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Differentiation of Human CD8⁺ T Cells from a Memory to Memory/Effector Phenotype¹

Hiroko Tomiyama,² Tomoko Matsuda,² and Masafumi Takiguchi³

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 character-

ized 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⁻CD45RA⁺ 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⁺ → CCR7⁺CD45RA⁻ → CCR7⁻CD45RA⁻ → 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 μ l 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 \pm SD	1.4 \pm 1.4	76.9 \pm 11.7	20.8 \pm 9.9	1.0 \pm 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 \pm SD	1.8 \pm 0.9	85.0 \pm 7.3	12.3 \pm 6.4	1.0 \pm 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 cells stained 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 ⁵¹Cr release assay as follows. Target cells (2×10^5) were incubated for 60 min with 100 μ Ci Na₂ ⁵¹Cr 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 (FACSVerse 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.

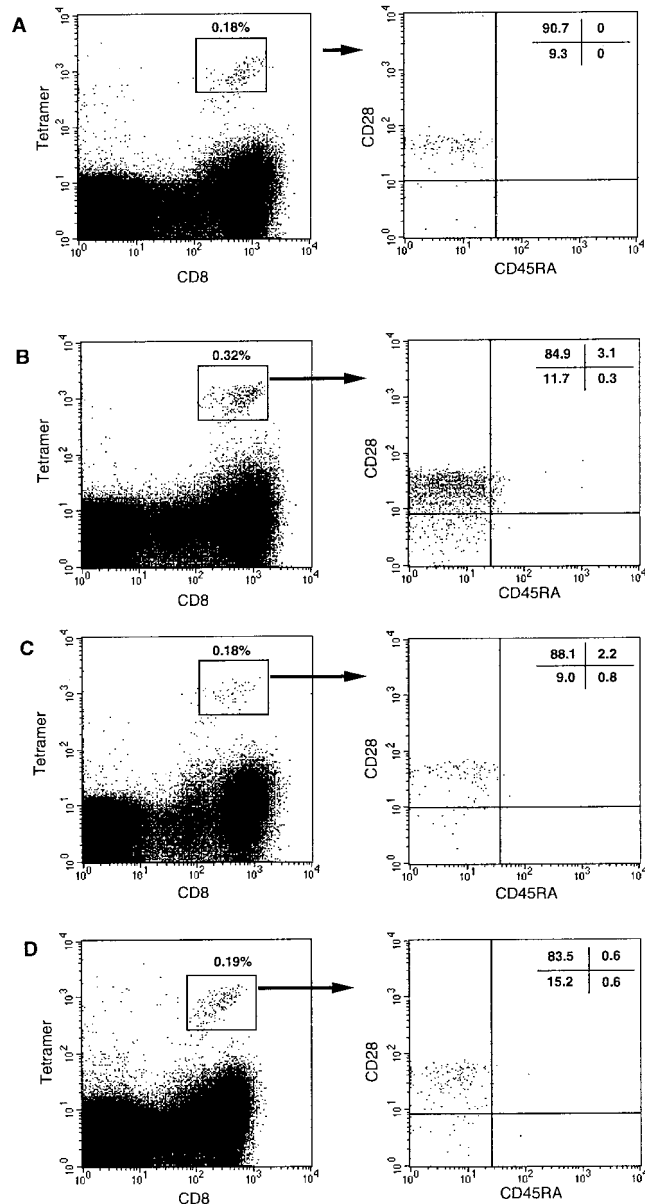


FIGURE 1. CD28/CD45RA phenotype of EBV-specific CD8⁺ T cells. PBMCs from individuals M14 (A) and U5 (B) were stained simultaneously with the EBV-1-A*1101 tetramer, anti-CD8 mAb, anti-CD28 mAb, and anti-CD45RA mAb. Similarly, PBMCs from individuals M14 (C) and A21 (D) were simultaneously stained with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

