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Functional expression of chemokine receptor CCR6 on human effector memory CD8$^+$ T cells

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Since CCR6 is a receptor for the chemokine CCL20, which is produced in tissues such as intestine and colon, it is thought that T cells expressing CCR6 are involved in mucosal immunity. The expression and function of CCR6 on human CD8$^+$ T cells have not well been analyzed, although it is known that this receptor is expressed on a subset of human CD8$^+$ T cells. We here characterize human CCR6$^+$ CD8$^+$ T cells. Multi-color flow cytometric analysis demonstrated that CCR6$^+$ cells are predominantly found among CD8$^+$ T cells having the memory phenotype. The expression of CCR6 is positively and negatively correlated with that of CCR5 and CCR7, respectively. CCR6$^+$ CD8$^+$ T cells express granzyme A and a low level of perforin but not granzyme B. In addition, a major population among these cells has the ability to produce IFN-γ and TNF-α but not IL-2. These results indicate that CCR6$^+$ CD8$^+$ T cells have characteristics of early effector memory cells rather than effector or central memory cells. A chemotaxis assay revealed that CCR6$^+$ CD8$^+$ T cells have the ability to migrate in response to CCL20, suggesting that these T cells migrate to tissues such as colon and are involved in mucosal immunity.

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Introduction

CD8$^+$ T cells are essential for immunity to many viral, bacterial and protozoal pathogens. Upon primary activation by foreign Ag, CD8$^+$ T cells follow a program of proliferation and differentiation into CTL armed with effector functions that enable pathogen clearance or containment [1, 2]. After the expansion phase, the majority of Ag-specific CD8$^+$ T cells undergo programmed cell death, leaving a population of memory CD8$^+$ T cells that swiftly proliferate upon secondary antigenic challenge. Effector CD8$^+$ T cells have the ability to kill their target through perforin (Per), granzyme and the Fas ligands [3]. However, memory CD8$^+$ T cells can proliferate and produce cytokines such as IL-2 and IFN-γ in response to antigen stimulation, although they have no ability to directly kill target cells [4, 5]. Phenotypic classification of memory and effector CD8$^+$ T cells has proven to be very useful in mouse and human immunological studies. In humans, the particular expression patterns of the costimulatory molecules CD27, CD28, and CD45RA or CD45RO are associated with naive, memory and effector function of CD8$^+$ T cells [4, 6, 7]. Effector and effector memory (T_{EM}) CD8$^+$ T cells, which were previously classified by the phenotypes of CD27$^-$CD28$^-$CD45RA$^{+/-}$ and CD27^{low}CD28$^-$CD45RA$^{+/-}$, respectively, were shown to both possess cytotoxic activity and the ability to produce cytokines [4–7].

Chemokines constitute a family of structurally related chemotactic cytokines that direct the migration of leukocytes throughout the body under both physiological and inflammatory conditions [8, 9]; they can be classified as homeostatic or inflammatory according to their function [10]. Homeostatic chemokines are expressed constitutively and appear to be responsible for the trafficking of lymphocytes under homeostatic

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Abbreviations: **ECD:** energy-coupled dye · **EBNA3B:** Epstein-Barr virus nuclear antigen 3B · **GraA/B:** granzyme A/B · **Per:** perforin · **T_{CM}:** central memory · **T_{EM}:** effector memory

conditions, while inflammatory chemokines are specifically up-regulated at sites of inflammation and are thought to play an important role in the recruitment of lymphocytes to peripheral tissues in response to immunological challenge. Chemokine receptors are also useful as surface markers to discriminate naive, memory and effector subsets in the human $CD8^+$ T cell population. For example, CCR7 is expressed on naive and memory $CD8^+$ T cells and acts as a receptor for homing of these cells to secondary lymphoid tissues [4, 8, 11]. CCR5 is predominantly expressed on memory $CD8^+$ T cells, and its expression decreases during differentiation from memory to effector $CD8^+$ T cells: $CD27^+CD28^+CD45RA^-$ → $CD27^+CD28^-CD45RA^-$ → $CD27^-CD28^-CD45RA^-$ [4, 8, 12]. A recent study demonstrated that CXCR1 is expressed on effector and T_{EM} $CD8^+$ T cells with the phenotypes $CD27^-CD28^-CD45RA^{+/-}$ and $CD27^{low}CD28^-CD45RA^{+/-}$ and that CXCR4 is predominantly expressed on $CD8^+$ T cells with the naive $CD27^+CD28^+CD45RA^+$ phenotype, with expression decreasing during differentiation from naive to memory and effector $CD8^+$ T cells [13, 14].

CCR6 is the unique receptor for the inflammatory chemokine MIP-3α/CCL20, which is expressed in intestine, colon, appendix, thymus, skin and liver [15, 16]. CCR6 has been identified on lymphoid but not myeloid cells in peripheral blood [17, 18]. $CCR6^+$ cells were more frequently found among $CD4^+$ T cells than among $CD8^+$ T cells [19]. Several studies revealed that CCR6 is highly expressed on immature dendritic cells (DC) but not on terminally differentiated DC [16, 18, 20]. A study using CCR6-deficient mice demonstrated not only deficiency and abnormalities of DC and $CD4^+$ T cells in mucosal and cutaneous tissues, respectively, but also showed that CCR6 play an important role in mucosal immunity [21], although another study showed the presence of $CD11b^+$ DC in dome regions of CCR6-deficient mice [22]. While the expression and function of CCR6 on $CD4^+$ T cells and DC have been studied, there has been no study analyzing the characteristics of $CD8^+$ T cells expressing CCR6.

In the present study, we investigated the expression and function of CCR6 on human $CD8^+$ T cells using multi-color flow cytometric analysis and a novel migration assay. The results show that $CCR6^+$ $CD8^+$ T cells are a subset with the T_{EM} phenotype and have the ability to migrate in response to MIP-3α/CCL20. The present findings suggested a role for $CCR6^+$ $CD8^+$ T cells in mucosal immunity.

Results

Surface expression of CCR6 on human $CD8^+$ T cells

To clarify the expression of CCR6 on human $CD8^+$ T cells, we first investigated the surface expression of CCR6 on $CD8^+$ T cells among PBMC from 22 healthy individuals using anti-CD8, anti-CD3 and anti-CCR6 mAb. A representative result of flow cytometric analysis is shown in Fig. 1A. Only 8.6% of the total $CD8^+$ T cells expressed CCR6 in individual U-16. The expression of CCR6 varied among the 22 individuals, with the percentage of $CCR6^+$ cells among the total $CD8^+$ T cell population ranging from 1.5 to 24.0% (mean ± SD, 7.6±4.5%; Fig. 1B). These results suggest that $CD8^+$ T cells may express CCR6 on a given $CD8^+$ T cell subset.

A previous study showed that $CD8^+$ T cells can be classified into naive cells, memory cells, T_{EM} cells and effector cells by the expression pattern of three cell surface markers, CD27, CD28 and CD45RA [4–7]. To identify the populations expressing CCR6, we investigated the surface expression of CCR6 on each CD27/CD28/CD45RA subset of $CD8^+$ T cells. PBMC were isolated from 9 healthy donors and then analyzed by flow cytometry with anti-CCR6, anti-CD8, anti-CD45RA, anti-CD27 and anti-CD28 mAb. A representative result is shown in Fig. 1C. The $CCR6^+$ cells were predominantly found in $CD27^+CD28^+CD45RA^-$ and $CD27^-CD28^+CD45RA^-$ subsets (Fig. 1D, $n=9$). We inversely analyzed the CD27/CD28/CD45RA expression on $CCR6^+$ $CD8^+$ T cells. Approximately 65% and 20% of $CCR6^+$ $CD8^+$ T cells had $CD27^{low}CD28^+CD45RA^-$ and $CD27^{high/-}CD28^+CD45RA^-$ phenotypes, respectively (Fig. 1E, F). Taken together, these results show that CCR6 is expressed on memory subsets.

