Three Memory Subsets of Human CD8$^+$ T Cells Differently Expressing Three Cytolytic Effector Molecules[1]

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Multicolor flow cytometric analysis for the expression of three effector molecules, i.e., perforin (Per), granzyme A (GraA), and granzyme B (GraB), in human CD8$^+$ T cells demonstrated that they included five subpopulations, implying the following pathway for the differentiation of CD8$^+$ T cells: Per$^-$GraA$^-$GraB$^-$→Per$^-$GraA$^+$GraB$^-$→Per^{low}GraA$^+$GraB$^-$→Per^{low}GraA$^+$GraB$^+$→Per^{high}GraA$^+$GraB$^+$. The analysis of the expression of these molecules in the subsets classified by the combination of the expression of CCR7 and CD45RA or by that of CD27, CD28, and CD45RA showed that functional CD8$^+$ T cell subsets could be partially identified by these phenotypic classifications. However, the functional subsets could be precisely identified by the classification using five cell surface markers or three cell surface markers and three cytolytic molecules. Per$^-$GraA$^-$GraB$^-$ and Per$^{-/low}$GraA$^+$GraB$^-$ cells were predominantly found in CCR5$^-$CCR7$^+$ and CCR5$^{high/low}$CCR7$^-$ subsets, respectively, of CD8$^+$ T cells expressing the CD27$^+$CD28$^+$CD45RA$^-$ phenotype, whereas Per^{low}GraA$^+$GraB$^+$ cells were found in the CCR5^{low}CCR7$^-$ subset of those expressing this phenotype and in a part of the CCR5$^{-/low}$CCR7$^-$ subset of those expressing the CD27$^{-/low}$CD28$^-$CD45RA$^{-/+}$ phenotype. Ex vivo EBV-specific CD8$^+$ T cells, which were Per$^{low/-}$GraA$^+$GraB$^{-/+}$ cells, hardly or very weakly killed the target cells, indicating that these were not effector T cells. These findings suggest that the Per$^-$GraA$^-$GraB$^-$, Per$^{-/low}$GraA$^+$GraB$^-$, and Per^{low}GraA$^+$GraB$^+$ cells were central memory, early effector memory, and late effector memory T cells, respectively. Per$^{-/low}$GraA$^+$GraB$^-$ cells gained GraB expression after TCR stimulation, indicating that early effector memory T cells could differentiate into late effector and effector T cells. The present study showed the existence of three memory subsets and the pathway for their differentiation. *The Journal of Immunology,* **2006, 177: 4330–4340.**

The CD8$^+$ T cells play a crucial role in immunity against viruses and tumors through their ability to secrete the various factors that suppress viral replication (1, 2) and kill the target cells (3). In a primary infection, naive CD8$^+$ T cells are primed in secondary lymph node and consequently proliferate and differentiate into effector and memory cells (4, 5). Peripheral effector CD8$^+$ T cells show direct effector function such as cytotoxic activity and cytokine production in response to their targets (6). In contrast, memory CD8$^+$ T cells do not have direct cytotoxic activity without prior activation, but can proliferate and secrete a large amount of cytokines as a response to Ag stimulation (6–8).

Previous studies showed that CD8$^+$ T cells change the expression of costimulatory molecules (CD27, CD28, and CD45RA) on their surface according to their differentiation and maturation (7, 9). Therefore, these molecules have been used for phenotypic classification of human CD8$^+$ T cells. Naive, memory, and effector CD8$^+$ T cells express CD27$^+$CD28$^+$CD45RA$^+$, CD27$^+$CD28$^+$CD45RA$^-$, and CD27$^-$CD28$^-$CD45RA$^{+/-}$ phenotypes, respectively. A previous study further demonstrated that CD8$^+$ T cells with the CD27$^-$CD28$^-$CD45RA$^{+/-}$ or CD27^{low}CD28$^-$CD45RA$^{+/-}$ phenotype have the ability to kill target cells (10).

Chemokine receptors play an important role in lymphocyte trafficking. They are also used to define the functional subsets of human CD8$^+$ T cells. Previous studies revealed that T cells could be classified based on their expressions of CD45RA and the chemokine receptor CCR7 that is associated with their ability to home in on secondary lymph nodes (11). They were classified into CCR7$^+$CD45RA$^+$ (naive), CCR7$^+$CD45RA$^-$ (central memory), CCR7$^-$CD45RA$^-$ (effector memory), and CCR7$^-$CD45RA$^+$ (effector). The CCR5 chemokine receptor is predominantly expressed on memory and effector memory CD8$^+$ T cells, and its expression decreases during the maturation process from memory to effector CD8$^+$ T cells (6, 10).

Perforin (Per)[3] and granzymes A/B (GraA/GraB) are key cytolytic effector molecules that are stored in cytolytic granules in effector CD8$^+$ T cells (12). They can be used as markers for effector CD8$^+$ T cells, because they are the actual functional molecules for killing target cells (13–15). A recent study showed that about one-half of the total human CD8$^+$ T cell population coexpressed both GraA and GraB, but that there was a small population that expressed either one or the other granzyme in CD8$^+$ T cells (16). These findings imply that human CD8$^+$ T cells do not simultaneously express these two effector molecules at the early stage of their peripheral differentiation. Further analysis of the coexpression of GraA/B and Per is expected to clarify the functional subsets of human CD8$^+$ T cells.

In the present study, we analyzed the coexpression of the effector molecules on the phenotypic subsets of human CD8$^+$ T cells by using advanced multicolor flow cytometry. In addition, we characterized a subset of CD8$^+$ T cells expressing GraA, but not GraB, to clarify the differentiation pathway from naive to effector CD8$^+$ T cells and among memory CD8$^+$ T cells.

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[3] Abbreviations used in this paper: Per, perforin; GraA, granzyme A; GraB, granzyme B; HCMV, human CMV; NCS, newborn calf serum.

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Materials and Methods

Blood samples

Blood samples were taken from healthy adult individuals. PBMCs were isolated from blood by using Ficoll-sodium metrizoate (ICN/Cappel).

Cells

C1R cells expressing HLA-A*1101 (C1R-A*1101) or HLA-A*0201 (C1R-A*0201) were previously generated (17) and were maintained in RPMI 1640 medium supplemented with 10% FCS and 0.15 mg/ml hygromycin B.

Monoclonal Abs

FITC-labeled anti-Per, PE-labeled anti-GraA, Alexa647-labeled anti-GraB, PE-Cy7-labeled anti-CCR7, FITC-labeled anti-CD27, Alexa405-labeled anti-CD45RA, allophycocyanin-labeled anti-CD28, FITC-labeled anti-CD27, allophycocyanin-Cy7-labeled anti-CD27, FITC-labeled anti-CD19 mAbs, and purified anti-CD28 mAbs were obtained from BD Biosciences. ECD-labeled anti-CD3, anti-CD45RA, and anti-CD28 mAbs were purchased from Beckman Coulter. FITC-labeled anti-CD3 mAb came from DakoCytomation. Cascade Blue- and Cascade Yellow-labeled anti-CD8 mAbs were made by conjugating Cascade Blue (Molecular Probes) and Cascade Yellow (Molecular Probes), respectively, with anti-CD8 mAb OKT8.

HLA class I tetramers

HLA class I-peptide tetrameric complexes (tetramers) were synthesized, as previously described (18). The human CMV (HCMV) CTL epitope (HCMV-1 pp65 495–503, NLVPMVATV (19)) and the EBV CTL epitope (EBV-3B 399–408, AVFDRKSDAK (20)) were used for refolding of HLA-A*0201 and HLA-A*1101 molecules, respectively. PE- or allophycocyanin-labeled streptavidin (Molecular Probes) at a molar ratio of 4:1 was used for generation of tetramers.

Flow cytometric analysis

Freshly isolated PBMCs were first stained with anti-CCR5 and anti-CCR7 mAbs for 30 min at room temperature, and subsequently with specific mAbs against surface markers at 4°C for 30 min. IgG isotype controls were used for negative controls. The cells were then washed twice with PBS containing 10% newborn calf serum (NCS; Summit Biotechnology). To determine the intracellular expression of Per, GraA, and GraB, we fixed cells with 4% paraformaldehyde PBS at 4°C for 20 min, and then made them permeable with PBS containing 0.1% saponin (Sigma-Aldrich) and 20% NCS (permeabilizing buffer) at 4°C for 10 min. The cells were stained with anti-Per, anti-GraA, and anti-GraB mAbs at 4°C for 20 min. Finally, the cells were washed three times in the permeabilizing buffer at 4°C. The corresponding IgG isotypes were used as negative controls. In some experiments, we used anti-CD19 mAb to identify B cells as a negative control for expression of the effector molecules.

To assess the expression of the effector molecules in virus-specific $CD8^+$ T cells, we mixed PBMC with the tetramers at a concentration of 0.01–0.02 mg/ml. After incubation at 37°C for 30 min, the cells were washed twice with 10% FCS (Sigma-Aldrich) containing RPMI 1640 and then stained with anti-CD8 mAb. Thereafter, they were fixed, made permeable, and then they were stained with anti-Per, anti-GraA, and anti-GraB mAbs.

The stained cells were analyzed by using a FACSAria (BD Biosciences). For determination of the effector molecule expressing cells in each $CD8^+$ T cell subset, all flow cytometric data were analyzed by using FlowJo software (Tree Star).