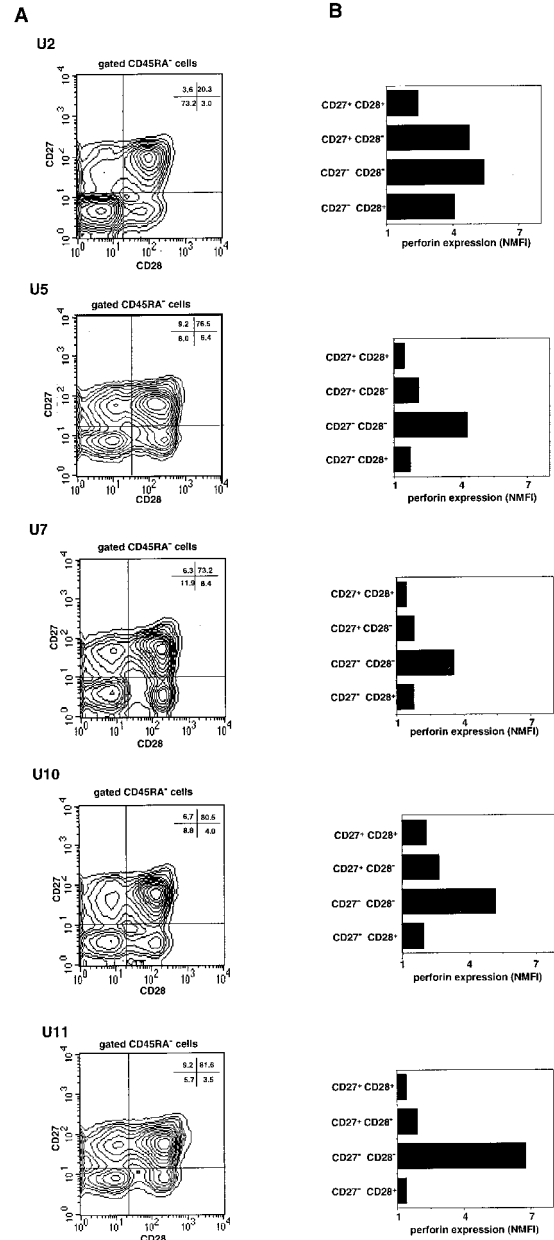


FIGURE 2. Frequency of, and perforin expression in, different CD27/CD28 subsets of CD45RA⁻CD8⁺ T cells. A, Frequency of different CD27/CD28 subsets of CD45RA⁻CD8⁺ T cells. CD8⁺ T cells were isolated from five individuals, U2, U5, U7, U10, and U11, and then stained with anti-CD27 mAb, anti-CD28 mAb, anti-CD45RA 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 ⁵¹Cr release was determined by measuring the cpm in supernatants from wells containing only target cells (cpm spn). Maximum ⁵¹Cr 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 × 10⁶) were dispensed into a U-bottom 96-well plate with or without 1 μM of an 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

