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Identification of Naive or Antigen-Experienced Human CD8$^+$ T Cells by Expression of Costimulation and Chemokine Receptors: Analysis of the Human Cytomegalovirus-Specific CD8$^+$ T Cell Response[1]

Mark R. Wills,[2] Georgina Okecha, Michael P. Weekes, Maher K. Gandhi, Patrick J. G. Sissons, and Andrew J. Carmichael

Human CMV (HCMV) infection provides an informative model of how long term human CD8$^+$ T cell memory is maintained in the presence of Ag. To clarify the phenotypic identity of Ag-experienced human CD8$^+$ T cells in vivo, we determined the expression of costimulation and chemokine receptors on Ag-specific CD8$^+$ T cells by quantifying individual virus-specific clones in different cell populations using TCR clonotypic probing. In healthy HCMV carriers, expanded CD8$^+$ clones specific for either HCMV tegument protein pp65 or immediate-early protein IE72 are found in both CD45RO^{high} cells and the subpopulation of CD45RA^{high} cells that lack the costimulatory molecule CD28. In contrast to previous suggested models of CD8$^+$ T cell memory, we found that in healthy virus carriers highly purified CD28$^-$CD45RA^{high}CCR7$^-$ cells are not terminally differentiated, because following stimulation in vitro with specific HCMV peptide these cells underwent sustained clonal proliferation, up-regulated CD45RO and CCR5, and showed strong peptide-specific cytotoxic activity. In an individual with acute primary HCMV infection, HCMV pp65-specific CD8$^+$ T cells are predominantly CD28$^-$CD45RO^{high}CCR7$^-$. During convalescence, an increasing proportion of pp65-specific CD8$^+$ T cells were CD28$^-$CD45RA^{high}CCR7$^-$. We conclude that naive human CD8$^+$ T cells are CD28$^+$CD45RA^{high}, express CCR7 but not CCR6, and are predominantly CD27$^+$ and L-selectin CD62 ligand-positive. The phenotype CD27$^+$CD45RA^{high} should not be used to identify naive human CD8$^+$ T cells, because CD27$^+$CD45RA^{high} cells also contain a significant subpopulation of CD28$^-$CD27$^+$ Ag-experienced expanded clones. Thus CD8$^+$ T cell memory to HCMV is maintained by cells of expanded HCMV-specific clones that show heterogeneity of activation state and costimulation molecular expression within both CD45RO^{high} and CD28$^-$CD45RA^{high} T cell pools. ***The Journal of Immunology,*** **2002, 168: 5455–5464.**

Following primary infection, the β-herpesvirus human CMV (HCMV)[3] establishes life-long infection with viral latency in cells of the myeloid lineage and intermittent shedding of infectious virions from mucosal surfaces. T cells play a crucial role in the control of HCMV; in HCMV-infected individuals, impairment of T cell responses (e.g., in advanced HIV infection or in allograft recipients who receive immunosuppressive drugs) is frequently followed by uncontrolled HCMV reactivation that leads to serious disease. HCMV provides an informative model of how long-term human CD8$^+$ T cell memory is maintained in presence of persistent viral Ag. Strong virus-specific CD8$^+$ T cell responses develop during primary infection and are maintained indefinitely. Healthy HCMV carriers have large populations of circulating HCMV-specific CD8$^+$ T cells, many of which recognize peptides derived from the virus structural protein pp65 (product of the *UL83* gene) (1) or the major immediate-early (IE) proteins expressed in infected cells (2). The CD8$^+$ T cell response against a defined HCMV peptide is typically dominated by relatively few individual clones that are greatly expanded and maintained in PBMC for long periods (3).

To understand the mechanisms by which T cell memory is generated and maintained, there is much interest in using the cell surface phenotype to distinguish between naive cells and Ag-experienced cells. For human T cells, it was initially proposed that expression of the high m.w. isoform of leukocyte common Ag, CD45RA, identified naive cells, whereas the low m.w. isoform, CD45RO, identified Ag-experienced cells (4, 5). However, analysis of the phenotypic distribution of Ag-specific CD8$^+$ T cells in virus carriers using either TCR clonotypic probing or class I MHC tetramers incorporating HCMV or EBV peptides has clearly demonstrated that these Ag-specific cells are distributed in both the CD45RO^{high} and CD45RA^{high} populations (6, 7). In long-term HCMV carriers, cells of an individual HCMV-specific CD8$^+$ T cell clone are present in both the CD45RA^{high} and CD45RO^{high} populations; the CD45RA^{high} population contributes 6- to 10-fold more than the CD45RO^{high} population to the total clone size in PBMC (8). The pool of CD45RA^{high} CD8$^+$ T cells thus contains a mixture of naive cells and Ag-experienced cells. Hamman et al. (9) have proposed that CD45RA^{high} cells, which express the TNFR family member CD27, represent naive cells, and that CD45RA^{high}CD27$^-$ cells, which have high levels of preformed perforin and shortened telomere length, are a terminally differentiated effector population. More recently, Sallusto et al. (10) described a subpopulation of CD45RA^{high}CD8$^+$ T cells that express the chemokine receptor CCR7, which favors homing to lymph

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[3] Abbreviations used in this paper: HCMV, human CMV; CD62L, CD62 ligand; IE, immediate-early; MIP-3β, macrophage inflammatory protein-3β; TC, TriColor.

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nodes through interaction with secondary lymphoid chemokine expressed on endothelial cells; these cells lack perforin, but upon stimulation produce large amounts of IL-2, but not IFN-γ. In contrast, CD45RA^{high}CCR7$^-$ cells express perforin and upon stimulation produce little IL-2 (10). The CD45RA^{high}CD27$^-$ cells characterized by Hamman et al. (9) appear to be a subpopulation within the CD45RA^{high}CCR7$^-$ cells.

Using MHC class I tetramers incorporating peptides of HIV or HCMV to identify Ag-specific CD8$^+$ T cells, Champagne et al. (11) found that tetramer-positive cells were present in both the CD45RA^{high}CCR7$^+$ and CD45RA^{high}CCR7$^-$ subpopulations of CD8$^+$ T cells and suggested that CD45RA^{high}CCR7$^-$ cells might be terminally differentiated effector cells. Faint et al. (12) reported that CD45RA^{high}CD8$^+$ T cells show a bimodal distribution of CD11a expression, and Ag-specific CD8$^+$ T cells (identified by MHC class I tetramers incorporating peptides of HCMV or EBV) were found within the CD11a^{high} subpopulation. They proposed that CD45RA^{high}CD11a^{low} cells are naive cells, and that CD45RA^{high}CD11a^{high} cells are a subset of Ag-experienced cells (12). Circulating melanoma specific CD8$^+$ T cells have been described in patients with metastatic melanoma; MART-1-specific cells are predominantly CD45RO$^+$, whereas tyrosinase-specific cells are predominantly CD45RA$^+$ and express perforin, but appear to have impaired effector function in vivo (13, 14).

In this study, we found that expression of the costimulatory molecule CD28 distinguishes two populations within the CD45RA^{high} CD8$^+$ T cell pool, and we used peptide-MHC tetramers and TCR clonotypic analysis to determine the distribution of virus-specific clones between the CD28$^+$CD45RA^{high} and CD28$^-$CD45RA^{high} subpopulations. We used four-color flow cytometry to analyze the expression of chemokine receptors and adhesion and costimulation molecules on the CD28$^+$CD45RA^{high}, CD28$^-$CD45RA^{high}, CD28$^+$CD45RO^{high}, and CD28$^-$CD45RO^{high} subpopulations. To determine whether CD8$^+$CD45RA^{high}CCR7$^-$ cells are terminally differentiated effector cells, we assessed the ability of purified CD8$^+$CD45RO^{high} and CD8$^+$CD45RA^{high}CCR7$^-$ cells to respond to stimulation with specific viral peptide and observed strong proliferative and peptide-specific cytotoxic responses. Our results show that CD8$^+$ T cell memory to HCMV is maintained by cells that show heterogeneity of activation state and costimulation molecule expression and are found within both the CD45RO^{high} and CD28$^-$CD45RA^{high} T cell pools.