Integrin $\alpha_4\beta_7$ acts as a key molecule for intestinal homing, and integrin $\alpha_4\beta_7$ expressing lymphocytes migrate preferentially to gut [23]. To identify the expression of integrin $\alpha_4\beta_7$ on memory $CCR6^+$ $CD8^+$ T cells, we analyzed integrin $\alpha_4\beta_7$ expression on the $CCR6^+$ and $CCR6^-$ subsets of $CD27^+CD28^+CD45RA^-$ $CD8^+$ T cells. The data from 7 individuals demonstrate that a significantly higher percentage of $CCR6^+CD27^+$ $CD28^+CD45RA^-$ $CD8^+$ T cells than $CCR6^-CD27^+$ $CD28^+CD45RA^-$ $CD8^+$ T cells express integrin $\alpha_4\beta_7$ (83.1±7.3% of $CCR6^+$ cells, 60.4±9.7% of $CCR6^-$ cells, $p=0.0004$; Fig. 1G). These results imply that $CCR6^+$ memory $CD8^+$ T cells in human PBMC preferentially migrate to mucosal tissues.

Co-expression of CCR6, CCR7 and CCR5 on CD8$^+$ T cells

CCR7 and CCR5 are predominantly expressed on naive and central memory CD8$^+$ T cells (T_{CM}) and T_{EM} CD8$^+$ T cells, respectively [4, 7, 11, 12]. To examine the correlation between expression CCR6 and that of the two other receptors, we investigated co-expression of CCR6 and CCR7 or CCR5 on CD8$^+$ T cells from 6 healthy donors. More than 90% of the CCR6$^+$ CD8$^+$ T cell subset expressed CCR5 at a high level, whereas the CCR6$^-$ CD8$^+$ T cell subset predominantly showed a CCR5$^-$ phenotype (Fig. 2A), indicating that CCR6 is expressed on CCR5$^+$ CD8$^+$ T cells. On the other hand, the CCR6$^+$ CD8$^+$ T cells failed to express CCR7 (Fig. 2A). The finding that CCR6$^+$ CD8$^+$ T cells predominantly express

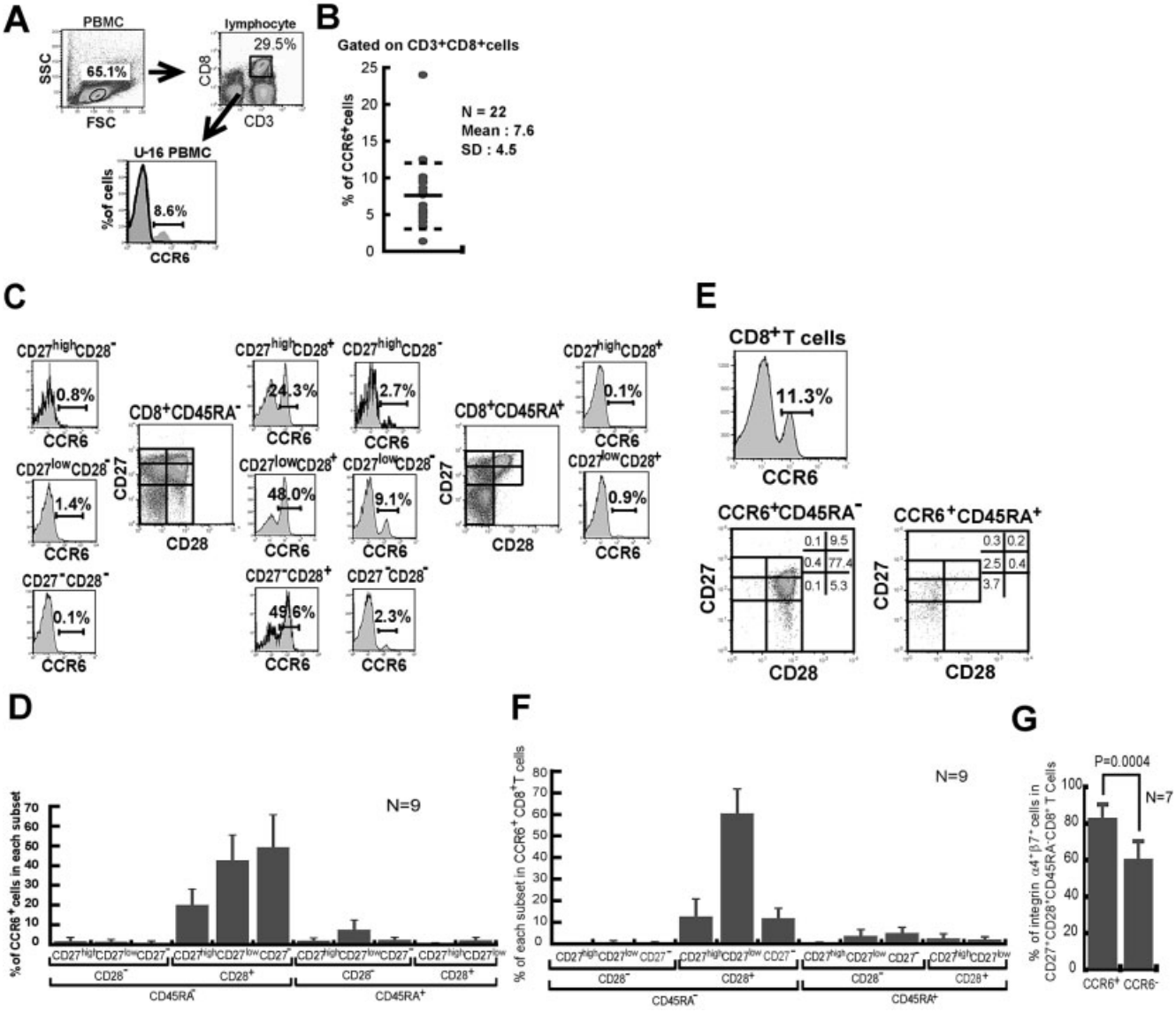

Figure 1. Surface expression of CCR6 on human CD8$^+$ T cells. (A) PBMC isolated from a healthy donor, U-16, were stained with Alexa Fluor®647-labeled anti-CCR6, ECD-labeled anti-CD3 and FITC-labeled anti-CD8 mAb and then analyzed by flow cytometry. The CD3$^+$CD8$^+$ T cells were analyzed for surface expression of CCR6. (B) Variation of CCR6 expression on CD3$^+$CD8$^+$ T cells from 22 healthy individuals. The mean percentage and SD of CCR6$^+$ cells among CD3$^+$CD8$^+$ T cells are presented. (C, D) Surface expression of CCR6 on CD27/CD28/CD45RA subsets of CD8$^+$ T cells. PBMC were isolated from 9 healthy donors and then stained with Cascade Blue-labeled anti-CD8, allophycocyanin (APC)-Cy7-labeled anti-CD27, FITC-labeled anti-CD28, ECD-labeled anti-CD45RA and Alexa Fluor®647-labeled anti-CCR6 mAb. Each CD27/CD28/CD45RA subset was gated and then analyzed for the expression of CCR6. For individual U-16, the percentage of CCR6$^+$ cells in each subset is shown in (C). The mean percentage and SD of CCR6$^+$ cells in each subset from 9 individuals are shown in (D). (E, F) Frequency of each CD27/CD28/CD45RA subset in the CCR6$^+$ CD8$^+$ T cell population. The frequency of each subset was analyzed and is shown for individual U-16 and 9 individuals in E and F, respectively. (G) The percentage and SD of integrin $\alpha_4\beta_7^+$ cells in the CCR6$^+$ and CCR6$^-$ subsets of CD27$^+$CD28$^+$CD45RA$^-$ CD8$^+$ T cells from 7 individuals are shown.

