Differentiation of Human CD8$^+$ T Cells from a Memory to Memory/Effector Phenotype[1]

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Previous studies of perforin expression and cytokine production in subsets of peripheral human CD45RA$^-$CD8$^+$ T cells with different CD28/CD27 phenotypes showed that CD28$^+$CD45RA$^-$CD8$^+$ and CD27$^+$CD45RA$^-$CD8$^+$ T cells have characteristics of memory T cells, whereas CD28$^-$CD45RA$^-$CD8$^+$ and CD27$^-$CD45RA$^-$CD8$^+$ T cells have characteristics of both memory and effector T cells. However, the differentiation pathway from memory CD8$^+$ T cells into memory/effector CD8$^+$ T cells has not been completely clarified. We investigated this differentiation pathway using EBV- and human CMV (HCMV)-specific CD8$^+$ T cells. Three subsets of CD45RA$^-$CD8$^+$ T cells were observed in both total CD8$^+$ T cells and EBV- or HCMV-specific CD8$^+$ T cells: CD27$^+$CD28$^+$, CD27$^+$CD28$^-$, and CD27$^-$CD28$^-$. A significant number of the CD27$^-$CD28$^+$ subset was observed in total CD8 T cells. However, this subset was barely detectable in EBV- or HCMV-specific CD8$^+$ T cells. Analysis of perforin expression and cytotoxic activity in the first three subsets suggested the following differentiation pathway: CD27$^+$CD28$^+$CD45RA$^-$→CD27$^+$CD28$^-$CD45RA$^-$→CD27$^-$CD28$^-$CD45RA$^-$. This was supported by the observation that the frequency of CCR5$^+$ cells and CCR7$^+$ cells decreased during this sequence. Analysis of CCR5 and CCR7 expression in the CD27$^+$CD28$^+$ memory cell subset demonstrated the presence of three CCR5/CCR7 populations: CCR5$^-$CCR7$^+$, CCR5$^+$CCR7$^+$, and CCR5$^+$CCR7$^-$. These findings suggested the following differentiation pathway: CD27$^+$CD28$^+$CD45RA$^-$ (CCR5$^-$CCR7$^+$→CCR5$^+$CCR7$^+$→CCR5$^+$CCR7$^-$)→CD27$^+$CD28$^-$CD45RA$^-$→CD27$^-$CD28$^-$CD45RA$^-$. The presence of a CD27$^-$CD28$^+$ subset with a CCR5$^+$CCR7$^-$ phenotype implies a specialized role for this subset in the differentiation of CD8$^+$ T cells. *The Journal of Immunology,* **2002, 168: 5538–5550.**

Memory and effector CD8$^+$ T cells play an important role in viral eradication through their ability to produce various factors involved in suppression of viral replication (1–3) as well as in cytolysis of virus-infected cells (4, 5). Studies of human memory CD8$^+$ T cells are important for vaccine development because identification of a vaccine that can effectively induce memory T cells is expected to prevent infection by various pathogens, including viruses, bacteria, and parasites. However, despite their clinical importance, the characteristics and differentiation of memory CD8$^+$ T cells remain unclear in both human and mouse.

CD27 and CD28 can be used as cell surface markers to discriminate naive and memory CD8$^+$ T cells from effector CD8$^+$ T cells (6–8). These molecules are well known as T cell costimulatory molecules. CD28 interacts with CD80(B7-1) and CD86(B7-2), which are expressed on APCs (9, 10), and has a dual role as an adhesion and a signaling molecule. Coengagement of CD28 with the TCR is required for T cell activation (11). CD28 is down-regulated after TCR stimulation (12). CD27 is less well characterized than CD28. Its expression is up-regulated upon TCR stimulation (13–15). The CD27 ligand CD70 is transiently up-regulated by Ag receptor stimulation on both T and B cells (16). CD27 ligation enhances TCR-induced expansion of both CD4$^+$ and CD8$^+$ T cells (17–18). A recent study suggested that CD27 is required for generation of T cell memory rather than for differentiation of CD8$^+$ T cells to effector cytotoxic T cells (19). CD45RA, which is one of two CD45 isoforms, can be also used as a marker of naive CD8$^+$ T cells (20). Recent studies using virus epitope-specific HLA tetramers demonstrated that CD45RA$^+$ CD8$^+$ T cells include effector T cells (21, 22), suggesting that naive CD8$^+$ T cells cannot be identified by the presence of CD45RA alone.

Previous studies using human CD8$^+$ T cells costained with an anti-CD45RA mAb and an anti-CD27 or anti-CD28 showed that CD8$^+$ T cells with a CD28$^+$CD45RA$^-$ or CD27$^+$CD45RA$^-$ phenotype express no or only a low level of perforin but have the ability to produce cytokines, while CD8$^+$ T cells with a CD28$^-$CD45RA$^+$ or CD27$^-$CD45RA$^+$ phenotype express a high level of perforin but have a limited ability to produce cytokines (8, 23). These observations strongly suggest that human CD8$^+$ T cells with a CD28$^+$CD45RA$^-$ or CD27$^+$CD45RA$^-$ phenotype are memory cells and that those with a CD28$^-$CD45RA$^+$ or CD27$^-$D45RA$^+$ phenotype are effector cells. As CD8$^+$ T cells with a CD28$^-$CD45RA$^-$ or CD27$^-$CD45RA$^-$ phenotype express a medium level of perforin, these cells are thought to be intermediate between memory and effector T cells, i.e., to be memory/effector T cells (23, 24).

CCR7 is a chemokine receptor that functions as a homing receptor in the migration of naive and memory CD8$^+$ T cells to secondary lymphoid tissues. A role for CCR7 in CD8$^+$ T cells has been demonstrated through the identification of CCR7$^+$

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$CD45RA^+$ naive $CD8^+$ T cells and $CCR7^+CD45RA^-$ and $CCR7^-CD45RA^-$ memory $CD8^+$ T cells (25). Furthermore, a recent study suggested the following differentiation lineage for Ag-specific $CD8^+$ T cells: $CCR7^+CD45RA^+ \rightarrow CCR7^+CD45RA^- \rightarrow CCR7^-CD45RA^- \rightarrow CCR7^-CD45RA^+$ (26). This study suggested that $CCR7^+CD45RA^+CD8^+$ and $CCR7^+CD45RA^-CD8^+$ T cells are precursor memory T cells while $CCR7^-CD45RA^-CD8^+$ and $CCR7^-CD45RA^+CD8^+$ T cells are preterminally differentiated and terminally differentiated cells, respectively. Thus, analysis of chemokine receptor expression may be useful for discriminating among naive, memory, and effector $CD8^+$ T cells.

In this study, we attempted to clarify the differentiation pathway of memory $CD8^+$ T cells to memory/effector $CD8^+$ T cells. We used HLA class I tetramers and flow cytometry to detect EBV- or human CMV (HCMV)[4]-specific $CD8^+$ T cells in healthy individuals. $Tetramer^+CD8^+$ T cells were analyzed for CD27, CD28, CD45RA, and perforin expression by four-color flow cytometric analysis. In addition, the surface expression of two chemokine receptors, CCR5 and CCR7, and the function (cytotoxic activity and cytokine production) of different CD27/CD28 subsets of $CD45RA^-CD8^+$ T cells were examined. These analyses enabled us to propose a pathway for the differentiation of memory $CD8^+$ T cells to memory/effector $CD8^+$ T cells.

Materials and Methods

Blood samples

Blood samples were taken from 18 HCMV-seropositive and EBV-seropositive healthy adult individuals (see Table II).

Peptides

The HLA-A*0201-restricted HCMV-specific CTL epitope pp65 495–503 (NLVPMVATV; HCMV-1) (27) and two HLA-A*1101-restricted EBV-specific CTL epitopes, EBV-1 (EBV 3B 416–424; IVTDFSVIK) and EBV-2 (EBV 3B 399–408; AVFDRKSDAK) (28), were previously identified. These peptides were generated using an automated peptide synthesizer (model PSSM-8; Shimazu, Kyoto, Japan) with F-moc strategy followed by cleavage. The purity of the synthesized peptides was examined by mass spectrometry.

[4] Abbreviations used in this paper: HCMV, human CMV; MFI, mean fluorescence intensity; NMFI, normalized MFI; MIP, macrophage-inflammatory protein.

Cells

C1R cells expressing HLA-A*1101 (C1R-A*1101) or HLA-A*0201 (C1R-A*0201) were previously generated (29) and were maintained in RPMI 1640 medium supplemented with 10% FCS and 0.15 mg/ml hygromycin B.

Monoclonal Abs

Anti-CD27-FITC mAb, anti-CD28-FITC mAb, anti-CD28-PE mAb, anti-CD28-CyChrome mAb, anti-CD28-allophycocyanin mAb, anti-CD45RA-CyChrome mAb, anti-CCR5-FITC mAb, anti-CCR7 mAb, and anti-perforin-PE mAb were obtained from BD PharMingen (San Diego, CA). Anti-CD8-FITC mAb and anti-CD8-allophycocyanin mAb were obtained from DAKO (Glostrup, Denmark).

HLA class I/EBV or HCMV peptide tetramers

HLA class I-peptide tetrameric complexes were synthesized as previously described (30). Briefly, recombinant HLA class I proteins (HLA-A*0201 and HLA-A*1101) and human β_2-microglobulin were produced in *Escherichia coli* cells transformed with the relevant expression plasmids. The H chain was modified by deletion of the transmembrane cytosolic tail and COOH-terminal addition of a sequence containing the BirA biotinylation site. Two EBV CTL epitopes (EBV-1 and EBV-2) and one HCMV CTL epitope (HCMV-1) were used for refolding of HLA class I molecules. The HLA class I-peptide complexes were refolded in vitro. The 45-kDa complexes were isolated using gel filtration on a Superdex G75 column (Amersham Pharmacia Biotech, Little Chalfont, U.K.). Purified complexes were biotinylated with BirA enzyme (Avidity, Denver, CO). The biotinylated complexes were purified using gel filtration first on a Superdex G75 column and then on a MonoQ column (Amersham Pharmacia Biotech). HLA class I/peptide tetrameric complexes (tetramers) were mixed with allophycocyanin-labeled streptavidin (BD PharMingen) at a molar ratio of 4:1.

Flow cytometric analysis

$CD8^+$ T cells were purified from cryopreserved or fresh PBMC using anti-CD8-coated magnetic beads (MACS CD8 Microbeads; Miltenyi Biotec, Bergisch Gladbach, Germany). The percentage of $CD8^+$ T cells in purified cells was >98%. A total of 1×10^6 $CD8^+$ T cells were mixed with tetramers at a concentration of 0.02–0.04 mg/ml. After incubation at 37°C for 30 min, the cells were washed once with RPMI/10% FCS, and then anti-CD27 mAb, anti-CD28 mAb, and anti-CD45RA mAb were added to the cell suspension. The cells were incubated at 4°C for 30 min, and then the cells were washed two times with PBS/10% FCS.