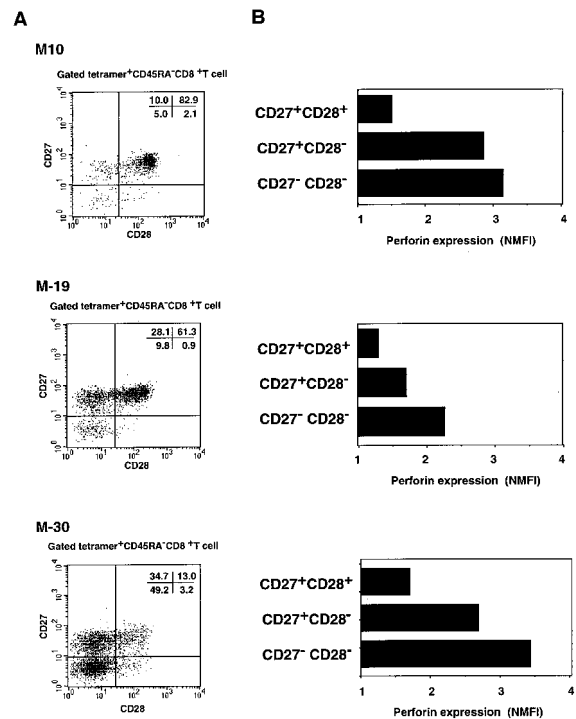


FIGURE 3. Frequency of, and perforin expression in, different CD27/CD28 subsets of EBV-specific and HCMV-specific CD45RA⁻CD8⁺ T cells. A, Frequency of different CD27/CD28 subsets of EBV-specific and HCMV-specific CD45RA⁻CD8⁺ T cells. CD8⁺ T cells were isolated from three individuals (M10, M19, and M30) 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