CCR5 but not CCR7 was confirmed by directly analyzing expression of the three receptors on $CD8^+$ T cells from 6 donors (Fig. 2B).

The fact that most $CCR6^+$ $CD8^+$ T cells were found in the $CD27^+CD28^+CD45RA^-$ memory subset and in the $CCR5^+CCR7^-$ subset strongly suggested that $CCR6^+$ $CD8^+$ T cells are memory T cells expressing the $CD27^+CD28^+CD45RA^-CCR7^-CCR5^+$ phenotype. To confirm the expression of the three chemokine receptors on memory $CD8^+$ T cells, we analyzed PBMC by 7-color flow cytometry; a representative result is shown in Fig. 2C. Approximately 40% of the $CCR7^-CCR5^+$ subset in the $CD27^{high}CD28^+CD45RA^-$ $CD8^+$ T cell population expressed CCR6. In contrast, small numbers of $CCR6^+$ cells were found in the $CCR7^+CCR5^-$ and $CCR7^-CCR5^-$ subsets. Similar findings were made for the $CD27^{low}CD28^+CD45RA^-$ memory $CD8^+$ T cell population, which contains more mature $CD8^+$ T cells than the $CD27^{high}CD28^+CD45RA^-$ memory subset [7]. These results were confirmed in experiments using PBMC from a total of 7 healthy individuals (data not shown). These findings confirm that CCR6 is expressed on $CD8^+$ T cells with the $CCR7^-CCR5^+CD27^{high/low}CD28^+CD45RA^-$ phenotype. These $CCR6^+$ $CD8^+$ T cells may have been T_{EM} T cells because they expressed CCR5 but not CCR7.

Expression of Per and granzymes A and B in $CCR6^+$ $CD8^+$ T cells

We investigated the expression of Per, granzyme A (GraA) and granzyme B (GraB) in both $CCR6^+$ and $CCR6^-$ subsets of $CD27^+CD28^+CD45RA^-$ $CD8^+$ T cells. In the $CD27^{high}CD28^+CD45RA^-$ subset, 85.1% of $CCR6^+$ cells expressed GraA and a low level of Per. In contrast, only 30.9% of the $CCR6^-$ subset expressed GraA and a low level of Per (Fig. 3). A similar finding was made for the $CD27^{low}CD28^+CD45RA^-$ memory subset. These results were confirmed in an experiment using cells from 3 different individuals (data not shown). The population expressing a low level of Per and GraA did

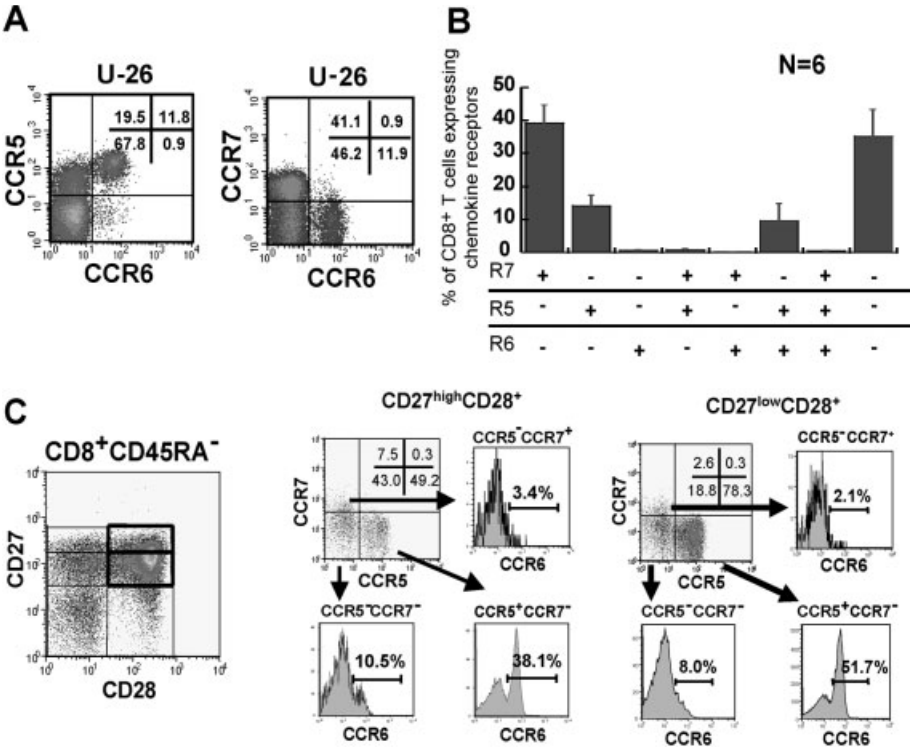

Figure 2. Co-expression of CCR7, CCR5 and CCR6 on $CD8^+$ T cells. (A) Co-expression of CCR6 and CCR5 or CCR7 on $CD8^+$ T cells. PBMC from 2 individuals were stained with PE-labeled anti-CD8, Alexa Fluor®647-labeled anti-CCR6, FITC-labeled anti-CCR5 and PE-Cy7-labeled anti-CCR7 mAb or with mouse IgG mAb as a negative control. The $CD8^+$ subset was gated and then analyzed for the expression of CCR6 and CCR5 or CCR7. The percentage of each CCR6/CCR5 or CCR6/CCR7 subset in the $CD8^+$ T cell population is shown in each plot. (B) Co-expression of CCR5, CCR7 and CCR6 on $CD8^+$ cells. The mean percentage and SD of $CD8^+$ T cells expressing different combinations of CCR6, CCR5 and CCR7 are shown. (C) Surface expression of CCR6 on CCR5/CCR7/CD27/CD28/CD45RA subsets of $CD8^+$ T cells. PBMC from 7 individuals were stained with Cascade Blue-labeled anti-CD8, ECD-labeled anti-CD45RA, allophycocyanin (APC)-Cy7-labeled anti-CD27, PE-labeled-anti-CD28, PE-Cy7-labeled anti-CCR7, FITC-labeled anti-CCR5 and Alexa Fluor®647-labeled anti-CCR6 or mouse IgG mAb as negative controls. The percentages of each CCR7/CCR5 subset in the $CD27^{high/low}CD28^+CD45RA^-$ $CD8^+$ T cell population and of $CCR6^+$ cells in each subset from a representative individual are shown in each plot.