To determine intracellular perforin expression in CD27/CD28 subsets of both tetramer-positive cells and total $CD8^+$ T cells, cells stained with anti-CD27 mAb, anti-CD28 mAb, and anti-CD45RA mAb were fixed with 4% paraformaldehyde at 4°C for 20 min, then permeabilized with PBS supplemented with 0.1% saponin containing 20% FCS (permeabilizing buffer) at 4°C for 10 min. Cells were washed with permeabilizing buffer and then resuspended in 100 μl of the same buffer. Anti-perforin mAb was added,

Table I. *CD28 and CD45RA expression in EBV-1-specific $CD8^+$ T cells isolated from healthy individuals with HLA-A*1101*

Individual	Percentage of Each Fraction				EBV-1 $Tetramer^+CD8^{+a}$	EBV-2 $Tetramer^+CD8^{+a}$
	$CD28^+CD45RA^+$	$CD28^+CD45RA^-$	$CD28^-CD45RA^-$	$CD28^-CD45RA^+$		
M-10	2.7	59.5	33.9	3.9	0.2	
M-14	0.0	90.7	9.3	0.0	0.2	
M-19	0.0	72.0	27.8	0.2	0.5	
E-2	1.6	81.1	17.3	0.0	0.1	
U-5	2.7	81.0	15.6	0.7	0.3	
Mean ± SD	1.4 ± 1.4	76.9 ± 11.7	20.8 ± 9.9	1.0 ± 1.7		
A-21	0.6	83.5	15.2	0.6		0.2
M-10	3.0	80.2	15.1	1.7		0.3
M-14	2.2	88.1	9.0	0.8		0.2
E-1	0.7	98.2	1.1	0.0		0.2
E-2	2.1	77.7	19.2	1.0		0.6
K-1	2.2	82.2	14.1	1.4		0.3
Mean ± SD	1.8 ± 0.9	85.0 ± 7.3	12.3 ± 6.4	1.0 ± 0.6		

[a] Percentage of $tetramer^+$ cells in total $CD8^+$ T cells.

the cell suspension was incubated at 4°C for 30 min, and the cells were washed three times in permeabilizing buffer at 4°C. PE-labeled mouse IgG1 was used as negative control.

The cells were finally resuspended in PBS containing 2% paraformaldehyde and then were analyzed using a FACSCalibur with CellQuest software (BD Biosciences, San Jose, CA). Between 70,000 and 200,000 gated events were acquired for analysis of intracellular perforin. Intracellular perforin expression is represented as the normalized mean fluorescence intensity (NMFI), which is the mean fluorescence intensity (MFI) of cellsstained with anti-perforin-PE mAb divided by the MFI of cells stained with the control mouse IgG1-PE mAb.

CTL assay

CTL activity was measured by a standard ^{51}Cr release assay as follows. Target cells (2×10^5) were incubated for 60 min with 100 μCi Na_2 ^{51}Cr in saline and then washed three times with RPMI 1640 medium containing 10% NCS. Labeled target cells (2×10^3/well) were added into U-bottom 96-well plates with the indicated amount of peptide. After incubation for 1 h, CD8$^+$ T cells purified by anti-CD8 mAb-coated beads or CD27$^-$CD28$^-$CD45RA$^-$CD8$^+$ and CD27$^+$CD28$^-$CD45RA$^-$CD8$^+$ T cells purified by a cell sorter (FACSVantage SE; BD Biosciences) were added and the mixtures were incubated for 6 h at 37°C. The supernatants were collected and analyzed with a gamma counter.

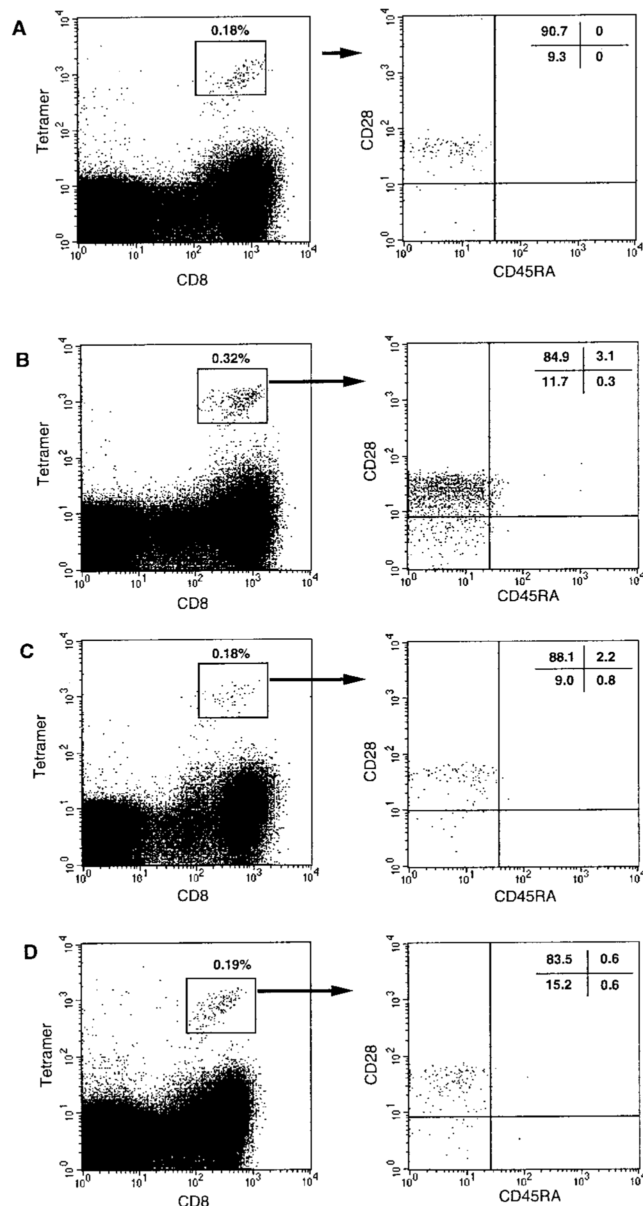


FIGURE 1. CD28/CD45RA phenotype of EBV-specific CD8$^+$ T cells. PBMCs from individuals M14 (*A*) and U5 (*B*) were stained simultaneously with the EBV-1-A*1101 tetramer, anti-CD8 mAb, anti-CD28 mAb, and anti-CD45RA mAb. Similarly, PBMCs from individuals M14 (*C*) and A21 (*D*) were simultaneously stained with the EBV-2-A*1101 tetramer, anti-CD8 mAb, anti-CD28 mAb, and anti-CD45RA mAb. Tetramer$^+$CD8$^+$ T cells were gated and then analyzed for CD28 and CD45RA expression

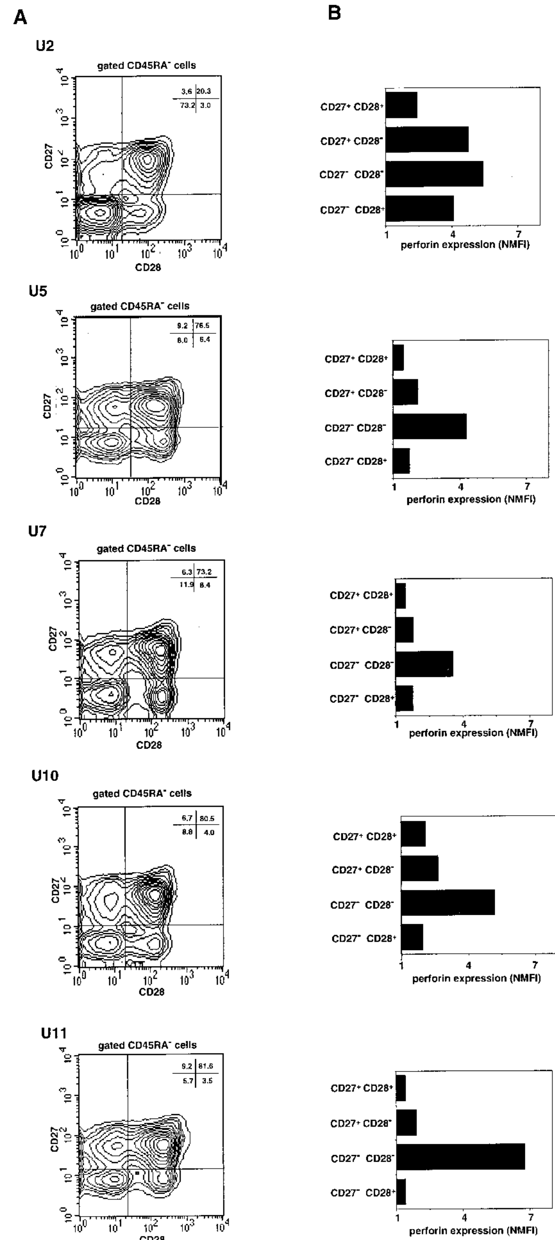


FIGURE 2. Frequency of, and perforin expression in, different CD27/CD28 subsets of CD45RA$^-$CD8$^+$ T cells. *A*, Frequency of different CD27/CD28 subsets of CD45RA$^-$CD8$^+$ T cells. CD8$^+$ T cells were isolated from five individuals, U2, U5, U7, U10, and U11, and then stained with anti-CD27 mAb, anti-CD28 mAb, anti-CD45RA mAb, and anti-perforin mAb or mouse IgG1 mAb. CD45RA$^-$ cells were gated and then analyzed for CD27 and CD28 expression. The percentage of each CD27/CD28 subset of CD45RA$^-$CD8$^+$ T cells is shown in each *upper right quadrant*. *B*, Perforin expression in different CD27/CD28 subsets of CD45RA$^-$CD8$^+$ T cells. The four CD27/CD28 subsets of CD45RA$^-$CD8$^+$ T cells were gated and then analyzed for perforin expression. Perforin expression in each CD27/CD28 subset is represented as the NMFI, i.e., MFI of cells stained with anti-perforin-PE mAb divided by the MFI of cells stained with control mouse IgG1-PE mAb.

Spontaneous ^{51}Cr release was determined by measuring the cpm in supernatants from wells containing only target cells (cpm spn). Maximum ^{51}Cr release was determined by measuring the cpm in supernatants from wells containing target cells in the presence of 2.5% Triton X-100 (cpm max). Specific lysis = ((cpm exp − cpm spn)/(cpm max − cpm spn)) × 100, where cpm exp is the cpm in supernatants of wells containing both target and effector cells.

Measurement of cytokine-producing cells by flow cytometry

To measure cytokine production in CD27/CD28 subsets of EBV- or HCMV-specific CD8$^+$ T cells, cells were purified from fresh PBMC isolated from healthy individuals who have EBV- or HCMV-specific CD8$^+$ T cells using anti-CD8-coated magnetic beads (MACS CD8 Microbeads; Miltenyi Biotec). CD8$^+$ T cells (2 × 10^6) were dispensed into a U-bottom 96-well plate with or without 1 μM of a EBV or HCMV peptide and incubated for 6 h. After the first 2 h of incubation, brefeldin A (10 μg/ml) was added to each well. The cells were then stained with a mixture of anti-CD27 mAb, anti-CD28 mAb, and anti-CD45RA mAb, fixed with 4% paraformaldehyde at 4°C for 20 min, and then permeabilized with PBS supplemented with 0.1% saponin containing 20% NCS (permeabilizing buffer) at 4°C for 10 min. Cells were resuspended in permeabilizing buffer and then stained with anti-IFN-γ mAb, anti-TNF-α mAb, or anti-macrophage-inflammatory protein (MIP)-1β mAb. The cells were finally resuspended in PBS containing 2% paraformaldehyde and then the percentage of intracellular IFN-γ-, MIP-1β-, or TNF-α-positive cells was analyzed by flow cytometry. To measure the number of EBV- or HCMV-specific CD8$^+$ T cells in each CD27/CD28 subset, CD8$^+$ T cells were stained with an EBV or HCMV tetramer and a mixture of anti-CD27 mAb, anti-CD28 mAb, and anti-CD45RA mAb. The percentage of cytokine-positive cells in tetramer-positive cells in each CD27/CD28 subset was determined.