Materials and Methods

Donors

Seven healthy HCMV seropositive laboratory donors were studied, all of whom were also EBV seropositive. In addition, two further EBV seropositive laboratory donors were studied. One patient with primary HCMV infection was also studied, in whom the diagnosis was confirmed serologically by detection of HCMV-specific IgM and subsequent isotype switching to HCMV-specific IgG (Public Health Laboratory Service, Addenbrookes Hospital, Cambridge, U.K.).

Viral peptides

The following peptides of HCMV pp65 were used: NLVPMVATV (aa 495–503), restricted through HLA-A2; TPRVTGGGAM (aa 417–426), restricted through HLA-B7; EFFWDANDIY (aa 511–525), restricted through HLA-B44; and VFPTKDVAL (aa 187–195), restricted through HLA-B35 (all supplied by Affiniti Research Products (Exeter, U.K.); >95% pure by HPLC). Peptides of HCMV IE72 were: CRVLCCYVL (aa 309–317), restricted through HLA-B7; and DELRRKMMYM (aa 198–207), restricted through HLA-B8 (2) (both gifts from Dr. F. Kern, Charitie, Humbolt University, Berlin, Germany). Peptides of EBV EBNA3C were: EENLLDFVRF (aa 281–290), restricted through HLA-B44.02; and PQPRAPIRPIPT (aa 880–891), restricted through HLA-B7 (supplied by Affiniti Research Products). All peptides were dissolved in RPMI and used at a 40 μg/ml final concentration (Table I).

Table I. *Viral peptides and MHC class I restriction used in this study*

Viral Protein	Peptide	HLA Restriction
HCMV pp65	NLVPMVATV aa 495–503	A*0201
HCMV pp65	TPRVTGGGAM aa 417–426	B*0702
HCMV pp65	EFFWDANDIY aa 511–525	B*4402
HCMV pp65	VFPTKDVAL aa 187–195	B*3503
HCMV IE72	CRVLCCYVL aa 309–317	B*0702
HCMV IE72	DELRRKMMYM aa 198–207	B*0801
EBV EBNA3c	EENLLDFVRF aa 281–290	B*4402
EBV EBNA3c	PQPRAPIRPIPT aa 880–891	B*0702

mAbs and surface phenotype analysis

mAbs were specific for CD3, CD4, CD8, CD14, CD16, CD19, CD25, CD27, CD28, CD45RO, CD45RA, CD56, CD57, CD62 ligand (CD62L), and HLA-DR (TCS Biologicals, Burlingdale, CA); anti-TCR Vβ3, -11, -13.1, -14, and -17 (Immunotech); and anti-chemokine receptors CXCR4, CCR5, CCR6, and CCR7 (BD Biosciences, Oxford, U.K.). Abs were conjugated to FITC, PE, TriColor (TC), or allophycocyanin. Because the anti-CCR7 was an unconjugated IgM, cells were stained with anti-IgM biotin (BD PharMingen), washed, and then further stained with streptavidin-Red 670 (Life Technologies, Paisley, U.K.). Before immunostaining for four-color analysis, CD8$^+$ cells were enriched from fresh PBMC by incubating PBMC with anti-CD8 MACS beads (Miltenyi Biotec, Auburn, CA), followed by separation on a BS-positive selection column. Because the anti-CD8 MACS beads did not occupy all the CD8 sites on the cells, the enriched cells were restained with anti-CD8-allophycocyanin (FL4) so that only high-expressing CD8$^+$ T cells were gated for subsequent analysis. We also used an MHC class I peptide tetramer of HLA-B7 containing HCMV pp65 peptide TPRVTGGGAM (gift from Dr. J. Lipolis, National Institutes of Health Core Tetramer Facility, Atlanta, GA).

Purification of cell subpopulations

For clonotypic probing analysis, PBMC were stained with anti-CD8, anti-CD45RA, and anti-CD28 to sort the CD8$^+$CD45RA$^-$ (CD45RO cells), CD8$^+$CD28$^-$CD45RA^{high}, and CD8$^+$CD28$^+$CD45RA^{high} T cell subpopulations using a FACSVantage cell sorter (BD Biosciences). To obtain the CD28$^-$CD27$^+$ and CD28$^-$CD27$^-$ subpopulations of CD8$^+$ T cells, PBMC were first depleted of CD16$^+$ NK cells (by incubation with anti-CD16 IgM (Leu-11b; BD Biosciences) followed by complement) and depleted of CD4$^+$ cells with anti-CD4-conjugated MACS microbeads (Miltenyi Biotech). Cells were then stained with anti-CD28-FITC and anti-CD27-PE and sorted into CD28$^-$CD27$^-$ and CD28$^-$CD27$^+$ subpopulations by a FACSVantage cell sorter. The purity of lymphocyte populations prepared by FACSVantage cell sorting or MACS microbeads was always >98%.

For functional studies, CD8$^+$CD45RO^{high} and CD8$^+$CD45RA^{high} cells were prepared from PBMC using negative cell sorting; PBMC were stained with FITC-conjugated anti-CD4, anti-CD19, anti-CD16, and anti-CD56 (to remove CD4$^+$ T cells, B cells, and NK cells, respectively) and either anti-CD45RA or anti-CD45RO and sorted for nonstained cells using a FACS Vantage cell sorter, yielding CD8$^+$CD45RO^{high} and CD8$^+$CD45RA^{high} T cell populations, respectively. CD45RA^{high}CD28$^-$CCR7$^-$CD8$^+$ T cells were also prepared by negative cell sorting, following staining with FITC-conjugated anti-CD4, anti-CD19, anti-CD16, anti-CD56, anti-CD45RO, anti-CD28, and anti-CCR7. Aliquots of the negative selected cells were restained with anti-CD8, anti-CD45RA, and anti-CD45RO to confirm their purity, which was >99% for CD45RA^{high} cells and 98–99% for CD45RO^{high} cells. For functional studies, CD28$^-$ cells were prepared from PBMC by negative selection using anti-CD28-FITC, followed by anti-FITC MACS microbeads (purity of lymphocyte populations, >98%).

For the functional studies, purified subpopulations of cells were stimulated in vitro with irradiated autologous peptide-pulsed PBMC in RPMI plus 10% FCS and 10% human AB serum plus 5 IU/ml human rIL-2 (provided by the Medical Research Council Centralized Facility for AIDS Reagents, National Institute of Biological Standards and Control) and cultured for 14 days, followed by assay of peptide-specific cytotoxicity as previously described (1, 3) and/or analysis by flow cytometry.

Generation of T cell clones and determination of TCR β-chain hypervariable sequence

T cell clones were generated from single-cell cultures by limiting dilution analysis followed by recloning as previously described (3). Total RNA was extracted from each clone, and first-strand cDNA was derived from this before PCR using a panel of 36 TCR Vβ family specific primers together with the corresponding C region-specific primer (synthesized by Genosys, Cambridge, U.K.) as previously described (3). The amplified PCR product from the clonal Vβ amplification was purified (Qiagen, Valencia, CA) and sequenced by automated DNA sequencing (Department of Biochemistry, University of Cambridge, Cambridge, U.K.).

