Down-regulation of CXCR4 expression on human CD8$^+$ T cells during peripheral differentiation

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Multi-color flow cytometric analysis on human CD8$^+$ T cell subsets revealed that CXCR4 is predominantly expressed on CD8$^+$ T cells with the naive CD27$^+$CD28$^+$CD45RA$^+$ phenotype, and is down-regulated during differentiation into those with an effector phenotype. The down-regulation of CXCR4 expression during peripheral differentiation was supported by the fact that the expression of CXCR4 on CD8$^+$ T cells was negatively correlated with that of perforin. The analysis of CCR5, CCR7, and CXCR4 co-expression further showed that CD8$^+$ T cells expressing a high level of CXCR4 are CCR7$^+$CCR5$^-$ naive or central memory subsets, and those expressing a low level of CXCR4 were included in the CCR7$^-$CCR5$^{+/-}$ memory/effector and effector subsets. Epstein Barr virus-specific CD8$^+$ T cells, which mostly express the memory phenotype, expressed CXCR4, while human cytomegalovirus-specific CD8$^+$ T cells, which mostly express the effector phenotype, partially expressed this receptor, showing that the expression of CXCR4 is also down-regulated during differentiation of viral antigen-specific CD8$^+$ T cells. The classification of human CD8$^+$ T cells based on the expression of these chemokine receptors should prove useful for studies that clarify the differentiation of human CD8$^+$ T cells.

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1 Introduction

CD8$^+$ T cells play an important role in viral eradication through their ability to produce various factors to suppress viral replication and to kill virus-infected cells [1–3]. Effector CD8$^+$ T cells have the ability to kill target cells through perforin, granzyme, and the Fas ligands [4, 5]. On the other hand, 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 [6–8]. 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 and CD28 as well as CD45RA or CD45RO are associated with naive, memory and effector function of CD8$^+$ T cells [7, 9–15]. Indeed, effector and memory/effector CD8$^+$ T cells that were classified by the phenotypes of CD27$^-$CD28$^-$CD45RA$^{+/-}$ and CD27^{low}CD28$^-$CD45RA$^{+/-}$, respectively, possess cytotoxic activity and the ability to produce cytokines [6, 7, 14, 15].

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Chemokine receptor signaling induces functional effects such as migration, rolling, sticking, the invasion and proliferation of granulocytes, monocytes and lymphocytes as well as increasing the intracellular calcium concentration in these cells [16–20]. Chemokine receptors are also useful as surface markers to discriminate naive, memory and effector subsets in human CD8$^+$ T cells. CCR7 is expressed on naive and memory CD8$^+$ T cells as a homing receptor to secondary lymphoid tissues [7, 15, 21, 22]. CCR5 is expressed on memory and memory/effector CD8$^+$ T cells and decreases during differentiation from memory to effector CD8$^+$ T cells; CD27$^+$CD28$^+$CD45RA$^-$ → CD27$^+$CD28$^-$CD45RA$^-$ → CD27$^-$CD28$^-$CD45RA$^-$ [7, 15, 23]. A recent study demonstrated that CXCR1 is expressed on effector and effector/memory CD8$^+$ T cells with phenotypes of CD27$^-$CD28$^-$CD45RA$^{+/-}$ and CD27^{low}CD28$^-$CD45RA$^{+/-}$, respectively, and that the expression of CXCR1 correlates with that of perforin [24].

CXCR4 is known to be a co-receptor for the entry of T-tropic HIV-1 into target cells [25]. Therefore, the expression of CXCR4 has been mostly studied in CD4$^+$ T cells. It is predominantly expressed on naive subsets of CD4$^+$ T cells [17, 26]. A previous study revealed that CXCR4 is expressed in both resting and activated human

CD8$^+$ T cells, and that the responsiveness for SDF-1 is diminished according to the following order: CD45RO$^-$CD62L$^+$ > CD45RO$^+$CD62L$^{+/-}$ > CD45RO$^-$-CD62L$^-$ subsets [17], implying that CXCR4 is predominantly expressed on naive CD8$^+$ T cells. However, the expression of this receptor on human CD8$^+$ T cells has not yet been investigated sufficiently.

In the present study, the expression of CXCR4 on CD8$^+$ T cell subsets was analyzed using three surface markers, CD27, CD28 and CD45RA. In addition, we investigated the correlation of the expression of this receptor with that of perforin, CXCR1, CCR7, and CCR5, and finally analyzed the expression of CXCR4 on EBV-specific and human (H) CMV-specific CD8$^+$ T cells using HLA-class I-peptide tetrameric complexes (tetramers).

2 Results

2.1 Surface expression of CXCR4 on CD8$^+$ T cells

To analyze the expression of CXCR4 on total CD8$^+$ T cells, PBMC from healthy individuals were stained with anti-CD8, anti-CD3 and anti-CXCR4 mAb. The CXCR4 expression on CD3$^+$CD8$^+$ subsets was measured by flow cytometry. A representative result is shown in Fig. 1. Approximately 70% of CD8$^+$ T cells expressed CXCR4. The results from five healthy individuals showed that 67–85% of CD8$^+$ T cells expressed CXCR4 (data not shown), suggesting that certain CD8$^+$ T cell populations may express CXCR4.