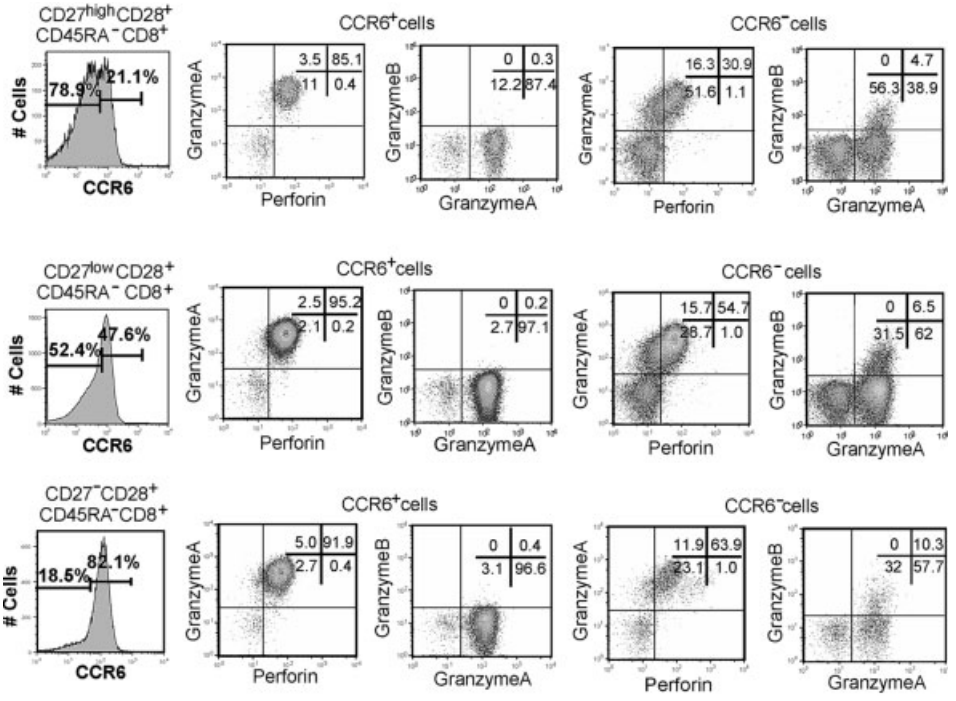

Figure 3. Differential expression of Per, GraA and GraB in CCR6$^+$ and CCR6$^-$ CD27/CD28/CD45RA subsets of CD8$^+$ T cells. CCR6$^+$ and CCR6$^-$ subsets of CD27$^{high/low/-}$CD28$^+$CD45RA$^-$CD8$^+$ T cells were analyzed for the expression of GraA and GraB or Per using FITC-labeled anti-Per and PE-labeled anti-GraA or FITC-labeled anti-GraA and PE-labeled anti-GraB mAb. The percentages of GraA/GraB and GraA/Per subsets among CCR6$^{+/-}$CD27$^{high/low/-}$CD28$^+$CD45RA$^-$ CD8$^+$ T cells are shown in each plot.

not carry GraB (Fig. 3), indicating that cells of the CCR6$^+$ subset among CD27$^+$CD28$^+$CD45RA$^-$ CD8$^+$ T cells were less differentiated than the CCR6$^-$ cells.

CCR6$^+$ CD8$^+$ T cells produce more IFN-γ and TNF-α than IL-2

To verify the difference in functional ability between the CCR6$^+$ and CCR6$^-$ subsets of CD8$^+$ T cells, we sorted the CCR6$^+$ and CCR6$^-$ subsets and then measured the amount of IFN-γ, IL-2 and TNF-α secreted from these sorted cells after having stimulated them with PMA and ionomycin. Representative data from individual U-26 are shown in Fig. 4A. Approximately 90% of the CCR6$^+$ subset produced IFN-γ and TNF-α, whereas only 30% of these cells produced IL-2. This stands in contrast to the production of these cytokines by the CCR6$^-$ subset. In three experiments using cells from 3 different individuals, a mean of 63.9% of the CCR6$^+$ subset secreted both IFN-γ and TNF-α, and 20.5% produced all three cytokines (Fig. 4B). We further compared cytokine production by CCR6$^+$ and CCR6$^-$ memory CD8$^+$ T cells to clarify functional differences. We sorted CCR6$^+$ and CCR6$^-$ subsets from CD27$^+$CD28$^+$CD45RA$^-$CD8$^+$ T cells from the same individual shown in Fig. 4A, because this individual had a sufficient number of these subsets for this study, and measured the amount of IFN-γ, IL-2 and TNF-α (Fig. 4C). Approximately 80% and 90% of the CCR6$^+$ memory subset produced IFN-γ and TNF-α, respectively, whereas 40% of these cells produced IL-2; 41.3% and 36.9% of CCR6$^+$CD27$^+$CD28$^+$CD45RA$^-$ CD8$^+$ T cells produced both IFN-γ and TNF-α and all three cytokines, respectively (Fig. 4D). In comparison, the number of CCR6$^-$CD27$^+$CD28$^+$CD45RA$^-$ CD8$^+$ T cells producing both IFN-γ and TNF-α was much lower. The same result was found in a different experiment using memory T cells from other individuals (data not shown). These findings indicate that CCR6 is expressed on two different functional memory subsets, one possessing the ability to produce IFN-γ and TNF-α and the other possessing the ability to produce IL-2, IFN-γ and TNF-α.

EBV-specific and HCMV-specific CD8$^+$ T cells lack CCR6 expression

Previous studies showed that most EBV-specific CD8$^+$ T cells express the CD27$^+$CD28$^+$CD45RA$^-$ memory phenotype in healthy individuals [24], suggesting that EBV-specific CD8$^+$ T cells may express CCR6. To investigate the CCR6, CCR5 and CCR7 expression on each CD27/CD28/CD45RA subset of EBV-specific CD8$^+$ T cells, we analyzed PBMC from healthy HLA-A*1101$^+$ or HLA-A*2402$^+$ individuals by 8-color flow cytometry with Alexa Fluor®647-labeled anti-CCR6, FITC-labeled anti-CCR5, PE-Cy7-labeled anti-CCR7, Alexa Fluor®405-