Quantitation of TCR clonotypes in phenotypically defined subpopulations

Complementary 15–20 mer oligonucleotide probes based on the TCR β-chain hypervariable region of immunodominant peptide-specific CTL clones were designed. Such probes are highly specific for individual CTL clonotypes (8). mRNA was extracted from purified subpopulations of cells, reverse transcribed into cDNA, and amplified in duplicate using TCR Vβ-specific PCR primers as described previously. A positive control sample from the original defined CTL clone and a negative control sample from the pooled PBMC of four HCMV- and HIV-seronegative donors were amplified simultaneously in duplicate using the same primers. Each PCR product was separated on an agarose gel and blotted onto a Zeta-probe nylon filter (Bio-Rad, Hercules, CA). After washing and prehybridization, the filter was incubated overnight with a γ-^{32}P end-labeled clonotypic probe in hybridization buffer. After washing, the amount of probe that had bound to each sample on the filter was quantitated using an Instant Imager (Beckman Coulter, Palo Alto, CA). The filter was stripped by soaking in 0.4 M NaOH, washed, and then rehybridized with a TCR β-chain constant region probe that detects all TCR sequences. In each subpopulation studied we calculated the relative abundance of the clonotype sequence as a proportion of all TCR sequences of the same Vβ family: relative abundance of clonotype sequence = 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))

In each purified subpopulation of cells, the clone size was calculated by multiplying the proportion of CD8$^+$ cells that had the corresponding Vβ segment (determined by flow cytometry) by the relative abundance of the clonotype sequence.

Results

HCMV-specific clones are abundant in CD28$^-$CD45RA^{high} cells, but not in CD28$^+$CD45RA^{high} cells

To determine the expression of CD28 within CD8$^+$CD45RA^{high} cells, PBMC from six HCMV-seropositive donors and two EBV-seropositive donors were stained with anti-CD8-allophycocyanin, anti-CD45RO-FITC, anti-CD45RA-TC, and anti-CD28-PE and analyzed by four-color flow cytometry. After gating on CD8$^+$ T cells, the relationship between the expression of CD45RA and CD28 for a single HCMV carrier is illustrated in Fig. 1*A*, in which four populations can be identified: 1) a circumscribed population that is CD28$^+$CD45RA^{high} (and CD45RB^{low} and CD45RO^{low}; data not shown), 2) a population that is CD28$^+$CD45RA$^{low/-}$CD45RO^{high}, 3) a small population that is CD28$^-$CD45RA$^-$CD45RO^{high}, and 4) a population that is CD28$^-$CD45RA^{high}CD45RO^{low}. The relative proportions of CD8$^+$ cells in each subpopulation varied little from subject to subject. To identify HCMV-specific cells, PBMC were also stained with anti-CD8-allophycocyanin, anti-CD45RA-FITC, anti-CD28-TC, and HLA-B7-HCMVpp65 peptide tetramer-PE. Fig. 1*B* shows the expression of CD28 and CD45RA after gating on tetramer-staining CD8$^+$ T cells. Tetramer-staining cells were predominantly CD28$^-$ and were almost entirely absent from the CD28$^+$CD45RA^{high} population.

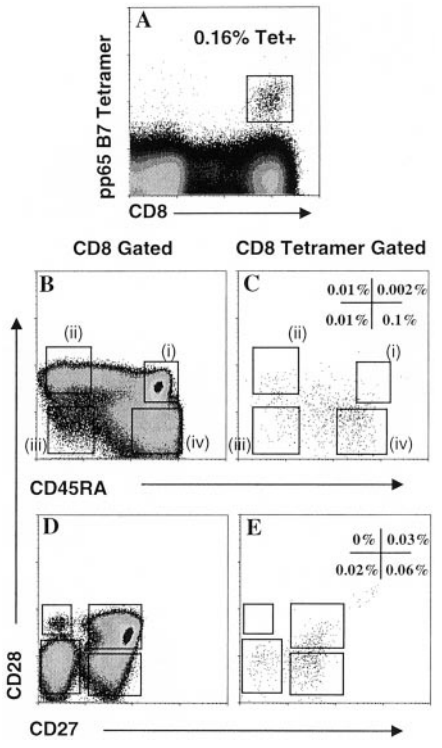

FIGURE 1. *A*, Expression of pp65 B7 tetramer. Expression of CD28 in relation to CD45RA and expression of CD28 in relation to CD27 gated on all CD8$^+$ T cells (*B* and *D*), or gated on HCMV pp65-specific CD8$^+$ T cells stained by HLA-B7-peptide tetramer (*D* and *E*) in PBMC of healthy HCMV carrier 009. The percentage of CD8$^+$ tetramer-positive cells in each region is shown.

We derived peptide-specific single-cell clones from each of the HCMV-seropositive donors, sequenced the hypervariable VDJ region of the TCR β-chain, and designed a clonotypic oligonucleotide probe for each immunodominant peptide-specific clonotype. Using quantitative clonotype probing, we determined the distribution of cells of each virus-specific clone within purified CD28$^-$CD45RA^{high} or CD28$^+$CD45RA^{high} subpopulations prepared from PBMC by cell sorting (Fig. 2). We calculated the number of cells of the clone in PBMC by multiplying the relative

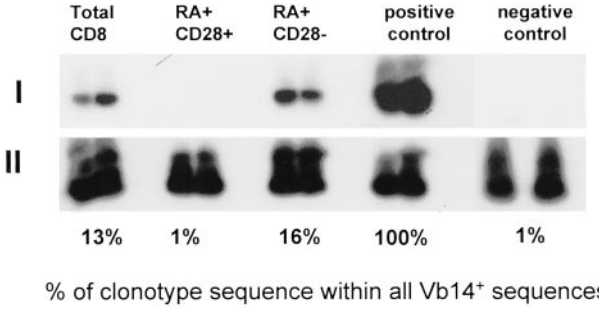

FIGURE 2. Oligonucleotide probing of a virus-specific clone in amplified cDNA from purified subpopulations of PBMC of healthy HCMV carrier 009. *I*, Probing with a labeled clonotypic probe specific for the hypervariable TCR β-chain sequence of a Vβ14$^+$ pp65 peptide-specific CD8$^+$ T cell clone. *II*, The filter was stripped and reprobed with a conserved constant region-specific probe that detects all amplified Vβ14$^+$ TCR sequences. The relative abundance of clonotype sequence as a proportion of all TCR sequences of the same Vβ14 family was calculated as described in the text.