Results

Predominant existence of the CD28$^+$CD45RA$^-$ subset in EBV-specific CD8$^+$ T cells from healthy individuals

HLA-A*1101 is a common allele in the Japanese population, and IVTDFSVIK (EBV-1) and AVFDRKSDAK (EBV-2) are known to be HLA-A*1101-restricted immunodominant epitopes. Therefore, we used these peptides to generate tetramers to analyze EBV-specific CD8$^+$ T cells in PBMC from EBV-seropositive healthy Japanese individuals with HLA-A*1101. Ex vivo flow cytometry analysis demonstrated that in 5 of the 10 individuals studied >0.1% of total CD8$^+$ T cells were EBV-1 tetramer$^+$, and that in 6 of the 10 individuals >0.1% of total CD8$^+$ T cells were EBV-2 tetramer$^+$ (Table I). Eight individuals had tetramer$^+$CD8$^+$ T cells for at least one of the EBV epitopes. Analysis of CD28 and CD45RA expression on the tetramer$^+$CD8$^+$ T cells demonstrated

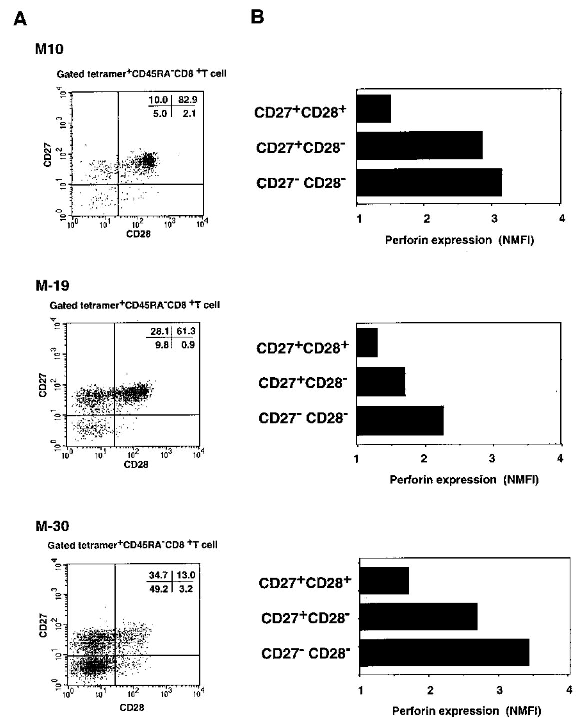


FIGURE 3. Frequency of, and perforin expression in, different CD27/CD28 subsets of EBV-specific and HCMV-specific CD45RA$^-$CD8$^+$ T cells. *A*, Frequency of different CD27/CD28 subsets of EBV-specific and HCMV-specific CD45RA$^-$CD8$^+$ T cells. CD8$^+$ T cells were isolated from three individuals (M10, M19, and M30) and then stained simultaneously with both EBV-1-A*1101 and EBV-2-A*1101 tetramers (M10 and M19) or HCMV-1-A*0201 tetramer (M30), and anti-CD27, anti-CD28 mAb, and anti-CD45RA mAb. Most tetramer$^+$ cells (>98%) were CD45RA$^-$. Tetramer$^+$CD45RA$^-$ T cells were gated and then analyzed for CD27 and CD28 expression. The percentage of each CD27/CD28 subset of CD45RA$^-$CD8$^+$ T cells is shown in each *upper right quadrant*. *B*, Perforin expression in different CD27/CD28 subsets of EBV-specific and HCMV-specific CD45RA$^-$CD8$^+$ T cells. The CD8$^+$ T cells were stained simultaneously with the EBV-1-A*1101 tetramers (M10 and M19) or HCMV-1-A*0201 tetramer (M30), and anti-CD27, anti-CD28 mAb, and anti-perforin mAb or mouse IgG1 mAb. Perforin expression in each CD27/CD28 subset of tetramer$^+$CD45RA$^-$CD8$^+$ T cells is represented as the NMFI, i.e., the MFI of cells stained with anti-perforin-PE mAb divided by the MFI of cells stained with mouse IgG1-PE mAb.

Table II. *Frequency of each CD27/CD28 phenotype in total CD45RA$^-$CD8$^+$ T cells*

Individual	HLA Class I Type	Percentage of CD45RA$^-$CD8$^+$ T Cells with Each CD27/CD28 Phenotype			
		CD27$^+$CD28$^+$	CD27$^+$CD28$^-$	CD27$^-$CD28$^-$	CD27$^-$CD28$^+$
A7	A*0201/A*2603, B*1501/B*4003	40.6	12.9	39.2	7.4
A16	A*0201/A*0206, B*3901/B*4001	33.8	8.3	54.6	3.4
A21	A*1101/A*1101, B*1501/B*1501	51.3	9.6	35.3	3.9
E1	A2/A11, B48/B55	75.4	9.1	7.7	7.8
E2	A11/A-, B62/B55 or B56	79.6	9.2	5.7	5.6
M10	A*1101/A*1101, B*5201/B*5401	76.1	6.5	7.2	10.2
M14	A*0206/A*1101, B*1501/B*1528	58.8	7.4	29.3	4.5
M19	A*1101/A*3303, B*4001/B*4403	67.6	15.5	10.7	6.1
M20	A*0201/A*2601, B*1501/B*4002	62.0	8.5	21.1	8.4
M26	A*0201/A*2402, B*1501/B*5502	77.3	8.9	10.2	3.6
M30	A*0201/A*3101, B*3501/B*5101	78.7	8.7	6.1	6.1
U2	A2/A33, B44/B62	27.7	3.3	65.4	3.4
U4	A*1101/A*2402, B*5201/B*5201	77.7	4.5	12.6	5.3
U5	A2/A11, B46/B62(15)	75.8	11.0	8.0	5.0
U7	A*2402/-, B*5201/B*4006	61.5	10.4	21.8	6.1
U9	A*0201/A*2402, B*5401/B61	27.5	11.9	59.6	0.8
U10	A24/A26, B52/B61	70.0	6.6	16.6	6.6
U11	A*2402/A*2602, B*1501/B*4801	75.8	11.1	8.1	5.1

that most individuals had a high number of tetramer$^+$CD8$^+$ T cells with a CD28$^+$CD45RA$^-$ memory phenotype but only a low number with a CD28$^-$CD45RA$^-$ memory/effector phenotype (Fig. 1 and Table I). Perforin expression was much lower in the tetramer$^+$CD8$^+$ T cells than in total CD8$^+$ T cells and HCMV-specific CD8$^+$ T cells (data not shown). These results indicate that most EBV-specific CD8$^+$ T cells in healthy individuals are memory CD8$^+$ T cells.

We also attempted to detect HCMV-specific CD8$^+$ T cells in PBMC from HCMV-seropositive healthy individuals with HLA-A2 using an HLA-A*0201 tetramer containing the HCMV epitope, pp65 495–503 (NLVPMVATV; HCMV-1). More than

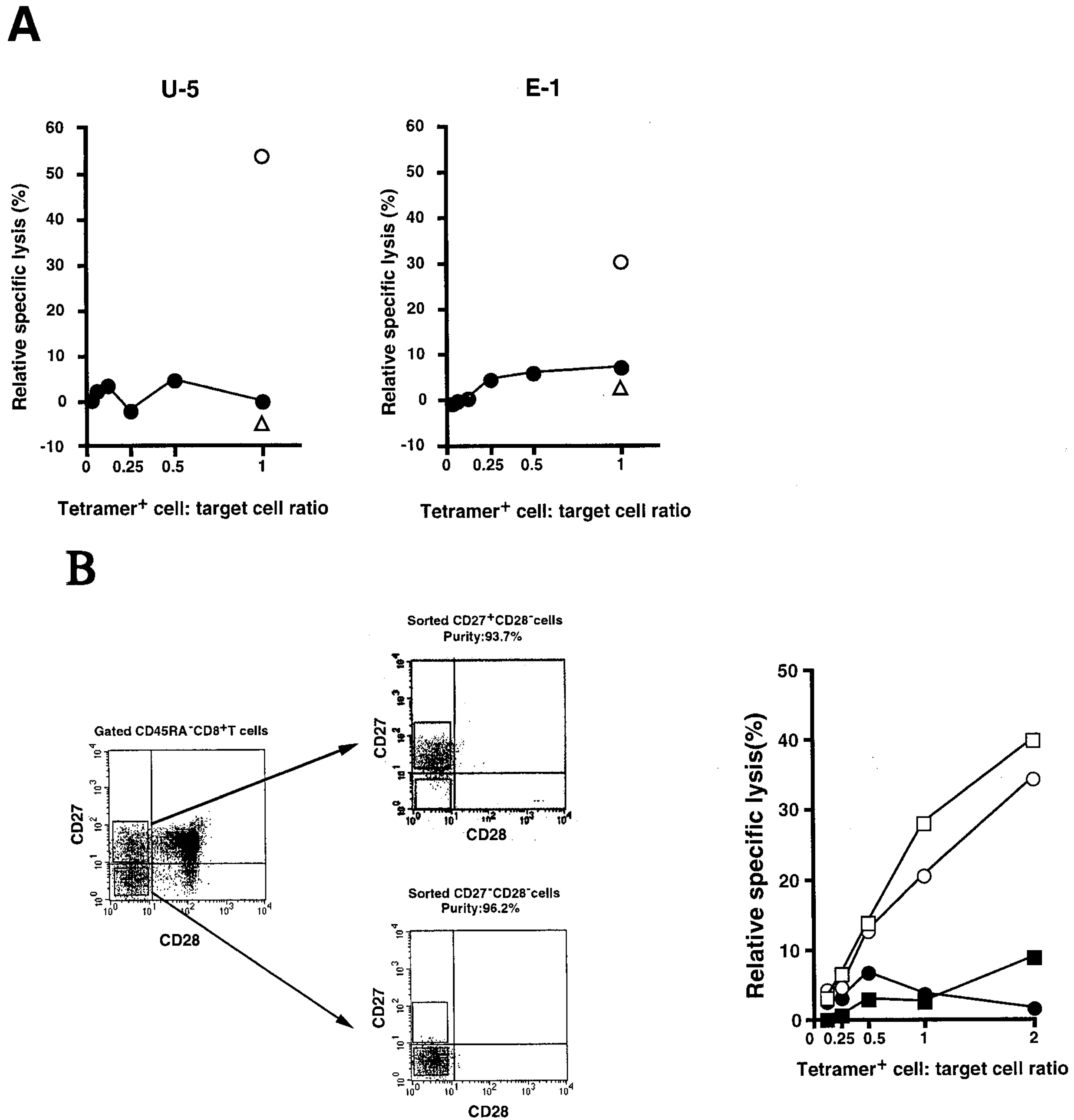


FIGURE 4. Cytotoxic activity of HCMV-1-specific and EBV-specific CD8$^+$ T cells. *A*, Cytotoxic activity of EBV-specific CD8$^+$ T cells. CD8$^+$ T cells were isolated from two individuals, U5 and E1. The percentage of HLA-A*1101-restricted, EBV-specific CD8$^+$ T cells was measured using EBV-1-A*1101 and EBV-2-A*1101 tetramers. The relative cytotoxic activity of the separated CD8$^+$ T cells for C1R-A*1101 cells pulsed with EBV-1 and EBV-2 peptides (●) was measured at different effector (tetramer$^+$ cells):target ratios. The relative cytotoxic activity of these cells for C1R cells pulsed with EBV-1 and EBV-2 peptides (△) and HLA-A*1101-restricted, EBV-1-specific CTL lines for C1R-A*1101 cells pulsed with EBV-1 and EBV-2 peptides (○) was measured at an effector (tetramer$^+$ cells):target ratio of 1:1. *B*, Cytotoxic activity of two CD27/CD28 subsets of HCMV-specific CD45RA$^-$CD8$^+$ T cells. *Left panel*, CD27$^+$CD28$^-$CD45RA$^-$CD8$^+$ and CD27$^-$CD28$^-$CD45RA$^-$CD8$^+$ T cells were isolated from the PBMC of individual M30 using a cell sorter. The percentage of HLA-A*0201-restricted, HCMV-1-specific CD8$^+$ T cells in each subset was measured using HCMV-1-A*0201 tetramer. *Right panel*, The cytotoxic activity of sorted cells for target cells pulsed with or without HCMV-1 peptide was analyzed. The relative cytotoxic activity of CD27$^+$CD28$^-$CD45RA$^-$CD8$^+$ T cells (○ and ●) and CD27$^-$CD28$^-$CD45RA$^-$CD8$^+$ T cells (□ and ■) for C1R-A*0201 (○ and □) and C1R cells (● and ■) was measured at different effector (tetramer$^+$ cells):target ratios.

