Phenotypic classification of human CD8+ T cells reflecting their function: inverse correlation between quantitative expression of CD27 and cytotoxic effector function

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Phenotypic classification of human CD8+ T cells using three cell surface markers, CD27, CD28 and CD45RA, was recently suggested to be useful for identification of naive, memory and effector CD8+ T cells. However, it still remains unclear whether such classification precisely reflects functional classification of CD8+ T cells. To clarify this, we characterized each CD27CD28CD45RA subset of total and human cytomegalovirus (HCMV)-specific CD8+ T cells by analyzing the expression of perforin and two chemokine receptors, CCR5 and CCR7, as well as their function. An inverse correlation between perforin and CD27 expression was found in all four CD28CD45RA subsets. Therefore, to achieve a phenotypic classification of CD8+ T cells that more precisely reflects their function, the CD27+ subset was divided into $CD27^{low}$ and $CD27^{high}$ subsets based on the expression level of CD27. Functional and flow cytometric analyses of CD27CD28CD45RA subsets showed that this phenotypic classification reflects functional classification of CD8+ T cells. HCMV-specific CD8+ T cells from healthy HCMV-seropositive individuals were predominantly found in effector and memory/effector subsets, indicating that HCMV-specific effector CD8+ T cells are actively induced by HCMV replication in healthy HCMV carriers. Phenotypic analyses of CD8+ T cells using this classification will enable the characterization of antigen-specific CD8+ T cells.

Received	14/8/03
Revised	20/1/04
Accepted	6/2/04

Key words: CTL / Cytomegalovirus / Memory / Effector

1 Introduction

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 [1–4] as well as in cytolysis of virus-infected cells [5, 6]. Effector cytotoxic CD8+ T cells have the ability to kill target cells through perforin and Fas ligands. CD8+ T cells that can kill target cells can be divided into two groups: effector cells and memory/effector cells. The former express a high level of perforin, have strong cytolytic activity, and produce low levels of cytokines such as IL-2 and IFN-γ, while the latter have medium levels of perforin, cytotoxic activity and produce high levels of cytokines [7, 8]. Memory CD8+ T cells fail to kill target cells but can proliferate and produce cytokines in response to antigen stimulation [7, 9].

[DOI 10.1002/eji.200324478]

Abbreviations: HCMV: Human cytomegalovirus **MFI:** Mean fluorescence intensity **NMFI:** Normalized MFI

Phenotypic classification of memory and effector CD8+ T cells is very useful in many human and animal immunological studies. In humans, previous studies suggested that particular expression patterns of costimulatory receptors CD27 and CD28 as well as CD45RA or CD45RO are associated with the naive, memory and effector function of human CD8+ T cells [8, 10–16]. Effector and memory/effector CD8+ T cells have a $CD28^-CD45RA^+$ or $CD27^-CD45RA^+$ and $CD28^-CD45RA^-$ or $CD27^-CD45RA^-$ phenotype, respectively, while naive and memory CD8+ T cells have a $CD28^+CD45RA^+$ or $CD27^+CD45RA^+$ and $CD28^+CD45RA^-$ or $CD27^+CD45RA^-$ phenotype, respectively [7, 15, 16]. In addition, a recent study of human CD8+ T cells with multi-color flow cytometric analysis showed that $CD27^+CD28^-CD45RA^-CD8^+$ T cells have cytotoxic activity and can effectively produce cytokines, suggesting that this phenotype reflects also memory/effector CD8+ T cells [8]. In another study, human CD8+ T cells were classified into four major subsets according to CD27 and CD28 expression: naive ($CD27^+CD28^+$), early ($CD27^+CD28^+$), intermediate ($CD27^+CD28^-$) and late ($CD27^-CD28^-$), though naive and

early subsets were mostly discriminated by the expression of CD45RA and CCR7 [17].

The chemokine receptor CCR7 is very useful for discriminating naive and memory $CD8^+$ T cells from memory/effector and effector $CD8^+$ T cells [8, 18, 19]. CCR7 functions as a homing receptor and is expressed in naive $CD8^+$ T cells and a subset of memory $CD8^+$ T cells. Previous studies revealed the following classification of $CD8^+$ T cells using CCR7 and CD45RA: naive ($CCR7^+CD45RA^+$), central/memory ($CCR7^+CD45RA^-$) and effector/memory ($CCR7^-CD45RA^{+/-}$) [16, 18, 19]. Recent studies showed that the CCR5 chemokine receptor, whose ligands are RANTES, macrophage inflammatory protein (MIP)-1α and MIP-1β, is expressed in memory, memory/effector and effector $CD8^+$ T cells [8, 20], with the number of $CCR5^+CD8^+$ T cells decreasing during differentiation of $CD27^+CD28^+CD45RA^-$ T cells to $CD27^-CD28^-CD45RA^-$ T cells [20]. These studies imply that these two chemokine receptors are also useful for classification of $CD8^+$ T cells.

A recent study of phenotypic classification using CD27 and CD28 suggested that three phenotypic subsets reflect the functional status of memory and effector $CD8^+$ T cells [17]. However, this study implied that this phenotypic classification of $CD8^+$ T cells is not appropriate because different phenotypic subsets were found in memory $CD8^+$ T cells responding to different viruses. An alternative interpretation of these findings is that this phenotypic classification of $CD8^+$ T cells was incomplete and that memory and effector $CD8^+$ T cells are induced at different stages by different viruses. Previous studies of human $CD8^+$ T cells using these cell surface markers might have only partially classified and analyzed human $CD8^+$ T cells. As these studies did not investigate human $CD8^+$ T cells by both multi-color flow cytometric analysis and functional analysis of each subset, the correlation between the functional subsets and the phenotypic subsets classified by these markers is still unclear.

In the present study, we attempted to classify human $CD8^+$ T cells using three cell surface markers, CD27, CD28, and CD45RA, as well as two ckemokine receptors, CCR5 and CCR7. The function of CD27CD28CD45RA subsets was further investigated to clarify whether our phenotypic classification precisely reflects functional classification. We propose the classification of human $CD8^+$ T cells using quantitative expression of CD27 in addition to qualitative expression of CD28 and CD45RA.

2 Results

2.1 Inverse correlation between perforin and CD27 expression in CD28CD45RA subsets of $CD8^+$ T cells

Perforin is often used as one marker for effector T cells when characterizing $CD8^+$ T cells, because it is a functional molecule for killing cells, which is one of the most important $CD8^+$ T cell functions [5, 7, 15]. To characterize each CD27CD28CD45RA subset of human $CD8^+$ T cells, we stained total $CD8^+$ T cells from eight healthy individuals with mAb specific for CD27, CD28 and CD45RA as well as a perforin-specific mAb, and then analyzed each subset for perforin expression level. First, we analyzed the correlation between perforin and CD27 or CD28 expression. Perforin expression was significantly different between cells expressing a high level of CD27 ($CD27^{high}$) and a low level of CD27 ($CD27^{low}$) ($p<0.005$), while there was no difference in perforin expression between cells expressing high and low levels of CD28 (data not shown). Therefore, we analyzed each CD27CD28CD45RA subset by dividing the $CD27^+$ cells into $CD27^{high}$ and $CD27^{low}$ cells. $CD27^{high}$ cells showed a lower level of perforin than $CD27^{low}$ cells in $CD27^+$ cells of all four CD28CD45RA subsets. Results from a representative individual and all eight healthy individuals are shown in Fig. 1A and B, respectively.

