clone was greater than the frequency of CTLp specific to the same peptide, which indicates that not all the CTL clones detected by the clonotype probing technique grew in LDA. It is recognized that the functional response requiring clonal expansion in vitro in LDA may underestimate the absolute number of Ag-specific cells as determined by peptide/MHC class I tetramer staining (37). Tetramer staining detects the aggregate of Ag-specific cells, but does not distinguish between individual CTL clonotypes. In this study we used clonotype probing rather than tetramer staining not only because we wished to study memory CTL responses specific for seven viral peptides restricted by five different MHC alleles, but also because the clonotype probing technique provides a higher level of resolution by allowing the study of the sizes of individual peptide-specific CTL clones over time and the segregation of individual clones into different phenotypic subpopulations.

Other observers have suggested that $CD28^-CD8^+$ cells as a population have impaired proliferative responses to in vitro mitogenic stimulation with PHA, anti-CD2, or anti-CD3 mAbs or a combination of the above (2, 3, 5, 6, 29, 33). In contrast, Gomez et al. observed significant proliferation of the $CD28^-CD8^+$ population in anti-EBV and anti-influenza T cell lines generated from PBMC of subjects responding to these viruses (38). Strong proliferation of $CD28^-CD8^+$ cells from HIV-infected subjects was also observed after in vitro restimulation with autologous blast cells that may have expressed HIV Ag (39). The proliferation of $CD28^-CD8^+$ T cells may therefore differ depending upon the conditions of stimulation in vitro. It is possible that $CD28^-CD8^+$ cells may be particularly susceptible to apoptosis upon stimulation with mitogen (5, 40). In our experiments upon stimulation with autologous irradiated PBMC pulsed with specific peptide, single $CD28^-$ Ag-specific memory CTLp were capable of undergoing at least 12–14 divisions necessary for detection of short term CTL clones in LDA. The frequency of gag, env, or pp65 peptide-specific CTLp within the CD28-depleted responder cells was similar to or higher than the frequency of CTLp specific for the same peptide in the undepleted responder cell population. From CD28-depleted responder cells we derived functional CTL clones possessing the same TCR β-chain sequence as clones derived from undepleted responder cells (data not shown), confirming that the large virus-specific T cell clones within the $CD28^-$ population also comprised functional cells. In four of the five donors, the estimated cloning efficiency (the LDA response as a proportion of peptide-specific CTL clone size) in the CD28-depleted population was similar to that in the undepleted population (Tables II and III). This indicates that the in vitro function of virus-specific $CD28^-CD8^+$ CTLp was comparable to that of the $CD8^+$ T cell population as a whole. It is possible that these $CD28^-CD8^+$ CTLp may express other molecules that are capable of providing costimulation in the absence of CD28, as appears to be the case in CD28 knockout mice (41). Therefore, at least during the asymptomatic phase of HIV infection, many virus-specific $CD28^-CD8^+$ CTLp were able to function in vitro, arguing against the suggestion that these cells are terminally differentiated (5). However, it is possible that during the course of advancing HIV disease, the function of virus-specific $CD28^-$ memory CTL clones may decline, and we are addressing this question in longitudinal studies.

In conclusion, by molecular analysis we found large clone sizes of individual virus-specific CTL clonotypes in $CD28^-CD8^+$ T cells of subjects asymptomatically infected with HIV and/or HCMV, and by functional assay we demonstrated that rather than being terminally differentiated, $CD28^-CD8^+$ cells contain high frequencies of functional virus-specific memory CTL clones.