Fluorescence-activated cell sorting

To purify $CD45RA^-CCR5^{high/low}CD8^+$ cells, we first isolated $CD8^+$ T cells from PBMCs by using anti-CD8-coated (clone: BW135/80) magnetic beads (Miltenyi Biotec). The isolated $CD8^+$ T cells (>98%) were further purified by the staining of anti-CD8 mAb (clone: OKT8) for FACS sorting. The $CD8^+$ T cells were stained with anti-CD8, anti-CCR5, and anti-CD45RA mAbs, and then $CD8^{high}CD45RA^-CCR5^{high}$ and $CD8^{high}CD45RA^-CCR5^{low}$ cells were sorted by using FACSAria (BD Biosciences).

$CD8^+$ T cell proliferation assay

Sorted $CD8^+$ T cell subsets were labeled with 0.5 μM CFSE (Molecular Probes) for 15 min at 37°C. CFSE-labeled cells were cultured for 5 days in anti-CD3 (5 μg/ml) and anti-CD28 (5 μg/ml) mAb-coated flat-bottom 96-well plates containing RPMI 1640 medium supplemented with 10% FCS and 200 U/ml human rIL-2 for 5 days. The cells were then stained with anti-CD3, anti-CD8, anti-GraA, and anti-GraB mAbs. To exclude the dead cells, we added 7-aminoactinomycin D (BD Biosciences) after the staining for cell surface markers. To assess the proliferation and the expression profile of GraA/B, cells were analyzed by using a FACSAria (BD Biosciences). For identification of each generation of proliferating cells and percentage of divided, the flow cytometric data were analyzed by use of the proliferation platform of the FlowJo software. Percentage of divided is the percentage of divided cells from the original sample. The percentage of the undivided cells was calculated from the percentage of divided.

Assay for cytotoxic activity

The cytotoxic activity of EBV-specific and HCMV-specific CTLs was measured by the standard ^{51}Cr release assay, as follows. Target cells (2×10^5) were incubated for 60 min with 100 μCi of $Na_2{}^{51}CrO_4$ in saline and then washed three times with 10% NCS-containing RPMI 1640. Labeled target cells (2×10^3/well) were added to U-bottom 96-well plates along with the corresponding peptide (1 μM). After a 1-h incubation, $CD8^+$ T cells purified by use of anti-CD8 mAb-coated magnetic beads were added, and the mixtures were then incubated for 6 h at 37°C. At the same time, the EBV-specific CTL clone and bulk CTLs were used for a positive control. The supernatants were then collected and analyzed with a gamma counter. The frequency of CMV-specific $CD8^+$ T cells among the total $CD8^+$ T cell population (1.3%) is 3.25 times higher than that of EBV-specific $CD8^+$ T cells (0.4%). To make an equal percentage of effector cells in the total cell population, we mixed purified autologous $CD4^+$ T cells with $CD8^+$ T cells in the ratio of 2.25:1. Then, the relative cytotoxic activity was measured. Each value of relative specific lysis was calculated by subtracting the specific lysis of the sample target cells prepulsed without peptide from that of target cells prepulsed with 1 μM epitope peptide.

Results

Coexpressions of three cytolytic effector molecules in human $CD8^+$ T cells

We investigated the coexpression of three cytolytic functional molecules, i.e., Per and GraA/B in human $CD8^+$ T cells. Total $CD8^+$ T cells from healthy individuals were stained by using mAbs specific for Per, GraA, or GraB. Representative results on the expression of these effector molecules are shown in Fig. 1*A*. GraA-positive ($GraA^+$) $CD8^+$ T cells and GraB-positive ($GraB^+$) $CD8^+$ T cells each had two major populations, one with a high level of expression (Per^{high}) and the other with a low level of it (Per^{low}). Per^{low} cells expressed GraA and comprised two subsets, $GraB^-$ and $GraB^+$. In contrast, Per^{high} cells expressed both GraA and GraB. $GraA^-$ cells were hardly detected in the $GraB^+CD8^+$ population. These results suggested that $CD8^+$ T cells have at least four populations: $Per^-GraA^-GraB^-$, $Per^{low}GraA^+GraB^-$, $Per^{low}GraA^+GraB^+$, and $Per^{high}GraA^+GraB^+$. Three-dimensional analysis of the expression of these three molecules confirmed that $CD8^+$ T cells had these four populations, and further suggested the existence of a small population of $Per^-GraA^+GraB^-$ (Fig. 1*B*). These results were confirmed in $CD8^+$ T cells from seven healthy individuals (data not shown).

Expression of the cytolytic effector molecules in each CCR7 CD45RA subset of human $CD8^+$ T cells

Previous studies using CCR7 and CD45RA markers showed that $CD8^+$ T cells could be divided into four populations: naive ($CCR7^+CD45RA^+$), central memory ($CCR7^+CD45RA^-$), memory effector ($CCR7^-CD45RA^-$), and effector ($CCR7^-CD45RA^+$) subsets (11). So, we analyzed the expression of the three effector molecules in each CCR7 CD45RA subset to evaluate whether there was a correlation between the functional and phenotypic categories. A representative result from one healthy individual and the summarized results of analysis of $CD8^+$ T cells from five healthy individuals are shown in Fig. 2, *A* and *B*, respectively. Human $CD8^+$ T cells were divided into naive, central memory, effector memory, and effector populations, based on the expression

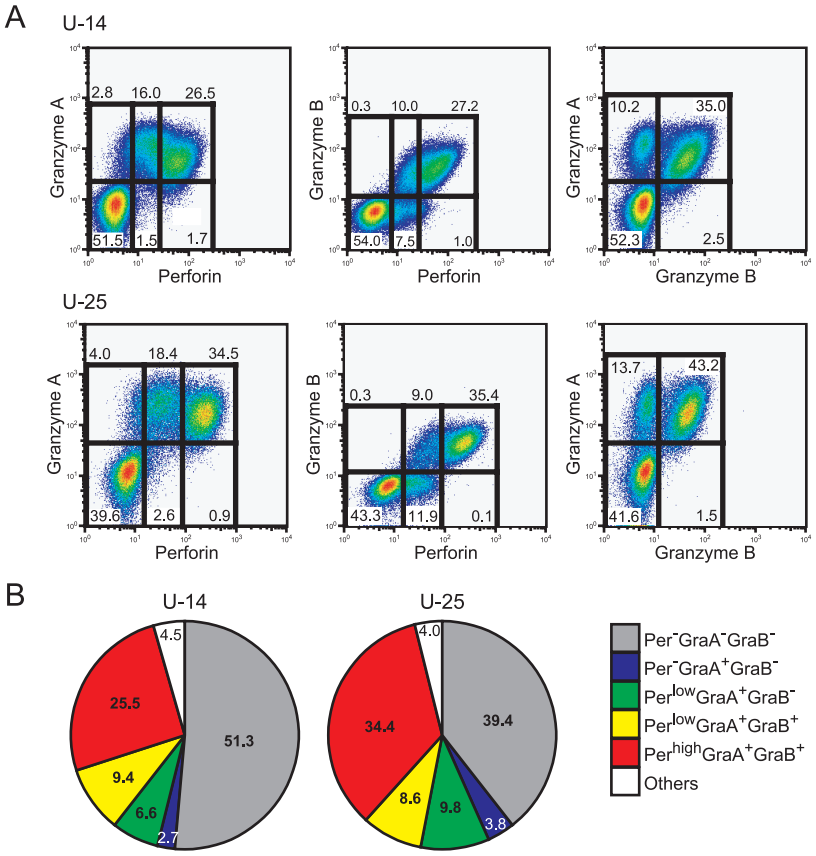

FIGURE 1. Expression of three cytolytic effector molecules in human CD8$^+$ T cells. *A*, Freshly isolated PBMCs from two healthy individuals (U-14 and U-25) were stained with anti-CD3, anti-CD8, anti-Per, anti-GraA, and anti-GraB mAbs. The CD8$^+$CD3$^+$ cells were gated and then analyzed by flow cytometry for their expression of each cytolytic effector molecule. The expression levels of Per and GraA or GraB and of GraA and GraB are shown. The values in each plot show the frequency of each subset in the CD8$^+$ T cell population. *B*, The expression of the three effector molecules in CD8$^+$ T cell was analyzed by using FlowJo software to determine the cell clusters that were expressing different effector molecules. The values in each pie chart showed the frequency of each subset in the CD8$^+$ T cell population.

of CD45RA and CCR7. The expression of the three effector molecules was compared among these four classical CD8$^+$ T cell subsets. Per^{low}GraA$^+$GraB$^+$ and Per^{high}GraA$^+$GraB$^+$ cells were predominantly found in effector and effector memory subsets, whereas naive CD8$^+$ T cells did not express any of the effector molecules. A large proportion of cells with the central memory phenotype was also negative for the effector molecules (81.2 ± 4.7%), although a small subset was Per$^{-/low}$GraA$^+$GraB$^-$ (17.3 ± 9.4%). The existence of Per$^-$GraA$^+$GraB$^-$ cells was clear in the central memory subset, unlike in the composite figure (Fig. 1*A*). Per^{low}GraA$^+$GraB$^+$ cells were evenly found in both the effector memory (18.3 ± 3.2%) and the effector subsets (21.7 ± 4.6%), whereas the percentage of Per^{high}GraA$^+$BraB$^+$ cells was higher in the effector subset than in the effector memory subset (52.1 ± 11.8 vs 31.3 ± 13.8%). The results indicate that both effector and effector memory CD8$^+$ T cell subsets consist of cells with a heterogeneous distribution of the effector molecules Per and GraA and GraB. Thus, these two phenotypically distinct categories do not represent functionally distinct subsets.