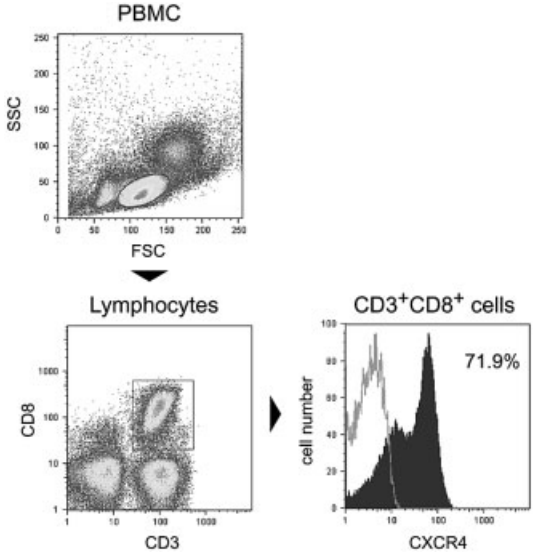

Fig. 1. Surface expression of CXCR4 on CD8$^+$ T cells. PBMC from a healthy donor, U-13, were stained with anti-CXCR4, anti-CD3 and anti-CD8 mAb. The CD3$^+$CD8$^+$ subset was gated, and then the surface expression of CXCR4 was analyzed by flow cytometry.

A recent study showed that CD8$^+$ T cells can be classified by the expression pattern of three cell surface markers, CD27, CD28 and CD45RA, as follows; naive cells: CD27$^+$CD28$^+$CD45RA$^+$, memory cells: CD27$^+$CD28$^+$-CD45RA$^-$, memory/effector cells: CD27^{low}CD28$^-$C-D45RA$^{+/-}$, and effector cells: CD27$^-$CD28$^-$CD45RA$^{+/-}$ [7]. To identify the populations expressing CXCR4, the surface CXCR4 expression on each CD27CD28CD45RA subset of CD8$^+$ T cells was investigated. CD8$^+$ T cells were isolated from eight healthy individuals, and then expression of CXCR4 was analyzed by four-color flow cytometric analysis with anti-CXCR4, anti-CD45RA, anti-CD27, and anti-CD28 mAb. A representative result is shown in Fig. 2A. The CXCR4$^+$ cells were predominantly found in the CD27$^+$CD28$^+$CD45RA$^+$ subset (>90%). The frequency of CXCR4$^+$ cells decreased according to the following order: CD27$^+$CD28$^+$CD45RA$^-$ → CD27^{low}-CD28$^-$CD45RA$^{+/-}$ → CD27$^-$CD28$^-$CD45RA$^{+/-}$ subsets. This result was confirmed by analysis of eight healthy individuals (Fig. 2B). These results suggest that CXCR4 is highly expressed on the naive subsets and that its surface expression is down-regulated during differentiation from memory to effector subsets.

2.2 Correlation between the expression of CXCR4 and perforin or CXCR1 in CD8$^+$ T cells

It is known that perforin is expressed in effector and memory/effector CD8$^+$ T cells [6, 14, 15]. Since CXCR4$^+$ cells were predominantly detected in CD8$^+$ T cells with naive and memory phenotypes, the assumption is that the expression of CXCR4 is negatively correlated with that of perforin. We investigated the correlation between the expression of CXCR4 and perforin on CD8$^+$ T cells from four healthy individuals. CD8$^+$ T cells were classified into three groups; CXCR4^{high}perforin$^-$, CXCR4^{low}perforin^{low}, and CXCR4$^-$perforin^{high} (Fig. 3A). These data indicate that the expression of CXCR4 is indeed negatively correlated with that of that of perforin.

Our recent study demonstrated that the surface expression of CXCR1 is positively correlated with that of perforin [24]. These findings suggest that the expression of CXCR4 correlates negatively with the expression of CXCR1. We therefore investigated the correlation between the CXCR4 and CXCR1 expressions on CD8$^+$ T cells from four healthy individuals (Fig. 3B). Most CXCR1$^-$CD8$^+$ T cells expressed a higher level of CXCR4, while a small population of CXCR1$^-$CD8$^+$ T cells expressed a lower level of or no CXCR4. In contrast,