0.1% of total CD8^{+} T cells were HCMV tetramer^{+} in 11 of 18 healthy individuals (data not shown). The tetramer^{+}CD8^{+} T cells were further analyzed to determine their CD28/CD45RA phenotype to clarify differentiation stage of these cells. In most individuals, two predominant effector phenotypes were observed in tetramer^{+}CD8^{+} T cells: CD28^{-}CD45RA^{-} and CD28^{-}CD45RA^{+}. An exception was observed in one individual (M-30), where >90% of tetramer^{+}CD8^{+} T cells were CD28^{-}CD45RA^{-} (data not shown). Therefore, the CD28/CD45RA phenotype of HCMV-specific CD8^{+} T cells differs from that of EBV-specific CD8^{+} T cells in healthy individuals.

CD45RA^{-}CD8^{+} T cells have four CD27/CD28 subsets

Previous studies suggested that CD8^{+} T cells with a CD28^{+}CD45RA^{-} or CD27^{+}CD45RA^{-} phenotype are memory T cells, whereas those with a CD28^{-}CD45RA^{-} or CD27^{-}CD45RA^{-} phenotype are memory/effector T cells. This classification is based on perforin expression: cells with the former phenotypes have no or only a low level of perforin while the latter have a higher level of perforin, but a lower level than that of CD28^{-}CD45RA^{+}CD8^{+} T cells (23). To clarify the differentiation pathway of memory (CD28^{+}CD45RA^{-} or CD27^{+}CD45RA^{-}) CD8^{+} T cells to memory/effector (CD28^{-}CD45RA^{-} or CD27^{-}CD45RA^{-}) CD8^{+} T cells, we analyzed the expression of both CD27 and CD28 in total CD45RA^{-}CD8^{+} T cells in PBMC isolated from 18 healthy individuals. In the majority of these individuals, cells with a CD27^{+}CD28^{+} phenotype formed a major population of total CD45RA^{-}CD8^{+} T cells, while three other phenotypes, CD27^{+}CD28^{-}, CD27^{-}CD28^{+}, and CD27^{-}CD28^{-}, formed a minor population (Fig. 2*A* and Table II). Exceptions were observed in three of the 18 individuals (A16, U2, and U9), where the CD27^{-}CD28^{-} subset was the major population (Table II). Perforin expression was lowest in the CD27^{+}CD28^{+} subset, highest in the CD27^{-}CD28^{-} subset, and intermediate in the CD27^{+}CD28^{-} and the CD27^{-}CD28^{+} subsets (Fig. 2*B*). This suggests that CD27^{+}CD28^{+}CD45RA^{-}CD8^{+} cells are memory T cells and that this subset may differentiate via the CD27^{+}CD28^{-} subset and/or the CD27^{-}CD28^{+} subset to the CD27^{-}CD28^{-} subset, which from its perforin expression level appears to have characteristics more typical of cytotoxic effector T cells than the other subsets.

To further investigate CD27/CD28 subsets in virus-specific CD45RA^{-}CD8^{+} T cells, we examined CD8^{+} T cells from individuals M10 and M19, who had a higher number of EBV-specific CD28^{-}CD45RA^{-}CD8^{+} T cells than the other individuals examined (Table I), and individual M30, who exclusively had HCMV-specific CD45RA^{-}CD8^{+} T cells. CD27 and CD28 expression was analyzed on the EBV tetramer^{+}CD45RA^{-} fraction of CD8^{+} T cells isolated from individuals M10 and M19. The percentage of CD27^{+}CD28^{+}, CD27^{+}CD28^{-}, and CD27^{-}CD28^{-} cells in EBV-specific CD8^{+} T cells was similar to that in total CD8^{+} T cells, but the percentage of CD27^{-}CD28^{+} cells in EBV-specific CD8^{+} T cells was much lower than that in total CD8^{+} T cells (Fig. 3*A*). Similarly, CD27^{+}CD28^{+}, CD27^{+}CD28^{-}, and CD27^{-}CD28^{-} subsets were observed in HCMV-specific CD8^{+} T cells from individual M30, while only a small number of CD27^{-}CD28^{+} cells was detected (Fig. 3*A*). Perforin expression in each EBV-specific and HCMV-specific CD45RA^{-}CD8^{+} T cell subset was similar to that observed in each total CD45RA^{-}CD8^{+} T cell subset, although expression in the CD27^{+}CD28^{-} subset appeared to be higher in EBV- and HCMV-specific CD8^{+} T cells than in total CD8^{+} T cells (Figs. 2*B* and 3*B*). These results support the idea that the CD27^{+}CD28^{+} and CD27^{-}CD28^{-} subsets are memory and memory/effector CD8^{+} T cells, respectively, and that the CD27^{+}CD28^{-} subset is an intermediate in the differentiation pathway from CD27^{+}CD28^{+} to CD27^{-}CD28^{-}.

Cytotoxic activity of different CD27/CD28 subsets of CD45RA^{-}CD8^{+} T cells

We evaluated the ex vivo cytotoxic activity of EBV-specific CD45RA^{-}CD8^{+} T cells with different CD27/CD28 phenotypes for target cells (C1R-A*1101 or autologous T cell blast) pulsed with EBV-1 or EBV-2. CD8^{+} T cells were isolated from PBMCs from two EBV-seropositive, healthy individuals (E1 and U5) with HLA-A*1101. Approximately 98 and 81% of EBV-specific CD8^{+} T cells from E1 and U5, respectively, had a CD27^{+}CD28^{+}CD45RA^{-} phenotype (Tables I and II). These CD8^{+} T cells exhibited no cytotoxic activity for HLA-A*1101^{+} target cells pulsed with EBV-1 and EBV-2 peptides at a tetramer^{+}CD8^{+} T cell:target ratio of 1:1 (Fig. 4*A*). These results indicate that CD27^{+}CD28^{+}CD45RA^{-}CD8^{+} T cells are not effector cytotoxic T cells.

To clarify the cytotoxic activity of the CD27^{+}CD28^{-} and CD27^{-}CD28^{-} subsets of CD45RA^{-}CD8^{+} T cells, we sorted these cells from PBMCs isolated from healthy individuals. We

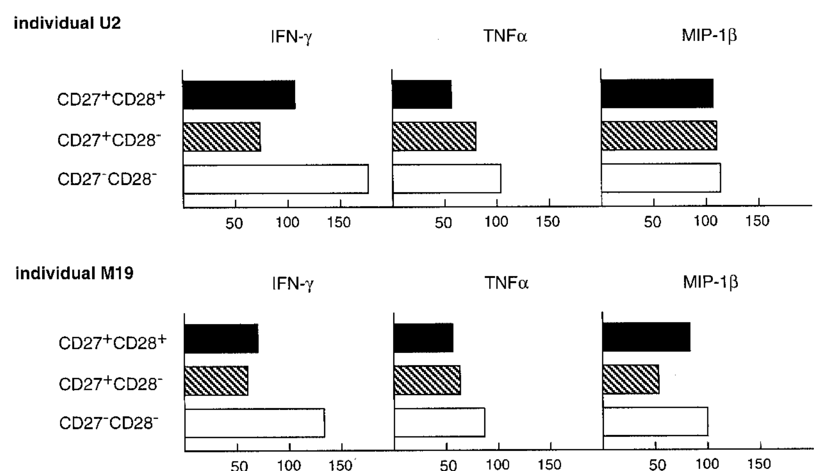


FIGURE 5. Cytokine production by CD27/CD28 subsets of EBV- or HCMV-specific CD8^{+} T cells. CD8^{+} T cells were isolated from fresh PBMC from individual M19, who has EBV-1-specific CD8^{+} T cells, and individual U2, who has HCMV-1-specific CD8^{+} T cells. CD8^{+} T cells from M19 and U2 were stimulated with EBV-1-specific and HCMV-specific peptides, respectively. Cells producing cytokines in different CD27/CD28 subsets were analyzed by flow cytometry. The number of EBV-specific and HCMV-specific CD8^{+} T cells was measured using EBV-1 tetramers and the HCMV-1 tetramer, respectively. The percentage of cytokine-producing cells in CD27/CD28 subsets of EBV-specific and HCMV-specific CD8^{+} T cells was calculated.

used PBMCs isolated from individual M30, whose HCMV-specific CD45RA$^-$CD8$^+$ T cells contained both subsets (Fig. 3*A*), because PBMCs containing both EBV-specific CD8$^+$ T cell subsets were not available for this experiment. Both the CD27$^+$CD28$^-$ and CD27$^-$CD28$^-$ subsets effectively killed target cells pulsed with an HCMV-1 peptide (Fig. 4*B*). These results are consistent with the perforin expression levels observed in these subsets.

Cytokine production in different CD27/CD28 subsets of CD45RA$^-$CD8$^+$ T cells

Cytokine production in CD27$^+$CD28$^+$, CD27$^+$CD28$^-$, and CD27$^-$CD28$^-$ subsets of CD45RA$^-$CD8$^+$ T cells was investigated using two EBV epitope peptides and an HCMV-1 peptide. Purified CD8$^+$ T cells from individuals U2 and M19 were analyzed for IFN-γ-, TNF-α-, and MIP-1β-producing cells 6 h after