The $CD27^{high}CD28^+CD45RA^-$ subset did not express perforin, while the $CD27^{low/-}CD28^+CD45RA^-$ subsets included a small number of cells expressing a low level of perforin. Perforin expression of the latter subsets was significantly higher than that of the former subset ($p<0.05$; Fig. 1B). Similarly, the $CD27^{high}CD28^+CD45RA^+$ subset did not express perforin, while the $CD27^{low}CD28^+CD45RA^+$ subset included a small number of cells expressing a low level of perforin. The $CD27^{low}CD28^-CD45RA^{+/-}$ subsets contained a large number of cells expressing a low level of perforin, while the $CD27^-CD28^-CD45RA^{+/-}$ subsets included a large number of cells expressing a high level of perforin. In both $CD45RA^+$ and $CD45RA^-$ populations, the $CD27^-CD28^-$ and the $CD27^{low}CD28^-$ subsets expressed significantly higher levels of perforin than the $CD27^{high}CD28^-$ subsets ($CD45RA^+$: $p<0.0005$ and $p<0.001$, respectively; $CD45RA^-$: $p<0.0005$ and $p<0.005$, respectively; Fig. 1B). These results suggest that the $CD27^-CD28^-$ subsets include cytotoxic effector T cells (effector subsets) while the $CD27^{low}CD28^-$ subsets include less differentiated effector T cells (memory/effector T cells).

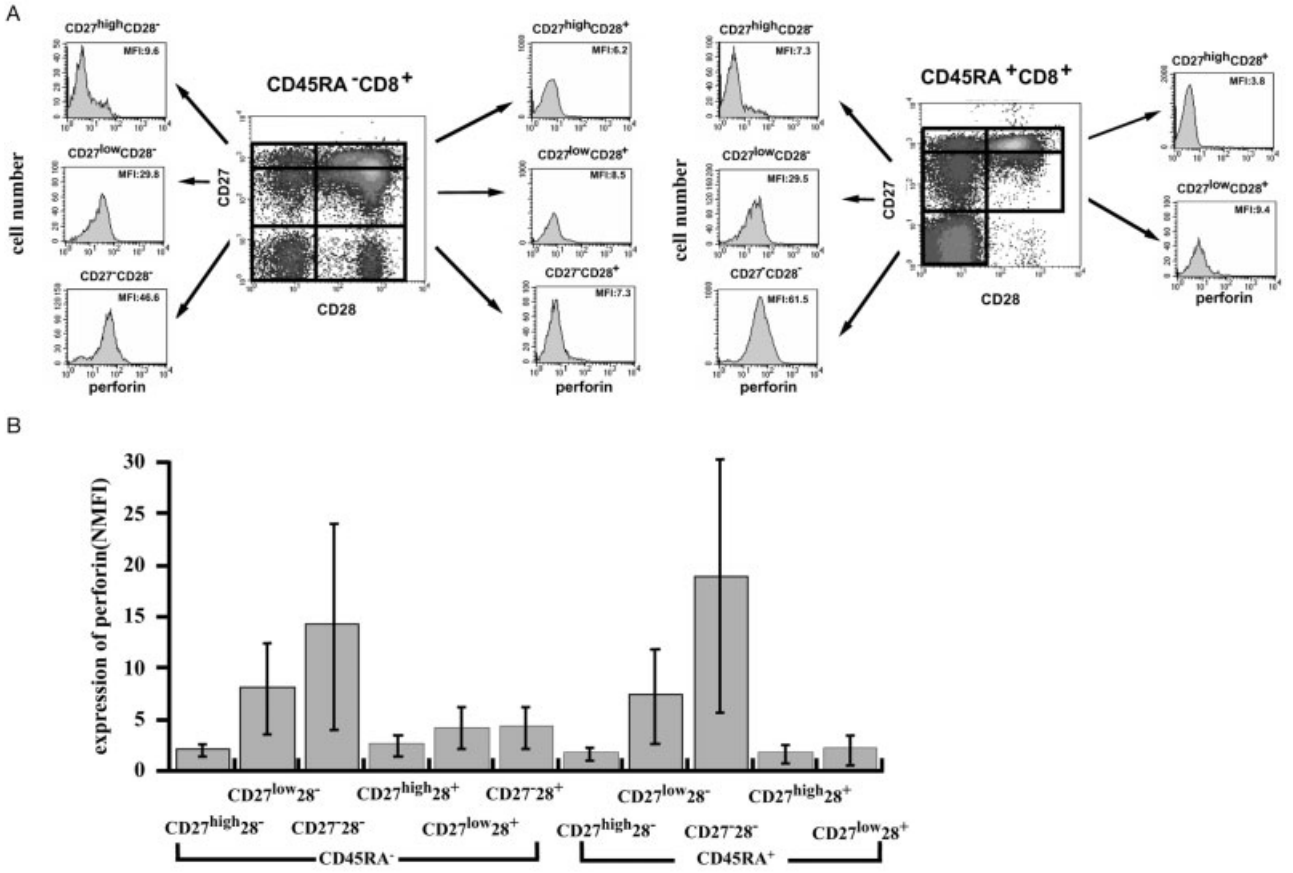

Fig. 1. Perforin expression in different CD27CD28CD45RA subsets of total $CD8^+$ T cells. (A) $CD8^+$ T cells were isolated from individual U13 and then stained with anti-CD27, anti-CD28, anti-CD45RA and anti-perforin mAb. $CD45RA^+$ and $CD45RA^-$ T cells were gated and then analyzed for CD27 and CD28 expression. Each population could be separated into five different subsets according to CD27 and CD28 expression ($CD27^{high}CD28^+$, $CD27^{low}CD28^+$, $CD27^{high}CD28^+$, $CD27^{low}CD28^+$, $CD27^-CD28^-$). $CD27^{high}$ and $CD27^{low}$ populations were divided by a quarter of maximum CD27 MFI. In this case, CD27 MFI 495 divided $CD27^{high}$ and $CD27^{low}$ populations because the maximum CD27 MFI is 1,981. The $CD27^-CD28^-$ subset was gated in CD8 T cells stained without antibodies. The CD27CD28CD45RA subsets were gated and then analyzed for perforin expression. Intracellular perforin expression in each CD27CD28CD45RA subset is represented in the upper right of each plot as MFI. (B) Intracellular perforin expression in each CD27CD28CD45RA subset from eight healthy individuals was analyzed. Intracellular perforin expression is represented as NMFI, which is the MFI of cells stained with anti-perforin-PE mAb divided by the MFI of cells stained with the control mouse IgG2b-PE mAb. Mean and standard deviation of NMFI from these eight individuals are shown.