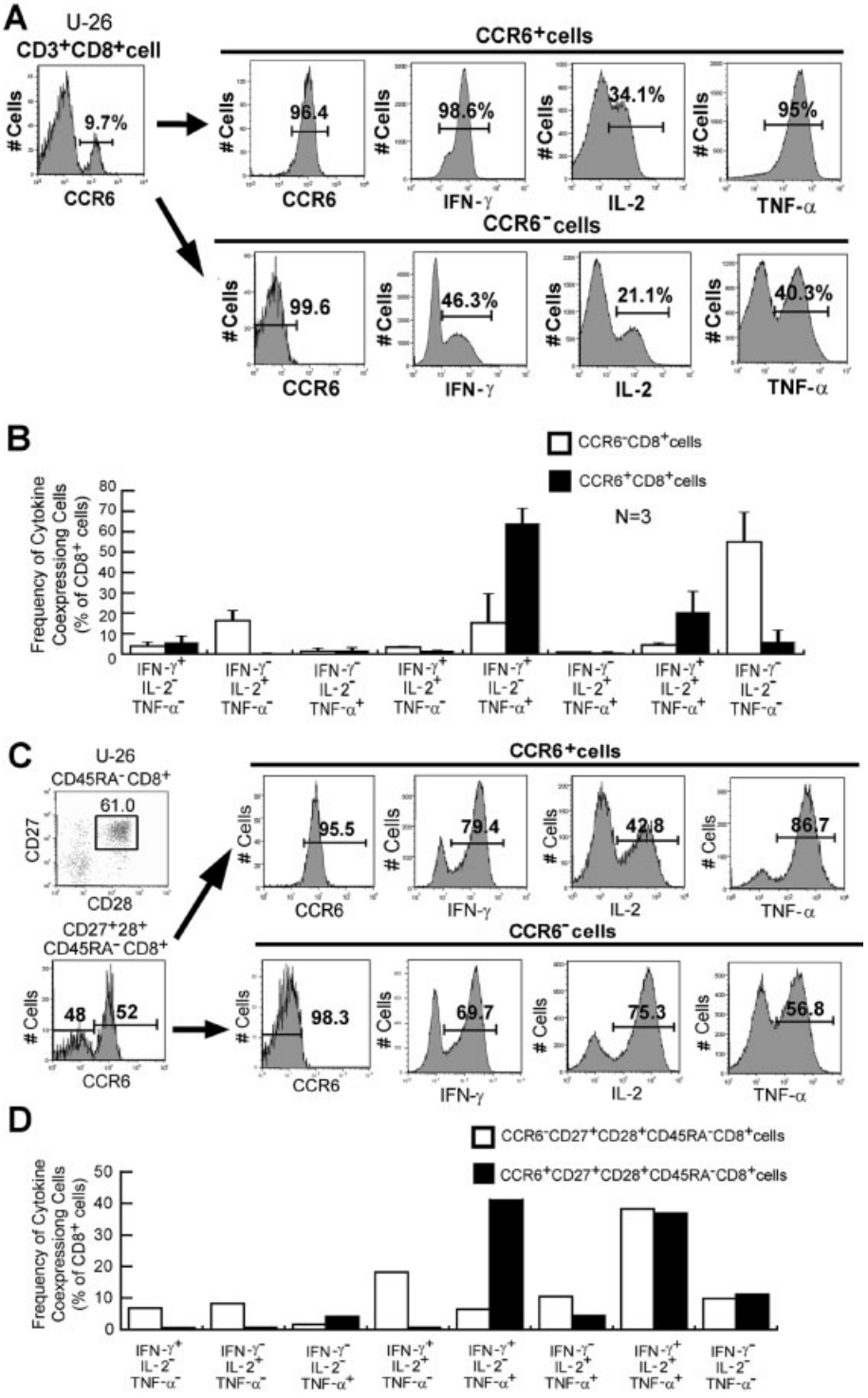

Figure 4. Cytokine production by CCR6$^+$ and CCR6$^-$ subsets of CD3$^+$CD8$^+$ T cells. (A) The potential for cytokine production by CCR6$^+$ and CCR6$^-$ CD8$^+$ T cells was investigated by measuring IFN-γ, IL-2 and TNF-α production by cells stimulated with PMA and ionomycin. IFN-γ-, IL-2- and TNF-α-producing cells were analyzed by flow cytometry using FITC-labeled anti-IFN-γ, PE-labeled anti-IL-2 and PE-Cy7-labeled anti-TNF-α mAb. Sorting purities of CCR6$^+$ CD3$^+$CD8$^+$ and CCR6$^-$ CD3$^+$CD8$^+$ T cells from individual U-26 were 96.4% and 99.6%, respectively. (B) Relative frequency of cells expressing IFN-γ, IL-2 and TNF-α in PBMC from 3 individuals. Data are expressed as the percentage of each CD8$^+$ T cell subset. The mean percentage and SD of each CD8$^+$ T cell subset producing each combination of cytokines are indicated. (C) Cytokine production by CCR6$^+$ and CCR6$^-$ memory subsets of CD8$^+$ T cells. CCR6$^+$ and CCR6$^-$ subsets of CD27$^+$CD28$^+$CD45RA$^-$ CD8$^+$ T cells from individual U-26 were purified using a cell sorter. Sorting purities of CCR6$^+$CD27$^+$CD28$^+$CD45RA$^-$ and CCR6$^-$CD27$^+$CD28$^+$CD45RA$^-$ CD8$^+$ T cells were 95.5% and 98.3%, respectively. (D) Relative frequency of cells expressing IFN-γ, IL-2 and TNF-α in CD27$^+$CD28$^+$CD45RA$^-$ CD8$^+$ subsets. Data are expressed as the percentage of each CD8$^+$ T cell subset.

labeled anti-CD45RA, allophycocyanin (APC)-Cy7-labeled anti-CD27, energy-coupled dye (ECD)-labeled anti-CD28 and Cascade Yellow-labeled anti-CD8 mAb as well as PE-labeled A*1101 or A*2402 tetramers. The results show that Epstein-Barr virus nuclear antigen 3B (EBNA3B, latent antigen)-specific $CD8^+$ T cells, which had the $CD27^{high/low}CD28^+CD45RA^-$ memory phenotype, express neither CCR6 nor CCR7 but do express CCR5 (Fig. 5A). On the other hand, only approximately 5% of EBV-BRLF1 (lytic antigen)-specific $CD8^+$ T cells expressed CCR6 (Fig. 5B). These results indicate that EBV-specific $CD8^+$ T cells predominantly have the $CCR7^-CCR5^+CCR6^-CD27^+CD28^+CD45RA^-$ phenotype.

We also analyzed the CCR6 expression on HLA-A*0201-restricted, HCMV-specific $CD8^+$ T cells from 2 healthy individuals by 8-color flow cytometry with the specific tetramer and seven mAb. The results show that HCMV-specific $CD8^+$ T cells, which had $CD27^-CD28^-CD45RA^{+/-}$ and $CD27^{low}CD28^{+/-}$ $CD45RA^{+/-}$ phenotypes, did not express CCR6 (Fig. 5C).

Migration of $CCR6^+$ $CD8^+$ T cells in response to CCL20 (MIP-3α)

To determine whether chemotaxis of $CCR6^+$ $CD8^+$ T cells can be induced by MIP-3α, we measured the chemotactic response of $CD27^+CD28^+CD45RA^-$ $CD8^+$ T cells to MIP-3α or SDF-1 using a TAXIScan holder [25]. Both $CD27^+CD28^+CD45RA^-$ $CD8^+$ T cells and $CD27^+CD28^+CD45RA^+$ $CD8^+$ T cells were sorted from the PBMC of a healthy individual, and migration of the sorted cells elicited by MIP-3α or SDF-1 was measured (Fig. 6). Among the $CD27^+CD28^+CD45RA^-$ $CD8^+$ T cells, 49.4% expressed CCR6, whereas only 0.5% of $CD27^+CD28^+CD45RA^+$ $CD8^+$ T cells expressed CCR6. As both cell populations expressed CXCR4 [14], SDF-1 was used as a positive control. The chemotaxis of $CD27^+CD28^+CD45RA^-$ $CD8^+$ T cells was induced by MIP-3α or SDF-1. In contrast, migration of $CD27^+CD28^+CD45RA^+$ $CD8^+$ T cells was induced by SDF-1 but not by MIP-3α. These results were confirmed in an experiment using cells from a different individual (data not shown). These findings indicate that $CCR6^+$ $CD8^+$ T cells expressing the $CD27^+CD28^+CD45RA^-$ $CD8^+$ phenotype show chemotactic activity in response to a CCR6 ligand.

Discussion

A previous study showed that CCR6 is expressed on a CD45RO-expressing subpopulation of human $CD4^+$ and $CD8^+$ T cells, though the frequency of $CCR6^+$ cells among $CD4^+$ T cells was much higher than that among $CD8^+$ T cells [19]. In addition, it was demonstrated that this chemokine receptor is expressed on the $CD28^+CD45RO^{high}$ subset of $CD8^+$ T cells [26]. These studies suggest that $CCR6^+CD8^+$ T cells are memory T cells. In the present study, we first analyzed the phenotype of $CCR6^+CD8^+$ T cells in detail to characterize these cells. CCR6 was predominantly expressed on human $CD8^+$ T cells with the $CD27^{low}CD28^+CD45RA^-$ memory or T_{EM} phenotype. In addition, most $CCR6^+CD8^+$ T cells expressed CCR5 but not CCR7. Previous studies revealed that CCR5 is expressed on a subset of T_{EM} $CD8^+$ T cells, while CCR7 is expressed on naive and T_{CM} $CD8^+$ T cells [4, 7, 12]. These phenotypic analyses together suggest that $CCR6^+$ $CD8^+$ T cells are T_{EM} T cells.