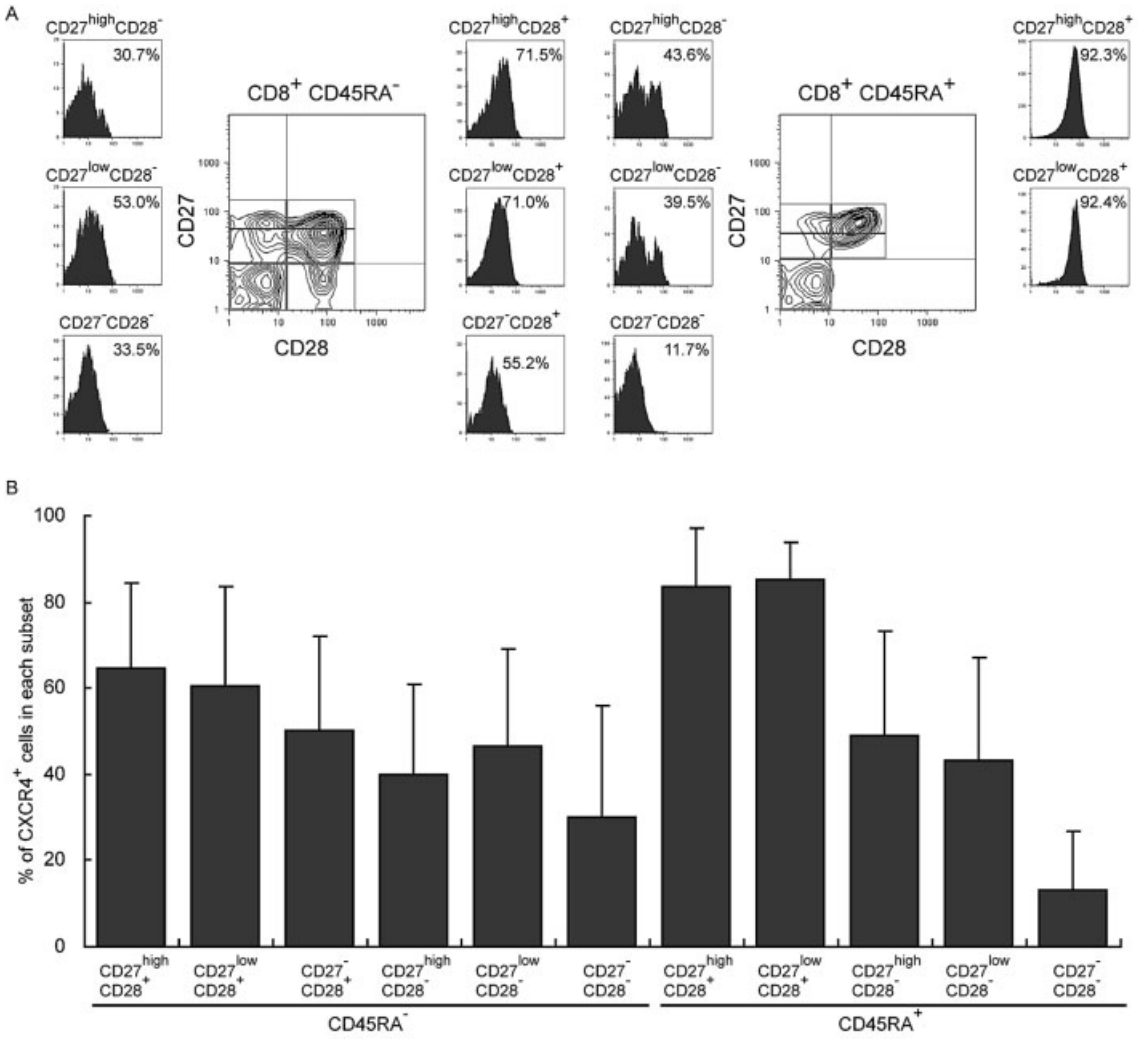

Fig. 2. Surface expression of CXCR4 on CD27CD28CD45RA subsets of $CD8^+$ T cells. (A) The frequency of $CXCR4^+$ cells in each CD27CD28CD45RA subset of $CD8^+$ T cells. $CD8^+$ T cells were isolated from one individual, U-13, and then stained with anti-CD27, anti-CD28, anti-CD45RA and anti-CXCR4 mAb. CD27CD28CD45RA subsets were gated and then the expression of CXCR4 on each subset was analyzed. The percentage of $CXCR4^+$ cells in each subset is shown in each plot. (B) The frequency of $CXCR4^+$ cells in each CD27CD28CD45RA subset of $CD8^+$ T cells from eight individuals. $CD8^+$ T cells were isolated from eight individuals and then stained with anti-CD27, anti-CD28, anti-CD45RA and anti-CXCR4 mAb. The mean percentage and SD of $CXCR4^+$ cells in each subset are shown.

$CXCR1^+CD8^+$ T cells expressed a low level of, or no, CXCR4. These findings indicate a reduced expression of CXCR4 on $CXCR1^+CD8^+$ T cells, but a considerable number of $CD8^+$ T cells lose both receptors.

2.3 The correlation between the expression of CXCR4 and CCR7 or CCR5 on $CD8^+$ T cells

CCR7 is expressed on naive $CD8^+$ T cells and a portion of memory $CD8^+$ T cells [7, 15, 21, 22], while CCR5 is predominantly expressed on memory $CD8^+$ T cells and a some effector $CD8^+$ T cells [15, 23, 27]. These observations suggest that the expression of CXCR4 is positively correlated with that of CCR7, but is not correlated with that of CCR5. To clarify the correlation of expression between CXCR4 and these two receptors, we investigated the co-expression of CXCR4 and CCR7 or CCR5 on $CD8^+$ T cells from healthy individuals. $CCR7^+CD8^+$ T cells expressed a high level of CXCR4 while $CCR7^-CD8^+$ T cells include both populations that express a low level of CXCR4 and no CXCR4 (Fig. 4A). These results show that CXCR4 is highly expressed on $CCR7^+CD8^+$ T cells, including naive and central memory $CD8^+$ T cells. On the other hand, analysis of $CD8^+$ T cells using anti-CXCR4 and CCR5 mAb showed that they