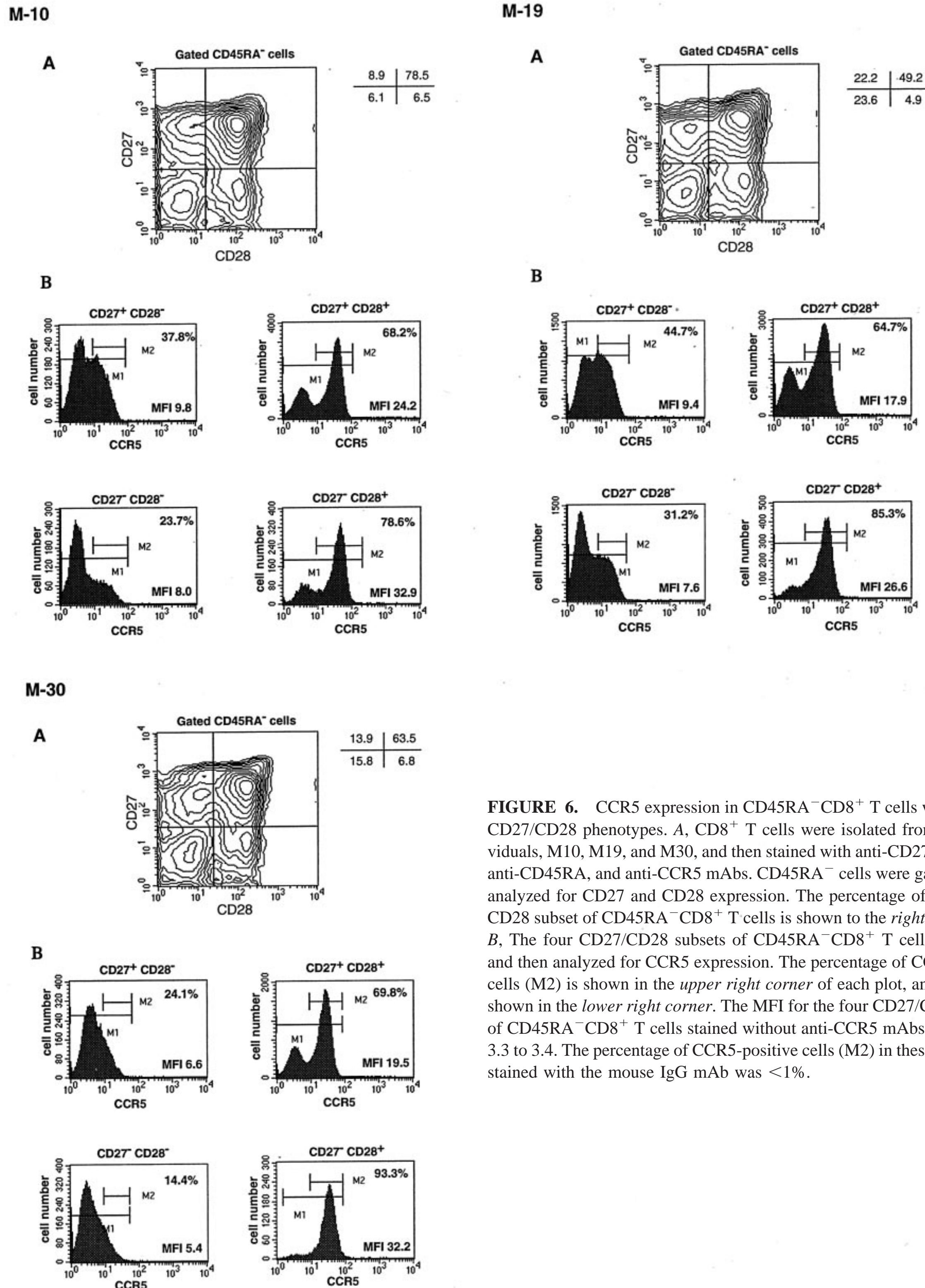


FIGURE 6. CCR5 expression in CD45RA$^-$CD8$^+$ T cells with different CD27/CD28 phenotypes. *A*, CD8$^+$ T cells were isolated from three individuals, M10, M19, and M30, and then stained with anti-CD27, anti-CD28, anti-CD45RA, and anti-CCR5 mAbs. CD45RA$^-$ cells were gated and then analyzed for CD27 and CD28 expression. The percentage of each CD27/CD28 subset of CD45RA$^-$CD8$^+$ T cells is shown to the *right* of each plot. *B*, The four CD27/CD28 subsets of CD45RA$^-$CD8$^+$ T cells were gated and then analyzed for CCR5 expression. The percentage of CCR5-positive cells (M2) is shown in the *upper right corner* of each plot, and the MFI is shown in the *lower right corner*. The MFI for the four CD27/CD28 subsets of CD45RA$^-$CD8$^+$ T cells stained without anti-CCR5 mAbs ranged from 3.3 to 3.4. The percentage of CCR5-positive cells (M2) in these subset cells stained with the mouse IgG mAb was $<$1%.

stimulation with the appropriate peptide(s). All three subsets from both individuals effectively produced these three cytokines, although cytokine-producing cells seemed to be more prevalent in the $CD27^-CD28^-$ subset than in other subsets (Fig. 5). These results indicate that these three subsets of HCMV-specific and EBV-specific $CD8^+$ T cells are able to effectively produce cytokines.

CCR5 expression in different CD27/CD28 subsets of $CD45RA^-CD8^+$ T cells

Our recent study showed that $CD28^+CD45RA^-CD8^+$ T cells express a high level of CCR5, and that this expression is decreased in $CD28^-CD45RA^-CD8^+$ T cells (31). To further investigate CCR5 expression in different CD27/CD28 subsets of $CD45RA^-CD8^+$ T cells, we stained total $CD8^+$ T cells from three individuals (M10, M19, and M30) with anti-CCR5, anti-CD27, anti-CD28, and anti-CD45RA mAb (Fig. 6). Approximately 60–70% of the $CD27^+CD28^+$ subset and 10–30% of the $CD27^-CD28^-$ subset were $CCR5^+$. The $CD27^+CD28^-$ subset showed an intermediate number of $CCR5^+$ cells that was much lower than that of the $CD27^+CD28^+$ subset. These results support the sequence $CD27^+CD28^+ \rightarrow CD27^+CD28^- \rightarrow CD27^-CD28^-$ for the differentiation of memory $CD8^+$ T cells to memory/effector $CD8^+$ T cells suggested by the observed perforin expression levels

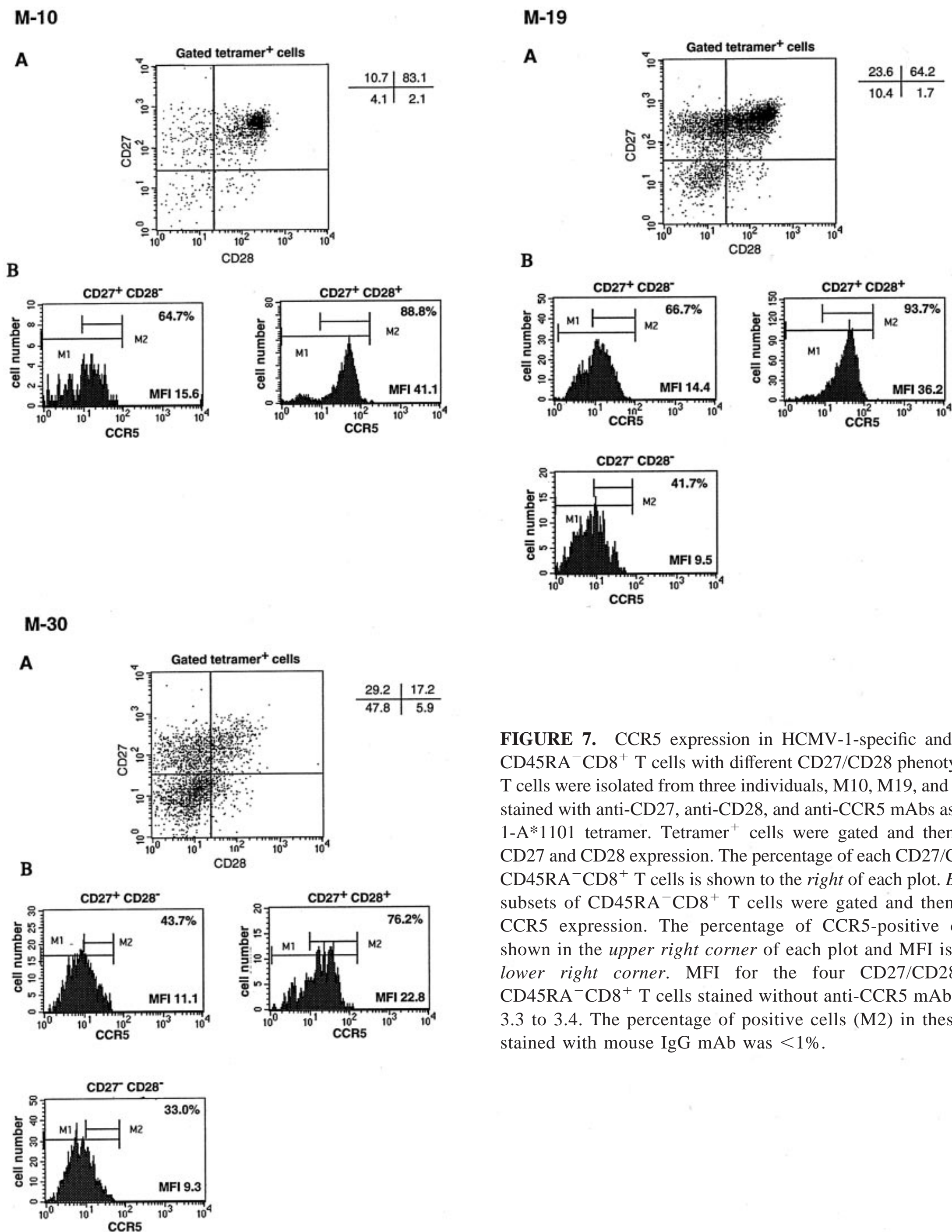
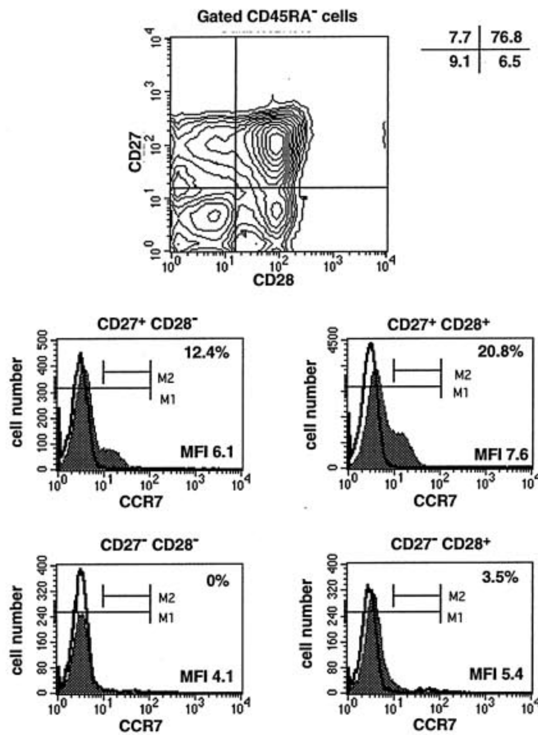
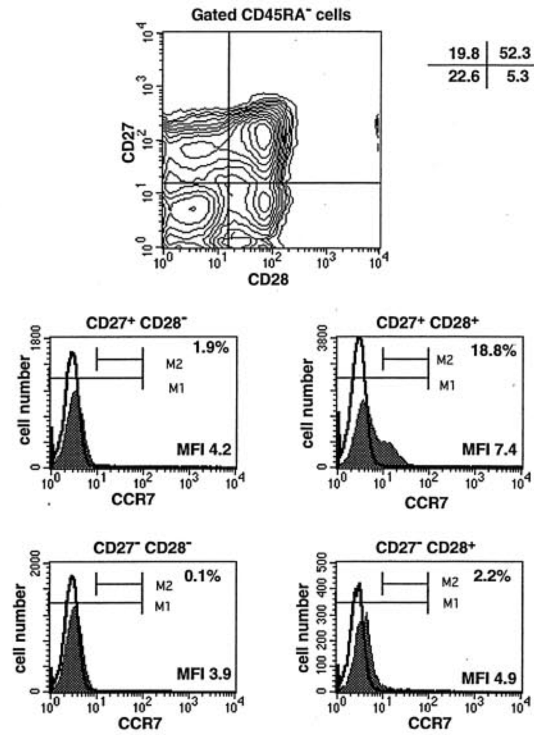


FIGURE 7. CCR5 expression in HCMV-1-specific and EBV-specific $CD45RA^-CD8^+$ T cells with different CD27/CD28 phenotypes. *A*, $CD8^+$ T cells were isolated from three individuals, M10, M19, and M30, and then stained with anti-CD27, anti-CD28, and anti-CCR5 mAbs as well as EBV-1-A*1101 tetramer. $Tetramer^+$ cells were gated and then analyzed for CD27 and CD28 expression. The percentage of each CD27/CD28 subset of $CD45RA^-CD8^+$ T cells is shown to the *right* of each plot. *B*, CD27/CD28 subsets of $CD45RA^-CD8^+$ T cells were gated and then analyzed for CCR5 expression. The percentage of CCR5-positive cells (M2) is shown in the *upper right corner* of each plot and MFI is shown in the *lower right corner*. MFI for the four CD27/CD28 subsets of $CD45RA^-CD8^+$ T cells stained without anti-CCR5 mAbs ranged from 3.3 to 3.4. The percentage of positive cells (M2) in these subset cells stained with mouse IgG mAb was $<1\%$.