We also analyzed the expression of CCR7 and CD45RA molecules in the five subsets determined by the different patterns of expression of the three effector molecules (Fig. 2, *C* and *D*). Pre$^-$GraA$^-$GraB$^-$ cells were predominantly of the naive phenotype (88.4 ± 4.2%), whereas a small proportion of these cells carried central memory (5.0 ± 0.9%) or effector memory (4.5 ± 2.2%) markers. The majority of the Per$^-$GraA$^+$GraB$^-$ cells expressed effector memory markers (63.0 ± 10.9%) and a smaller proportion of them expressed central memory markers (16.7 ± 5.6%). Of Per^{low}GraA$^+$GraB$^+$cells, 63.9 ± 14.6% expressed effector memory markers. Both Per^{low}GraA$^+$GraB$^+$ and Per^{high}GraA$^+$GraB$^+$ cells expressed effector or effector memory phenotype at a ratio of 4:6, confirming that these two phenotypic categories did not represent functionally different subsets.

Expression of the effector molecules in CD27 CD28 CD45RA subsets of human CD8$^+$ T cells

We previously showed that the classification of human CD8$^+$ T cells in terms of CD27, CD28, and CD45RA markers is useful to identify naive, memory, and effector cells (6, 10). To re-evaluate this classification, by using seven-color flow cytometric analysis, we further analyzed the expression of the three effector molecules in subsets classified by their expression of CD27, CD28, and CD45RA. The expression of these effector molecules in each CD27 CD28 subset of CD45RA$^-$ and CD45RA$^+$CD8$^+$ T cells from a representative individual is shown in Fig. 3, *A* and *B*, respectively. In addition, a summary of data from six individuals is represented in Fig. 3*C*. CD8$^+$ T cells expressing the CD27$^{high/low}$CD28$^+$CD45RA$^-$ memory phenotypes included Per$^-$GraA$^-$GraB$^-$, Per$^-$GraA$^+$GraB$^-$, Per^{low}GraA$^+$GraB$^-$, and Per^{low}GraA$^+$GraB$^+$ cells, indicating that this population contained central memory and effector memory T cells. The CD27^{low}CD28$^+$CD45RA$^-$ subset included many more Per^{low} GraA$^+$GraB$^+$ cells (22.8 ± 6.4%) than the CD27^{high} CD28$^+$CD45RA$^-$ subset (8.1 ± 3.8%), supporting a previous finding that the former subset was a more mature population than the latter one (10). These cells with the CD27^{high}CD28$^+$ CD45RA$^+$ naive phenotype did not express any effector molecules. Because these cells express CCR7 (10), this result is consistent with that for CCR7$^+$CD45RA$^+$ cells shown in Fig. 2. CD8$^+$ T cells expressing the CD27^{low}CD28$^-$CD45RA$^{+/-}$ phenotype expressed the three effector molecules with the same pattern as those expressing CD27$^-$CD28$^-$CD45RA$^{+/-}$, the effector phenotype, suggesting that both subsets mostly included effector T cells. Because the CD27$^-$CD28$^-$CD45RA$^{+/-}$ subsets contained much more Per^{high}GraA$^+$GraB$^+$ cells than CD27^{low} CD28$^-$CD45RA$^{+/-}$ subsets, the former subsets are thought to be

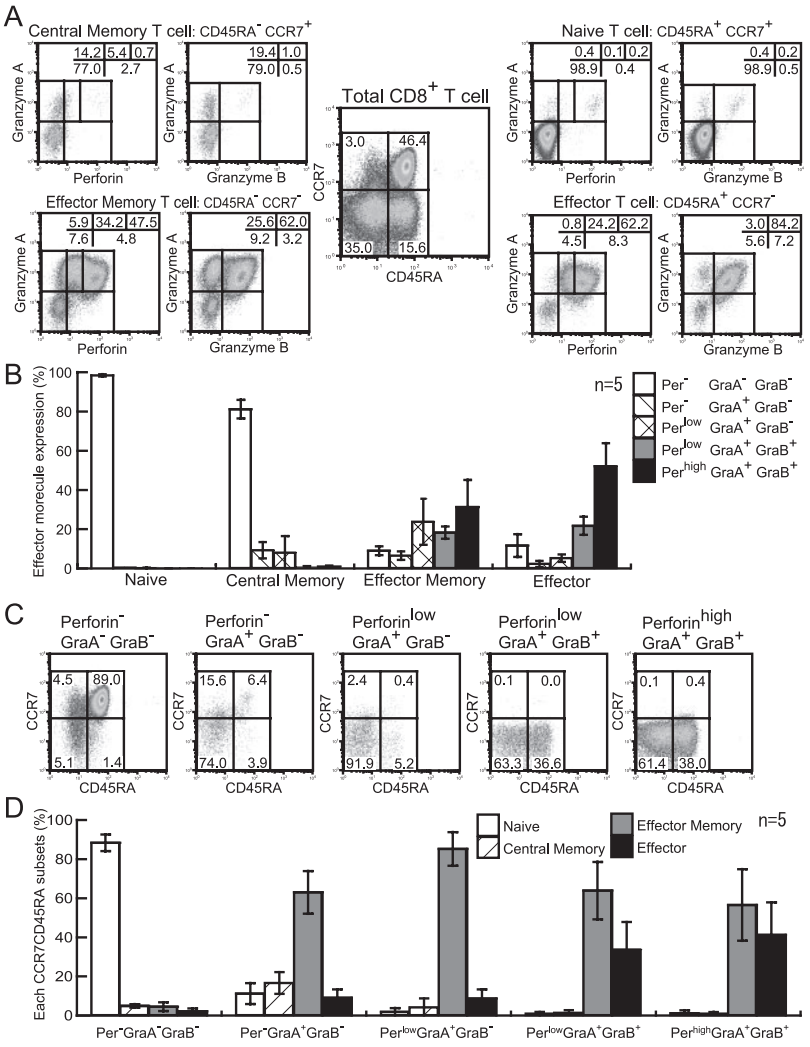

FIGURE 2. Different expression of three cytolytic effector molecules in CCR7 CD45RA subsets of $CD8^+$ T cells. *A*, Expression of three cytolytic effector molecules in CCR7 CD45RA subsets. PBMCs from individual U-14 were stained with anti-CD3, anti-CD8, anti-CCR7, anti-Per, anti-GraA, and anti-GraB mAbs. Based on the Per expression, $CD8^+$ T cells were divided into three populations, i.e., cells not expressing Per (Per^-), cells expressing a low level of it (Per^{low}), and cells expressing a high level of Per (Per^{high}). Per^{high} and Per^{low} $CD8^+$ T cells were divided by a line at one-seventh of the maximum Per fluorescence intensity. $CD8^+CD3^+$ cells were gated and analyzed for the correlation between the expressions of two of the three effector molecules in each CCR7 CD45RA $CD8^+$ T cell subset. *B*, Frequency of cells expressing the effector molecules in each CCR7 CD45RA subset of $CD8^+$ T cells from five individuals. The mean percentage and SD of each population are shown. *C*, Phenotypes of cells expressing different effector molecules. We gated the $CD8^+$ T cells based on the expression level of Per, GraA, and GraB. Five different populations were analyzed for their CCR7 and CD45RA expression. *D*, Frequency of cells expressing each CCR7 CD45RA phenotype in five subsets expressing different effector molecules of $CD8^+$ T cells from five individuals. The mean percentage and SD of each population are shown. The values in each plot indicate the frequency of each population.

more differentiated cells than the latter ones. Interestingly, both $CD27^{high}CD28^-CD45RA^+$ and $CD27^{high}CD28^-CD45RA^-$ subsets included a large number of $Per^-GraA^-GraB^-$ cells, suggesting that these subsets were closely related to the naive or central memory subset. Taken together, central memory T cells can be hardly discriminated from effector T cells by this phenotypic classification.

$CD27^{high}CD28^+CD45RA^-$ and $CD27^{low}CD28^+CD45RA^-$ subsets include both $CCR7^+$ and $CCR7^-$ cells, whereas the $CD27^-CD28^+CD45RA^-$ subset does not express CCR7 (10). Because $CD27^-$ cells were only a minor population in the $CD28^+CD45RA^-$ subset, we analyzed only $CD27^{high/low}$ $CD28^+CD45RA^-$ subsets. We evaluated the expression of effector molecules in $CCR7^+$ and $CCR7^-$ subsets of $CD27^{high/low}$ $CD28^+CD45RA^-$ cells by staining $CD8^+$ T cells with mAbs specific for CD28, CD45RA, CCR7, and the effector molecules (Fig. 4). The $CCR7^-CD28^+CD45RA^-$ subset included a large number of $Per^{low}GraA^+GraB^-$ cells and a small number of $Per^-GraA^+GraB^-$ and $Per^-GraA^-GraB^-$ ones. In contrast, ~60% of the $CCR7^+CD28^+CD45RA^-$ subset were $Per^-GraA^-GraB^-$ cells, whereas the rest were $Per^-GraA^+GraB^-$ or $Per^{low}GraA^+GraB^-$ cells. These results suggested that $CCR7^+$ and $CCR7^-$ cells in the $CD27^{high/low}CD28^+CD45RA^-$ subsets predominantly included central memory and effector memory T cells, respectively.