Table II. *The percentage of clonotype sequence as a proportion of all TCR sequences of the same Vβ family in purified subpopulations of CD28$^-$CD45RA^{high} or CD28$^+$CD45RA^{high} cells*

Donor	Peptide	% Clonotype Sequence[a]			Clone Size Per Million Cells of Each Phenotype		Clone Size Per Million CD8$^+$ Cells in Peripheral Blood		
		Total CD8 (%)	CD28$^-$ CD45RA (%)	CD28$^+$ CD45RA (%)	CD28$^-$ CD45RA	CD28$^+$ CD45RA	Total CD8	CD28$^-$ CD45RA	CD28$^+$ CD45RA
009	HCMV pp65 (aa 417–426)	12	15	0	6170	0	6435	3320	0
	HCMV IE (aa 309–317)	27	21	0	5380	150	400	1480	40
013	HCMV IE (aa 198–207)	27	51	3	18960	530	8850	8040	200
017	HCMV pp65 (aa 187–195)	28	22	1	5500	10	9530	1610	4
018	HCMV pp65 (aa 265–278)	23	12	1	4100	270	14170	2330	150
022	EBV EBNA3c (aa 281–290)	22	40	2	22120	50	4645	4170	15

[a] The percentage clonotype sequence as a proportion of all TCR sequences of the same Vβ family in each purified subpopulation.

proportion of clonotype sequence within all TCR sequences of the same Vβ family by the proportion of CD8$^+$ cells that express the same Vβ segment determined by flow cytometry. As we had previously found, individual CTL clones that recognized HCMV peptides could be very large (Table II). Clones specific for either HCMV pp65 or IE72 were very abundant in the CD28$^-$ CD45RA^{high} subpopulation, but were almost entirely absent from the CD28$^+$CD45RA^{high} subpopulation (clonotype detection range, 0–3%; close to the limit of detection of the assay within the purity achieved by cell sorting). For comparison with another persistent herpesvirus, we also derived EBV peptide-specific CD8$^+$ CTL clones, designed clonotypic probes, and used these to determine the distribution of EBV-specific clones within the same purified T cell subpopulations. An HLA-B4402-restricted EBNA3C-specific clone from one subject was also very abundant in the CD45RO^{high} and the CD28$^-$CD45RA^{high} subpopulations, but not in the CD28$^+$CD45RA^{high} subpopulation (Table II). HLA-B7-restricted EBNA3C-specific clones from three other subjects were almost entirely distributed within CD45RO^{high} cells (data not shown).

HCMV-specific clonotypes are abundant in both CD28$^-$CD27$^+$ and CD28$^-$CD27$^-$ cells

Using four-color flow cytometry, we studied the expression of CD28, CD27, and CD45RA on peripheral blood CD8$^+$ T cells (Fig. 3). In six healthy HCMV carriers, the proportion of CD8$^+$ T cells that were CD28$^+$CD27$^+$ ranged from 37 to 66%, the proportion that were CD28$^+$CD27$^-$ ranged from 1 to 3%, the proportion that were CD28$^-$CD27$^+$ ranged from 9 to 26%, and the proportion that were CD28$^-$CD27$^-$ ranged from 24 to 38%. Thus, the vast majority of CD28$^+$CD8$^+$ cells were also CD27$^+$; the CD28$^+$CD27$^-$ population was always small. Among CD27$^+$ cells, the level of expression of CD27 on CD28$^+$ cells was generally higher than that on CD28$^-$ cells. We analyzed the expression of CD45RA, CCR7, and CD62L after gating on either CD8$^+$CD28$^+$ or CD8$^+$CD28$^-$ cells. The CD8$^+$CD28$^-$CD27$^+$ cells were predominantly CD45RA^{high}, were almost all CCR7$^-$, and were 25–65% CD62L$^+$ (data not shown). The CD8$^+$CD28$^-$CD27$^-$ cells were also predominantly CD45RA^{high} (Fig. 3), were almost all CCR7$^-$, and were 10–55% CD62L$^+$ (data not shown).

We examined the distribution of individual clonotypes in the CD28$^-$CD27$^-$ and CD28$^-$CD27$^+$ subpopulations of CD8$^+$ T cells. Virus-specific clonotype sequences often accounted for a large proportion of all TCR sequences of the same Vβ family (Fig. 4). For most clones, cells of the clone were distributed in both the CD28$^-$CD27$^-$ and CD28$^-$CD27$^+$ subpopulations, and in general, the clone size in 10^6 cells of each subpopulation was greater in the CD28$^-$CD27$^+$ subpopulation than in the CD28$^-$CD27$^-$ subpopulation (Table III). In healthy HCMV carrier 017, an individual HCMV-specific clone 17A was almost entirely partitioned in the CD28$^-$CD27$^-$ subpopulation, in which it comprised 38–48% of Vβ17$^+$ sequences; in the CD28$^-$CD27$^+$ population, this clonotype was consistently very low, close to the limit of detection of the assay (determined by the binding of the clonotypic probe to the negative control sample; Fig. 4*B*). When purified CD28$^-$ PBMC from this donor were stimulated in vitro with irradiated

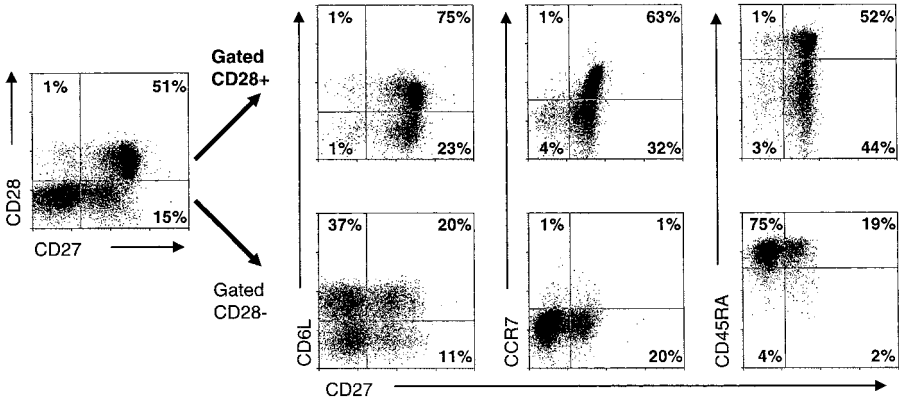

FIGURE 3. Expression of CD27 in relation to CD62L, CCR7, or CD45RA gated on either CD28$^+$CD8$^+$ T cells or CD28$^-$CD8$^+$ T cells in PBMC of healthy HCMV carrier 013.

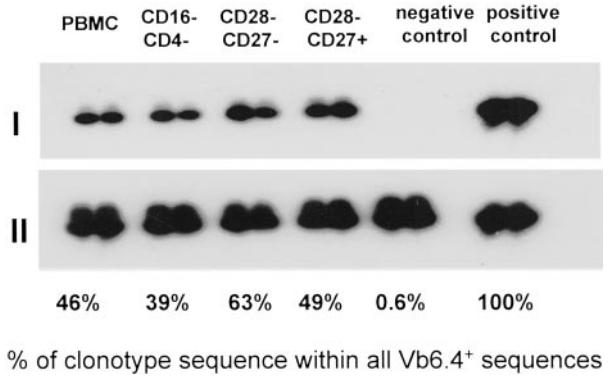

FIGURE 4. Oligonucleotide probing of a virus-specific clone in amplified cDNA from purified subpopulations of PBMC. *I*, Probing with a labeled clonotypic probe specific for the hypervariable TCR β-chain sequence of a Vβ6.4$^+$ pp65 peptide aa 511–525-specific CD8$^+$ T cell clone of healthy HCMV carrier 011. *II*, The filter was stripped and reprobed with a conserved constant region-specific probe that detects all amplified Vβ6.4$^+$ TCR sequences.

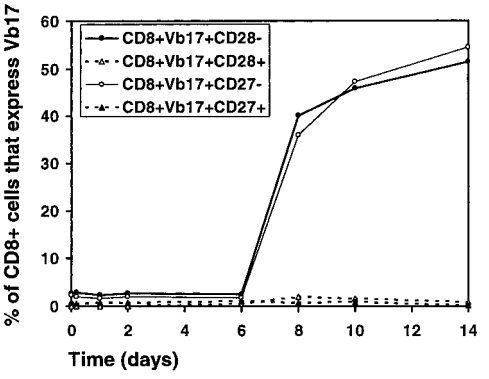

FIGURE 5. Kinetics of proliferation of CD28$^-$CD27$^-$Vβ17$^+$ CD8$^+$ T cells following in vitro stimulation of CD28$^-$ PBMC from healthy HCMV carrier 017 with specific HCMV pp65 peptide aa 187–195 (representative of two independent experiments).

autologous peptide-pulsed PBMC and cultured for 14 days in the presence of exogenous IL-2, there was a strong proliferative response of Vβ17$^+$CD8$^+$ T cells that remained CD28$^-$ and CD27$^-$ (Fig. 5).