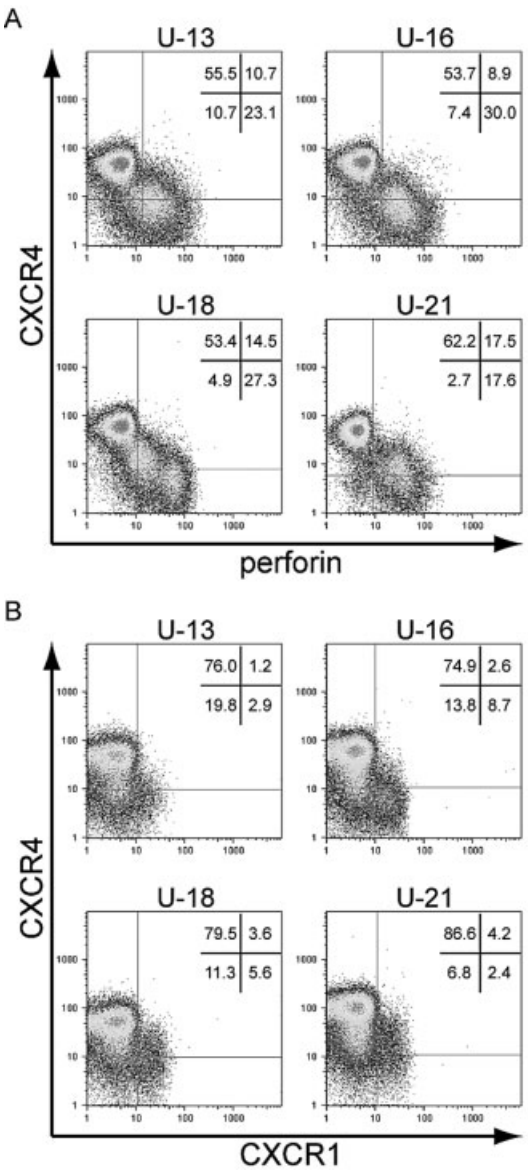

Fig. 3. Expression of perforin or CXCR1 on/in CXCR4$^{+/-}$ CD8$^+$ T cells. (A) PBMC from four individuals were stained with anti-CD8, anti-CD3, anti-perforin and anti-CXCR4 mAb, or mouse IgG mAb as an isotype control. The CD3$^+$CD8$^+$ subset was gated and then analyzed for the expression of CXCR4 and perforin. The percentage of each CXCR4 perforin subset in CD3$^+$CD8$^+$ cells is shown in each plot. (B) PBMC from four individuals were stained with anti-CD8, anti-CD3, anti-CXCR1 and anti-CXCR4 mAb, or mouse IgG mAb as isotype controls. The CD3$^+$CD8$^+$ subset was gated and then analyzed for the expression of CXCR4 and CXCR1. The percentage of each CXCR4CXCR1 subset in CD3$^+$CD8$^+$ cells is shown in each plot.

include three populations, CCR5$^-$CXCR4^{high}, CCR5$^+$-CXCR4$^{low/-}$, and CCR5$^{low/-}$CXCR4$^-$ (Fig. 4B). We hypothesized that the CCR5$^-$CXCR4^{high} population includes CCR7$^+$ naive and central memory CD8$^+$ T cells, because they express high level of CXCR4 but not CCR5 [7, 15, 27], and that other CCR5CXCR4 populations include both memory/effector and effector T cells. To investigate these CCR7/CXCR4 and CCR5/CXCR4 populations in detail, we directly analyzed the expression of these three receptors on CD8$^+$ T cells from the same individual using three mAb specific for CXCR4, CCR5, and CCR7. A representative result is shown in Fig. 4C. CD8$^+$ T cells were found to include five populations; CCR7$^+$CCR5$^-$CXCR4^{high}, CCR7$^-$CCR5$^+$CXCR4^{low}, CCR7$^-$CCR5$^+$CXCR4$^-$, CCR7$^-$CCR5$^-$CXCR4^{low}, and CCR7$^-$CCR5$^-$CXCR4$^-$. CCR7$^+$CCR5$^-$CXCR4^{high} is the dominant population, and is found in half of total CD8$^+$ T cells, while each other population is found in approximately only 10% of total CD8$^+$ T cells. The same results were found in CD8$^+$ T cells from three other individuals (data not shown). The CCR7$^+$CCR5$^-$CXCR4^{high} population includes naive and a portion of memory cells, while the other populations were found to include the following populations; a CCR7$^-$CCR5$^+$CXCR4^{low} population that includes memory and memory/effector cells, and CCR7$^-$CCR5$^+$CXCR4$^-$, CCR7$^-$CCR5$^-$CXCR4^{low}, and CCR7$^-$CCR5$^-$CXCR4$^-$ populations that include memory/effector and effector cells.