Cytolytic effector molecules such as Per and the serine proteases GraA and GraB are useful to identify functional subsets of human $CD8^+$ T cells [5, 6]. Previous studies showed that Per is expressed in T_{EM} and effector $CD8^+$ T cells [7]. We recently found that these molecules are expressed step-wise during differentiation from T_{EM} to effector $CD8^+$ T cells according to the following pathway: $Per^-GraA^-GraB^- \rightarrow Per^-GraA^+GraB^- \rightarrow Per^{low}GraA^+GraB^- \rightarrow Per^{low}GraA^+GraB^+ \rightarrow Per^{high}GraA^+GraB^+$ [27]. The $Per^{low}GraA^+GraB^-$ subset of $CD8^+$ T cells predominantly expressed CCR5 [27]. In the present study, we showed that $CCR6^+$ $CD8^+$ T cells in the $CD27^{low}CD28^+CD45RA^-$ subset express a low level of Per and GraA but not GraB, suggesting that the $CCR6^+$ $CD8^+$ T cells do not have the ability to kill target cells. Indeed, we recently found that $Per^{low}GraA^+GraB^-$ fail to kill target cells [27].

Previous studies demonstrated that $CD8^+$ T cells displaying the $CD45RA^+CD27^-$ effector phenotype produce IFN-γ but not IL-2 [5, 28] and that $CCR7^+CD45RA^-$ T_{EM} $CD8^+$ cells, but not $CCR7^-CD45RA^-$ T_{EM} $CD8^+$ cells, contain a large number of cells producing both IL-2 and IFN-γ [28]. These findings indicate that T_{EM} $CD8^+$ T cells possess the ability to produce IFN-γ but not IL-2. In the present study, we demonstrated that most of the $CCR6^+$ $CD8^+$ T cells secrete both IFN-γ and TNF-α but not IL-2 and that a significant number (approximately 20%) produce all three cytokines. A similar finding was found for $CCR6^+$ memory $CD8^+$ T cells, although the number of cells producing all three cytokines was greater. Thus, $CCR6^+$ $CD8^+$ T cells include two different functional subsets. Since $CCR6^+$ $CD8^+$ cells do not express CCR7, they are not T_{CM} cells. The subset with the ability to produce all three cytokines may contain less matured memory T cells than the subset possessing the ability to produce only IFN-γ and TNF-α; these $CCR6^+$ $CD8^+$ T cells might be early T_{EM} T cells, because they express a low level of Per and do not express GraB.

Many previous studies demonstrated that EBV-specific $CD8^+$ T cells in healthy individuals have the

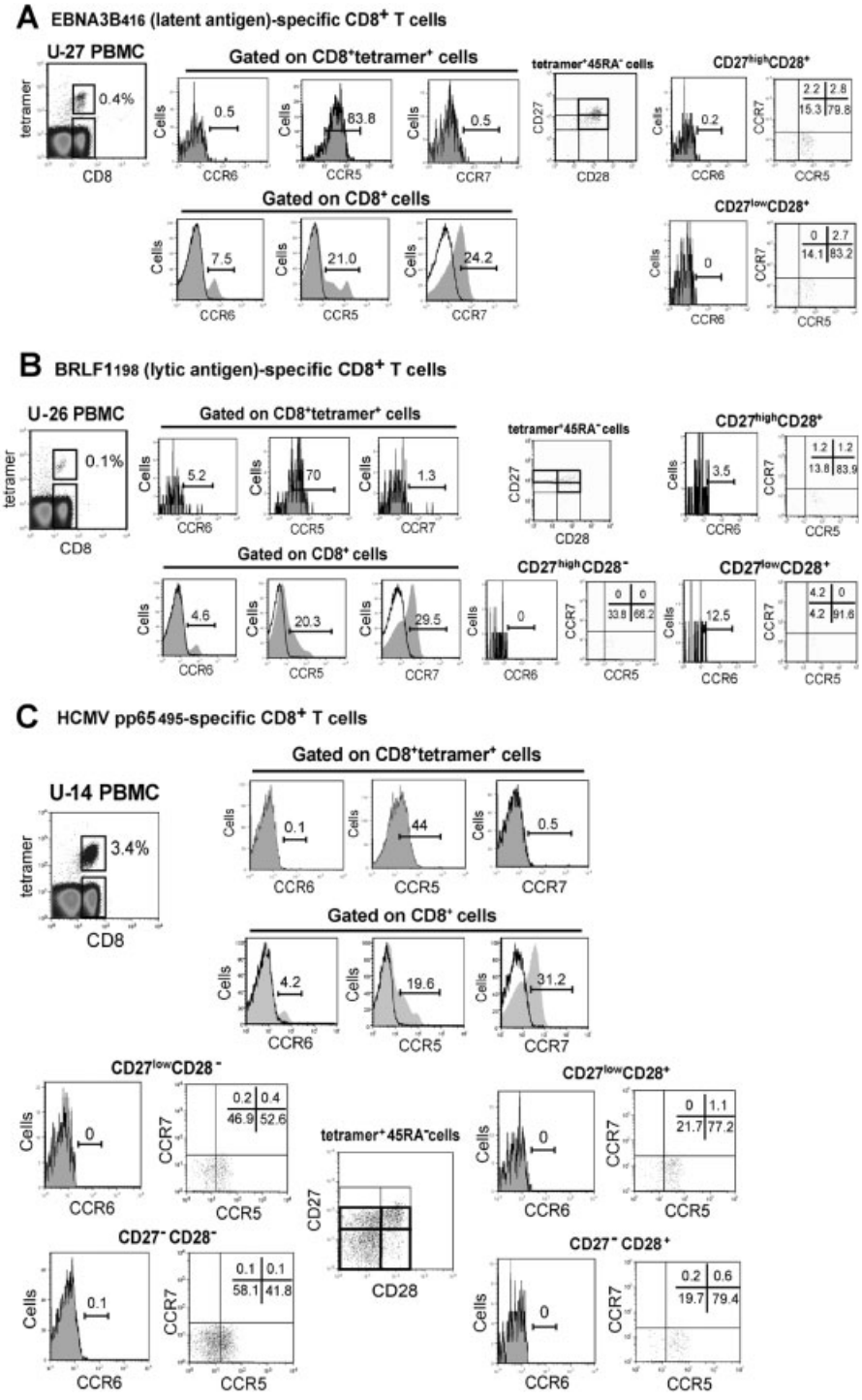

Figure 5. Surface expression of CCR6 on EBV-specific CD8$^+$ T cells. (A) Surface expression of CCR6 on EBNA3B 416-specific CD8$^+$ T cells. PBMC from individual U-27 having HLA-A*1101 were stained with Cascade Yellow-labeled anti-CD8 mAb, Alexa Fluor®405-labeled anti-CD45RA, allophycocyanin (APC)-Cy7-labeled anti-CD27, ECD-labeled anti-CD28, PE-Cy7-labeled anti-CCR7, FITC-labeled anti-CCR5 and Alexa Fluor®647-labeled anti-CCR6 mAb as well as PE-labeled HLA-A*1101-restricted EBNA3B 416-specific tetramer. The percentage of tetramer$^+$ cells in the CD8$^+$ T cell population is shown. The CCR7/CCR5/CD27/CD28/CD45RA subsets of the CD8$^+$ tetramer$^+$ subset were gated and then analyzed for the expression of CCR6. The percentage of each CCR7/CCR5 subset in CD27$^{high/low}$CD28$^+$CD45RA$^-$ CD8$^+$ tetramer$^+$ cell populations and of CCR6$^+$ cells in each subset are shown in each plot. (B) The surface expression of CCR6 on BRLF1 198-specific CD8$^+$ T cells. PBMC from individual U-26 having HLA-A*2402 were examined using the same mAb as in Fig. 5A, with the HLA-A*2402-restricted BRLF1 198-specific tetramer replacing the HLA-A*1101-restricted EBNA3B 416-specific tetramer. (C) Surface expression of CCR6 on HCMV-specific CD8$^+$ T cells. PBMC from individual U-14 having HLA-A*0201 were examined using the same mAb as in Fig. 5A, with the HLA-A*0201-restricted pp65 495-specific tetramer replacing the HLA-A*1101-restricted EBNA3B 416-specific tetramer.