Distinctive patterns of chemokine receptor expression on CD28$^+$CD45RA^{high} and CD28$^-$CD45RA^{high} subpopulations of CD8$^+$ T cells

Recirculation of T cells among blood, secondary lymphoid tissue, and peripheral tissues is an essential part of anti-viral immune surveillance. The complex trafficking of lymphocytes is partly regulated by specific chemokines that bind to chemokine receptors expressed on T cells, and memory and naive T cells would be expected to express distinctive patterns of chemokine receptors. We used four-color flow cytometry to analyze the chemokine receptor expression on CD8$^+$CD28$^+$CD45RA^{high}, CD8$^+$CD28$^+$CD45RO^{high}, CD8$^+$CD28$^-$CD45RO^{high}, and CD8$^+$CD28$^-$CD45RA^{high} T cells in PBMC derived from six donors, four of whom were both HCMV-seropositive and EBV-seropositive, using Abs against CCR5, CCR6, CCR7, and CXCR4 shown for a representative donor (Fig. 6). For each of the four T cell subpopulations, the results were generally consistent between individual donors and between the HCMV-seropositive and HCMV-seronegative donors (Table IV).

CD8$^+$CD28$^+$CD45RA^{high} cells were the most homogeneous population, whereas CD8$^+$CD28$^+$CD45RO^{high} T cells showed bimodal distributions of expression of CCR5, CCR6, CCR7, and CD62L; this heterogeneity of expression of different surface receptors could potentially give rise to a very complex repertoire of chemokine receptor expression among individual CD8$^+$CD28$^+$CD45RO^{high} T cells. CD8$^+$CD28$^-$CD45RA^{high} cells showed variable expression of CXCR4 expression between donors, although the absolute level of CXCR4 expression on CD8$^+$CD28$^-$CD45RA^{high} cells was low compared with that in the other subpopulations. Many CD8$^+$CD28$^-$CD45RA^{high} cells expressed CD57; the expression of CD57 was consistently higher in the HCMV-seropositive donors (66–76%) compared with the HCMV-seronegative donors (13–20%).

Table III. *The percentage of clonotype sequence as a proportion of all TCR sequences of the same Vβ family in purified subpopulations of CD28$^-$CD27$^-$ and CD28$^-$CD27$^+$ cells*

Donor	Peptide	Clonotype		Date of Sample	% Clonotype Sequence[a]			Clone Size Per Million CD8$^+$ Cells of Each Phenotype		Clone Size Per Million CD8$^+$ Cells in Peripheral Blood		
					Total CD8$^+$ cells (%)	Sorted cells		Sorted cells		Total CD8$^+$ cells	Sorted cells	
						CD28$^-$ CD27$^-$ (%)	CD28$^-$ CD27$^+$ (%)	CD28$^-$ CD27$^-$	CD28$^-$ CD27$^+$		CD28$^-$ CD27$^-$	CD28$^-$ CD27$^+$
009	HCMV pp65 (aa 417–426)	09A	Vβ14	07/99	10	9	9	3,200	6,300	7,200	1,000	1,500
011	HCMV pp65 (aa 495–503)	11A	Vβ17	07/99	20	73	60	20,000	35,000	12,000	4,800	3,200
	HCMV pp65 (aa 511–525)	11B	Vβ6.4	07/99	38	62	48	ND	ND	ND	ND	ND
017	HCMV pp65 (aa 187–195)	17A	Vβ17	08/99	12	47	0.5	15,000	220	6,000	4,000	20
				03/00	9	38	1.0	16,000	10	4,400	3,900	1
018	HCMV pp65 (aa 495–503)	18A	Vβ13.1	04/00	0.6	1.3	4	160	330	90	60	60
	HCMV pp65 (aa 265–274)	18D	Vβ5.1	04/00	15	6	44	3,500	9,500	6,200	1,300	1,700

[a] The percentage clonotype sequence as a proportion of all TCR sequences of the same Vβ family in each purified subpopulation.

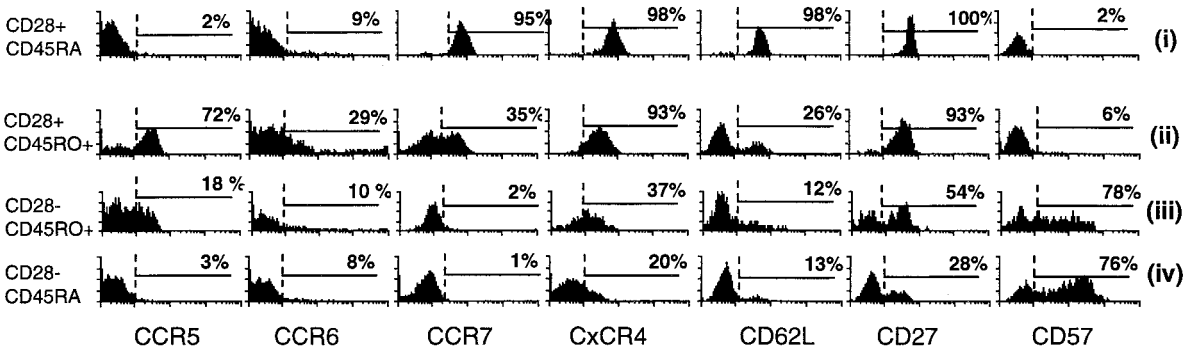

FIGURE 6. Expression of chemokine receptors, CD62L, CD27, and CD57 on the four subpopulations of $CD8^+$ T cells $CD28^+CD45RA^{high}$, $CD28^+CD45RO^{high}$, $CD28^-CD45RO^{high}$, and $CD28^-CD45RA^{high}$ in PBMC of healthy HCMV carrier 009.

In healthy virus carriers, $CD8^+CD28^-CD45RA^{high}CCR7^-$ cells are not terminally differentiated

We have previously shown that both the $CD45RO^{high}$ and $CD45RA^{high}CD8^+$ T cell populations can respond to defined HCMV peptides by proliferation and differentiation into peptide-specific cytotoxic effector T cells that are $CD45RO^{high}CD45RA^{low}$ (8). On the basis of short term culture it has recently been suggested that $CD8^+CD45RA^+CCR7^-$ cells might be terminally differentiated effector cells (11). To test this hypothesis, we purified $CD8^+CD28^-CD45RA^{high}CCR7^-$ cells by negative selection to avoid ligation of cell surface CD8 or CD45RA, and as a positive control we also purified $CD8^+CD45RO^{high}$ cells. Each purified population of cells was stimulated in vitro with irradiated autologous peptide-pulsed PBMC and cultured for 14 days in the presence of exogenous IL-2, followed by assay of peptide-specific cytotoxicity and analysis of surface expression of CD28, CD45RA, CD45RO, CCR5, CCR6, and CCR7 by flow cytometry.

As expected, following stimulation with peptide the $CD8^+CD45RO^{high}$ cells proliferated, generating a large population of tetramer-positive effector cells that up-regulated CD45RO and showed strong peptide-specific cytotoxicity. These effector cells showed varying levels of CD28 expression, but were $CCR5^{high}$, $CCR6^-$, and $CCR7^-$ (Fig. 7). Following stimulation with peptide, purified $CD8^+CD28^-CD45RA^{high}CCR7^-$ cells also proliferated and differentiated into peptide-specific cytotoxic effector cells. These effector cells down-regulated CD45RA, up-regulated CD45RO, and remained $CD28^-$; they were also $CCR5^{high}$, $CCR6^-$, and $CCR7^-$.