2.4 Surface expression of CXCR4 on EBV-specific and HCMV-specific CD8$^+$ T cells

The expression of CXCR4 on EBV- and HCMV-specific CD8$^+$ T cells was investigated. Most EBV-specific CD8$^+$ T cells express the CD27$^+$CD28$^+$CD45RA$^-$ memory phenotype [7, 28–30], while HCMV-specific CD8$^+$ T cells have CD27$^-$CD28$^-$CD45RA$^{+/-}$ effector phenotype or CD27^{low}CD28$^-$CD45RA$^{+/-}$ memory/effector phenotype [11, 15, 30]. Therefore, it is hypothesized that the former cells for the most part express CXCR4, while the latter do in part. We examined the CXCR4 expression on EBV-specific and HCMV-specific CD8$^+$ T cells using HLA-A*1101- and HLA-A*0206-tetramers, respectively. PBMC from HLA-A*0206$^+$ or HLA-A*1101$^+$ healthy individuals were stained with the combination of anti-CD8, anti-CXCR4 mAb and the tetramer, and with that of anti-CD8, anti-CD27, anti-CD28, anti-CD45RA mAb and the tetramer. All EBV-specific CD8$^+$ T cells, which mostly have the CD27$^+$CD28$^+$CD45RA$^-$ phenotype, expressed CXCR4 (Fig. 5A). On the other hand, approximately 35–43% of HCMV-specific CD8$^+$ T cells did not express CXCR4 (Fig. 5B). Approximately 40–50% of HCMV-specific CD8$^+$ T cells displayed a CD27$^-$CD28$^-$CD45RA$^{+/-}$ effector phenotype, which is in agreement with the percentage of CXCR4-negative cells in the HCMV-specific CD8$^+$ T cells.

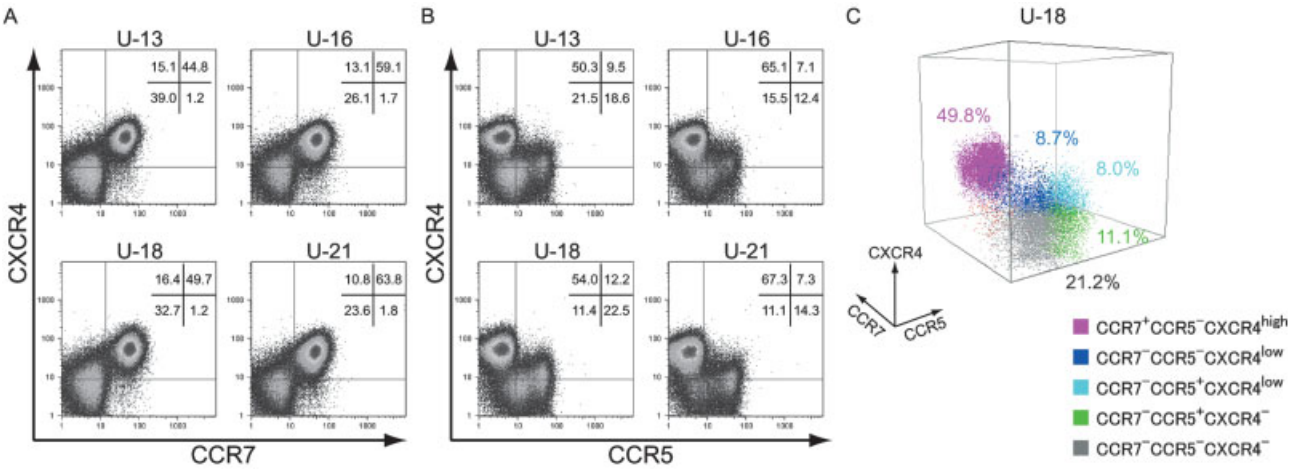

Fig. 4. Surface expression of CCR7 or CCR5 on CXCR4$^{+/-}$ CD8$^+$ T cells. (A) PBMC from four individuals (the same individuals analyzed in Fig. 3) were stained with anti-CD8, anti-CCR7 and anti-CXCR4 mAb, or mouse IgG mAb as negative control. The CD8$^+$ subset was gated and then analyzed for the expression of CXCR4 and CCR7. The percentage of each CXCR4CCR7 subset in CD8$^+$ cells is shown in each plot. (B) PBMC from the same four individuals were stained with anti-CD8, anti-CCR5 and anti-CXCR4 mAb, or mouse IgG mAb as negative control. The CD8$^+$ subset was gated and then analyzed for the expression of CXCR4 and CCR5. The percentage of each CXCR4CCR5 subset in CD8$^+$ cells is shown in each plot. (C) The co-expression of CXCR4, CCR5 and CCR7 on CD8$^+$ cells was examined using anti-CD8, anti-CCR5, anti-CCR7 and anti-CXCR4 mAb, or mouse IgG mAb as negative control. The CD8$^+$ subset was gated and then analyzed for the expression of CXCR4, CCR5 and CCR7 on CD8$^+$ cells using Paint-A-Gate PRO^{TM} software. The percentage of each subset in CD8$^+$ cells is shown.