2.2 Correlation between IFN-γ production and perforin expression in CD27CD28CD45RA subsets of $CD8^+$ T cells

The ability of each CD27CD28CD45RA subset to produce IFN-γ was investigated in total $CD8^+$ T cells from three healthy individuals by measuring IFN-γ production after stimulation with anti-CD3 mAb. A significant number of IFN-γ-producing cells were found in seven subsets: $CD27^{low}CD28^-CD45RA^-$, $CD27^-CD28^-CD45RA^-$, $CD27^{low}CD28^-CD45RA^+$, $CD27^-CD28^-CD45RA^+$, $CD27^-CD28^+CD45RA^-$, $CD27^{low}CD28^+CD45RA^-$ and $CD27^{low}CD28^+CD45RA^+$ (Fig. 2A, B). Of these subsets, the $CD27^-CD28^-CD45RA^-$ subset contained the highest number of IFN-γ-producing cells. Three subsets, $CD27^{low}CD28^-CD45RA^{+/-}$ and $CD27^-CD28^-CD45RA^+$, had a higher number of IFN-γ-producing cells than the $CD27^-CD28^+CD45RA^-$ and $CD27^{low}CD28^+CD45RA^{+/-}$ subsets. These results indicate that IFN-γ-producing cells are predominantly found in four subsets ($CD27^-CD28^-CD45RA^{+/-}$ and $CD27^{low}CD28^-CD45RA^{+/-}$) including a higher number of $perforin^+$ cells, though $CD28^-CD45RA^-$ subsets produce much more IFN-γ than $CD28^-CD45RA^+$ subsets.

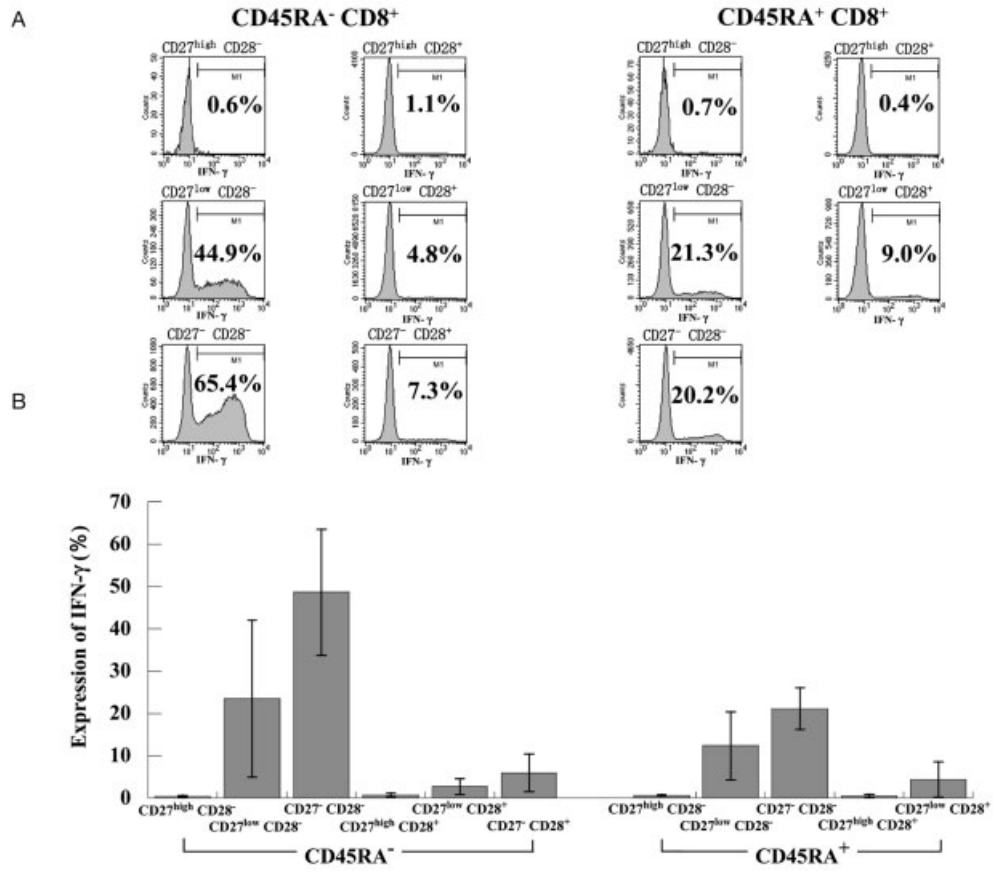

Fig. 2. Anti-CD3 mAb-induced cytokine production by CD8$^+$ T cells in different CD27CD28CD45RA subsets. (A) PBMC isolated from healthy individual U4 were cultured in F-bottom 24-well plates coated with or without anti-CD3 mAb (2 μg/ml) in R10 medium for 6 h. IFN-γ-producing cells in different CD27CD28CD45RA subsets of total CD8$^+$ T cells were analyzed by flow cytometry. In the absence of anti-CD3 mAb, each subset of total CD8$^+$ T cells contained less than 0.6% IFN-γ-producing cells. (B) Mean percentage and standard deviation of IFN-γ-producing cells in different CD27CD28CD45RA subsets of total CD8$^+$ T cells from three healthy individuals.

2.3 CCR5 expression decreases during maturation of CD8$^+$ T cells

CCR7 is expressed in naive CD8$^+$ T cells and central memory CD8$^+$ T cells [18, 19]. Recent studies have shown that CCR5 is expressed in memory and effector CD8$^+$ T cells and that the number of CCR5$^+$ cells decreased during differentiation from the CD27$^-$CD28$^-$CD45RA$^-$ subset to the CD27$^+$CD28$^-$ CD45RA$^-$ subset [8, 21]. These chemokine receptors may be useful to discriminate between naive, memory and effector CD8$^+$ T cells. To further investigate CCR5 and CCR7 expression in different CD27CD28CD45RA subsets of total CD8$^+$ T cells, we stained total CD8$^+$ T cells from seven healthy individuals with anti-CD27, anti-CD28, anti-CD45RA and anti-CCR5 or anti-CCR7 mAb.