References

1. June, C. H., J. A. Bluestone, L. M. Nadler, and C. B. Thompson. 1994. The B7 and CD28 receptor families. *Immunol. Today 15:321.*
2. Azuma, M., J. H. Phillips, and L. L. Lanier. 1993. $CD28^-$ T lymphocytes: antigenic and functional properties. *J. Immunol. 150:1147.*
3. Borthwick, N. J., M. Bofill, W. M. Gombert, A. N. Akbar, E. Medina, K. Sagawa, M. C. Lipman, M. A. Johnson, and G. Janossy. 1994. Lymphocyte activation in HIV-1 infection. II. Functional defects of CD28-T cells. *AIDS 8:431.*
4. Brinchmann, J. E., J. H. Dobloug, B. H. Heger, L. L. Haaheim, M. Sannes, and T. Egeland. 1994. Expression of costimulatory molecule CD28 on T cells in human immunodeficiency virus type 1 infection: functional and clinical correlations. *J. Infect. Dis. 169:730.*
5. Lewis, D. E., D. S. Tang, A. Adu Oppong, W. Schober, and J. R. Rodgers. 1994. Anergy and apoptosis in $CD8^+$ T cells from HIV-infected persons. *J. Immunol. 153:412.*
6. Vingerhoets, J. H., G. L. Vanham, L. L. Kestens, G. G. Penne, R. L. Colebunders, M. J. Vandenbruaene, J. Goeman, P. L. Gigase, M. De Boer, and J. L. Ceuppens. 1995. Increased cytolytic T lymphocyte activity and decreased B7 responsiveness are associated with CD28 down-regulation on $CD8^+$ T cells from HIV-infected subjects. *Clin. Exp. Immunol. 100:425.*
7. Monteiro, J., F. Batliwalla, H. Ostrer, and P. K. Gregersen. 1996. Shortened telomeres in clonally expanded CD28-$CD8^+$ T cells imply a replicative history that is distinct from their $CD28^+$ $CD8^+$ counterparts. *J. Immunol. 156:3587.*
8. Mugnaini, E. N., A. Spurkland, T. Egeland, M. Sannes, and J. E. Brinchmann. 1998. Demonstration of identical expanded clones within both $CD8^+CD28^+$ and $CD8^+CD28^-$ T cell subsets in HIV type 1-infected individuals. *Eur. J. Immunol. 28:1738.*
9. Quinnan, G. V., Jr., W. H. Burns, N. Kirmani, A. H. Rook, J. Manischewitz, L. Jackson, G. W. Santos, and R. Saral. 1984. HLA-restricted cytotoxic T lymphocytes are an early immune response and important defence mechanism in cytomegalovirus infections. *Rev. Infect. Dis. 6:156.*
10. Garboczi, D. N., P. Ghosh, U. Utz, Q. R. Fan, W. E. Biddison, and D. C. Wiley. 1996. Structure of the complex between human T-cell receptor, viral peptide and HLA-A2. *Nature 384:134.*
11. Borysiewicz, L. K., S. Graham, J. K. Hickling, P. D. Mason, and J. G. Sissons. 1988. Human cytomegalovirus-specific cytotoxic T cells: their precursor frequency and stage specificity. *Eur. J. Immunol. 18:269.*
12. Peters, B. S., E. J. Beck, S. Anderson, D. Coleman, R. Coker, J. Main, C. Migdal, J. R. Harris, and A. J. Pinching. 1991. Cytomegalovirus infection in AIDS: patterns of disease, response to therapy and trends in survival. *J. Infect. 23:129.*
13. Wills, M. R., A. J. Carmichael, K. Mynard, X. Jin, M. P. Weekes, B. Plachter, and J. G. Sissons. 1996. The human cytotoxic T-lymphocyte (CTL) response to cytomegalovirus is dominated by structural protein pp65: frequency, specificity, and T-cell receptor usage of pp65-specific CTL. *J. Virol. 70:7569.*
14. Weekes, M. P., M. R. Wills, K. Mynard, A. J. Carmichael, and J. G. P. Sissons. 1999. The memory cytotoxic T-lymphocyte (CTL) response to human cytomegalovirus infection contains individual peptide-specific CTL clones that have undergone extensive expansion in vivo. *J. Virol. 73:2099.*