$Per^{-/low}GraA^+GraB^-$ cells predominantly exist in the $CCR5^+$ subset

$Per^{-/low}GraA^+GraB^-$ cells were found predominantly in the $CCR7^-$ subset of $CD8^+$ T cells expressing the effector memory phenotype. Previous studies demonstrated that CCR5 is predominantly expressed in memory $CD8^+$ T cells that do not express CCR7 (21). These findings imply that $Per^{-/low}GraA^+GraB^-$ cells may express CCR5. To clarify CCR5 expression on $Per^{-/low}$ $GraA^+GraB^-$ cells, we stained PBMC from six healthy individuals with mAbs specific for CD3, CD8, CCR5, GraA, and GraB (Fig. 5, *A* and *B*). $GraA^+GraB^-$ cells are thought to be $Per^{-/low}$ $GraA^+GraB^-$ cells, because $Per^{high}GraA^+GraB^-$ cells were hardly detected. The result showed that $Per^{-/low}GraA^+GraB^-$ cells were found predominantly among the $CD8^+$ T cells expressing a high level of CCR5 ($CCR5^{high}$) or a part of those expressing a low level of it ($CCR5^{low}$). $CCR5^{high}$ cells were predominantly found among the $CD8^+$ T cells expressing the $CD27^{high/low}$ $CD28^+CD45RA^-$ memory phenotype, whereas $CCR5^{low}$ cells were detected among both $CD8^+$ T cells expressing the $CD27^{high/low}$ $CD28^+CD45RA^-$ memory phenotype and those expressing the $CD27^-CD28^-CD45RA^-$ effector phenotype (Fig. 5*C*). These findings together suggest that $Per^{-/low}GraA^+GraB^-$ cells exist among $CCR5^{high/low}CD8^+$ T cells expressing the $CD27^{high/low}$ $CD28^+CD45RA^-$ memory phenotype. Because the results shown

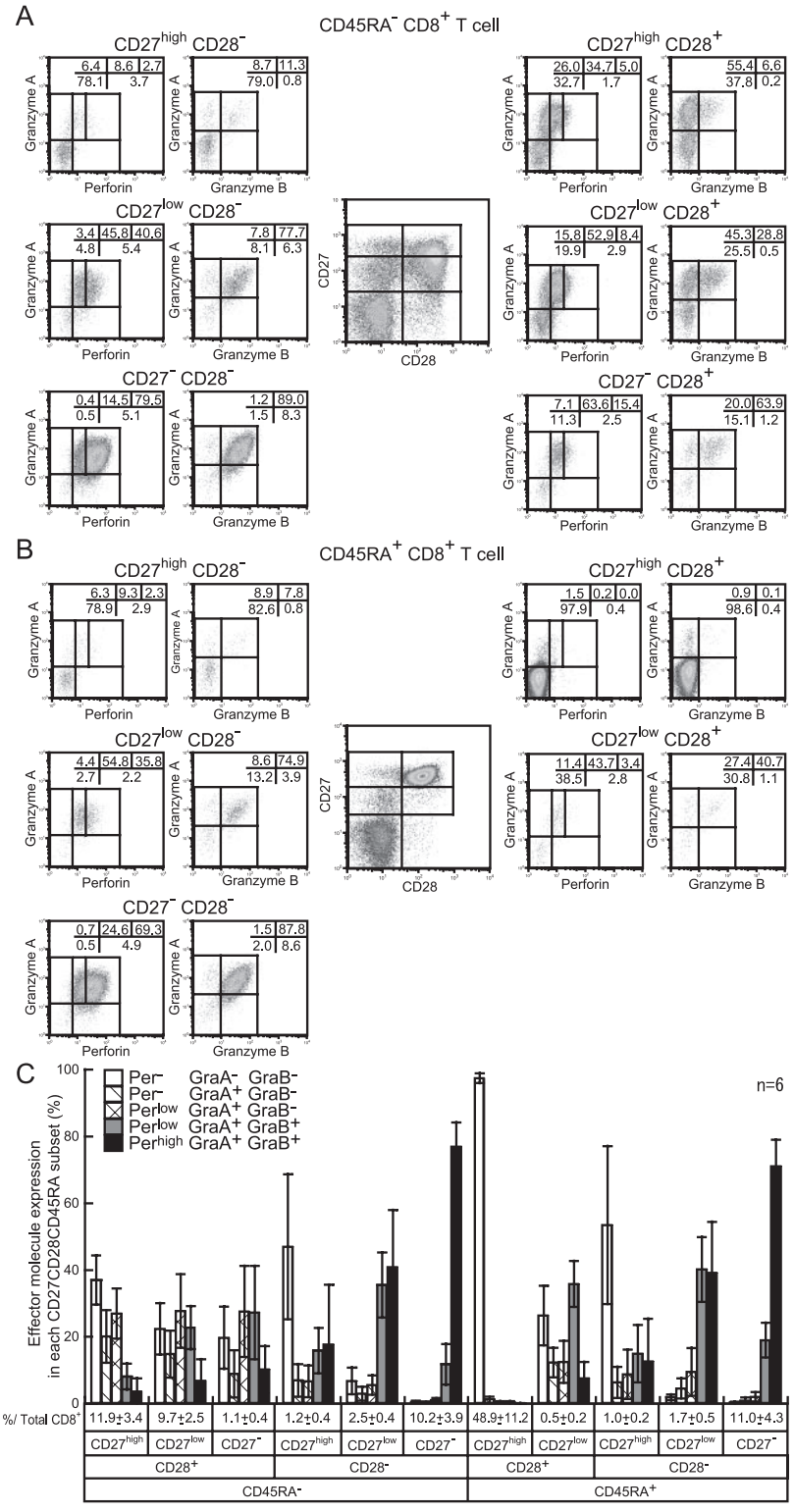

FIGURE 3. Different expression of the three effector molecules in CD27 CD28 CD45RA subsets of $CD8^+$ T cells. *A* and *B*, PBMCs from individual U-14 were stained with anti-CD3, anti-CD8, anti-CD27, anti-CD28, anti-CD45RA, anti-GraA, and anti-Per or anti-GraB mAbs. They were then analyzed by seven-color flow cytometry. Each CD27 CD28 CD45RA subset of $CD8^+$ T cells was gated, and the coexpression of Per/GraA and GraB/GraA is shown for each subset. The values in each plot show the frequency of each population. *C*, Frequency of cells expressing the effector molecules in each CD27 CD28 CD45RA subset of $CD8^+$ T cells from five individuals. The percentage of cells expressing the various combinations of the three effector molecules was calculated from the data of the combinations of the two effector molecules. The mean percentage and SD of cells expressing these effector molecules are shown.

in Fig. 5, *A* and *B*, included effector T cells, we added CD28 and CD45RA markers to the analysis to exclude effector $CD8^+$ T cells. We stained PBMC from two individuals with mAbs specific for CD28, CD45RA, CD8, CCR5, GraA, and GraB. Because effector $CD8^+$ T cells were included in the $CD28^-CD45RA^-$ subset, the $CD28^+CD45RA^-$ subset was analyzed. $GraA^+GraB^-$ cells were predominantly found in $CCR5^{high}CD28^+$ and $CCR5^{low}CD28^+$ subsets, and $GraA^-GraB^-$ in the $CCR5^-CD28^+$ subset (Fig. 5*D*). Conversely, we analyzed CCR5 expression on the $GraA^+GraB^-$ subset of $CD45RA^-CD8^+$ T cells. Most of the $GraA^+GraB^-$ cells were $CCR5^{high}CD28^+$ and $CCR5^{low}CD28^+$ (Fig. 5*E*), confirming that $Per^{-/low}GraA^+GraB^-$ cells existed in $CCR5^{high/low}CD8^+$ T cells expressing the $CD27^{high/low}CD28^+CD45RA^-$ memory phenotype. The seven-color flow cytometric analysis using mAbs specific for CD3, CD8, CD45RA, CCR5, CCR7, GraA, and GraB confirmed that $GraA^+GraB^-CD45RA^-CD8^+$ T cells expressed $CCR7^-CCR5^{high/low}$ phenotype (Fig. 5*F*). Thus, $Per^{-/low}GraA^+GraB^-CD8^+$ T cells predominated in the $CCR7^-CCR5^{high/low}$

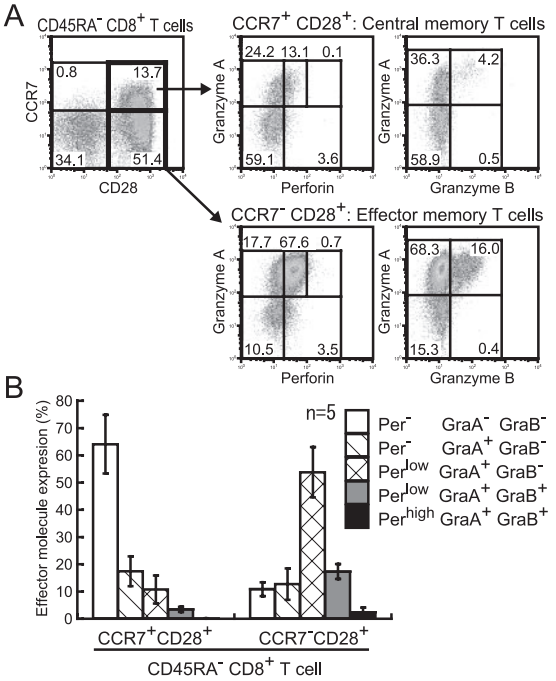

FIGURE 4. Expression of the three effector molecules in $CD8^+$ T cells with $CCR7^{+/-}CD45RA^-CD28^+$ memory subsets. *A*, PBMCs from individual U-25 were stained with anti-CD3, anti-CD8, anti-CCR7, anti-CD28, anti-CD45RA, anti-GraA, anti-Per, or anti-GraB mAbs. $CCR7^+$ $CD45RA^-CD28^+$ and $CCR7^-CD45RA^-CD28^+$ $CD8^+$ T cells were gated to assess the coexpression of Per/GraA and GraB/GraA. The values in each plot show the frequency of each population. *B*, The different effector molecule expression in $CCR7^{+/-}CD45RA^-CD28^+$ subsets from five individuals was analyzed using seven-color flow cytometric analysis. The frequency of cells expressing these effector molecules was calculated by using the data on the coexpression of Per/GraA and GraB/GraA. The mean percentage and SD of each population are shown.