Representative results from individual U13 and results from seven healthy individuals are shown in Fig. 3A and B, respectively. CCR7 was expressed on CD27^{high}CD28$^+$CD45RA$^{+/-}$ subsets, but not on four subsets containing perforin$^+$ effector cells (CD27^{low}CD28$^-$CD45RA$^{+/-}$ and CD27$^-$CD28$^-$CD45RA$^{+/-}$). The CD27^{high}CD28$^-$CD45RA$^+$ subset also included CCR7$^+$ cells but not CCR5$^+$ cells, suggesting that this subset may have characteristics of naive T cells. The CD27^{high}CD28$^+$CD45RA$^-$ subset as well as two subsets, CD27^{low}CD28$^+$CD45RA$^+$ and CD27^{high}CD28$^-$CD45RA$^-$, included both CCR7$^+$ cells and CCR5$^+$ cells, although the frequency of these cells varied between these subsets. The CD27^{low}CD28$^+$CD45RA$^+$ subset included a larger number of CCR7$^+$ cells and a smaller number of CCR5$^+$ cells than the CD27^{high}CD28$^+$CD45RA$^-$ memory subset, suggesting that the CD27^{low}CD28$^+$CD45RA$^+$ subset has characteristics of immature memory T cells rather than the CD27^{high}CD28$^+$CD45RA$^-$ memory subset. The CD27^{high}CD28$^-$CD45RA$^-$ subset included a smaller number of CCR7$^+$ cells than the CD27^{high}CD28$^+$CD45RA$^-$

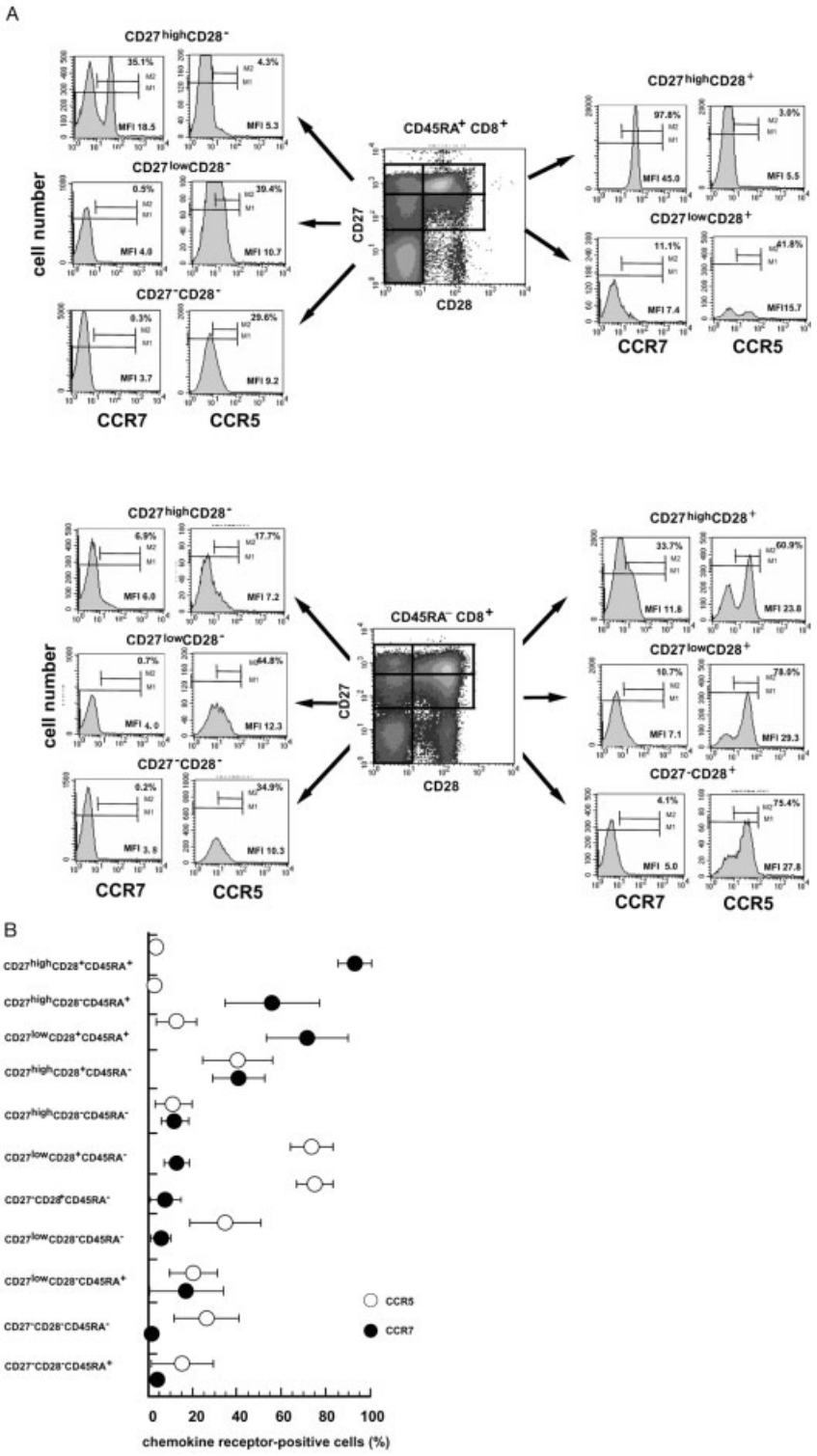

Fig. 3. CCR5 and CCR7 expression on each CD27CD28CD45RA subset of total CD8+ T cells. (A) CD8+ T cells were isolated from individual U13 and then stained with anti-CD27, anti-CD28, anti-CD45RA and anti-CCR5 or anti-CCR7 mAb. CD45RA+ cells were gated and then analyzed for CD27 and CD28 expression. The three CD27CD28 subsets of CD45RA−CD8+ T cells were further gated for analysis of CCR5 expression. The percentage and MIF of CCR5+ or CCR7+ cells are shown in the upper right and lower right of each plot, respectively. The MFI for CD8+ T cells stained without anti-CCR5 or anti-CCR7 mAb ranged from 3.0 to 3.5. (B) Mean percentage and standard deviation of CCR5+ or CCR7+ cells in each CD27CD28CD45RA subset from eight healthy individuals.

memory subset, suggesting that the former has characteristics of mature memory T cells rather than the latter.

The $CD27^{low}CD28^{-}CD45RA^{+/-}$ subsets as well as the $CD27^{-}CD28^{-}CD45RA^{+/-}$ subsets expressed CCR5 but not CCR7, though the frequency of $CCR5^{+}$ cells was higher in the $CD27^{low}$ subsets than in the $CD27^{-}$ subsets. The $CD27^{-}$ subsets are thought to be more differentiated effector cells than the $CD27^{low}$ subsets because the former subsets express higher level of perforin than the latter subsets. Thus, these results support the idea that the number of $CCR5^{+}$ cells decreases during maturation to effector T cells. The $CD27^{-}CD28^{+}CD45RA^{-}$ subset also expressed CCR5 but not CCR7, implying that this is an intermediate subset between the $CD27^{low}CD28^{+}CD45RA^{-}$ and $CD27^{-}CD28^{-}CD45RA^{-}$ subsets.