15. Koup, R. A., J. T. Safrit, Y. Cao, C. A. Andrews, G. McLeod, W. Borkowsky, C. Farthing, and D. D. Ho. 1994. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J. Virol. 68:4650.*
16. Borrow, P., H. Lewicki, X. Wei, M. S. Horwitz, N. Peffer, H. Meyers, J. H. Nelson, J. E. Gairin, B. H. Hahn, M. B. A. Oldstone, et al. 1997. Antiviral pressure exerted by HIV-1 specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. *Nat. Med. 3:205.*
17. Ogg, G., X. Jin, S. Bonhoeffer, P. R. Dunbar, M. A. Nowak, S. Monard, J. P. Segal, Y. Cao, S. L. Rowland-Jones, V. Cerundolo, et al. 1998. Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. *Science 279:2103.*
18. Carmichael, A., X. Jin, P. Sissons, and L. Borysiewicz. 1993. Quantitative analysis of the human immunodeficiency virus type 1 (HIV-1)-specific cytotoxic T lymphocyte (CTL) response at different stages of HIV-1 infection: differential CTL responses to HIV-1 and Epstein-Barr virus in late disease. *J. Exp. Med. 177:249.*
19. Kabat, E. A., and T. T. Wu. 1991. Identical V region amino acid sequences and segments of sequences in antibodies of different specificities: relative contributions of V_H and V_L genes, minigenes, and complementarity-determining regions to binding of antibody-combining sites. *J. Immunol. 147:1709.*
20. Wills, M. R., A. J. Carmichael, M. P. Weekes, K. Mynard, G. Okecha, R. Hicks, and J. G. P. Sissons. 1999. Human virus-specific $CD8^+$ CTL clones revert from $CD45RO^{high}$ to $CD45RA^{high}$ in vivo: $CD45RA^{high}$ $CD8^+$ T cells comprise both naive and memory cells. *J. Immunol. 162, In press.*●●
21. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*, Chapt. 9. Cold Spring Harbor Laboratory Press, Cold Spring Harbor..
22. Jin, X., M. Wills, J. G. P. Sissons, and A. Carmichael. 1998. Progressive loss of IL-2-expandable HIV-1-specific cytotoxic T lymphocytes during asymptomatic HIV infection. *Eur. J. Immunol. 28:3564.*
23. Wilson, J. D. K., G. S. Ogg, R. L. Allen, P. J. R. Goulder, A. Kelleher, A. K. Sewell, C. A. O'Callaghan, S. L. Rowland-Jones, M. F. C. Callan, and

A. J. McMichael. 1998. Oligoclonal expansions of $CD8^+$ T cells in chronic HIV infection are antigen specific. *J. Exp. Med. 188:785.*

24. Kalams, S. A., R. P. Johnson, A. K. Trocha, M. J. Dynan, H. S. Ngo, R. T. D'Aquila, J. T. Kurnick, and B. D. Walker. 1994. Longitudinal analysis of T cell receptor (TCR) gene usage by human immunodeficiency virus 1 envelope-specific cytotoxic T lymphocyte clones reveals a limited TCR repertoire. *J. Exp. Med. 179:1261.*
25. Argaet, V. P., C. W. Schmidt, S. R. Burrows, S. L. Silins, M. G. Kurilla, D. L. Doolan, A. Suhrbier, D. J. Moss, E. Kieff, T. B. Sculley, et al. 1994. Dominant selection of an invariant T cell antigen receptor in response to persistent infection by Epstein-Barr virus. *J. Exp. Med. 180:2335.*
26. Utz, U., D. Banks, S. Jacobson, and W. E. Biddison. 1996. Analysis of the T-cell receptor repertoire of human T-cell leukemia virus type 1 (HTLV-1) Tax-specific $CD8^+$ cytotoxic T lymphocytes from patients with HTLV-1-associated disease: evidence for oligoclonal expansion. *J. Virol. 70:843.*