3 Discussion

The present study demonstrated that CXCR4 is predominantly expressed on CD8$^+$ T cells of the naive and memory CD27CD28CD45RA phenotypes, while its expression is down-regulated during differentiation from CD8$^+$ T cells with the memory phenotype to those with the effector phenotype. In addition, the expression of CXCR4 was negatively correlated with that of perforin. These results indicate that the expression of CXCR4 is down-regulated during the differentiation to effector CD8$^+$ T cells. However, the precise functional role of this receptor on naive and memory CD8$^+$ T cells still remains unknown. A recent study demonstrated that central memory CD8$^+$ T cells in *plt/plt* mice that do not express CCR7 ligands in secondary lymphoid organs have the ability of rolling and sticking in the high endothelial venules (HEVs) of subiliac lymph nodes, and this sticking could be blocked by an anti-CXCL12 mAb, suggesting that CXCR4 on central memory CD8$^+$ T cells is involved in at least some of these functions [31]. CD45RO$^-$CD62L$^+$ naive and CD45RO$^+$CD62L$^{+/-}$ memory CD8$^+$ T cell subsets showed increased calcium flux in response to SDF-1 after stimulation with anti-CD3 antibodies, but unstiumlated CD8$^+$ T cells including these subsets did not respond to SDF-1 [17], suggesting that CXCR4 may effectively function on memory and memory/effector CD8$^+$ T cells expressing CXCR4 at the moment or just after these cells recognize antigens. Thus, these studies provide some evidence concerning the role of CXCR4 on CD8$^+$ T cells expressing this receptor. However, the precise functional role of CXCR4 on CD8$^+$ T cells still remains unclear.

The analysis using three chemokine receptors, CCR5, CCR7, and CXCR4, makes it evident that CD8$^+$ T cells consist of at least five populations: CCR7$^+$CCR5$^-$CXCR4^{high}, CCR7$^-$CCR5$^+$CXCR4^{low}, CCR7$^-$CCR5$^+$CXCR4$^-$, CCR7$^-$CCR5$^-$CXCR4^{low}, and CCR7$^-$CCR5$^-$CXCR4$^-$. CCR7$^+$CCR5$^-$CD8$^+$ T cells, which are naive and central memory cells, express a high level of CXCR4. CD27$^+$CD28$^+$CD45RA$^+$ naive T cells are CCR7$^+$CCR5$^-$, while CD27$^+$CD28$^+$CD45RA$^-$ memory T cells include three CCR7CCR5 populations, CCR7$^+$CCR5$^-$, CCR7$^-$CCR5$^+$, and CCR7$^-$CCR5$^-$ (our unpublished observations). Since CD27$^+$CD28$^+$CD45RA$^-$ memory T cells with CCR7$^+$CCR5$^-$ are thought to be less mature cells than those with other CCR7CCR5 phenotypes, it is likely that CCR7$^+$CCR5$^-$CD8$^+$ T cells are naive and central memory cells. Thus, the finding that CCR7$^+$CCR5$^-$CD8$^+$ T cells express a high level of CXCR4 indicates that CXCR4 is highly expressed on naive and central memory CD8$^+$ T cells. CCR7$^-$CD8$^+$ T cells are mature memory (memory/effector) or effector cells. In addition, CCR5 is dominantly expressed on memory CD8$^+$ T cells with the phenotype CD27$^+$CD28$^+$CD45RA$^-$, and its expression is down-regulated during the maturation into effector cells [7, 15, 27]. These findings suggest