$CD27^{high/low}CD28^+CD45RA^-$ effector memory subset. These results taken together show that the memory subset included cells at three different stages, i.e., $Per^-GraA^-GraB^-$, $Per^{-/low}GraA^+GraB^-$, and $Per^{low}GraA^+GraB^+$, which we consider to correspond to central memory, early effector memory, and late effector memory T cells, respectively. $Per^-GraA^-GraB^-$ and $Per^{-/low}GraA^+GraB^-$ predominantly existed in $CCR5^-CCR7^+$ and $CCR5^{high/low}CCR7^-$ subsets, respectively, of $CD27^{high/low}$ $CD28^+CD45RA^-$ T cells, whereas $Per^{low}GraA^+GraB^+$ did so in the $CCR5^{low}CCR7^-$ subset of $CD27^{high/low}CD28^+CD45RA^-$ and $CD27^{low}CD28^-CD45RA^-$ T cells.

$Per^{-/low}GraA^+GraB^-$ cells express GraB after activation and proliferation in vitro

We suspected that $Per^{-/low}GraA^+GraB^-$ cells differentiated into $Per^{low/high}GraA^+GraB^-$ cells during their maturation. To elucidate this possibility, we investigated the in vitro maturation of $Per^{-/low}GraA^+GraB^-$ cells. $CD8^+$ T cells with the $CCR5^{high}$ $CD45RA^-$ and $CCR5^{low}CD45RA^-$ phenotypes were sorted because the former cells are mostly $Per^{-/low}GraA^+GraB^-$, whereas the latter includes more mature cells. These sorted cells were labeled with CFSE before being stimulated with anti-CD3 and anti-CD28 mAbs for 5 days. After a 5-day culture period, the expression of GraA and GraB in the cells was measured (Fig. 6). The sorted $CCR5^{high}CD45RA^-CD8^+$ T cells included $GraA^+GraB^-$ cells at 94.2% purity. The expression of GraB in the $CCR5^{high}$ $CD45RA^-$ cells increased during in vitro proliferation, whereas that of GraA decreased. In contrast, the sorted $CCR5^{low}$ $CD45RA^-CD8^+$ T cells included $GraA^+GraB^+$, $GraA^+GraB^-$, and $GraA^-GraB^-$ cells at 59.9, 28.2, and 11.5%, respectively. The expression of GraA in the $CCR5^{low/-}CD45RA^-$ cells slightly decreased during in vitro proliferation. That of GraB increased after the first division, but did not change after the second division. These results suggest that the $GraA^+GraB^-CD8^+$ T cells can obtain GraB expression during their proliferation.

However, because 7.3% of the sorted $CCR5^{high}CD45RA^-$ cells were $GraA^+GraB^+$ cells, it remains possible that the $GraB^+$ cells in the culture were derived from contaminating $GraA^+GraB^+$ cells. To evaluate this possibility, we calculated how many cells that divided and expressed $GraA^+GraB^+$ were derived from the $GraA^+GraB^+$ cells in the original $CCR5^{high}CD45RA^-$ cell population. In each generation of the dividing $CCR5^{high}CD45RA^-$ cell population, 3.5% (generation 0: undivided), 11.3.% (generation 1: divided one time), 8.6% (generation 2: divided two times), 4.2% (generation 3: divided three times), 2.3% (generation 4: divided four times), 2.7% (generation 5: divided five times), and 2.4% (generation 6: divided six times) of the total population of stimulated cells were $GraA^+GraB^+$ cells in each generation of the divided cells (Fig. 6). The calculation made by the FlowJo software showed that $GraA^+GraB^+$ cells were derived from 21.6% of original $CCR5^{high}CD45RA^-$ cells. This percentage is much higher than the percentage (7.3%) of $GraA^+GraB^+$ cells among the sorted $CCR5^{high}CD45RA^-$ cells, indicating that more than half of the $GraA^+GraB^+$ cells among the stimulated cells were derived from $GraB^-$ cells in the population of sorted $CCR5^{high}CD45RA^-$ cells. These results support the idea that $Per^{-/low}GraA^+GraB^-$ cells obtain GraB expression in response to TCR stimulation. However, the increased expression of Per was not found in $CCR5^{high}CD45RA^-$ cells after these cells had been stimulated with anti-CD3 and anti-CD28 mAbs (data not shown).

EBV-specific $CD27^{high/low}CD28^+CD45RA^-$ $CD8^+$ T cells express $Per^{-/low}GraA^+GraB^{-/+}$

Previous studies revealed that HLA-A*1101-restricted EBV-3B 399–408-specific $CD8^+$ T cells in healthy individuals express the $CD27^+CD28^+CD45RA^-$ phenotype and that they also predominantly express CCR5, but not CCR7 (6), suggesting that these EBV-specific $CD8^+$ T cells have characteristics more similar to those of differentiated memory T cells than to those to central memory T cells. To clarify the differentiation stage of the EBV-specific $CD8^+$ T cells, we stained PBMC from two healthy individuals (U-5 and U-27) with HLA-A*1101 tetramers specific for EBV-3B 399–408 as well as with anti-CD8, anti-Per, anti-GraA, and anti-GraB mAbs. The results showed that EBV-specific $CD8^+$ T cells expressed $Per^{-/low}GraA^+GraB^-$ and $Per^{low}GraA^+GraB^+$ (Fig. 7*A*). In contrast, it is well known that HCMV-specific T cells in healthy individuals express the $CD27^{low/-}CD28^-CD45RA^{-/+}$ phenotype and have characteristics of effector T cells (6, 10). Analysis of HCMV-pp65 495–503-specific T cells from a healthy individual showed that they expressed $Per^{high}GraA^+GraB^+$ and $Per^{low}GraA^+GraB^+$ (Fig. 7*B*).

Next, we investigated whether these EBV-3B 399–408-specific and HCMV pp65 495–503-specific $CD8^+$ T cells had the ability to kill target cells. This ability of these $CD8^+$ T cells was evaluated by using $CD8^+$ T cells isolated from PBMCs of three healthy individuals. EBV 399–408-specific $CD8^+$ T cells from individual U-5 very weakly killed the target cells, whereas those from individual U-27 failed to kill them. In contrast, HCMV pp65 495–503-specific $CD8^+$ T cells, EBV 399–408-specific CTLs in bulk culture, and an EBV 399–408-specific CTL clone effectively killed the target cells (Fig. 7*C*). These results strongly suggest that