2.4 Human cytomegalovirus-specific $CD8^{+}$ T cells express a memory/effector or effector phenotype

The human cytomegalovirus (HCMV) epitope pp65 495–503 NLVPMVATV (HCMV-1) is an HLA-A*0201-restricted immunodominant epitope [22–24] that is also presented by HLA-A*0206 (our unpublished observation). We generated both HCMV-1-A*0201 and HCMV-1-A*0206 tetramers using this epitope peptide in order to analyze HCMV-specific $CD8^{+}$ T cells from HCMV-seropositive healthy individuals with HLA-A*02 (A*0201 and A*0206). *Ex vivo* flow cytometry analysis using these tetramers demonstrated that approximately 1.0% of total $CD8^{+}$ T cells were $tetramer^{+}$ in 13 of 20 healthy individuals (data not shown). We further analyzed the CD27CD28CD45RA phenotypes of HCMV-specific $CD8^{+}$ T cells. HCMV-specific $CD8^{+}$ T cells showed various phenotypes in eight healthy individuals. They included the $CD27^{-}CD28^{-}CD45RA^{+/-}$ subsets as well as the $CD27^{low}CD28^{+}CD45RA^{-}$ and $CD27^{low}CD28^{-}CD45RA^{+/-}$ subsets (Table 1). The ratio of HCMV-specific $CD8^{+}$ T cells to total $CD8^{+}$ T cells was higher in the $CD27^{low}CD28^{-}CD45RA^{+/-}$ and $CD27^{-}CD28^{-}CD45RA^{+}$ subsets than in other subsets (Table 1), indicating that the relative number of HCMV-specific $CD8^{+}$ T cells increased in these three subsets. This suggests that in healthy individuals, HCMV-specific effector $CD8^{+}$ T cells are always induced from HCMV-specific memory T cells.

We analyzed CCR5 and CCR7 expression in HCMV-specific $CD8^{+}$ T cells by five-color flow cytometry analysis using the HCMV-1-A*0201 tetramer and anti-CD27, anti-CD28, anti-CD45RA and anti-CCR5 or anti-CCR7 mAb (Fig. 4). $CCR7^{+}$ cells were not detected in any subset of HCMV-specific $CD8^{+}$ T cells. The number of $CCR5^{+}$ cells decreased following the same sequence as observed in total $CD8^{+}$ T cells: $CD27^{low}CD28^{+}CD45RA^{-} \rightarrow CD27^{low}CD28^{-}CD45RA^{-} \rightarrow CD27^{-}CD28^{-}CD45RA^{-} \rightarrow CD27^{low}CD28^{-}CD45RA^{+} \rightarrow CD27^{-}CD28^{-}CD45RA^{+}$. This result indicates that the number of $CCR5^{+}$ cells decreases during maturation to effector T cells in HCMV-specific $CD8^{+}$ T cells.

Table 1. Frequency of each CD27CD28CD45RA subset in HCMV-specific and total $CD8^{+}$ T cells from eight healthy individuals

subsets	Percentage of each CD27CD28CD45RA subset in total $CD8^{+}$ T cells (%±SD) [1]	Percentage of each CD27CD28CD45RA subset in $tetramer^{+}$ $CD8^{+}$ T cells (%±SD) [2]	[2]/[1]
$CD27^{high}CD28^{+}CD45RA^{+}$	32.31±19.96	0.63±0.40	0.02±0.02
$CD27^{low}CD28^{+}CD45RA^{+}$	2.77±2.61	0.34±0.29	0.28±0.44
$CD27^{high}CD28^{-}CD45RA^{+}$	1.27±1.22	0.33±0.61	0.49±1.18
$CD27^{low}CD28^{-}CD45RA^{+}$	2.50±1.98	7.27±8.69	3.26±3.02
$CD27^{-}CD28^{-}CD45RA^{+}$	20.20±14.30	21.00±13.76	1.19±0.98
$CD27^{high}CD28^{+}CD45RA^{-}$	5.60±2.97	1.69±1.37	0.41±0.44
$CD27^{low}CD28^{+}CD45RA^{-}$	9.44±6.15	9.59±11.37	1.09±0.83
$CD27^{-}CD28^{+}CD45RA^{-}$	1.40±0.57	1.46±0.70	1.04±0.42
$CD27^{high}CD28^{-}CD45RA^{-}$	0.84±0.49	0.70±0.68	1.08±1.38
$CD27^{low}CD28^{-}CD45RA^{-}$	2.11±0.54	18.64±14.72	9.17±7.02
$CD27^{-}CD28^{-}CD45RA^{-}$	12.58±11.49	28.99±15.03	4.23±5.56