A





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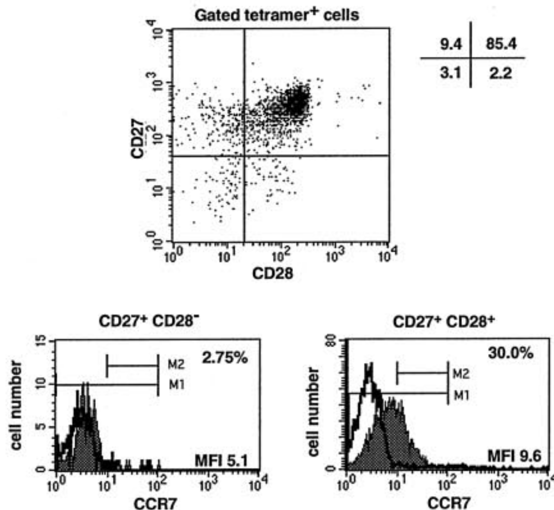

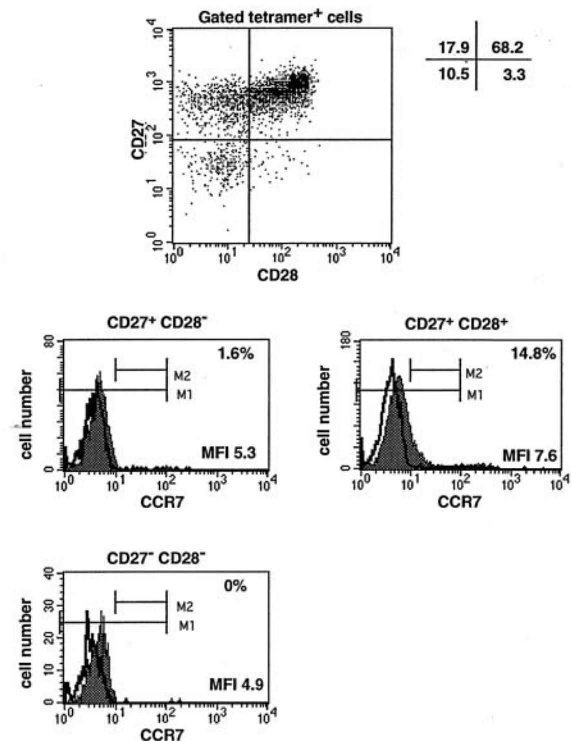

FIGURE 8. CCR7 expression of total and EBV-specific CD45RA$^-$CD8$^+$ T cells with different CD27/CD28 phenotype. *A*, CCR7 expression in total CD45RA$^-$CD8$^+$ T cells with different CD27/CD28 phenotypes. CD8$^+$ T cells were isolated from two individuals, M10 and M19, and then stained with anti-CD27, anti-CD28, anti-CD45RA, and anti-CCR7 mAbs (shaded histograms) or an isotype control mouse IgM mAb (open histograms). CD45RA$^-$ cells were gated and then analyzed for CD27 and CD28 expression. The percentage of each CD27/CD28 subset of CD45RA$^-$CD8$^+$ T cells is shown to the

in these subsets. However, the $CD27^-CD28^+$ subset had the highest number of $CCR5^+$ cells of the four subsets (Fig. 6), suggesting that this subset may have characteristics more similar to memory T cells.

To clarify CCR5 expression in these subsets in EBV- and HCMV-specific CD8 T cells, we stained $CD8^+$ T cells purified from three HLA-A*1101^+ or HLA-A*0201^+ individuals (M10, M19, and M30), in whom most $tetramer^+CD8^+$ T cells were $CD45RA^-$ (Table I), with tetramers and anti-CD27, anti-CD28, and anti-CCR5 mAbs. CCR5 expression was similar to that observed in total $CD8^+$ T cells, although the percentage of $CCR5^+$ cells in each subset was higher in $tetramer^+CD8^+$ T cells than in total $CD8^+$ T cells (Fig. 7). These results indicate that the number of $CCR5^+$ cells decreased during differentiation from memory ($CD27^+CD28^+CD45RA^-$) $CD8^+$ T cells to memory/effector ($CD27^-CD28^-CD45RA^-$) $CD8^+$ T cells, and support the idea that the $CD27^+CD28^-$ subset is intermediate between the $CD27^+CD28^+$ and $CD27^-CD28^-$ subsets in the differentiation pathway.

CCR7 expression in $CD45RA^-CD8^+$ T cells with a $CD27^+CD28^+$ phenotype

A recent study showed that CCR7 is expressed on $CD27^+CD45RA^+$ and $CD27^+CD45RA^-$ but not on $CD27^-CD45RA^+$ $CD8^+$ T cells (32), suggesting that CCR7 is expressed only on naive and memory $CD8^+$ T cells. In addition, our recent study showed that $CD28^+CD45RA^-CD8^+$ T cells have three CCR5/CCR7 subsets: $CCR5^-CCR7^+$, $CCR5^+CCR7^+$, and $CCR5^+CCR7^-$ (31). Because most $CD28^+CD45RA^-$ cells in total $CD8^+$ T cells express CD27 (Table II), it is likely that $CD27^+CD28^+CD45RA^-CD8^+$ T cells contain both $CCR7^+$ and $CCR7^-$ populations. Indeed, flow cytometry analysis of different CD27/CD28 subsets of total $CD45RA^-CD8^+$ T cells showed that the $CD27^+CD28^+$ subset contains both $CCR7^+$ and $CCR7^-$ populations (Fig. 8*A*). In contrast, most cells in both the $CD27^+CD28^-$ and $CD27^-CD28^-$ subsets were $CCR7^-$. These results, together with those of CCR5 expression in these subsets of $CD45RA^-CD8^+$ T cells, suggest that $CD27^+CD28^+CD45RA^-CD8^+$ T cells have three subsets, $CCR5^-CCR7^+$, $CCR5^+CCR7^+$, and $CCR5^+CCR7^-$, while $CD27^+CD28^-CD45RA^-CD8^+$ and $CD27^-CD28^-CD45RA^-CD8^+$ T cells have two subsets, $CCR5^+CCR7^-$ and $CCR5^-CCR7^-$.

Similar results were observed in the $CD27^+CD28^+$ subset of EBV-specific $CD45RA^-CD8^+$ T cells from two individuals, although the percentage of $CCR7^+$ cells differed between the two (Fig. 8*B*). A higher number of $CCR7^+$ cells was observed in individual M10 compared with M19, suggesting that the $CD27^+CD28^+$ subset of EBV-specific $CD8^+$ T cells is more differentiated in individual M19 than M10. Only 10–15% of $tetramer^+CD8^+$ cells in M10 were $CD27^+CD28^-CD45RA^-$ or $CD27^-CD28^-CD45RA^-$, while ~30% of $tetramer^+CD8^+$ T cells in M19 expressed these phenotypes (Figs. 3, 7*A*, and 8*B*), also suggesting that EBV-specific $CD8^+$ T cells are more differentiated in M19 than in M10.

The $CD27^-CD28^+$ subset does not express CCR7 (Fig. 8*A*), while most of this subset expresses CCR5 (Fig. 7). This suggests that this subset is more differentiated than $CD27^+CD28^+CD45RA^-CD8^+$ T cells but less differentiated than $CD27^+CD28^-CD45RA^-CD8^+$ and $CD27^-CD28^-CD45RA^-CD8^+$ T cells.

Discussion

Previous studies showed that human $CD8^+$ T cells with a $CD28^+CD45RA^-$ or $CD27^+CD45RA^-$ phenotype are memory T cells, those with a $CD28^-CD45RA^+$ or $CD27^-CD45RA^+$ phenotype are effector T cells, and those with a $CD28^-CD45RA^-$ or $CD27^-CD45RA^-$ phenotype are intermediate between memory and effector $CD8^+$ T cells (memory/effector $CD8^+$ T cells) (8, 24). In this study, we further analyzed CD27 and CD28 phenotypes in total and virus-specific $CD45RA^-CD8^+$ T cells. The $CD27^+CD28^+$ subset had no or only a very low level of perforin, whereas the $CD27^-CD28^-$ subset expressed a medium level of perforin. Thus, it is likely that these subsets are memory and memory/effector $CD8^+$ T cells, respectively. Although the differentiation pathway from $CD27^+CD28^+$ to $CD27^-CD28^-$ is unclear, $CD27^+CD28^-$ and $CD27^-CD28^+$ subsets are candidates for intermediate stages in this pathway. Indeed, perforin expression in these two subsets was intermediate between that in the $CD27^+CD28^+$ and $CD27^-CD28^-$ subsets. Interestingly, the number of cells in the $CD27^-CD28^+$ subset was much lower in EBV-specific and HCMV-specific $CD8^+$ T cells than in total $CD8^+$ T cells in three individuals, M10, M19, and M30 (Fig. 3 and Table II). The same results were found in HCMV-specific $CD8^+$ T cells from other healthy individuals (our unpublished observation). A recent study showed that most growing populations of EBV-specific $CD8^+$ T cells in PBMC from patients with acute EBV infection express both a $CD27^+CD28^-CD45RO^+$ and $CD27^-CD28^-CD45RO^+$ phenotype (33). These findings suggest the following hypotheses: 1) the $CD27^-CD28^+$ subset is an intermediate in a subdominant pathway of differentiation from $CD27^+CD28^+$ to $CD27^-CD28^-$ subsets (this subset might be resting memory T cells); and 2) the $CD27^-CD28^+$ subset is an intermediate in the differentiation pathway of $CD8^+$ T cells specific for particular Ags from $CD27^+CD28^+$ to $CD27^-CD28^-$.