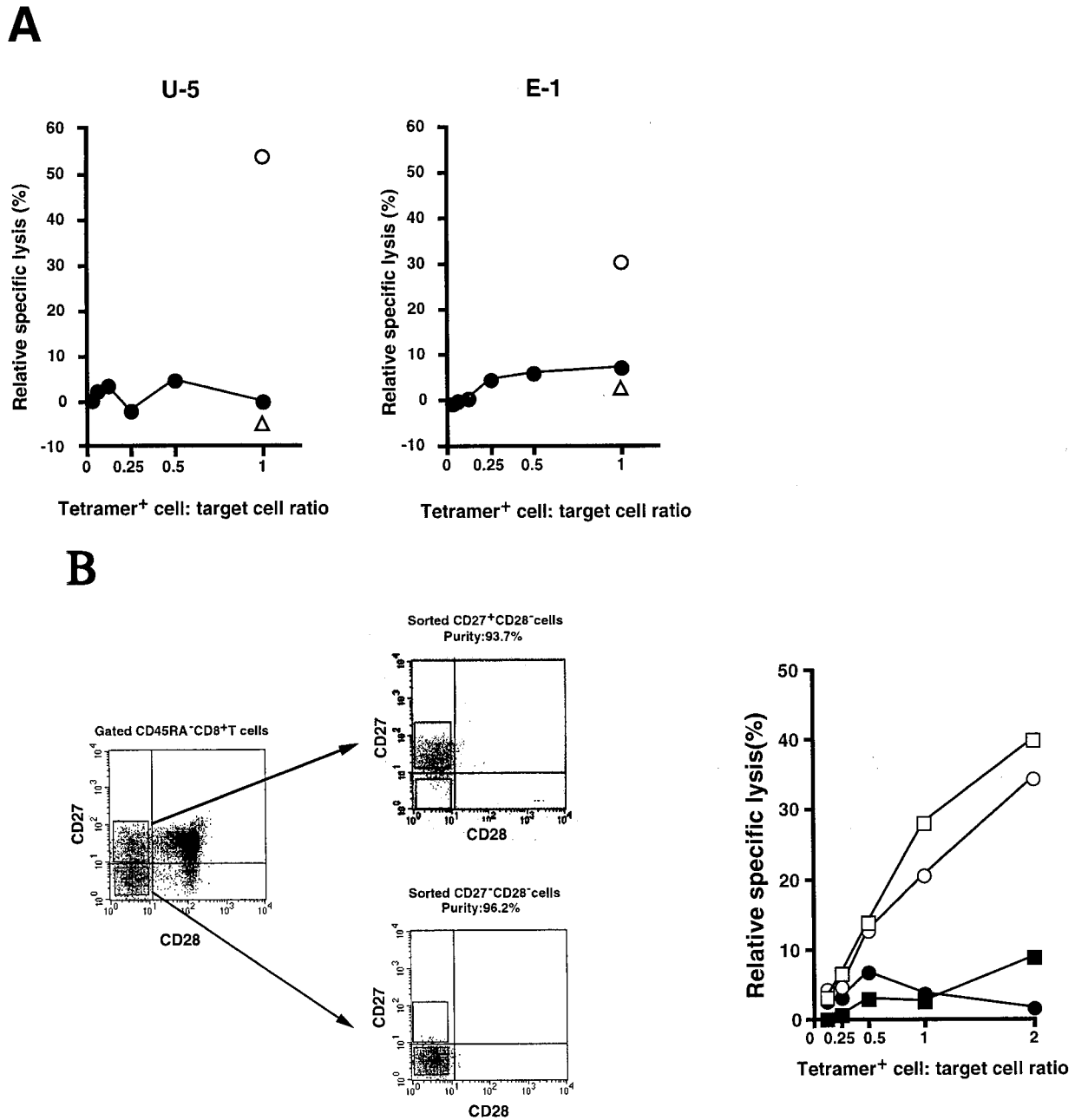


FIGURE 4. Cytotoxic activity of HCMV-1-specific and EBV-specific CD8⁺ T cells. **A**, Cytotoxic activity of EBV-specific CD8⁺ T cells. CD8⁺ T cells were isolated from two individuals, U5 and E1. The percentage of HLA-A*1101-restricted, EBV-specific CD8⁺ T cells was measured using EBV-1-A*1101 and EBV-2-A*1101 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. 2A 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. 2B). 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 indi-

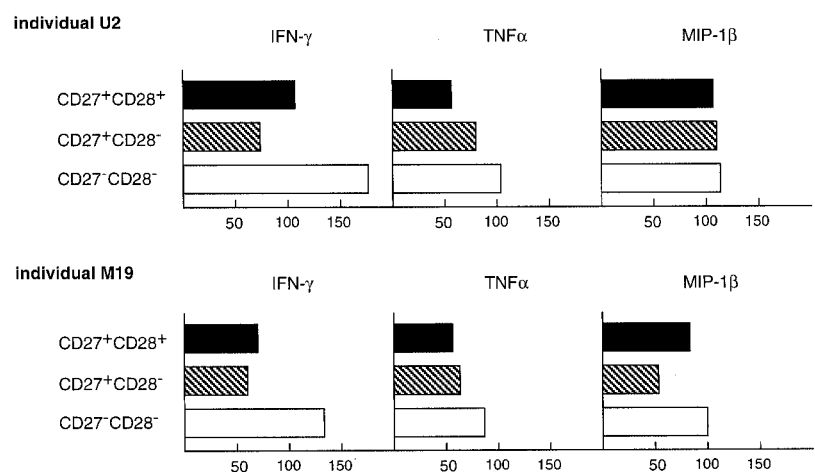
viduals M10 and M19, who had a higher number of EBV-specific CD28⁻CD45RA⁻CD8⁺ T cells than the other individuals examined (Table I), and individual M30, who exclusively had HCMV-specific CD45RA⁻CD8⁺ T cells. CD27 and CD28 expression was analyzed on the EBV tetramer⁺CD45RA⁻ fraction of CD8⁺ T cells isolated 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. 3A). 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. 3A). 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. 2B and 3B). 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. 4A). 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.



Percentage of cytokine-producing cells in CD27/CD28 subsets of tetramer⁺ CD8⁺ T cells

used PBMCs isolated from individual M30, whose HCMV-specific CD45RA⁻CD8⁺ T cells contained both subsets (Fig. 3A), 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. 4B). 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-1 β -producing cells 6 h after