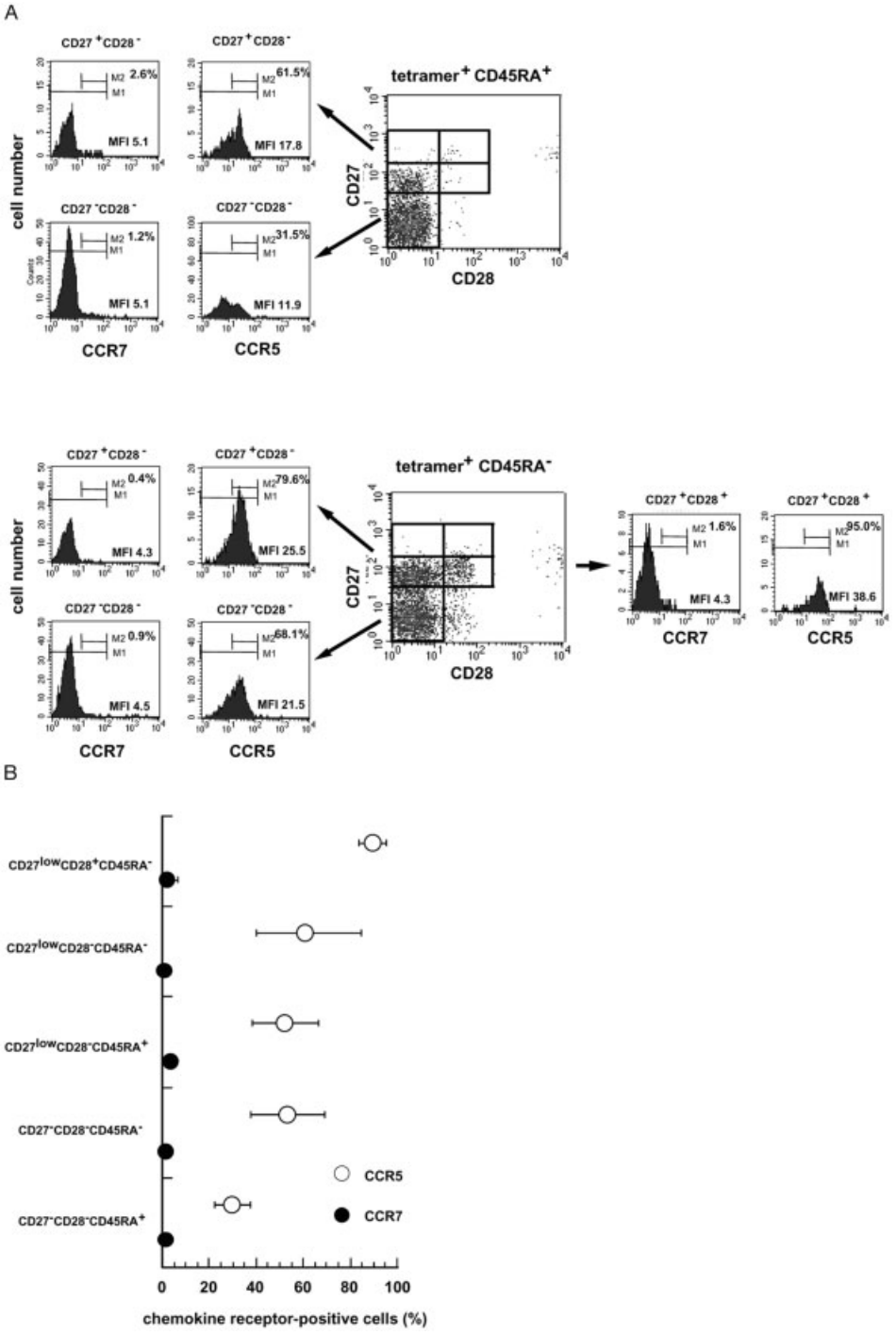

Fig. 4. CCR5 and CCR7 expression on HCMV-1-specific CD45RA+CD8+ T cells with different CD27CD28 phenotypes. (A) CD8+ T cells were isolated from individual U2, and then stained with anti-CD27, anti-CD28, anti-CD45RA and anti-CCR5 or anti-CCR7 mAb as well as HCMV-1-A*0206 tetramer. Tetramer+ cells were gated and then analyzed for CD27, CD28 and CD45RA expression. The five CD27CD28CD45RA subsets in which tetramer+ cells were found were gated and then analyzed for CCR5 or CCR7 expression. The percentage of CCR5+ or CCR7+ cells (M2) is shown in the upper right of each plot and the MFI is shown in the lower right. MFI for the five subsets stained without mAb and with mouse IgM mAb (isotype control for anti-CCR7 mAb) ranged from 3.5 to 4.5 and from 3.3 to 4.5, respectively. (B) Mean percentage and standard deviation of CCR5+ and CCR7+ cells in five CD27CD28CD45RA subsets of tetramer+CD8+ T cells from three healthy individuals.

2.5 A CD27^{low}CD28^{-}CD45RA^{+} subset of HCMV-specific CD8^{+} T cells has cytolytic activity

Although our previous study showed that HCMV-1-specific CD27^{+}CD28^{-}CD45RA^{-}CD8^{+} T cells have cytotoxic activity against target cells pulsed with HCMV-1 peptide [8], it remains unknown whether the CD27$^{+(low)}$CD28^{-}CD45RA^{+} subset is able to kill target cells. We first investigated perforin expression of the CD27^{low}CD28^{-}CD45RA$^{+/-}$ and CD27^{-}CD28^{-}CD45RA$^{+/-}$ subsets in HCMV-specific CD8^{+} T cells using mAb against CD27, CD45RA and perforin as well as the HCMV-1-A*0201 or HCMV-1-A*0206 tetramer. All four CD27CD45RA subsets expressed a high level of perforin though perforin expression in both CD27^{-}CD28^{-} CD45RA$^{+/-}$ subsets was higher than in the CD27^{low}CD28^{-} CD45RA$^{+/-}$ subsets (data not shown). These results suggest that HCMV-specific CD27^{low}CD28^{-}CD45RA^{+}CD8^{+} T cells also have cytotoxic activity.

We therefore investigated the ability of the CD27^{low}CD28^{-}CD45RA^{+} subset of HCMV-specific CD8^{+} T cells to kill target cells. The CD27^{+}CD28^{-}CD45RA^{+} and CD27^{-}CD28^{-}CD45RA^{+} subsets of total CD8^{+} T cells from individuals M20 and U9 were sorted (Fig. 5A), and then their cytolytic activity for HLA-A*0201^{+} target cells pulsed with HCMV-1 peptide was examined at various tetramer^{+}CD8^{+} T cell to target ratios. Cells from both