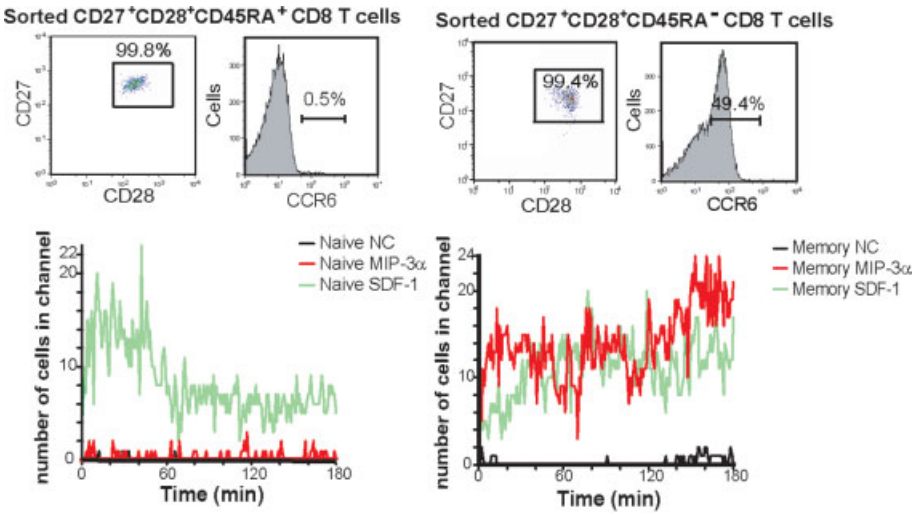

Figure 6. Migration of CD27/CD28/CD45RA subsets of $CD8^+$ T cells in response to MIP-3α. $CD8^+$ T cells isolated from healthy individual U-24 were stained with anti-CD27, anti-CD28, anti-CD45RA and anti-CCR6 mAb. $CD27^+CD28^+CD45RA^-$ and $CD27^+CD28^+CD45RA^+$ subsets were then isolated using a cell sorter. The percentage of each CD27/CD28 subset in the $CD45RA^{+/-}$ $CD8^+$ T cell population is shown in each plot. Chemotaxis of these subsets was measured using the TAXIScan (Effector Cell Institute). The migration of $CD27^+CD28^+CD45RA^-$ T cells (right panel at bottom) and $CD27^+CD28^+CD45RA^+$ T cells (left panel at bottom) induced by 100 μg/mL MIP-3α (red line), SDF-1 (green line) or 0.1% BSA/Hepes RPMI buffer (NC, black line) as a negative control is shown. Sorting purities of $CD27^+CD28^+CD45RA^-$ and $CD27^+CD28^+CD45RA^+$ T cells were 97.2% and 98.1%, respectively.

$CD27^+CD28^+CD45RA^-$ memory phenotype [4, 24]. EBV latent antigen-specific $CD8^+$ T cells express CD45RO and CD62L but not CD69 [24], whereas they express a low level of Per and no GraB and fail to kill target cells [4, 27]. These findings suggest that EBV latent antigen-specific $CD8^+$ T cells are immature memory T cells. However, the present study showed that in 5 healthy individuals, both EBV-EBNA3B- and EBV-BRLF1-specific $CD8^+$ T cells that express the $CD27^{high/low-}CD28^+CD45RA^-$ memory phenotype express neither CCR6 nor CCR7 but do express CCR5. Such findings indicate that these EBV antigen-specific $CD8^+$ T cells are not T_{CM} T cells but have characteristics of T_{EM} T cells. We found that some of EBV-BRLF1-specific $CD8^+$ T cells expressed CCR6 after they were stimulated with specific peptide (our unpublished observation). This implies that a portion of the T_{EM} $CD8^+$ T cells that express CCR6 after stimulation with antigen-presenting cells in lymph node migrate to tissues such as colon and are involved in mucosal immunity.

Several methods have been developed to measure the chemotaxis of cells *in vitro*. The most widely used methods are Boyden's chamber technique and its various modifications. In principle, these methods utilize a chamber whose upper and lower compartments are separated by a filter. Cells and a chemoattractant are induced into the upper and lower compartments, respectively. In this assay, the number of cells that migrate onto the lower surface of the filter membrane or into the lower compartment is counted, directly or indirectly. However, it is impossible to observe the cell migration directly. In addition, since Boyden's chamber requires a large number (more than 5×10^4) of cells, it is very difficult to use this method for analysis of a restricted number of cells. Because $CCR6^+$ $CD8^+$ T cells represent only 6% of the total $CD8^+$ T cell population, we used the KK-chamber in the present study, as it enables one to analyze the migration of only 100 cells [25]. This chamber also enables real-time horizontal analysis of chemokines. We measured the chemotactic activity of sorted $CCR6^+$ and $CCR6^-$ subsets of $CD27^+CD28^+CD45RA^-$ $CD8^+$ T cells using MIP-3α as a chemokine in a TAXIScan holder and definitively showed that chemotaxis of the $CCR6^+$ subset, but not the $CCR6^-$ subset, is induced using MIP-3α. Thus, this method proved useful for analysis of the migration of a restricted number of cells.

CCR6 is expressed on immature DC cultured in medium containing GM-CSF, IL-4 and TGF-β1 [29]. This receptor is down-regulated during the maturation process, while CCR7 is up-regulated [30]. Since MIP-3α is expressed in intestinal mucosa and cutaneous tissues, it is thought that CCR6 plays a critical role in lymphocyte homeostasis in the intestinal mucosa and cutaneous tissues. Indeed, studies using CCR6-deficient mice have demonstrated reduced migration of $CD4^+$ T cells into the gut mucosa [21]. A previous study showed that MIP-3α is produced in epithelial cells of normal human colon and that its production is up-regulated by inflammatory cytokines such as TNF-α and IL-1 [31]. MIP-3α production was significantly up-regulated in colonic tissues from patients with active

Crohn's disease [32]. In addition, in epithelial cells from normal human colon, some T cells expressed CCR6 [31]. MIP-3α/CCL20 is constitutively expressed at low levels in normal human skin. However, the production of MIP-3α in keratinocytes was strongly enhanced by inflammatory cytokines. The induction of MIP-3α was markedly up-regulated in chronic inflammatory skin disorders such as psoriasis and atopic dermatitis [33]. Furthermore, CCR6 mRNA was significantly increased in PBMC from psoriatic patients compared to normal PBMC. Similarly, psoriatic skin-homing T cells expressed higher levels of functional CCR6 protein on their surface in comparison to skin-homing T cells from control patients [34]. These studies suggest that MIP-3α/CCL20 indeed plays an important role in the recruitment of skin-homing $CCR6^+$ T cells and immature DC to inflamed skin. The present study demonstrated the effective migration of $CCR6^+$ $CD8^+$ T cells. Taken together, these studies suggest that $CCR6^+$ $CD8^+$ T_{EM} T cells can migrate to the intestinal mucosa and cutaneous tissues, where they are involved in T cell homeostasis as well as in immunity.