During acute primary HCMV infection, most pp65-specific $CD8^+$ T cells are $CD28^-CD45RO^+CCR7^-$ and during convalescence they revert to $CD28^-CD45RA^-CCR7^-$

During acute primary HCMV infection in a single subject we detected activated pp65 peptide-specific cytotoxic $CD8^+$ T cells in unstimulated PBMC; at a standard E:T cell ratio, the magnitude of peptide-specific cytotoxicity was greatest at the peak of symptoms (3 wk after the onset of symptoms) and diminished to low levels by 8 wk after the onset of symptoms (8). We analyzed the expression of CD28 and CD45RO on tetramer-positive $CD8^+$ T cells in cryopreserved cells obtained from the same subject during and after acute primary HCMV infection. At the peak of symptoms (3 wk after the onset of symptoms) 80% of the tetramer-positive $CD8^+$ cells were $CD28^-CD45RO^+$ and $CCR7^-$ (Fig. 8). During convalescence there was a progressive expansion of the $CD28^-CD45RA^{high}CCR7^-$ tetramer-positive population, which may reflect apoptotic death of some highly activated $CD28^-CD45RO^+$ cells and/or redistribution of $CD28^-CD45RA^{high}$ cells from inflamed tissues to the circulation after resolution of acute disseminated HCMV infection. These findings are in agreement with our previous observation in this subject that cells of an immunodominant pp65-specific $CD8^+$ clone were initially abundant in $CD45RO^+$ cells, but became progressively enriched in $CD45RA^+$ cells during convalescence, consistent with clonal reversion from $CD45RO^+$ to $CD45RA^+$ with time (8).

Discussion

We found that in healthy HCMV carriers, expanded HCMV-specific $CD8^+$ T cell clones are distributed in both $CD45RO^{high}$ and $CD28^-CD45RA^{high}$ populations and are abundant in both $CD27^+CD28^-$ and $CD27^-CD28^-$ cells. Neither HCMV-specific nor EBV-specific clones are found in the $CD28^+CD45RA^{high}$ T cell population. We found that in healthy virus carriers, highly purified $CD28^-CD45RA^{high}CCR7^-$ cells are not terminally differentiated, because following stimulation in vitro with specific HCMV peptide these cells underwent sustained clonal proliferation, up-regulated CD45RO and CCR5, and showed strong peptide-specific cytotoxic activity. In an individual with acute primary HCMV infection, pp65-specific $CD8^+$ T cells were predominantly

Table IV. *The proportion of $CD8^+$ cells in each subpopulation that express CCR5, CCR6, CCR7, CXCR4, CD62L, CD27, or CD57*[a]

Cell Population	% of Positive Cells in Each Subpopulation						
	CCR5	CCR6	CCR7	CXCR4	CD62L	CD27	CD57
$CD28^+CD45RA^{high}$	1–3	7–10	95–99	97–99	91–98	98–100	1–4
$CD28^+CD45RO^{high}$	52–79	29–54	35–42	92–98	18–31	85–96	1–8
$CD28^-CD45RO^{high}$	12–18	10–12	2–4	37–58	12–31	47–54	66–78
$CD28^-CD45RA^{high}$	1–3	1–12	1–3	2–58	13–60	2–28	13–76

[a] Range of values observed in seven healthy HCMV carriers.

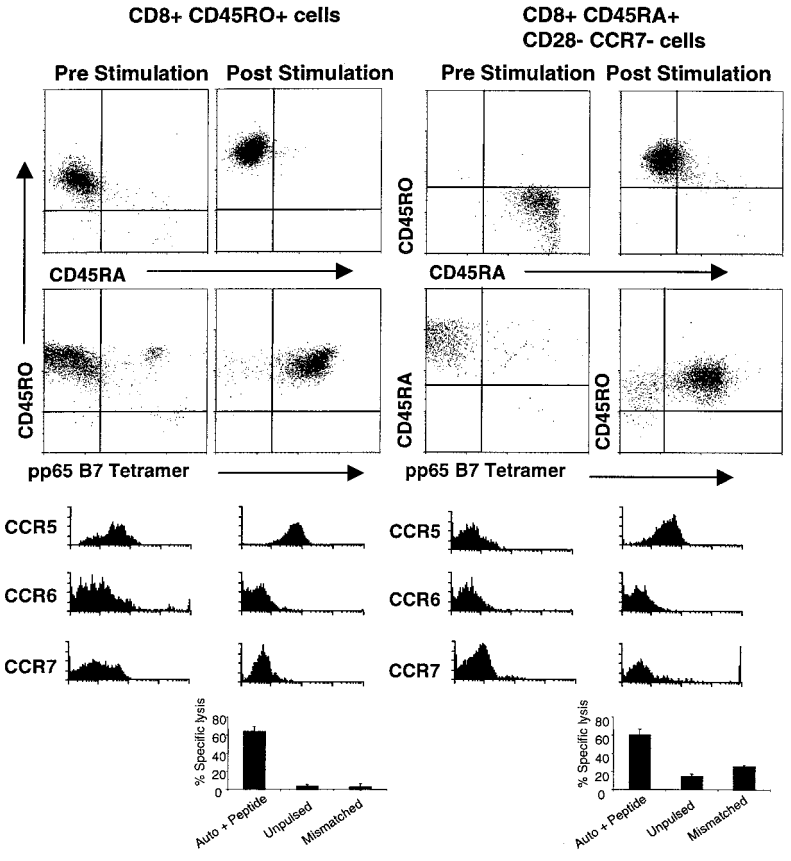

FIGURE 7. In vitro stimulation of purified $CD8^+$ $CD45RO^+$ (*left panels*) or $CD8^+CD28^-CD45RA^+CCR7^-$ cells (*right panels*) with autologous irradiated peptide-pulsed PBMC of healthy HCMV carrier 013 leads to peptide-specific proliferation and differentiation. Expression of CD45 isoforms and chemokine receptors and HCMV pp65-specific tetramer staining are shown gated on $CD8^+$ T cells before and 14 days after stimulation. Peptide-specific cytotoxic activity was also assayed 14 days after stimulation at an E:T cell ratio of 10:1 against ^{51}Cr-labeled autologous or MHC mismatched lymphoblastoid target cells pulsed with HCMV pp65 peptide or unpulsed.

$CD28^-CD45RO^{high}$ and almost entirely lacked expression of CCR7; during convalescence, an increasing proportion of pp65-specific $CD8^+$ T cells were $CD28^-CD45RA^{high}CCR7^-$.