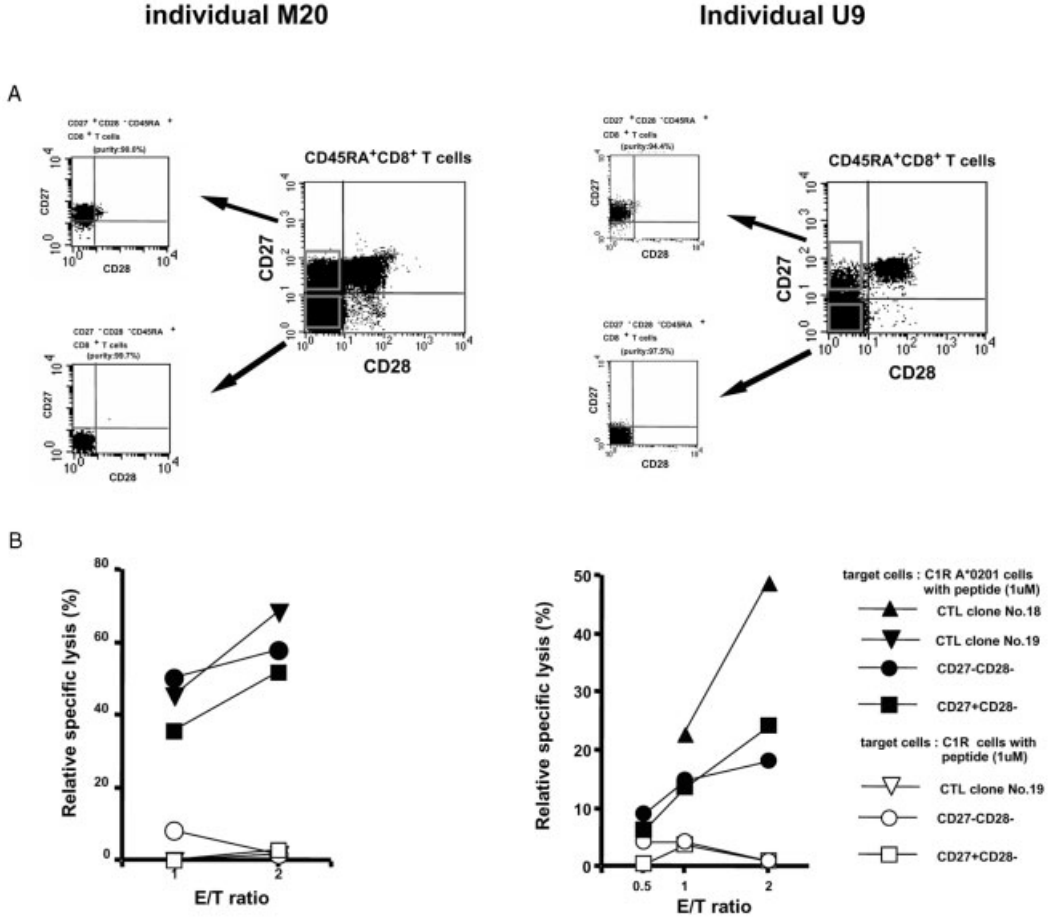

Fig. 5. Cytotoxic activity of CD27^{+} and CD27^{-} populations of HCMV-specific CD28^{-}CD45RA^{+}CD8^{+} T cells. (A) Isolation of CD27^{+}CD28^{-} and CD27^{-}CD28^{-} subsets of total CD45RA^{+}CD8^{+} T cells. CD8^{+} T cells were isolated from individuals M20 and U9 using MACS. CD27^{+}CD28^{-}CD45RA^{+}CD8^{+} and CD27^{-}CD28^{-}CD45RA^{+}CD8^{+} T cells were then isolated from the CD8^{+} T cells 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. (B) Cytotoxic activity of two CD27CD28 subsets of HCMV-specific CD45RA^{+}CD8^{+} T cells. The relative cytotoxic activity of the CD27^{-}CD28^{-} (filled circles) and CD27^{+}CD28^{-} (filled squares) subsets as well as HCMV-1-specific CTL clones (filled triangles) for C1R-A*0201 cells pulsed with HCMV-1 peptide was measured at different effector (tetramer^{+} cells) to target ratios. The relative cytotoxic activity of CD27^{-}CD28^{-} (empty circles) and CD27^{+}CD28^{-} (empty squares) subsets as well as CTL clones (empty triangles) for C1R cells pulsed with HCMV-1 peptides was also measured at different effector (tetramer^{+} cells) to target ratios.

subsets effectively killed target cells pulsed with the HCMV-1 peptide (Fig. 5B). Since HCMV-1-specific CD8$^+$ T cells in the CD27$^+$CD28$^-$CD45RA$^+$ subset showed a CD27^{low}CD28$^-$CD45RA$^+$ phenotype in these individuals (Table 1), these results together indicate that HCMV-1-specific CD27^{low}CD28$^-$CD45RA$^+$CD8$^+$ T cells have cytotoxic activity. Thus, the present study together with a previous study [8] showed that both CD27^{low} CD28$^-$CD45RA$^+$ and CD27^{low}CD28$^-$CD45RA$^-$ subsets of HCMV-1-specific CD8$^+$ T cells have cytotoxic activity.

3 Discussion

Recent studies demonstrated that perforin$^+$CD8$^+$ T cells express a CD27$^-$ phenotype [17, 25]. The present study confirmed these studies and further showed an inverse correlation between perforin and CD27 expression. The CD27^{low}CD28$^-$CD45RA$^{+/-}$ and CD27$^-$CD28$^-$CD45RA$^{+/-}$ subsets showed cytolytic activity and expressed significantly higher levels of perforin than other subsets, indicating that these subsets are mostly cytotoxic effector T cells. The former subsets may have characteristics of immature effector T cells (memory/effector T cells) since the CD27^{low}CD28$^-$CD45RA$^{+/-}$ subsets expressed lower levels of perforin than the CD27$^-$CD28$^-$CD45RA$^{+/-}$ subsets. Thus, we here showed that CD8$^+$ T cells with cytotoxic effector function could be effectively discriminated from other CD8$^+$ T cells by this classification using CD28, CD45RA and quantitative expression of CD27.

It is hardly possible to discriminate naive CD8$^+$ T cells from memory CD8$^+$ T cells by perforin expression because both CD8$^+$ T cells do not express perforin. On the other hand, two chemokine receptors, CCR7 and CCR5, are useful to discriminate between naive, memory and effector CD8$^+$ T cells, because CCR7 and CCR5 are expressed in naive CD8$^+$ T cells and central memory CD8$^+$ T cells [18, 19] and in memory CD8$^+$ T cells and a part of effector CD8$^+$ T cells [21], respectively. A previous study showed that EBV-specific CD8$^+$ T cells expressed CD27$^+$CD28$^+$CD45RA$^-$ phenotype (both CD27^{high}CD28$^+$CD45RA$^-$ and CD27^{low}CD28$^+$CD45RA$^-$) as well as both CCR7 and CCR5, whereas they failed to kill target cells ([8], our unpublished observation), suggesting that the CD27$^+$CD28$^+$CD45RA$^-$ subset is a memory CD8$^+$ T cell subset. However, the CD27^{low}CD28$^+$ CD45RA$^-$ subset included a larger number of cells expressing a low level of perforin than the CD27^{high}CD28$^+$CD45RA$^-$ subset. In addition, the former subset included a large number of CCR5$^+$CCR7$^-$ cells while the latter included both CCR5$^+$ cells and CCR7$^+$ cells. These findings indicate that the CD27^{low}CD28$^+$ CD45RA$^-$ subset is a more differentiated memory subset than the CD27^{high}CD28$^+$CD45RA$^-$ subset.

The CD27^{high}CD28$^+$CD45RA$^+$ subset included a large number of CCR7$^+$CCR5$^-$ cells while the CD27^{low} CD28$^+$CD45RA$^+$ and CD27^{high}CD28$^+$CD45RA$^-$ subsets included both CCR5$^+$ cells and CCR7$^+$ cells. The CD27^{high}CD28$^+$CD45RA$^+$ subset had a higher number of CCR7$^+$ cells than the CD27^{low}CD28$^+$CD45RA$^-$ subset, implying that the latter subset represents more mature T cells than the former. Thus, these four subsets can be thought of as follows. The CD27^{high}CD28$^+$CD45RA$^+$ subset mostly includes naive T cells. The CD27^{low} CD28$^+$CD45RA$^+$ subset might include both naive cells and memory T cells. The CD27^{high}CD28$^+$CD45RA$^-$ memory subset mostly includes undifferentiated memory T cells, while the CD27^{low}CD28$^+$CD45RA$^-$ memory subset includes differentiated memory T cells.

The CD27^{high}CD28$^-$CD45RA$^{+/-}$ subsets mostly included cells not expressing perforin, suggesting that these subsets are naive or memory T cells. The CD27^{high} CD28$^-$CD45RA$^+$ subset included a large number of CCR7$^+$CCR5$^-$ cells and showed much weaker proliferation ability than memory, memory/effector and effector subsets (data not shown), while the CD27^{high}CD28$^-$ CD45RA$^-$ subset included a small number of CCR7$^+$ cells and CCR5$^+$ cells. These findings imply that these subsets carry intermediate characteristics between memory and naive subsets though the CD27^{high}CD28$^-$CD45RA$^-$ subset has more mature characteristics than the CD27^{high}CD28$^-$CD45RA$^+$ subset.

A recent study of CD45RA$^-$ subset showed that the percentage of CCR5$^+$ cells was the highest in the CD27$^+$CD28$^+$CD45RA$^-$ memory CD8$^+$ T cell subset, and decreased during the following differentiation sequence: CD27$^+$CD28$^+$CD45RA$^-$ $\rightarrow$ CD27$^+$CD28$^-$CD45RA$^-$ $\rightarrow$ CD27$^-$CD28$^-$CD45RA$^-$ [8]. The present study analyzed all CD27CD28CD45RA subsets and further demonstrated that the percentage of CCR5$^+$ cells decreased according to the following sequence: CD27^{high}CD28$^+$CD45RA$^-$ and CD27^{low}CD28$^+$CD45RA$^-$ $\rightarrow$ CD27^{low}CD28$^-$CD45RA$^{+/-}$ $\rightarrow$ CD27$^-$CD28$^-$CD45RA$^{+/-}$, supporting that CCR5 expression decreases during differentiation from memory CD8$^+$ T cells to effector CD8$^+$ T cells. As CCR5 functions as a chemokine receptor for RANTES and MIP-1β [20], this receptor may play an important role in migration of memory and memory/effector CD8$^+$ T cells to inflammation sites. Indeed, our previous study showed effective migration of memory and effector subsets in response to RANTES [21].