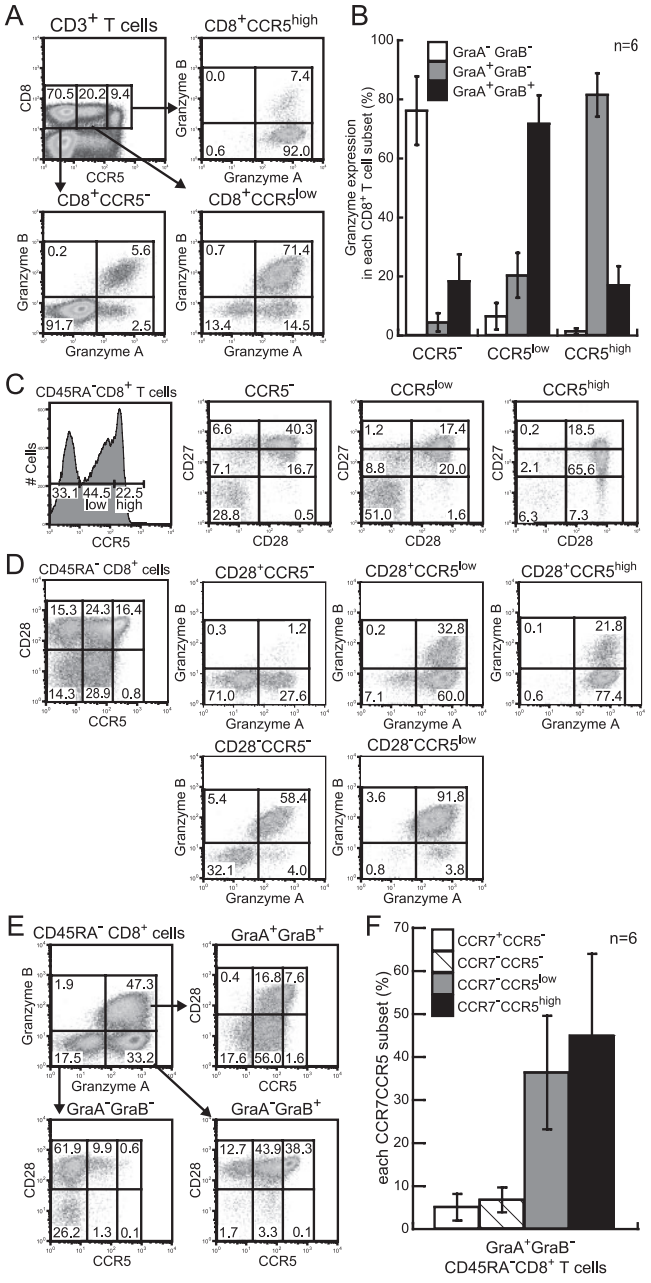

FIGURE 5. GraA$^+$GraB$^-$CD8$^+$ T cells predominate in CCR5$^{high/low}$ CCR7$^-$ subsets of memory CD8$^+$ T cells. *A*, Different expression of GraA/B in CCR5$^{high/low/-}$ subsets of CD8$^+$ T cells. PBMCs from individual U-16 were stained with anti-CD3, anti-CD8, anti-CCR5, anti-GraA, and anti-GraB mAbs. Based on the CCR5 expression, CD3$^+$CD8$^+$ T cells were divided into three populations, i.e., cells not expressing CCR5 (CCR5$^-$), cells expressing a low level of CCR5 (CCR5^{low}), and cells expressing a high level of it (CCR5^{high}). CCR5^{high} and CCR5^{low} CD8$^+$ T cells were divided by a line representing one-third of the maximum CCR5 fluorescence intensity. Coexpression of GraA/B in each CD8$^+$ T cell subset is shown. *B*, PBMCs from six healthy individuals were analyzed to determine the frequency of the cells expressing GraA/B among CCR5$^{high/low/-}$CD8$^+$ T cells. The mean and SD of each population are given. *C*, CD27/CD28 phenotype according to CCR5 expression on CD8$^+$ T cells. PBMCs were stained with anti-CD3, anti-CD8, anti-CCR5, anti-CD27, anti-CD28, and anti-CD45RA mAbs. CCR5$^{high/low/-}$ cells in CD45RA$^-$CD8$^+$ T cells were gated and then analyzed for the coexpression of CD27 and CD28. *D*, Coexpression of GraA and GraB in each CCR5CD28 subset of CD45RA$^-$CD8$^+$ T cells. PBMCs were stained with anti-CD8, anti-CD45RA, anti-CD28, anti-CCR5, anti-GraA, and anti-GraB mAbs. Coexpression of GraA and GraB in each CCR5CD28 subset is depicted. *E*, The CCR5 and CD28 expression on GraAGraB subset in CD45RA$^-$CD8$^+$ T cells. The GraA$^+$GraB$^+$, GraA$^+$GraB$^-$, and GraA$^-$GraB$^-$ subsets of CD45RA$^-$CD8$^+$ T cells were gated, and then the expression of CCR5 and CD28 in each subset was determined. *F*, The CCR5 and CCR7 expression on the GraA$^+$GraB$^-$ subset of CD45RA$^-$CD8$^+$ T cells. PBMCs from six individuals were stained with anti-CD3, anti-CD8, anti-CD45RA, anti-CCR5, anti-CCR7, anti-GraA, and anti-GraB mAbs. The GraA$^+$GraB$^-$ subset of CD45RA$^-$CD8$^+$ T cells was gated, and then the expression of CCR5 and CCR7 was analyzed. The mean percentage and SD of each CCR5 CCR7 subset in the GraA$^+$GraB$^-$CD45RA$^-$CD8$^+$ T cell population are shown.

the Per expression in effector cells was associated with their cytotoxic activity. To confirm this, we analyzed the correlation between Per or GraA expression in tetramer$^+$CD8$^+$ T cells and their CTL activity. The results of this correlation study on EBV-specific and HCMV-specific CTLs from five individuals showed a positive correlation between the Per expression and cytolytic activity (r = 0.89), but no correlation between the GraA expression and cytolytic activity (r = 0.02; Fig. 7*E*).

EBV 399–408-specific CTLs in bulk culture, which were made by stimulating PBMCs from the same individual with the EBV epitope peptide and then culturing them for 2 wk, expressed Per^{low}GraA$^+$GraB$^+$ (Fig. 8*A*). They also showed much stronger cytolytic activity than ex vivo CTLs. These results indicate that the EBV-specific CD8$^+$ T cell population, which included Per$^{-/low}$ GraA$^+$GraB$^-$ and Per^{low}GraA$^+$GraB$^+$ cells, hardly or very weakly killed target cells, but that they could effectively kill them when activated with epitope peptides. HCMV-pp65 495–503 bulk CTLs were also analyzed (Fig. 8*B*). These results also revealed that the bulk CTLs, which were stimulated with the HCMV peptide to undergo proliferation and activation, had much stronger cytotoxic activity than ex vivo CTLs. These results indicate that activated CTLs had a strong cytotoxic activity even though they expressed a low level of Per.

Discussion

Recent studies showed that human CD8$^+$ T cells include three different subsets expressing GraA and GraB, i.e., GraA$^-$GraB$^-$, GraA$^+$GraB$^-$, and GraA$^+$GraB$^+$ (16, 22). In the present study analyzing the expression of Per, GraA, and GraB, we further demonstrated that they comprised five major distinct subsets: Per$^-$GraA$^-$GraB$^-$, Per$^-$GraA$^+$GraB$^-$, Per^{low}GraA$^+$GraB$^-$, Per^{low}GraA$^+$GraB$^+$, and Per^{high}GraA$^+$GraB$^+$. Because significant numbers of Per$^-$GraA$^+$GraB$^+$ and Per$^+$GraA$^-$GraB$^+$ cells were not found in the human CD8$^+$ T cell population, we speculated human CD8$^+$ T cells to acquire the expression of these effector molecules stepwise during peripheral differentiation according to the following sequence: Per$^-$GraA$^-$GraB$^-$→ Per$^-$GraA$^+$GraB$^-$→ Per^{low}GraA$^+$GraB$^-$→Per^{low}GraA$^+$GraB$^+$→ Per^{high}GraA$^+$GraB$^+$.

Several models of the phenotypic classification of human CD8$^+$ T cells have been proposed (7, 9–11, 13, 21, 23). However, it is unclear whether these models actually reflect the effector function. To evaluate the proposed phenotypic classifications, we analyzed the expression of three effector molecules among the subsets in two well-known models using both CCR7 and CD45RA (11), and CD27, CD28, and CD45RA (7, 9, 10). Naive and central memory cells were mostly identified by the phenotypic classification using CCR7 and CD45RA. However, the effector memory (CCR7$^-$CD45RA$^-$) and effector (CCR7$^-$CD45RA$^+$) subsets contained both Per^{low}GraA$^+$GraB$^+$ and Per^{high}GraA$^+$GraB$^+$ cells, indicating that effector memory cells cannot be discriminated from effector cells by this phenotypic classification. In the other

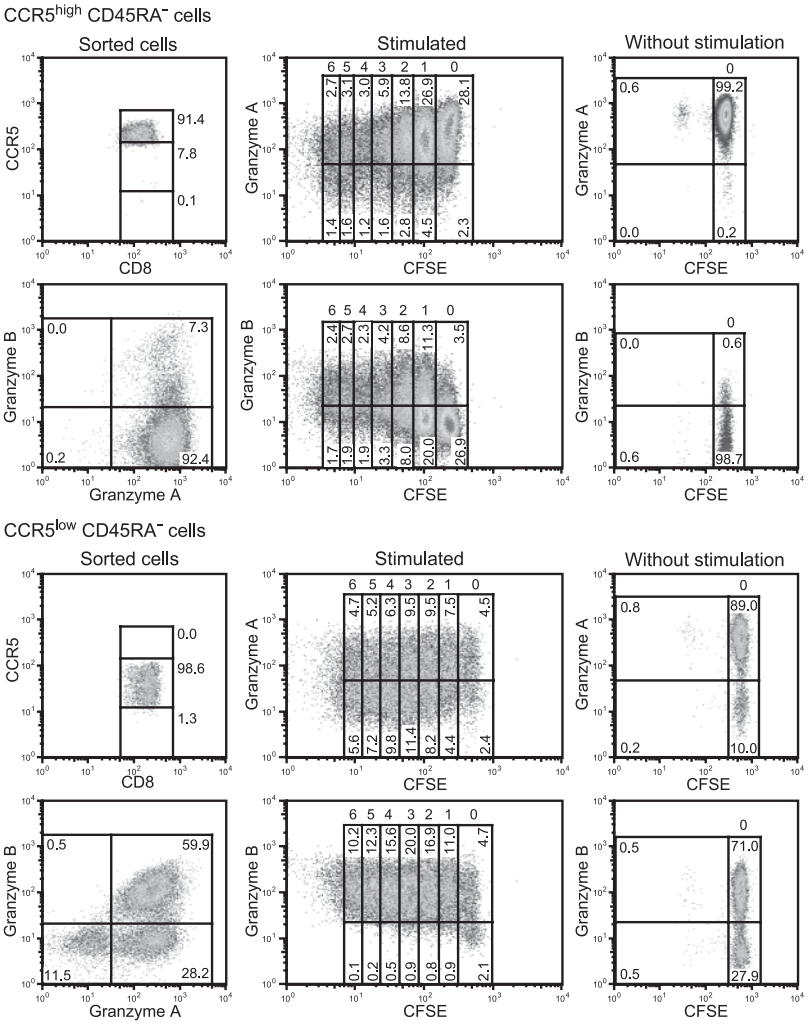

FIGURE 6. GraA$^+$GraB$^-$CD8$^+$ T cells acquire GraB according to their activation and proliferation. CCR5^{high}CD45RA$^-$ and CCR5^{low}CD45RA$^-$ CD8$^+$ cells were sorted from individual U-16. The sorted CD8$^+$ T cell subsets were stained with anti-CD3, anti-CD8, anti-GraA, and anti-GraB mAbs to confirm the GraA/B expression (*left plot*). Other sorted CD8$^+$ T cells were labeled with CFSE, and were then stimulated with anti-CD3 and anti-CD28 mAbs coated on plates (*central plot*: stimulated) or cultured in noncoated plates (*right plot*: without stimulation) for 5 days. For assessment of the proliferation and the expression profile of GraA/B, the cells were then stained with anti-CD3, anti-CD8, anti-GraA, and anti-GraB mAbs. The CD3$^+$CD8$^+$ cells were gated, and thereafter, the sequential decrease in CFSE fluorescence intensity and the expression of GraA/B in them were examined. The number above each gate represents the generation number. The values in each gate showed the percentage of each population in the two sorted subsets of CD3$^+$CD8$^+$ T cell.