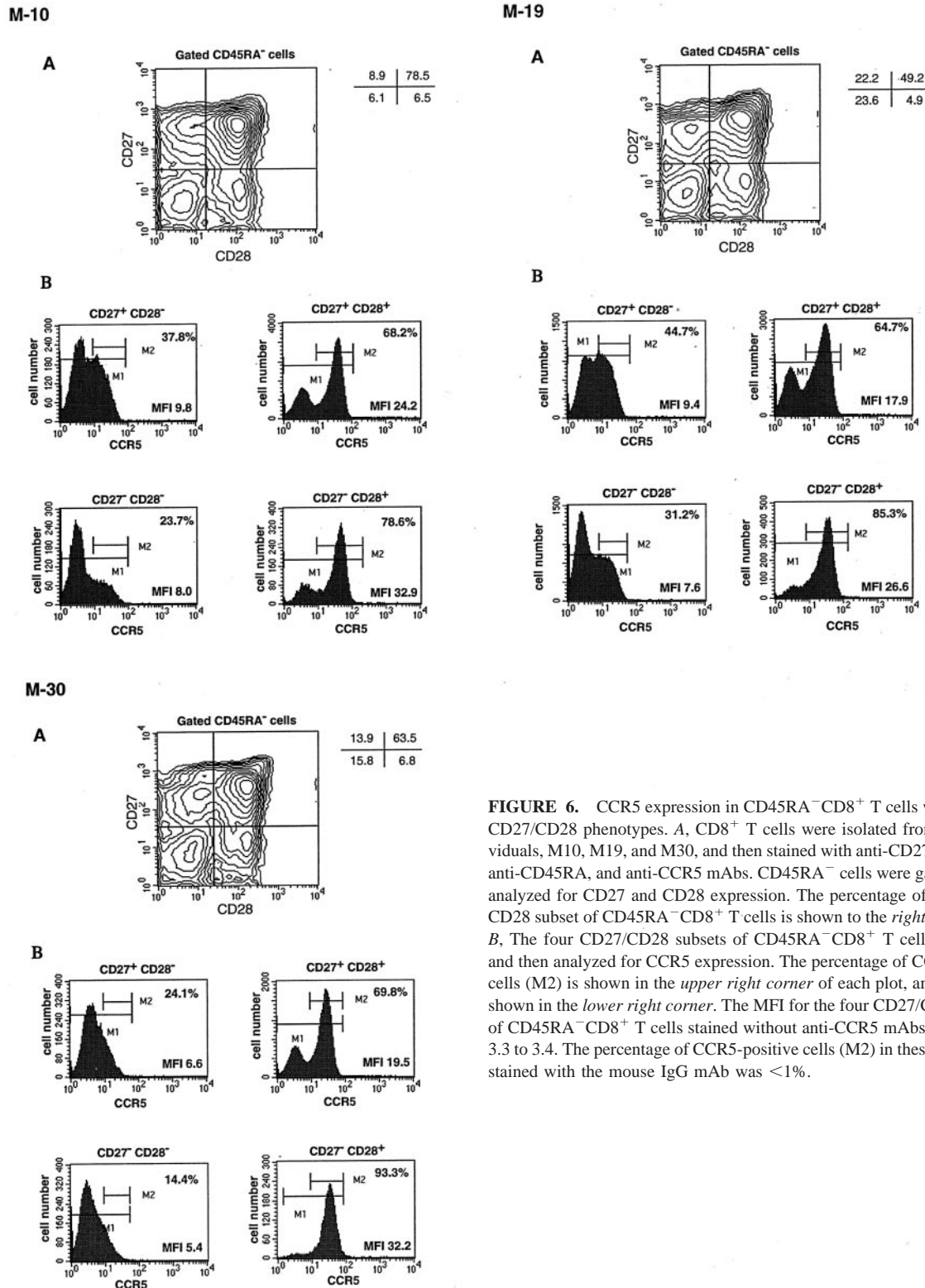


FIGURE 6. CCR5 expression in CD45RA⁻CD8⁺ T cells with different CD27/CD28 phenotypes. **A**, CD8⁺ T cells were isolated from three individuals, M10, M19, and M30, and then stained with anti-CD27, anti-CD28, anti-CD45RA, and anti-CCR5 mAbs. CD45RA⁻ cells were gated and then analyzed for CD27 and CD28 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⁺ → CD27⁺CD28⁻ → CD27⁻CD28⁻ for the differentiation of memory CD8⁺ T cells to memory/effector CD8⁺ T cells suggested by the observed perforin expression levels