IFN-γ-producing cells were predominantly found in effector and memory/effector subsets, while a very small number of IFN-γ-producing cells were found in memory subsets. A previous study showed that memory CD8$^+$ T cells can produce IFN-γ [7]. This difference may result

from different experimental conditions: in the present study, the $CD8^+$ T cells were stimulated with anti-CD3 mAb, whereas they were stimulated with PMA and ionomycin or with PMA and anti-CD3 mAb in a previous study. Interestingly, our data showed that the number of cytokine-producing cells in the $CD27^{low/-}CD28^-CD45RA^+$ subset was much lower than that in the $CD27^{low/-}CD28^-CD45RA^-$ subset. This indicates that IFN-γ production does not exactly correlate with perforin expression. Further analysis of production of other cytokines is required to characterize these CD27CD28CD45RA subsets.

Most EBV-specific $CD8^+$ T cells have a $CD27^+CD28^+CD45RA^-$ memory phenotype [8, 17, 26, 27], while HCMV-specific $CD8^+$ T cells express various phenotypes, from memory to effector phenotype [12, 17, 24]. Here we show that HCMV-specific $CD8^+$ T cells are at various differentiation stages, but predominantly express four effector and memory/effector phenotypes (Table 1). In addition, we show that these HCMV-specific $CD8^+$ T cells had cytolytic activity. These observations indicate that HCMV-specific $CD8^+$ T cells have effector function in healthy individuals. The $CD27^{low}CD28^-CD45RA^+$, $CD27^{low}CD28^-CD45RA^-$ and $CD27^-CD28^-CD45RA^-$ subsets relatively increased in HCMV-specific $CD8^+$ T cells, implying that HCMV actively replicates and induces HCMV-specific effector $CD8^+$ T cells from memory $CD8^+$ T cells in healthy individuals.

In the present study, we show an inverse correlation between perforin and CD27 expression. Human $CD8^+$ T cells were classified based on quantitative expression of CD27 in addition to qualitative expression of CD28 and CD45RA. This phenotypic classification enabled a more precise reflection of the function of $CD8^+$ T cells than previous phenotypic classification using qualitative expression of these three markers. According to this classification, HCMV-specific $CD8^+$ T cells observed in healthy individuals were identified as effector and memory/effector T cells. Further characterization of these subsets will contribute to the studies concerning the role of $CD8^+$ T cells in patients with various diseases.

4 Materials and methods

4.1 Blood samples

Blood samples were taken from healthy, HCMV-seropositive adult individuals with HLA-A*02 (A*0201 and A*0206).

4.2 Peptides

The HLA-A*0201-restricted HCMV-specific CTL epitope, pp65 495–503 NLVPMVATV (HCMV-1), was previously identified [22]. This peptide was generated using an automated peptide synthesizer with 9-fluorenylmethoxycarbonyl (Fmoc) strategy followed by cleavage. The purity of the synthesized peptide was examined by HPLC and mass spectrometry.

4.3 Cells

C1R cells expressing HLA-A*0201 (C1R-A*0201) were previously generated [28], and were maintained in RPMI 1640 medium supplemented with 10% FCS and 0.15 mg/ml hygromycin B. Two HLA-A*02-restricted, HCMV-1-specific CTL clones were recently established, and were maintained in RPMI 1640 medium supplemented with 10% FCS and 200 IU/ml r-human IL-2.

4.4 Monoclonal antibodies

Anti-CD27-FITC, anti-CD28-FITC, anti-CD28-PE, anti-CD28-Cy-Chrome, anti-CD28-APC, anti-CD45RA-Cy-Chrome, anti-CCR5-FITC, anti-CCR7 and anti-perforin-FITC mAb were obtained from Pharmingen International (San Diego, CA). Anti-CD45RA-ECD was obtained from Immunotech (Marseille, France). Anti-CD8-FITC, anti-CD8-APC and anti-IFN-γ-FITC mAb were obtained from Dako A/S (Glostrup, Denmark).

4.5 HLA class I-HCMV-1 peptide tetramers

HLA class I-peptide tetrameric complexes were synthesized as previously described [29]. The HCMV CTL epitope (HCMV-1) was used for refolding of HLA class I molecules. The HLA class I-peptide complexes were refolded *in vitro* and the resulting 45-kDa complexes were isolated using gel filtration on a Superdex G75 column (Amersham Pharmacia Biotech, Little Chalfont, GB). Purified complexes were biotinylated using 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 (HCMV-1-A*0201 and HCMV-1-A*0206 tetramers) were mixed with APC-labeled streptavidin (Molecular Probes, Inc., Eugene, OR) or APC-Cy7-labeled streptavidin (Caltag Laboratories, Burlingame, CA) at a molar ratio of 4:1.

4.6 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%. $CD8^+$ T cells (1×10^6) were mixed with HCMV-1-A*0201 or HCMV-1-A*0206 tetramer 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, anti-CD28, anti-CD45RA, anti-CCR5 or anti-CCR7 mAb was 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 CD27CD28CD45RA subsets of both $tetramer^+$ cells and total $CD8^+$ T cells, cells stained with anti-CD27, anti-CD28 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, the cell suspension was incubated at 4°C for 30 min, and the cells were then 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 analyzed using a FACSCalibur or FACSVantage SE with Cell Quest software (Becton Dickinson, 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. Statistical analysis of perforin expression was performed with Statview 4.02 (Abacus Concepts, Berkeley, CA) using Mann-Whitney's U-test.

4.7 Assay for cytotoxic activity

Cytotoxic 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 various amounts 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) were added and the mixtures were incubated for 6 h at 37°C. The supernatants were then collected and analyzed with a gamma counter.

4.8 Measurement of cytokine-producing cells by flow cytometry

To measure cytokine production in CD27CD28CD45RA subsets of total $CD8^+$ T cells, $CD8^+$ T cells were purified from fresh PBMC from healthy individuals using anti-CD8-coated magnetic beads (MACS CD8 Microbeads). $CD8^+$ T cells were cultured in F-bottom 24-well plates coated with or without anti-CD3 mAb (2 μg/ml) in R10 medium 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, anti-CD28 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. The cells were finally resuspended in PBS containing 2% paraformaldehyde and then the percentage of intracellular IFN-γ^+ cells was analyzed by flow cytometry.

Acknowledgements: This research was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sport and Culture, the Government of Japan and a grant from Japan Health Science Foundation. The authors thank Sachiko Sakai for secretarial assistance.

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