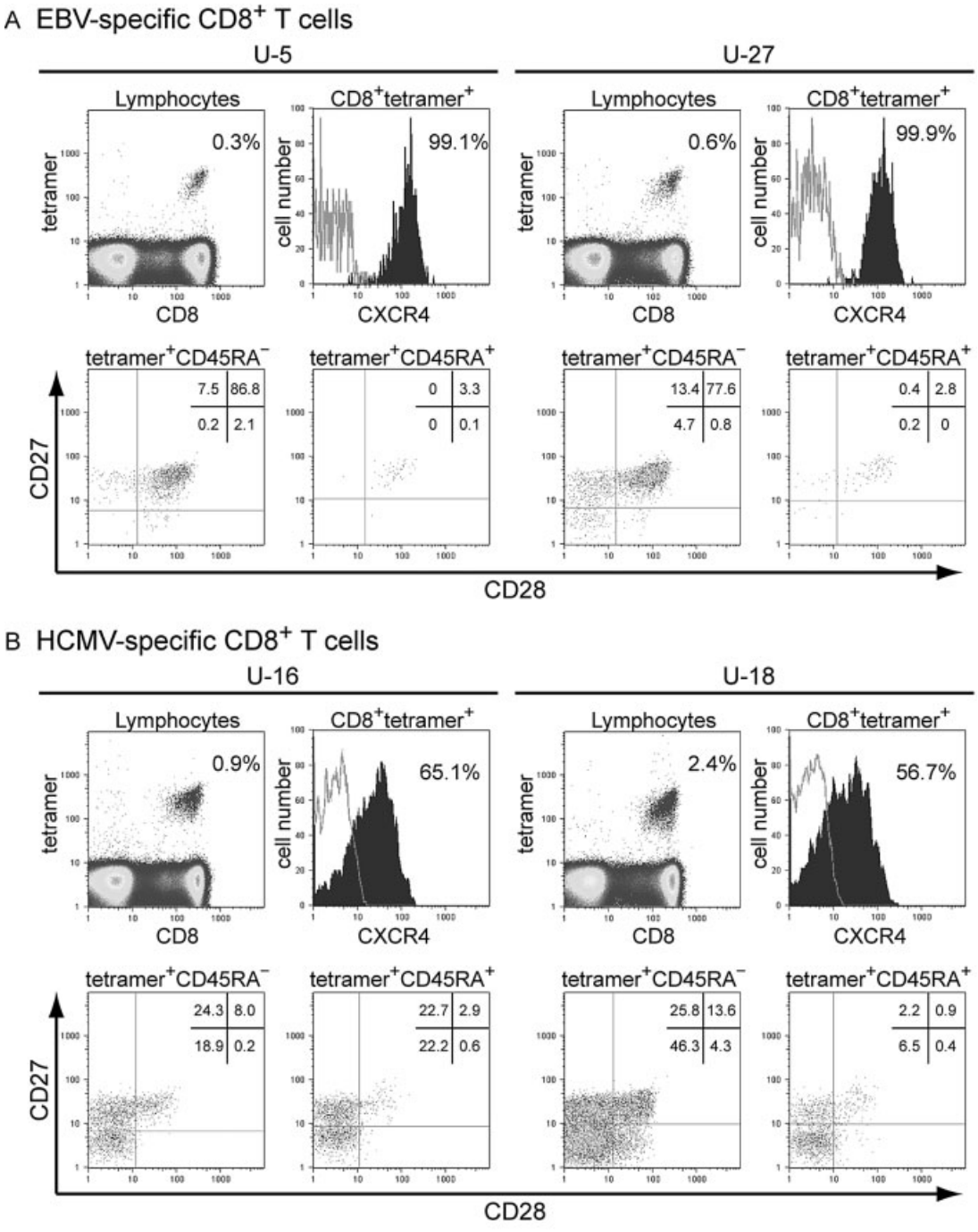

Fig. 5. Surface expression of CXCR4 on EBV-specific and HCMV-specific CD8$^+$ T cells. (A) The surface expression of CXCR4 on EBV-specific CD8$^+$ T cells. PBMC from two individuals (U-5 and U-27) with HLA-A*1101 were stained with anti-CD8 mAb, anti-CXCR4 mAb and the HLA-A*1101 tetramer. CD8$^+$ tetramer$^+$ cells were gated and then analyzed for the expression of CXCR4. The percentage of tetramer$^+$ subsets in CD8$^+$ T cells were shown. CD8$^+$ T cells from the same individuals were also stained with anti-CD27, anti-CD28, anti-CD45RA and anti-CXCR4 mAb, and with the HLA-A*1101 tetramer. The expression of CD27, CD28 and CD45RA on CD8$^+$ tetramer$^+$ cells from each individual is shown in each plot. (B) Surface expression of CXCR4 on HCMV-specific CD8$^+$ T cells. PBMC from two individuals (U-16 and U-18) with HLA-A*0206 were stained with anti-CD8 mAb, anti-CXCR4 mAb and the HLA-A*0206 tetramer. CD8$^+$ tetramer$^+$ cells were gated and then analyzed for the expression of CXCR4. The percentage of tetramer$^+$ subsets in CD8$^+$ T cells is shown. CD8$^+$ T cells from the same individuals were also stained with anti-CD27, anti-CD28, anti-CD45RA and anti-CXCR4 mAb, and with the HLA-A*0206 tetramer. The expression of CD27, CD28 and CD45RA on the CD8$^+$ tetramer$^+$ cells from each individual is shown in each plot.

that the CCR7$^-$CCR5$^+$CXCR4^{low} population may be more immature cells than the other three populations, CCR7$^-$CCR5$^+$CXCR4$^-$, CCR7$^-$CCR5$^-$CXCR4^{low}, and CCR7$^-$CCR5$^-$CXCR4$^-$. However, the specific maturation/differentiation status of these three populations remains unclear. Further analysis of these three receptors on CD27CD28CD45RA subsets using multi-color flow cytometric analysis should enable a better characterization of the CCR7$^-$CCR5$^+$CXCR4$^-$, CCR7$^-$CCR5$^-$CXCR4^{low}, and CCR7$^-$CCR5$^-$CXCR4$^-$ subsets. The expression of the chemokine receptors on human CD8$^+$ T cells is summarized in Fig. 6.

Previous studies have reported that EBV-specific CD8$^+$ T cells, which are unable to kill target cells, have a memory phenotype (CD27$^+$CD28$^+$CD45RA$^-$) and that HCMV-specific CD8$^+$ T cells, which do have cytotoxic activity, have effector and memory/effector phenotypes (CD27$^-$CD28$^-$CD45RA$^{+/-}$ and CD27^{low}CD28$^-$CD45RA$^{+/-}$, respectively). The present study shows that EBV-specific CD8$^+$ T cells for the most part express CXCR4, while its expression decreased on HCMV-specific CD8$^+$ T cells (Fig. 5A, B). These findings indicate that surface CXCR4 is down-regulated during the maturation of virus-specific CD8$^+$ T cells.

In the present study, it has been shown that CXCR4 is highly expressed on naive and central memory CD8$^+$ T cells, and its expression is down-regulated during the maturation into effector cells. The expression pattern of this receptor is useful for functional classification of human CD8$^+$ T cells in conjunction with other chemokine receptors. The classification of human CD8$^+$ T cell subsets using the expression pattern of these chemokine receptors will contribute to the effort to clarify the steps in the differentiation pathway of human peripheral CD8$^+$ T cells.

4 Materials and methods

4.1 Blood samples

Blood samples were taken from healthy adult individuals. For analysis of HCMV-specific and EBV-specific CD8$^+$ T cells, samples were obtained from HCMV-seropositive adult individuals with HLA-A*0206 and EBV-seropositive adult individuals with HLA-A*1101, respectively.