Perforin expression in the $CD27^+CD28^-$ subset of total $CD45RA^-CD8^+$ T cells seems to vary among individuals. The level of perforin expression in the $CD27^+CD28^-$ subset was similar to that of the $CD27^-CD28^-$ subset in individual U2, but similar to that of the $CD27^+CD28^+$ subset in individuals U5, U7, U10, and U11 (Fig. 2). Similar findings were observed in EBV-specific $CD8^+$ T cells. The $CD27^+CD28^-$ subset of EBV-specific $CD8^+$ T cells from individual M19 expressed a lower level of perforin than the same subset from individual M30 (Fig. 3). As the sorted $CD27^+CD28^-$ subset of HCMV-specific $CD8^+$ T cells from M30, which expressed a relatively higher level of perforin, effectively killed target cells, this subset should include effector cytotoxic T cells. A recent study also showed that

right of each plot. The four CD27/CD28 subsets of $CD45RA^-CD8^+$ T cells were gated and then analyzed for CCR7 expression. The percentage of CCR7-positive cells (M2) is shown in the *upper right corner* of each plot, and the MFI is shown in the *lower right corner*. The MFI for the four CD27/CD28 subsets of $CD45RA^-CD8^+$ T cells stained with mouse IgM mAb ranged from 3.2 to 3.5. The percentage of positive cells (M2) in these subsets was <2.5%. *B*, CCR7 expression of EBV-specific $CD45RA^-CD8^+$ T cells with different CD27/CD28 phenotypes. *Upper panels*, $CD8^+$ T cells were isolated from two individuals, M10 and M19, and then stained with anti-CD27, anti-CD28, and anti-CCR7 mAbs as well as EBV-1-A*1101 tetramers. $Tetramer^+$ cells were gated and then analyzed for CD27 and CD28 expression. The percentage of each CD27/CD28 subset of $CD45RA^-CD8^+$ T cells is shown to the *right* of each plot. *Lower panels*, CD27/CD28 subsets of $CD45RA^-CD8^+$ T cells were gated and then analyzed for CCR7 expression (shaded histograms) or an isotype control mouse IgM mAb (open histograms). The percentage of CCR7-positive cells (M2) is shown in the *upper right corner* of each plot and the MFI is shown in the *lower right corner*. The MFI for the four CD27/CD28 subsets of $CD45RA^-CD8^+$ T cells stained with mouse IgM mAb ranged from 3.2 to 3.9. The percentage of positive cells (M2) in these subsets was <1%.

the CD27$^+$CD28$^-$CD45RO$^+$ subset of EBV-specific CD8$^+$ T cells has cytotoxic activity (33). Thus, it is likely that this subset contains memory/effector CD8$^+$ T cells which can kill target cells but may include a wide range of subpopulations between memory and memory/effector phenotypes or various combinations of memory and memory/effector cells in different individuals.

A previous study showed that CD27$^+$CD45RA$^-$CD8$^+$ and CD27$^-$CD45RA$^-$CD8$^+$ T cells effectively produce IFN-γ and TNF-α after strong and nonspecific stimulation with PMA and ionomycin (8). Recent studies showed effective production of IFN-γ from both CD28$^+$ and CD28$^-$ fractions and from both CD45RA$^+$ and CD45RA$^-$ fractions in EBV-specific CD8$^+$ T cells after epitope peptide stimulation (34). In addition, a recent study showed that perforin expression and cytokine production segregated into subpopulations of CD8$^+$ T cells: perforin-positive cells express CD45RA but neither CD27 nor CD28, while IFN-γ-producing cells have both CD28(CD27)$^+$ and CD28(CD27)$^-$ phenotypes and a CD45RA$^-$ phenotype (35). These studies imply that the CD27$^+$CD28$^+$, CD27$^+$CD28$^-$, and CD27$^-$CD28$^-$ subsets of CD45RA$^-$CD8$^+$ T cells can effectively produce cytokines. Indeed, we showed in this work that these subsets effectively produced IFN-γ, TNF-α, and MIP-1β after stimulation with epitope peptides. Thus, the CD27$^+$CD28$^-$ and CD27$^-$CD28$^-$ subsets have cytotoxic activity and can produce cytokines, whereas the CD27$^+$CD28$^+$ subset can only produce cytokines.

Our recent study (31) showed that CD28$^+$CD45RA$^-$CD8$^+$ T cells include three subsets, CCR5$^-$CCR7$^+$, CCR5$^+$CCR7$^+$, and CCR5$^+$CCR7$^-$, and suggested that these subsets differentiate in the following sequence: CCR5$^-$CCR7$^+$$\rightarrow$CCR5$^+$CCR7$^+$$\rightarrow$CCR5$^+$CCR7$^-$. The present study further clarified CCR5/CCR7 expression in CD27$^+$CD28$^+$CD45RA$^-$CD8$^+$ T cells. The CD27$^+$CD28$^+$ subset, which is memory CD8$^+$ T cells, has both positive and negative populations for CCR5 and CCR7 expression. These findings suggest that CD27$^+$CD28$^+$CD45RA$^-$CD8$^+$ T cells also have three CCR5/CCR7 subsets that differentiate in the following sequence: CCR5$^-$CCR7$^+$$\rightarrow$CCR5$^+$CCR7$^+$$\rightarrow$CCR5$^+$CCR7$^-$ (Fig. 9).

We demonstrated that the number of CCR5$^+$ cells in the CD27$^+$CD28$^-$ subset is intermediate between that in the CD27$^+$CD28$^+$ and the CD27$^-$CD28$^-$ subsets. These findings further support the idea that the CD27$^+$CD28$^-$ subset is an intermediate between the CD27$^+$CD28$^+$ and CD27$^-$CD28$^-$ subsets. In contrast, as the CD27$^-$CD28$^+$ subset includes a small number of CCR5$^-$CCR7$^-$ cells and a large number of CCR5$^+$CCR7$^-$ cells, this subset may have characteristics between the CD27$^+$CD28$^+$ and CD27$^+$CD28$^-$ subsets (Fig. 9). The fact that the CD27$^-$CD28$^+$ subset expresses a lower level of perforin than the CD27$^+$CD28$^-$ subset also supports the idea that the CD27$^-$CD28$^+$ subset has characteristics close to memory CD8$^+$ T cells.

The CD27$^+$CD28$^-$ and CD27$^-$CD28$^-$ subsets fail to express CCR7, while ~25–45% of the former subset and 15–30% of the latter subset were CCR5$^+$ (Fig. 6). This indicates that both subsets have two populations, CCR5$^+$CCR7$^-$ and CCR5$^-$CCR7$^-$, and that the number of CCR5$^+$CCR7$^-$ cells decreases during differentiation from the CD27$^+$CD28$^-$ subset to the CD27$^-$CD28$^-$ subset. The number of CCR5$^+$CCR7$^-$ cells further decreased in the CD28$^-$CD45RA$^+$ effector CD8$^+$ T cell population (31). Because CCR5 is a receptor for RANTES, MIP-1α, and MIP-1β (36–39), it may play a role in the migration of memory CD8$^+$ T cells to secondary lymph nodes and inflammatory sites. Indeed, recent studies showed accumulation of CCR5$^+$ T cells at inflammatory sites in inflammatory kidney diseases (40), inflammatory bowel diseases (41), multiple sclerosis (42, 43), and rheumatoid arthritis (44). In contrast, binding of CCR5 ligands and CD3/CD28 costimulations causes down-regulation of CCR5 surface expression (45–47). These findings suggest that some CCR5$^+$ T cells may stop expressing CCR5 following migration to inflammatory sites and recognition of epitopes presented by MHC molecules.

A recent study demonstrated that both the CD27$^+$CD28$^-$ and CD27$^-$CD28$^-$ subsets of CD45RO$^+$CD8$^+$ T cells expand in acute EBV-infected individuals and that both subsets have cytotoxic activity (34). This study also showed that, in a CD3 mAb-mediated, redirected cytotoxicity assay, a CD27$^+$CD28$^+$CD45RO$^+$ subset from a healthy individual had no cytotoxic activity while this subset from a patient with acute EBV infection had cytotoxic activity. The present study showed that this subset in EBV-specific CD8$^+$ T cells from EBV-seropositive healthy individuals showed low perforin expression and no cytotoxic activity (Figs. 3 and 4). These findings suggest that this subset of EBV-specific CD8$^+$ T cells in patients with acute EBV infection may contain populations with cytotoxic effector function that are difficult to discriminate by analysis of CD27/CD28/CD45RA phenotypes.

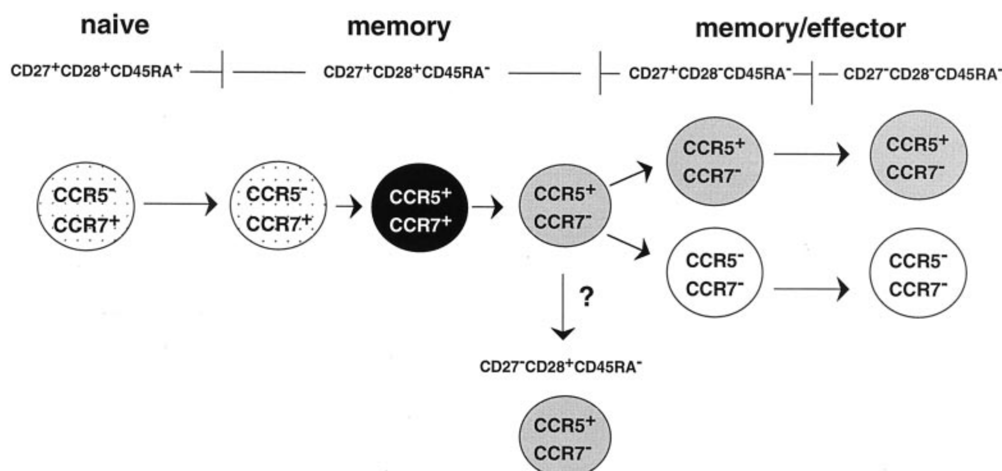

FIGURE 9. Proposed differentiation pathway for CD27$^+$CD28$^+$CD45RA$^-$ memory CD8$^+$ T cells to CD27$^-$CD28$^-$CD45RA$^-$ memory/effector CD8$^+$ T cells.

Previous studies have analyzed the expression of surface molecules on EBV-specific CD8$^+$ T cells from EBV-seropositive healthy individuals by flow cytometry using HLA class I tetramers and mAb against these molecules (48, 49). These studies showed that these cells express CD27, CD28, CD45RO, and CD62L, although the percentage of EBV-specific CD8$^+$ T cells expressing these molecules varied among individuals. Using HLA-A*1101 latent epitope tetramers, the present study showed that most EBV-specific CD8$^+$ T cells from EBV-seropositive healthy individuals have a CD27$^+$CD28$^+$ CD45RA$^-$ memory phenotype. A recent study revealed that EBV lytic epitope-specific CD8$^+$ T cells heterogeneously express CD28 and CD45RA/RO, whereas most EBV latent epitope-specific CD8$^+$ T cells express CD28 and CD45RO (34). This suggests that EBV latent epitope-specific CD8$^+$ T cells are memory T cells while EBV lytic epitope-specific CD8$^+$ T cells include both memory and memory/effector or effector T cells. The use of two HLA-A*1101 latent epitope tetramers in the present study also showed that ~80% of EBV-specific CD8$^+$ T cells express a (CD27$^+$)CD28$^+$CD45RA$^-$ memory phenotype in EBV-seropositive healthy individuals. However, this varied from 60 to 98% among individuals (Table I).

In summary, we investigated the expression of perforin and two chemokine receptors as well as the function (cytotoxicity and cytokine production) of CD27/CD28 subsets of CD45RA$^-$CD8$^+$ T cells. The data suggested a differentiation pathway for memory CD8$^+$ T cells into memory/effector CD8$^+$ T cells. Further detailed analysis of each step in the differentiation pathway of memory CD8$^+$ T cells is important for our understanding of immune memory in CD8$^+$ T cells.