The current study adds to the evidence that the cells of a single expanded Ag-experienced $CD8^+$ T cell clone are very heterogeneous in phenotype in vivo, reflecting many states of activation/differentiation (3, 8). Naive cells are $CD28^+CD45RA^{high}CCR7^+$ $CD62L^+CD27^+$ and $CD11a^{low}$. Following activation by Ag, all daughter cells permanently up-regulate CD11a (proposed in Ref. 12) and up-regulate CD45RO, at least initially. Some daughter cells remain $CD28^+CD45RO^+CCR7^+$; these cells have been termed central memory cells (10). $CD45RO^+CCR7^-$ cells have been termed effector memory cells (10). Our results show that these cells are, in fact, made up of two subpopulations of daughter cells, namely $CD28^+CD45RO^+CCR7^-$ and $CD28^-CD45RO^+$ $CCR7^-$, that also differ in the expression of CD57. The precise relationship among these three different populations of $CD45RO^+$ cells requires further study; it is unclear whether $CD28^-$ $CD45RO^+$ cells can arise directly from activated naive cells or via intermediate $CD28^+CD45RO^+$ cells. $CD28^-CD45RO^+CCR7^-$ cells predominate in peripheral blood during acute primary HCMV infection and revert to $CD28^-CD45RA^+CCR7^-$ cells in convalescence; on $CD28^-CD45RA^+$ cells, the pattern of surface expression of a wide range of chemokine receptors and adhesion molecules closely resembles that on $CD28^-CD45RO^+$ cells. The factors that lead to the transition from $CD28^-CD45RO^+$ to $CD28^-CD45RA^+$ are not yet understood. Our results show that when $CD28^-CD45RA^+CCR7^-$ cells are activated by peptide Ag in vitro, they proliferate, remain $CD28^-$, and up-regulate CD45RO and CCR5. The circulating $CD28^-CD45RO^+CCR7^-$ population, some of which express CCR5, is relatively rich in HCMV tetramer-positive cells (Fig. 1) and may include cells that have recently been activated by exposure to HCMV Ag in vivo.

The generation of phenotypic diversity within the clonal progeny of a single virus-specific cell may be an instructive process as a result of differences in the activation state of APC to which the naive cell and later daughter cells are exposed (15, 16) and differences in the cytokine milieu during their activation and differentiation (17). An alternative possibility is that activation of a single $CD8^+$ T cell might give rise to daughter cells of different phenotypes by a stochastic process. In either case, the diversity of phenotypes may be modified by subsequent selection of those daughter cells whose activation state and/or homing pathway are best suited to control the virus at a given site. In the case of a persistent virus that infects cells in different tissues, we speculate that those viral Ags expressed in nonlymphoid tissues such as the intestine or respiratory tract may evoke specific $CD8^+$ T cells that have a different phenotype and pathway of recirculation compared with other viral Ags expressed in lymphoid tissues. Whereas $CD8^+$ T cell clones specific for the HCMV structural protein pp65 are abundant in $CD28^-CD45RA^{high}$ cells, it is interesting that in the same donor the phenotype of expanded $CD8^+$ T cell clones specific for EBV EBNA3C expressed in latently infected B cells was dominated by $CD45RO^+$ cells and included very few $CD28^-CD45RA^{high}$ cells.

Our finding of the same clonotype in both $CD28^-CD27^+$ and $CD28^-CD27^-$ subpopulations confirms that these subsets represent different activation states of the same lineage (18). Loss of CD27 expression on $CD8^+$ T cells is associated with increased expression of perforin and granzyme B and cytotoxic activity in the absence of prior restimulation in vitro. It has been suggested that these $CD27^-$ cells might be terminally differentiated and possibly incapable of further cell division (18). Our results (Fig. 5) show that following stimulation with specific peptide, the $CD28^-CD27^-$ population contains cells that are capable of sustained proliferation in vitro; the ability of $CD28^-CD27^-$ cells to proliferate

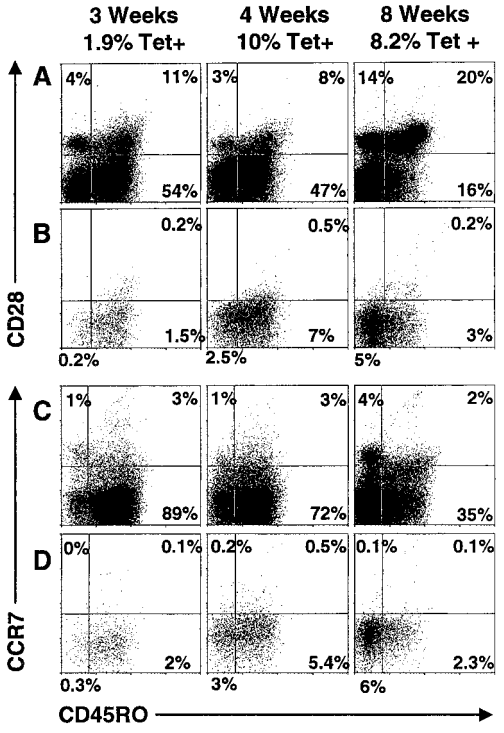

FIGURE 8. In acute primary HCMV infection, most circulating HCMV pp65-specific CD8$^+$ T cells are CD28$^-$CD45RO$^+$ and, during convalescence, they revert to CD28$^-$CD45RA$^+$. PBMC obtained at 3, 4, and 8 wk after the onset of symptoms were analyzed by four-color flow cytometry. The expression of CD28 in relation to CD45RO, the percentage of CD8$^+$ cells in each quadrant (*A*), and the percentage of CD8$^+$ pp65 B7 tetramer-positive cells in each quadrant (*B*), the expression of CCR7 in relation to CD45RO, the percentage of CD8$^+$ cells in each quadrant (*C*), and the percentage of CD8$^+$ pp65 B7-tetramer positive cells in each quadrant (*D*) are shown.

in vitro may depend on the specific experimental conditions used, in particular the provision of autologous APC and exogenous IL-2 (19). It has previously been suggested that the CD27$^+$CD45RA^{high} population of human CD8$^+$ T cells may represent naive cells (9). However, we found that expanded pp65-specific clones were abundant in both CD27$^+$CD28$^-$ and CD27$^-$CD28$^-$ cells. Thus, for human CD8$^+$ T cells the phenotype CD27$^+$CD45RA^{high} should not be used to identify naive cells; CD27$^+$CD45RA^{high} cells do contain naive cells, but also contain a significant subpopulation of CD28$^-$CD27$^+$ Ag-experienced expanded clones. In general, among CD8$^+$ T cells that express CD27, those cells that are CD28$^+$ express a higher level of CD27 compared with CD28$^-$ cells (Fig. 3). Our results are consistent with those of Kern et al. (20), who demonstrated that the CD27^{int} population contained many more HCMV-specific cells than the CD27^{high} population. In the paper that proposed that CD27$^+$CD45RA^{high} cells might represent naive CD8$^+$ T cells, the CD27$^+$CD45RA^{high} population did, in fact, contain a significant population of CD27^{int} cells that stained positively for intracellular perforin and granzyme B (Fig. 6 of Ref. 9), which probably correspond to the Ag-experienced CD27$^+$CD28$^-$ clones we describe.

Our results disagree with the recent suggestion that CD45RA^{high} CCR7$^-$ cells might be terminally differentiated effector cells (11). Champagne et al. (11) reported that CD45RA$^+$CCR7$^-$ cells failed to proliferate in response to stimulation with a combination of anti-CD3 and anti-CD28; this is not surprising, because as we have shown almost all CD45RA$^+$CCR7$^-$ cells lack the expression of CD28. In contrast, when we stimulated purified CD28$^-$ CD45RA$^+$CCR7$^-$ cells with fresh autologous peptide-pulsed APC, we observed strong proliferative responses accompanied by up-regulation of CD45RO. This difference in functional response is probably due to experimental conditions. We used fresh CD28$^-$CD45RA$^+$CCR7$^-$ cells and studied the response over 14 days because of the kinetics of proliferation of CD28$^-$ cells in response to peptide stimulation (Fig. 5), whereas in equivalent experiments Champagne et al. (11) used cryopreserved cells and studied the response for the first 96 h only.