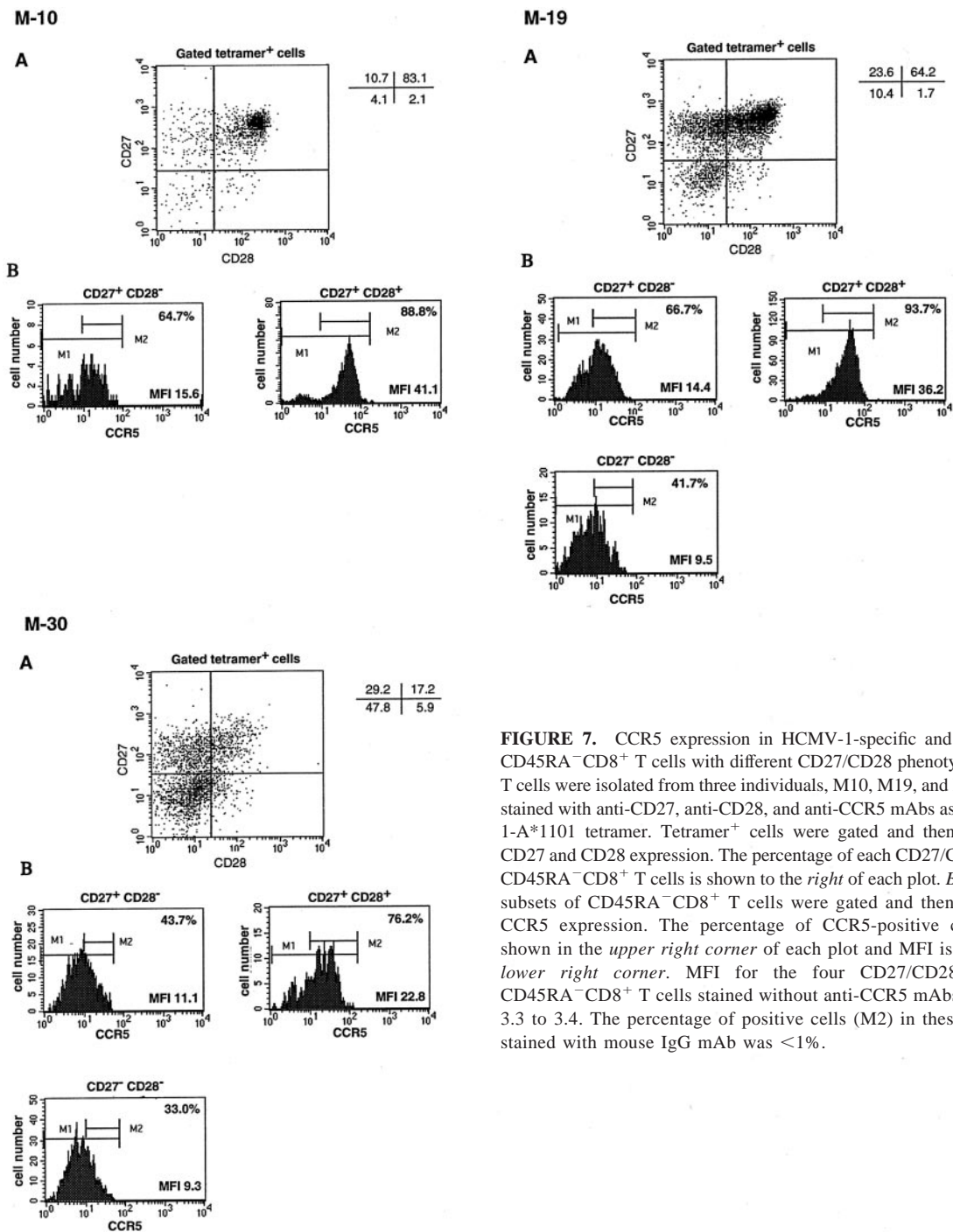


FIGURE 7. CCR5 expression in HCMV-1-specific and EBV-specific CD45RA⁻CD8⁺ T cells with different CD27/CD28 phenotypes. **A**, CD8⁺ T cells were isolated from three individuals, M10, M19, and M30, and then stained with anti-CD27, anti-CD28, and anti-CCR5 mAbs