$CD8^+$ memory T cell differentiation and the lineage relationships between T_{CM} and T_{EM} T cells *in vivo* were analyzed in mice after infection with LCMV [35]. In this model, T_{EM} cells converted to T_{CM} cells, but not *vice versa*, when the cells were monitored in the absence of antigen. $CD8^+$ T_{EM} cells are memory cells that have lost their constitutive expression of CCR7 and express characteristic sets of chemokine receptors and adhesion molecules that are required for homing to inflamed tissues [11]. T_{EM} cells are characterized by rapid effector function as compared with T_{CM} cells. $CD8^+$ T_{EM} cells express Per and possess killing activity following antigenic stimulation and can also produce IFN-γ [36]. Within the tissues, T_{CM} and T_{EM} cells show characteristic patterns of distributions. T_{EM} cells are the larger population in lung, liver and gut, whereas lymph nodes and tonsils are enriched in T_{CM} cells [37]. On the other hand, there is a restricted knowledge with regard to human memory $CD8^+$ T cells. Human $CD8^+$ T_{EM} cells are proposed to be a population between T_{CM} cells, which are able to secrete IL-2, and effector T cells that possess killing activity [4–6, 38]. In addition, classification using markers such as CD27, CD28 and CD45RA, or CCR7 and CD45RA, could not precisely identify the human $CD8^+$ T_{EM} population (our unpublished observation). The present study adds a new marker for memory $CD8^+$ T cells. CCR6, together with CCR5 and CCR7, is useful for the classification of $CD27^{+/-}CD28^+CD45RA^-$ memory subsets. CCR7 discriminates T_{CM} T cells from T_{EM} T cells, whereas $CCR7^-$ T_{EM} cells include different subsets, *i.e.* $CCR5^+CCR6^+$, $CCR5^{low}CCR6^-$ and $CCR5^-CCR6^-$. We propose that $CCR6^+$ $CD8^+$ T cells are early T_{EM} cells in the periphery, since these cells were found in the $CCR5^+CCR7^-$ subsets of $CD27^+CD28^+CD45RA^-CD8^+$ T cells and expressed GraA and a low level of Per but no GraB.

In the present study, we characterized $CCR6^+$ $CD8^+$ T cells. These cells have characteristics of early T_{EM} T cells, with the ability to produce cytokines but not to kill target cells. They also have the ability to migrate in response to MIP-3α. Thus, our present results strongly suggest that these cells can migrate to tissues such as colon and are involved in mucosal immunity. Further analysis of these cells will clarify the role of $CCR6^+$ $CD8^+$ T cells in mucosal immunity as well as in various diseases such as autoimmune colitis.

Materials and methods

Blood samples

Blood samples were taken from healthy adult individuals. For analysis of EBV-specific and HCMV-specific $CD8^+$ T cells, samples were obtained from EBV-seropositive individuals with HLA-A*1101 or HLA-A*2402 and individuals with HLA-A*0201, respectively. Kumamoto University Ethical Committee approval was received for this study, including HLA DNA typing, and the informed consent of all participating subjects was obtained.

Antibodies and flow cytometric analysis

Allophycocyanin (APC)-Cy7-labeled anti-CD27, FITC/PE-labeled anti-CD28, FITC-labeled anti-CCR5, PE-Cy7-labeled anti-CCR7, Alexa Fluor®405-labeled anti-CD45RA, Alexa Fluor®647-labeled anti-CCR6, FITC-labeled anti-Per, FITC/PE-labeled anti-GraA, PE-labeled anti-GraB, PE-labeled anti-integrin β7 chain and FITC/PE-labeled anti-mouse IgG mAb were obtained from BD Biosciences (San Diego, USA). FITC-labeled anti-CD3 mAb was purchased from Dako Cytomation (Glostrup, Denmark). ECD-labeled anti-CD3, ECD-labeled anti-CD45RA, ECD-labeled anti-CD28 mAb and FITC-labeled anti-CD49d (integrin α4 chain) were obtained from Immunotech (Marseille, France). Cascade Blue-labeled/Cascade Yellow-labeled anti-CD8 mAb were prepared by conjugating Cascade Blue and Cascade Yellow (Molecular Probes, Eugene, USA) with anti-CD8 mAb OKT8.

HLA class I tetramer

HLA class I-peptide tetrameric complexes (tetramers) were synthesized as previously described [39]. Two EBV CTL epitopes (EBNA3B 416–424, IVTDFSVIK [40] and BRLF1 198–206, TYPVLEEMF [41]) and an HCMV CTL epitope (HCMV-1 pp65 495–503, NLVPMVATV [42]) were used for the refolding of HLA-A*1101 or HLA-A*2402 and HLA-A*0201 molecules, respectively. PE-labeled streptavidin was used for generation of the tetramers (Molecular Probes).

Cytokine production by CCR6$^+$ and CCR6$^-$ subsets of CD3$^+$CD8$^+$ T cells

To measure cytokine production by CCR6$^+$ and CCR6$^-$ subsets of CD3$^+$CD8$^+$ T cells, we purified CD8$^+$ T cells from PBMC using anti-CD8-coated magnetic beads (Miltenyi Biotec, Gladbach, Germany). The purified CD8$^+$ T cells (>98%) were stained with anti-CD3, anti-CD8 and anti-CCR6 mAb, and the CCR6$^+$ CD3$^+$CD8$^+$ and CCR6$^-$ CD3$^+$CD8$^+$ T cells were separated using a cell sorter (FACSAria; BD Biosciences, San Jose, CA). Sorted T cell subsets were cultured for 6 h in flat-bottomed 96-well plates with or without PMA (5 ng/mL)/ionomycin (500 ng/mL) in RPMI containing 10% fetal calf serum (R10 medium), and brefeldin A (10 μg/mL) was then added to each well. The cells were fixed with 4% paraformaldehyde at 4°C for 20 min and then permeabilized at 4°C for 20 min by treatment with 0.1% saponin containing 10% newborn calf serum (permeabilizing buffer). The cells were resuspended in the same buffer and then stained with anti-IFN-γ, anti-IL-2 and TNF-α mAb at RT for 30 min. Thereafter they were washed three times in the permeabilizing buffer at 4°C. We also used FITC-, PE- and PE-Cy7-labeled mouse IgG as isotype controls. The cells were finally resuspended in PBS containing 2% paraformaldehyde, and the cytokine profile was analyzed by LSR II flow cytometry. To measure cytokine production by CCR6$^+$ or CCR6$^-$ subsets of CD27$^+$CD28$^+$CD45RA$^-$ CD8$^+$ T cells, we performed the same assay.

Migration assay

The chemotaxis of T cells was measured using a TAXIScan holder (Effector Cell Institute, Tokyo, Japan) as previously demonstrated [25]. Chemokines used in the experiments were 100 μg/mL human recombinant MIP-3β and 100 μg/mL SDF-1α (PeproTech House, London, England). When the chemotaxis of sorted cells was examined, the glass plate was coated with RPMI/40% FCS before assembly of the KK-chamber. After 180 min, the number of sorted cells that migrated toward the middle of channel was counted using a TAXIScan analyzer (Effector Cell Institute, Tokyo, Japan).

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