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References

1. Guidotti, L. G., and F. V. Chisari. 1996. To kill or to cure: options in host defense against viral infection. *Curr. Opin. Immunol. 8:478.*
2. Cocchi, F., A. L. DeVico, A. Garzino-Demo, S. K. Arya, R. C. Gallo, and P. Lusso. 1995. Identification of RANTES, MIP-1α, and MIP-1β as the major HIV-suppressive factors produced by CD8$^+$ T cells. *Science 270:1811.*
3. Levy, J. A., C. E. Mackewicz, and E. Barker. 1996. Controlling HIV pathogenesis: the role of the noncytotoxic anti-HIV response of CD8$^+$ T cells. *Immunol. Today 17:217.*
4. Liu, C. C., C. M. Walsh, and J. D. Young. 1995. Perforin: structure and function. *Immunol. Today 16:194.*
5. Trapani, J. A., V. R. Sutton, and M. J. Smyth. 1999. CTL granule: evolution of vesicles essential for combating virus infections. *Immunol. Today 20:351.*
6. Azuma, M., J. H. Phillips., and L. L. Lanier. 1993. CD28$^-$ T lymphocytes: antigenic and functional properties. *J. Immunol. 150:1147.*
7. Weekes, M. P., A. J. Carmichael, M. R. Wills, K. Mynard, and J. G. Sissons. 1999. Human CD28$^-$CD28$^+$ T cells contain greatly expanded functional virus-specific memory CTL clones. *J. Immunol. 162:7569*.
8. Hamann, D., P. A. Baars, M. H. Rep, B. Hooibrink, S. R. Kerkhof-Garde, M. R. Klein, and R. A. van Lier. 1997. Phenotypic and functional separation of memory and effector human CD8$^+$ T cells. *J. Exp. Med. 186:1407.*
9. Lenschow, D. J., T. L. Walunas, and J. A. Bluestone. 1996. CD28/B7 system of T cell costimulation. *Annu. Rev. Immunol. 14:233.*
10. Greenfield, E. A., K. A. Nguyen, and V. K. Kuchroo. 1998. CD28/B7 costimulation: a review. *Crit. Rev. Immunol. 18:389.*
11. Viola, A., and A. Lanzavecchia. 1996. T cell activation determined by T cell receptor number and tunable thresholds. *Science 273:104.*
12. Lake, R. A., R. E. O'Hehir, A. Verhoef, and J. R. Lamb. 1993. CD28 mRNA rapidly decays when activated T cells are functionally anergized with specific peptide. *Int. Immunol. 5:461.*
13. Van Lier, R. A., J. Borst, T. M. Vroom, H. Klein, P. Van Mourik, W. P. Zeijlemaker, and C. J. Melief. 1987. Tissue distribution and biochemical and functional properties of Tp55 (CD27), an novel T cell differentiation antigen. *J. Immunol. 139:1589.*
14. Hintzen, R. Q., R. de Jong, S. M. A. Lens, and R. A. W. van Lier. 1994. CD27: marker and mediator of T-cell activation. *Immunol. Today 15:307.*
15. Gravestein, L. A., J. D. Nieland, A. M. Kruisbeek, and J. Borst. 1995. Novel mAbs reveal potent co-stimulatory activity of murine CD27. *Int. Immunol. 7:551.*
16. Oshima, H., H. Nakano, C. Nohara, T. Kobata, A. Nakajima, N. A. Jenkins, D. J. Gilbert, N. G. Copeland, T. Muto, H. Yagita, and K. Okumura. 1998. Characterization of murine CD70 by molecular cloning and mAb. *Int. Immunol. 10:517.*
17. Kobata, T., K. Agematsu, J. Kameoka, S. F. Schlossman, and C. Morimoto. 1994. CD27 is a signal-transducing molecule involved in CD45RA$^+$ naive T cell costimulation. *J. Immunol. 153:5422.*
18. Hintzen, R. Q., S. M. Lens, K. Lammers, H. Kuiper, M. P. Beckmann, and R. A. van Lier. 1995. Engagement of CD27 with its ligand CD70 provides a second signal for T cell activation. *J. Immunol. 154:2612.*
19. Hendriks, J., L. A. Gravestein, K. Tesselaar, R. A. van Lier, T. N. Schumacher, and J. Borst. 2000. CD27 is required for generation and long-term maintenance of T cell immunity. *Nat. Immunol. 1:433.*
20. Okumura, M., Y. Fujii, K. Inada, K. Nakahara, and H. Matuda. 1993. Both CD45RA$^+$ and CD45RA$^-$ subpopulations of CD8$^+$ T cells contain cells with high levels of lymphocyte function-associated antigen-1 expression, a phenotype of primed T cells. *J. Immunol. 150:429.*
21. Gillespie, G. M., M. R. Wills, V. Appay, C. O'Callaghan, M. Murphy, N. Smith, P. Sissons, S. Rowland-Jones, J. I. Bell, and P. A. Moss. 2000. Functional heterogeneity and high frequencies of cytomegalovirus-specific CD8$^+$ T lymphocytes in healthy seropositive donors. *J. Virol. 74:8140.*
22. Callan, M. F., L. Tan, N. Annels, G. S. Ogg, J. D. Wilson, C. A. O'Callaghan, N. Steven, A. J. McMichael, and A. B. Ricknson. 1998. Direct visualization of antigen-specific CD8$^+$ T cells during the primary immune response to Epstein-Barr virus in vivo. *J. Exp. Med. 187:1395.*
23. Sobao, Y., H. Tomiyama, S. Nakamura, H. Sekihara, K. Tanaka, and M. Takiguchi. 2001. Visual demonstration of hepatitis C virus-specific memory CD8$^+$ T-cell expansion in patients with acute hepatitis C. *Hepatology 33:287.*
24. Hamann, D., M. T. L. Roos, and R. A. W. van Lier. 1999. Faces and phases of human CD8$^+$ T-cell development. *Immunol. Today 20:177.*
25. Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature 401:708.*
26. Champagne, P., G. S. Ogg, A. S. King, C. Knabenhans, K. Ellefsen, M. Nobile, V. Appay, G. P. Rizzardi, S. Fleury, M. Lipp, et al. 2001. Skewed maturation of memory HIV-specific CD8 T lymphocytes. *Nature 410:106.*
27. 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.*
28. Gavioli, R., M. G. Kurilla, P. O. de Campos-Lima, L. E. Wallace, R. Dolcetti, R. J. Murray, A. B. Rickinson, and M. G. Masucci. 1993. Multiple HLA-A11-restricted cytotoxic T-lymphocyte epitopes of different immunogenicities in the Epstein-Barr virus-encoded nuclear antigen 4. *J. Virol. 67:1572.*
29. Karaki, S., A. Kariyone, N. Kato, K. Kano, Y. Iwakura, and M. Takiguchi. 1993. HLA-B51 transgenic mice as recipients for production of polymorphic HLA-A, B-specific antibodies. *Immunogenetics 37:139.*
30. Altman, J. D., P. A. Moss, P. J. Goulder, D. H. Barouch, M. G. McHeyzer-Williams, J. I. Bell, A. J. McMichael, and M. M. Davis. 1996. Phenotypic analysis of antigen-specific T lymphocytes. *Science 274:94.*
31. Fukada, K., Y. Sobao, H. Tomiyama, S. Oka, and M. Takiguchi. 2002. Functional expression of the chemokine receptor CCR5 on virus epitope-specific memory and effector CD8$^+$ T cells. *J. Immunol. 168:2225.*
32. Campbell, J. J., K. E. Murphy, E. J. Kunkel, C. E. Brightling, D. Soler, Z. Shen, J. Boisvert, H. B. Greenberg, M. A. Vierra, S. B. Goodman, et al. 2001. CCR7 expression and memory T cell diversity in humans. *J. Immunol. 166:877.*
33. Roos, M. T., R. A. van Lier, D. Hamann, G. J. Knol, I. Verhoofstad, D. van Baarle, F. Miedema, and P. T. Shellekens. 2000. Changes in the composition of circulation CD8$^+$ T cells subsets during acute Epstein-Barr and human immunodeficiency virus infections in humans. *J. Infect. Dis. 182:451.*
34. Hislop, A. D., N. H. Gudgeon, M. F. Callan, C. Fazou, H. Hasegawa, M. Salmon, and A. B. Rickinson. 2001. EBV-specific CD8$^+$ T cell memory: relationships between epitope specificity, cell phenotype, and immediate effector function. *J. Immunol. 167:2019.*
35. Sandberg, J. K., N. M. Fast, and D. F. Nixon. 2001. Functional heterogeneity of cytokines and cytolytic effector molecules in human CD8$^+$ T lymphocytes. *J. Immunol. 167:181.*
36. Raport, C. J., J. Gosling, V. L. Schweichkert, P. W. Gray, and I. F. Charo. 1996. Molecular cloning and functional characterization of a novel human CC chemokine receptor (CCR5) for RANTES, MIP-1β, and MIP-1α. *J. Biol. Chem. 271: 17161.*
37. Combadiere, C., S. K. Ahuja, H. L. Tiffany, and P. M. Murphy. 1996. Cloning and functional expression of CC CKR5, a human monocyte CC chemokine receptor selective for MIP-1α, MIP-1β, and RANTES. *J. Leukocyte Biol. 60:147.*
38. Rollins, B. J. 1997. Chemokines. *Blood 90:909.*
39. Ward, S. G., K. Bacon, and J. Westwick. 1998. Chemokines and T lymphocytes: more than an attraction. *Immunity 9:1.*
40. Segerer, S., M. Mack, H. Regele, D. Kerjaschki, and D. Schlondörff. 1999. Expression of the C-C chemokine receptor 5 in human kidney diseases. *Kidney Int. 56:52.*

41. Agace, W. W., A. I. Roberts, L. Wu, C. Greineder, E. C. Ebert, and C. M. Parker. 2000. Human intestinal lamina propria and intraepithelial lymphocytes express receptors specific for chemokines induced by inflammation. *Eur. J. Immunol. 30:819.*
42. Balashov, K. E., J. B. Rottman, H. L. Weiner, and W. W. Hancock. 1999. CCR5$^+$ and CXCR3$^+$ T cells are increased in multiple sclerosis and their ligands MIP-1α and IP-10 are expressed in demyelinating brain lesions. *Proc. Natl. Acad. Sci. 96:6873.*
43. Sorensen, T. L., M. Tani, J. Jensen, V. Pierce, C. Lucchinetti, V. A. Folcik, S. Qin, J. Rottman, F. Sellebjerg, R. M. Strieter, et al. 1999. Expression of specific chemokines and chemokine receptors in the central nervous system of multiple sclerosis patients. *J. Clin. Invest. 103:807.*
44. Suzuki, N., A. Nakajima, S. Yoshino, K. Matsushima, H. Yagita, and K. Okumura. 1999. Selective accumulation of CCR5$^+$ T lymphocytes into inflamed joints of rheumatoid arthritis. *Int. Immunol. 11:553.*
45. Carroll, R. G., J. L. Riley, B. L. Levine, Y. Feng, S. Kaushal, D. W. Ritchey, W. Bernstein, O. S. Weislow, C. R. Brown, E. A. Berger, et al. 1997. Differential regulation of HIV-1 fusion cofactor expression by CD28 costimulation of CD4$^+$ T cells. *Science 276:273.*
46. Riley, J. L., R. G. Carroll, B. L. Levine, W. Bernstein, D. C. St. Louis, O. S. Weislow, and C. H. June. 1997. Intrinsic resistance to T cell infection with HIV type 1 induced by CD28 costimulation. *J. Immunol. 158:5545.*
47. Mack, M., J. Cihak, C. Simonis, B. Luckow, A. E. Proudfoot, J. Plachy, H. Bruhl, M. Frink, H.-J. Anders, V. Vielhauer, et al. 2001. Expression and characterization of the chemokine receptors CCR2 and CCR5 in mice. *J. Immunol. 166:4697.*
48. Tan, L. C., N. Gudgeon, N. E. Annels, P. Hansasuta, C. A. O'Callaghan, S. Rowland-Jones, A. J. McMichael, A. B. Rickinson, and M. F. Callan. 1999. A reevaluation of the frequency of CD8$^+$ T cells specific for EBV in healthy virus carriers. *J. Immunol. 162:1827.*
49. Tussey, L., S. Speller, A. Gallimore, and R. Vessey. 2000. Functionally distinct CD8$^+$ memory T cell subsets in persistent EBV infection are differentiated by migratory receptor expression. *Eur. J. Immunol. 30:1823.*