classification, the naive and effector subsets could be discriminated from other subsets by the phenotypic classification using CD27, CD28, and CD45RA. In contrast, the memory subset (CD27$^{high/low}$CD28$^+$CD45RA$^-$) included Per$^-$GraA$^-$GraB$^-$, Per$^-$GraA$^+$GraB$^-$, Per^{low}GraA$^+$GraB$^-$, and Per^{low}GraA$^+$GraB$^+$ cells, indicating that effector memory cells cannot be discriminated from central memory cells or effector cells by this phenotypic classification. These two phenotypic classifications have often been used in various studies on infectious diseases and cancers to characterize Ag-specific human CD8$^+$ T cells (13, 24–29). However, the present study demonstrated that the functional subsets of human CD8$^+$ T cells cannot be identified by using these classifications.

A recent study showed that Ag-specific effector CD8$^+$ T cells expressing both GraA and GraB are induced by vaccination, and that 1 year later after immunization the number of specific CD8$^+$ T cells expressing GraA is increased (22), indicating that memory CD8$^+$ T cells preferentially express GraA, but not GraB. This was supported by the present study showing that Per$^{-/low}$GraA$^+$GraB$^-$ cells were predominantly found among the CD8$^+$ T cells expressing the CD27$^+$CD28$^+$CD45RA$^-$ memory phenotype. We further showed that CD8$^+$ T cells with this memory phenotype included three subsets: CCR5$^{high/low}$CCR7$^-$, CCR5$^-$CCR7$^+$, and CCR5$^-$CCR7$^-$. It is thought that the CCR5$^-$CCR7$^+$ subset represents central memory cells because this subset expressed Per$^-$GraA$^-$GraB$^-$ (Figs. 4*B* and 5*E*). In contrast, Per$^{-/low}$GraA$^+$GraB$^-$ cells were predominantly detected in the CCR5$^{high/low}$CCR7$^-$ subset (Fig. 5, *E* and *F*), suggesting that this subset is mostly composed of early effector memory cells. Per^{low}GraA$^+$GraB$^-$ and Per^{low}GraA$^+$GraB$^+$ cells were found in the CCR5$^-$CCR7$^-$ subset, indicating that this subset included both early and late effector memory cells. Per$^{-/low}$GraA$^+$GraB$^-$ cells gained the expression of GraB after stimulation with anti-CD3 and anti-CD28 Abs. This result supports the idea that the CCR5^{high}CCR7$^-$ subset expressing Per$^{-/low}$GraA$^+$GraB$^-$ is an intermediate one between the CCR5$^-$CCR7$^+$ central memory subset expressing Per$^-$GraA$^-$GraB$^-$ and the late effector memory subset expressing Per^{low}GraA$^+$GraB$^+$. Thus, the results of the present study indicate that the phenotypic classification using five cell surface markers (CD27, CD28, CD45RA, CCR5, and CCR7) or three cell surface makers (CD27, CD28, and CD45RA) and the three cytolytic molecules is much more useful to identify naive, central memory, early effector memory, late effector memory, and effector CD8$^+$ T cells than the other two classifications (Fig. 9).

A previous study demonstrated that CD8$^+$ T cells have the ability to effect granule-dependent killing in GraA-deficient mice (30). In contrast, CD8$^+$ T cells and NK cells completely lost their ability to kill in this manner in Per-deficient mice (31). CTL derived from GraB-deficient mice showed reduced killing activity and a profound defect in their ability to induce rapid DNA fragmentation and apoptosis in target cells (32). These studies suggest that the expression of Per and GraB in CD8$^+$ T cells is critical for killing of target cells. Therefore, we speculate that Per^{high}GraA$^+$GraB$^+$ and Per^{low}GraA$^+$GraB$^+$ CD8$^+$ T cells have the ability to kill target cells, whereas Per$^{-/low}$GraA$^+$GraB$^-$CD8$^+$ T cells do not have

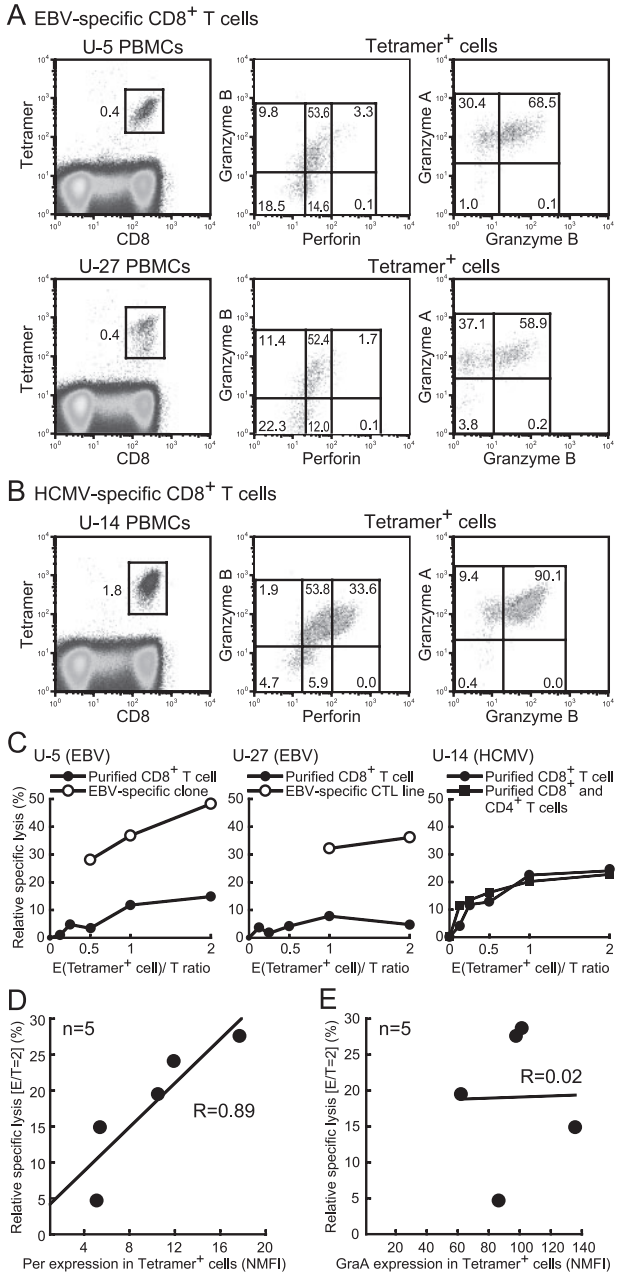

FIGURE 7. Expression of effector molecules in EBV-specific and HCMV-specific CD8$^+$ T cells and their cytolytic activity. *A*, Expression of the effector molecules in EBV-specific CD8$^+$ T cells. PBMCs isolated from two healthy individuals (U-5 and U-27) were stained with anti-CD8, anti-Per, anti-GraA, anti-GraB, and EBV-A*1101 tetramer. Tetramer$^+$ cells were gated and then analyzed for expression of these effector molecules. The percentage of tetramer$^+$ cells in CD8$^+$ T cell was shown. *B*, Expression of the effector molecules in HCMV-1-specific CD8$^+$ T cells. PBMCs isolated from a healthy individual U-14 were stained with anti-CD8, anti-Per, anti-GraA, anti-GraB, and HCMV-1-A*0201 tetramer. Tetramer$^+$ cells were gated and then analyzed for expression of effector molecules. *C*, Cytotoxic activity of EBV- and HCMV-1-specific CD8$^+$ T cells. CD8$^+$ T cells were purified from three individuals, and then the relative cytotoxic activity of purified CD8$^+$ T cells (●) for C1R-A*1101 cells pulsed with the EBV CTL epitope peptide (U-5 and U-27) or C1R-A*0201 cells pulsed with the HCMV-1 CTL epitope peptide (U-14) was measured at different effector (tetramer$^+$ cells) to target ratios. To clarify the effect of nonspecific T cells as physical barrier on killing of specific CTLs, CD4$^+$ T cells purified from U-14 were added into CD8$^+$ T cells and then the relative cytotoxic activity was measured (■). It was compared with that of purified CD8$^+$ T cells alone (●). Each E:T ratio was calculated from the frequency of tetramer$^+$ cells in the total CD8$^+$ T cell population. An EBV-specific CTL clone and EBV-specific CTL line (○) were used as positive controls. Each value of relative specific lysis was calculated by subtracting the specific lysis of the sample target cells without peptide from that of target cells with peptide. *D*, Correlation between Per expression and cytolytic activity in tetramer$^+$ cells. The effector molecule expression and cytotoxic activity of EBV- and HCMV-1-specific CD8$^+$ T cells from five healthy individuals were analyzed in the same manner as in *A–C*. The Per expression is represented as NMFI, which is the mean fluorescence intensity (MFI) of cells stained with anti-Per mAb divided by the MFI of cells stained with the isotype control mAb. Then, the Per expression and relative specific lysis at an E:T ratio of 2:1 were plotted. The linear correlation coefficient was $r = 0.89$. *E*, Lack of correlation between GraA expression and cytolytic activity in tetramer$^+$ cells. The GraA expression and relative specific lysis at an E:T ratio of 2:1 were plotted. The GraA expression is also presented as NMFI based on the isotype control. The linear correlation coefficient was $r = 0.02$.