27. Silins, S. L., S. M. Cross, S. L. Elliott, S. J. Pye, S. R. Burrows, J. M. Burrows, D. J. Moss, V. P. Argaet, and I. S. Misko. 1996. Development of Epstein-Barr virus-specific memory T cell receptor clonotypes in acute infectious mononucleosis. *J. Exp. Med. 184:1815.*
28. Batliwalla, F., J. Monteiro, D. Serrano, and P. K. Gregersen. 1996. Oligoclonality of $CD8^+$ T cells in health and disease: ageing, infection, or immune regulation? *Hum. Immunol. 48:68.*
29. Fitzgerald, J. E., N. S. Ricalton, A. C. Meyer, S. G. West, H. Kaplan, C. Behrendt, and B. L. Kotzin. 1995. Analysis of clonal $CD8^+$ T cell expansions in normal individuals and patients with rheumatoid arthritis. *J. Immunol. 154:3538.*
30. Hingorani, R., I. H. Choi, P. Akolkar, B. Gulwani Akolkar, R. Pergolizzi, J. Silver, and P. K. Gregersen. 1993. Clonal predominance of T cell receptors within the $CD8^+$ $CD45RO^+$ subset in normal human subjects. *J. Immunol. 151:5762.*
31. Maryanski, J. L., C. V. Jongeneel, P. Bucher, J. L. Casanova, and P. R. Walker. 1996. Single-cell PCR analysis of TCR repertoires selected by antigen in vivo: a high magnitude CD8 response is comprised of very few clones. *Immunity 4:47.*
32. Monteiro, J., R. Hingorani, I. H. Choi, J. Silver, R. Pergolizzi, and P. K. Gregersen. 1995. Oligoclonality in the human $CD8^+$ T cell repertoire in normal subjects and monozygotic twins: implications for studies of infectious and autoimmune diseases. *Mol. Med. 1:614.*
33. Morley, J. K., F. M. Batliwalla, R. Hingorani, and P. K. Gregersen. 1995. Oligoclonal $CD8^+$ T cells are preferentially expanded in the $CD57^+$ subset. *J. Immunol. 154:6182.*
34. Posnett, D. N., R. Sinha, S. Kabak, and C. Russo. 1994. Clonal populations of T cells in normal elderly humans: the T cell equivalent to "benign monoclonal gammapathy" [published erratum appears in J Exp Med 1994 Mar 1;179(3): 1077]. *J. Exp. Med. 179:609.*
35. Schwab, R., P. Szabo, J. S. Manavalan, M. E. Weksler, D. N. Posnett, C. Pannetier, P. Kourilsky, and J. Even. 1997. Expanded $CD4^+$ and $CD8^+$ T cell clones in elderly humans. *J. Immunol. 158:4493.*
36. Wang, E. C., P. A. Moss, P. Frodsham, P. J. Lehner, J. I. Bell, and L. K. Borysiewicz. 1995. $CD8^{high}CD57^+$ T lymphocytes in normal, healthy individuals are oligoclonal and respond to human cytomegalovirus. *J. Immunol. 155:5046.*
37. Murali-Krishna, K., J. D. Altman, M. Suresh, D. J. Sourdive, A. J. Zajac, J. D. Miller, J. Slansky, and R. Ahmed. 1998. Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. *Immunity 8:177.*
38. Gomez, A., I. Bourgault, E. Gomard, F. Picard, and J. P. Levy. 1989. Role of different lymphocyte subsets in human anti-viral T cell cultures. *Cell. Immunol. 118:312.*
39. Fiorentino, S., M. Dalod, D. Olive, J. G. Guillet, and E. Gomard. 1996. Predominant involvement of $CD8^+$ $CD28^-$ lymphocytes in human immunodeficiency virus-specific cytotoxic activity. *J. Virol. 70:2022.*
40. Boudet, F., H. Lecoeur, and M. L. Gougeon. 1996. Apoptosis associated with ex vivo down-regulation of Bcl-2 and up-regulation of Fas in potential cytotoxic $CD8^+$ T lymphocytes during HIV infection. *J. Immunol. 156:2282.*
41. DeBenedette, M. A., A. Shahinian, T. W. Mak, and T. H. Watts. 1997. Costimulation of $CD28^-$ T lymphocytes by 4–1BB ligand. *J. Immunol. 158:551.*