The adhesion molecule and chemokine receptor expression by CD28$^+$CD45RA^{high} cells is that expected of naive CD8$^+$ T cells, namely, high expression of CD62L, CCR7, and CXCR4, which favor recirculation through lymph nodes via interaction with high endothelial addressins, secondary lymphoid chemokine or macrophage inflammatory protein-3β (MIP-3β), and stromal cell-derived factor-1, respectively. CD28$^+$CD45RO$^+$ cells have a complex pattern of homing molecule expression; some express one or more receptors that favor recirculation through lymph nodes (CD62L with or without CCR7), while others constitutively express CCR5 and/or CCR6, which would favor recruitment into inflamed tissues where MIP-1α, MIP-1β, and RANTES or MIP-3α are expressed. Almost all CD28$^-$CD45RA^{high} cells lack CCR7, most express little or no CXCR4, and a minority express CD62L, which suggests that recirculation through uninflamed lymph nodes may be less efficient; almost all CD28$^-$CD45RA^{high} cells lack constitutive expression of CCR5 and CCR6. Faint et al. (12) reported a greater proportion of HCMV tetramer-positive CD45RA^{high} cells in cells derived from

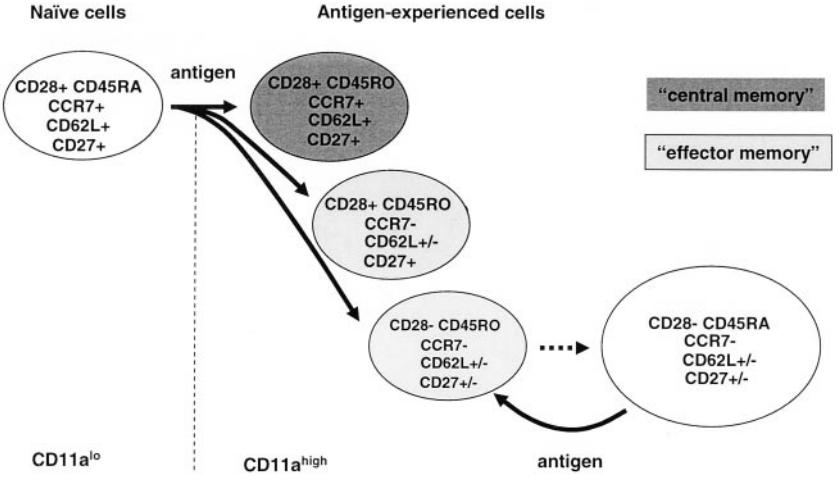

FIGURE 9. A model of the differentiation of human CD8$^+$ T cells. Following activation of a CD28$^+$CD45RA^{high} naive cell by Ag, CD11a is up-regulated permanently, and CD45RO is up-regulated at least initially. Whether CD28$^{minus]}$ CD45RO$^+$ cells arise directly from activated naive cells or via CD28$^+$CD45RO$^+$ cells is unclear. With time, CD28$^-$CD45RO$^+$ cells revert to CD28$^-$CD45RA$^{high;}$ upon subsequent re-exposure to Ag, CD28$^-$CD45RA^{high}CCR7$^-$ cells proliferate and give rise to CD28$^-$CD45RO$^+$ cells.

liver compared with cells from lymph node, consistent with preferential recirculation of $CD28^{-}CD45RA^{high}$ cells through nonlymphoid tissues. The up-regulation of CCR5 on $CD28^{-}CD45RA^{high}$ cells following activation in vitro suggests that these cells may express additional homing receptors when they migrate into tissues in which viral Ag is expressed during HCMV reactivation from latency.

It remains an important challenge to relate the phenotype of a $CD8^{+}$ T cell to its functional role in vivo. From many years there has been a binary functional classification of Ag-experienced T cells as either effector cells or memory cells (21). In this classification effector cells are generated early in the immune response and then decline rapidly, show immediate ex vivo cytotoxicity, and upon transfer into recipients can control acute infection, but not chronic infection, whereas memory cells are generated later in the immune response, do not show immediate ex vivo cytotoxicity, and upon transfer into recipients fail to control acute infection, but do control chronic infection, because they proliferate in response to Ag and differentiate into effector cells. An important limitation of this classification is that the effector activity of a given cell is an all-or-none property, whereas subsequent experimental data from purified populations of Ag-specific T cells assayed directly ex vivo indicate that the cytotoxic activity of an Ag-specific T cell in vivo is, in fact, a quantitative variable. Following the primary immune response, the magnitude of cytotoxic activity per cell varies between Ag-specific T cells purified from different tissues at a given time point (22), and in the spleen cytotoxic activity per cell tends to decrease (by up to 8-fold) with time after Ag exposure (23). Similarly, compared with that seen during primary HCMV infection (8), the magnitude of virus-specific cytotoxicity per cell shown by tetramer-positive $CD8^{+}$ T cells purified from PBMC of healthy carriers of HCMV was modest (7). In unstimulated $CD8^{+}$ T cells in PBMC of healthy adults, staining of intracellular perforin also shows a continuous spectrum, with highest levels in $CD27^{-}CD45RA^{+}$ cells (9) or $CD11a^{high}CD45RA^{+}$ cells (12). Thus in long term memory, Ag-experienced daughter cells of a single clone exhibit a spectrum of states of activation/differentiation that include quantitative (rather than qualitative) differences in levels of cytotoxicity. Because the mutually exclusive categories of effector cell and memory cell are no longer adequate to describe the complexity of Ag-experienced cells, the term effector memory cell has been introduced to describe effector cells found in long term memory, which may differ from the effector cells in acute virus infection in their susceptibility to apoptosis and capacity for proliferation. The term effector memory cells was originally applied to $CD45RO^{+}CCR7^{-}$ cells (10), but this nomenclature may lead to confusion, because $CD45RO^{+}CCR7^{-}$ cells are made up of both $CD28^{+}$ and $CD28^{-}$ cells, and effector-phenotype cells are also abundant in $CD27^{-}CD45RA^{+}$ cells (9) or $CD11a^{high}CD45RA^{+}$ cells (12). Rather than descriptive terms, we favor a more precise classification based directly on the expression of sets of surface molecules that reflect the diversity of cellular differentiation.

There is also heterogeneity among the activated effector T cells generated in acute virus infection. Many acutely activated cytotoxic cells (in blood, spleen, or infected peripheral tissues) fail to proliferate in vitro and die rapidly by apoptosis (24). However, a subpopulation of Ag-specific cytotoxic cells can survive in vitro and give rise to long term memory in vivo (25). Cytotoxic effector cells generated by strong antigenic stimulation in vitro can also give rise to progeny that are capable of sustained proliferation in vivo (26). It will be of particular interest to identify the phenotype(s) of the subpopulation of effector cells in acute virus infection in vivo that is destined not to die, but to give rise to long term memory. Based on the results presented in this paper, our current model of human $CD8^{+}$ T cell differentiation is illustrated in Fig. 9.

We conclude that naive human $CD8^{+}$ T cells are $CD28^{+}CD45RA^{high}$, express CCR7 but not CCR6, and are predominantly $CD27^{+}$ and L-selectin $CD62L^{+}$. The phenotype $CD27^{+}CD45RA^{high}$ should not be used to identify naive human $CD8^{+}$ T cells, because in addition to naive cells, $CD27^{+}CD45RA^{high}$ cells contain a significant subpopulation of $CD28^{-}CD27^{+}$ Ag-experienced expanded clones. $CD8^{+}$ T cell memory to HCMV is maintained by cells of expanded HCMV-specific clones that show heterogeneity of activation state and costimulation molecule expression within both $CD45RO^{high}$ and $CD28^{-}CD45RA^{high}$ T cell pools, which may make complementary contributions to the aggregate memory response in vivo.

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