it. Indeed, the HCMV-specific CD8$^+$ T cells, which were composed of Per^{high}GraA$^+$GraB$^+$ and Per^{low}GraA$^+$GraB$^+$ cells, effectively killed target cells. In contrast, EBV-specific CD8$^+$ T cells, which included Per$^{-/low}$GraA$^+$GraB$^-$ and Per^{low}GraA$^+$GraB$^+$ cells, hardly or very weakly killed target cells, but they effectively killed them when they were activated with epitope peptides. These results support the idea that Per$^{-/low}$GraA$^+$GraB$^-$ had no ability to kill target cells because the expressions of GraB and Per were lost or decreased, and further reveal that Per^{low}GraA$^+$GraB$^+$ cells show different abilities, weak and strong, to kill target cells in resting and activated stages, respectively. Activation of Per^{low}GraA$^+$GraB$^+$ T cells might allow easier release of granules so that they can effectively kill target cells.

Previous studies using ex vivo PBMC from HIV-1-infected donors and healthy donors showed that EBV-specific and HIV-1-specific CD8$^+$ T cells, which had a low frequency of Per$^+$ cells, failed to kill target cells (6, 14, 33), suggesting that Per expression is required for the killing activity of CD8$^+$ T cells. The study also showed that EBV-specific CD8$^+$ T cells, which are mostly Per$^+$ cells, failed to kill target cells (14), implying that the expression level of Per and that of GraB are important for killing activity of CD8$^+$ T cells. Indeed, the present study showed that the expression of Per was critical for this killing activity of CD8$^+$ T cells. Thus, CD8$^+$ T cells obtained cytotoxic function when they highly expressed Per and GraB. However, EBV-specific CTL clones and lines, which can effectively kill target cells, expressed Per^{low}GraA$^+$GraB$^+$ (Fig. 8), indicating that CD8$^+$ T cells expressing Per^{low}GraA$^+$GraB$^+$ also have cytotoxic function when they are activated.

A previous study showed that EBV-specific CD8$^+$ T cells expressed the CCR5$^+$CCR7$^-$ or CCR5$^-$CCR7$^-$ phenotype and CD27$^+$CD28$^+$CD45RA$^-$ one, and failed to kill target cells (6), suggesting that they were effector memory T cells having no cytotoxic function. In contrast, HCMV-specific CD8$^+$ T cells expressed the CCR5$^-$CCR7$^-$CD27$^{-/low}$CD28$^-$CD45RA$^{-/+}$ phenotype and effectively killed target cells (10), suggesting that they were effector T cells. In the present study, we divided memory CD8$^+$ T cells into three subtypes, central (Per$^-$GraA$^-$GraB$^-$), early effector memory (Per$^{low/-}$GraA$^+$GraB$^-$), and late effector memory (Per^{low}GraA$^+$GraB$^+$) T cells. EBV-specific CD8$^+$ T cells included both early effector memory and late effector memory T cells, whereas HCMV-specific CD8$^+$ T cells included both late effector memory and effector T cells. Thus, the present study analyzing three effector molecules afforded a more precise characterization of these T cells, and the result obtained suggests that the presence of Per^{high}GraA$^+$GraB$^+$ T cells is critical for the killing activity of HCMV-specific CD8$^+$ T cells.

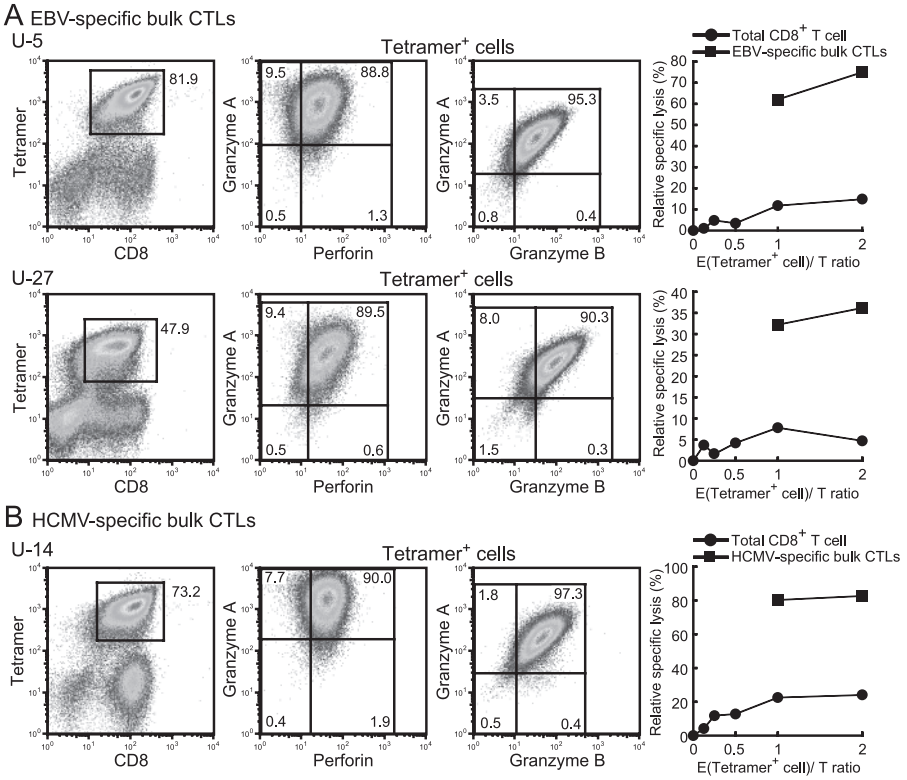

FIGURE 8. Expression of effector molecules in EBV-specific or HCMV-specific bulk CTLs and their cytolytic activity. *A*, EBV-specific CTLs. PBMCs isolated from EBV-seropositive donors U-5 and U-27 were cultured for 2 wk after stimulation with the EBV CTL epitope peptide to establish bulk CTLs. For assessment of the expression profile of Per, GraA, and GraB in the EBV-specific bulk CTLs, the cells were then stained with EBV-A*1101 tetramer, anti-CD8, anti-Per, anti-GraA, and anti-GraB mAbs. The tetramer$^+$ CD8$^+$ cells were gated, and the coexpression of Per/GraA and GraB/GraA was examined. For comparison of the cytotoxic activity between ex vivo CD8$^+$ T cells and bulk CTLs, CD8$^+$ T cells were purified from individuals U-5 and U-27, and then the relative cytotoxic activity of the purified CD8$^+$ T cells (●) and bulk CTLs (■) toward C1R-A*1101 cells pulsed with the EBV CTL epitope peptide was measured at different effector (tetramer$^+$ cells) to target ratios. *B*, HCMV-specific CTLs. PBMCs isolated from HCMV-seropositive donor U-14 were cultured for 2 wk after stimulation with the HCMV CTL epitope peptide to establish bulk CTLs. These cells were then treated in the same manner as in *A*. HCMV-A*0201 tetramer was used to detect the HCMV-specific CTLs. The relative cytotoxic activity of purified CD8$^+$ T cells (●) and bulk CTLs (■) for C1R-A*0201 cells pulsed with the HCMV CTL epitope peptide was measured at different effector (tetramer$^+$ cells) to target ratios.

In the present study, we characterized a subset of CD8$^+$ T cells expressing different effector molecules. The classification of human CD8$^+$ T cells by using the three cell surface markers and the three cytolytic molecules or the five cell surface markers reflected their effector function. Further detailed analysis of human memory CD8$^+$ T cell subsets may be expected to allow clarification of the functional differences among the memory subsets. In addition, the use of this phenotypic classification of human CD8$^+$ T cells from patients with various diseases such as HIV-1 infection and other chronic infections will provide important information on the pathogenesis of these diseases and lead to better therapy of them.

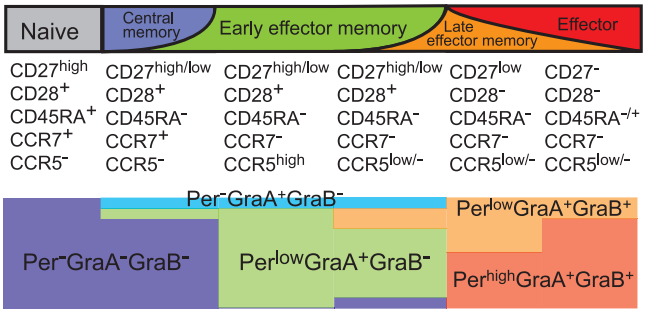

FIGURE 9. Expression of cell surface markers and cytolytic effector molecules during differentiation of human CD8$^+$ T cells.

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Disclosures

The authors have no financial conflict of interest.

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