as well as EBV-1-A*1101 tetramer. Tetramer⁺ 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%.

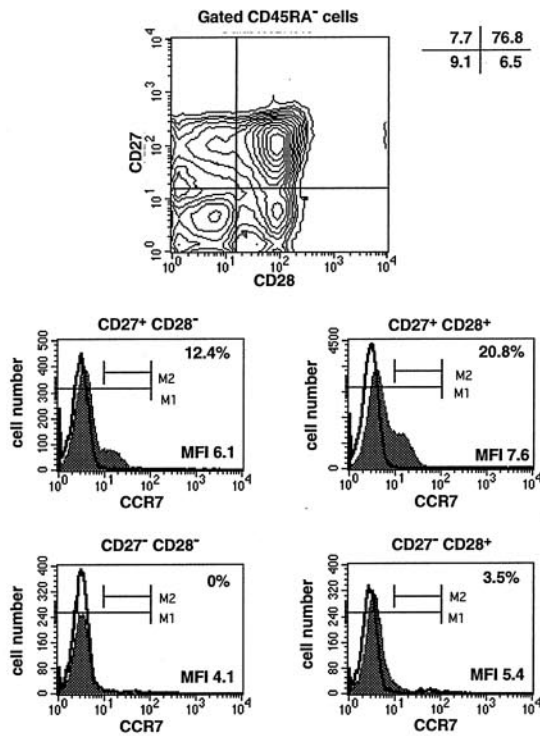
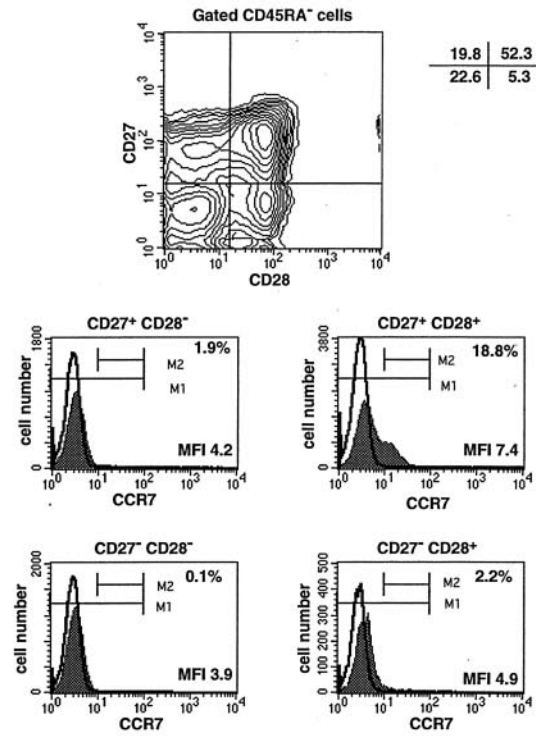
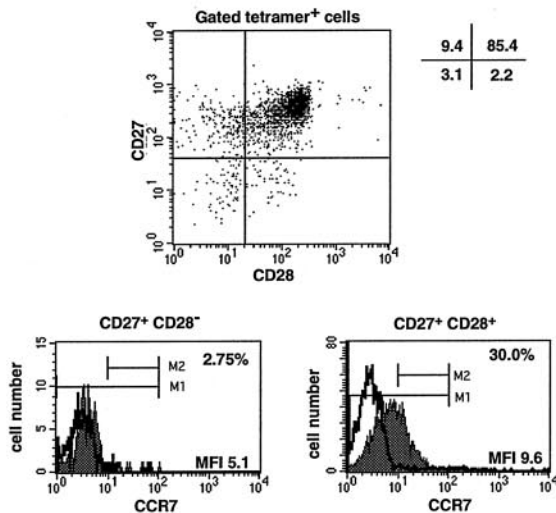
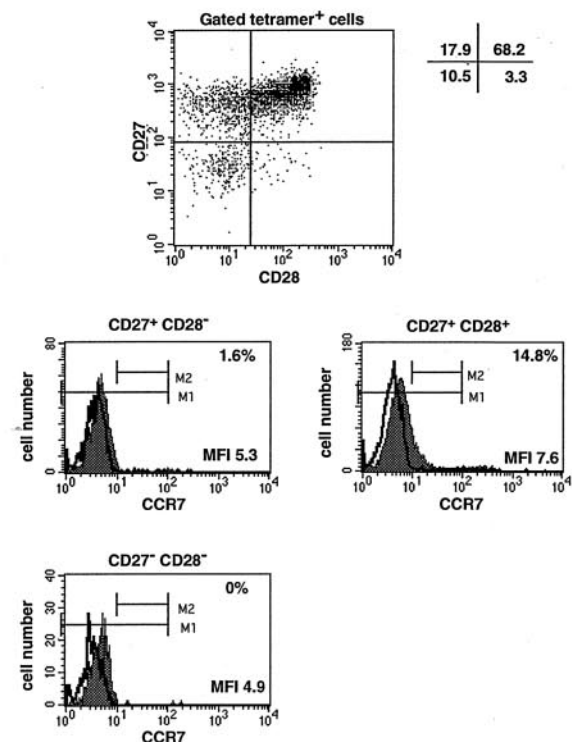
A**M10****M19****B****M10****M19**

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. 8A). 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. 8B). 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, 7A, and 8B), 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. 8A), 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-1 β 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-1 α , and MIP-1 β (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.

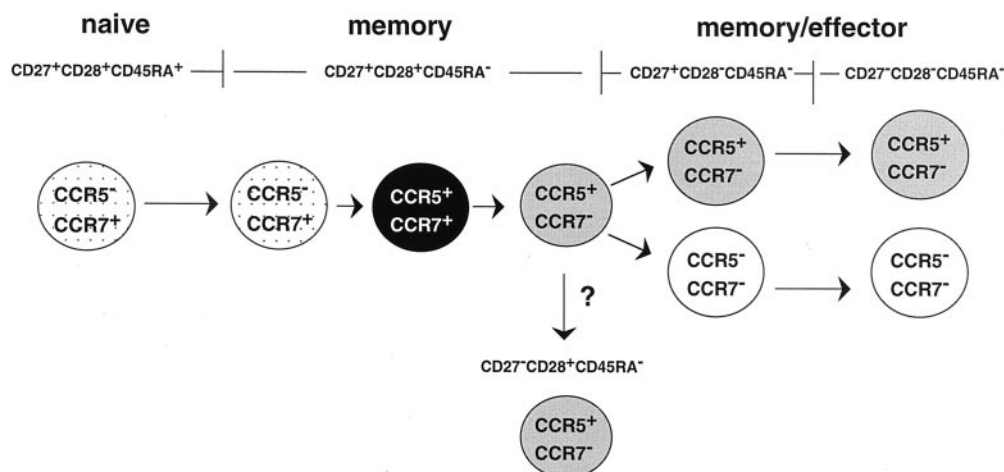


FIGURE 9. Proposed differentiation pathway for CD27⁺CD28⁺CD45RA⁻ memory CD8⁺ T cells to CD27⁻CD28⁻CD45RA⁻ memory/effector CD8⁺ T cells.

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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