4.2 Antibodies

Anti-CD27 FITC-labeled mAb, anti-CXCR4 PE-labeled mAb, anti-CD28 APC-labeled mAb, anti-CD8 PerCP-labeled mAb, anti-CD3 PerCP-labeled mAb, anti-CXCR1 APC-labeled mAb, anti-CD28 APC-labeled mAb, anti-CCR5 FITC-labeled mAb, anti-CCR7 PE-Cy7-labeled mAb, anti-mouse-IgG FITC- and PE-labeled mAb and anti-perforin FITC-labeled mAb were obtained from BD Biosciences (San Diego, CA). Anti-CD45RA ECD-labeled mAb was purchased from Immunotech (Marseille, France). Anti-CD8 Cascade Blue-labeled mAb was made by conjugating Cascade Blue (Molecular Probes, Eugene, OR) with anti-CD8 mAb OKT8.

4.3 HLA-class I tetramer

HLA-class I-peptide tetrameric complexes (tetramer) were synthesized as previously described [15]. The HCMV CTL epitope (HCMV-1 pp65 495-503: NLVPMVATV) and the EBV CTL epitope (EBNA3B 416-424: IVTDFSVIK) were used for

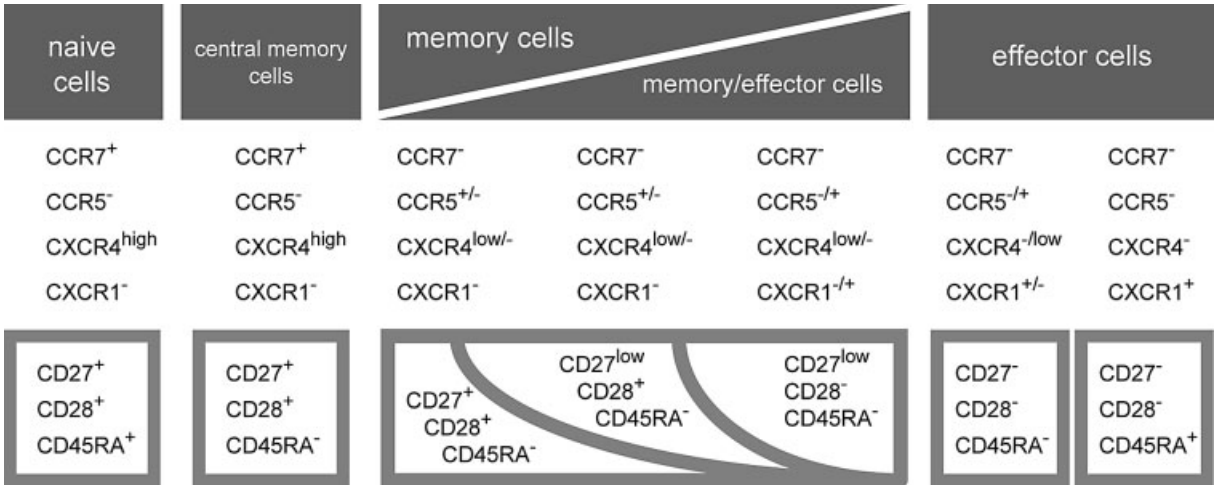

Fig. 6. Summary of the expression of chemokine receptors on human CD8$^+$ T cells.

the refolding of HLA-A*0206 and HLA-A*1101 molecules, respectively. PE-labeled streptavidin (Molecular Probes) was used for generation of the tetramers.

4.4 Flow cytometric analysis

PBMC from healthy individuals were stained with anti-CD3 and anti-CD8 mAb for 30 min at 4°C and were then washed twice with PBS containing 10% newborn calf serum (PBS/10% NCS). The cells were pre-treated with 1 μg human IgG per 1×10^5 cells for 15 min at room temperature, and then stained with anti-CXCR4 mAb for 20 min at room temperature. After washing twice with PBS/10% NCS, the percentage of CXCR4$^+$CD8$^+$ T cells in the total CD8$^+$ T cells was measured using a FACSCalibur (BD Biosciences).

To investigate the CXCR4 expression in each CD27CD28CD45RA subset of total CD8$^+$ T cells, we purified CD8$^+$ T cells from PBMC using anti-CD8-coated magnetic beads (Miltenyi Biotec, Gladbach, Germany). Purified CD8$^+$ T cells (>98%) were stained with anti-CD27, anti-CD28 and anti-CD45RA mAb at 4°C for 30 min, and were then washed twice with PBS/10% NCS. The blocking of the Fc receptor and staining of surface CXCR4 were carried out as described above. The percentage of CXCR4$^+$ cells in each subset was measured using a FACSCalibur.

To examine intracellular perforin expression in CXCR4$^+$ and CXCR4$^-$ subsets of total CD8$^+$ T cells, we stained PBMC with anti-CD8 and anti-CXCR4 mAb and then fixed them with 4% paraformaldehyde at 4°C for 20 min. The cells were permeabilized at 4°C for 10 min with PBS containing with 0.1% saponin and 20% NCS (permeabilizing buffer). The cells were washed with the permeabilizing buffer and then resuspended in 50 μl of the same buffer. After staining the cells with anti-perforin mAb at 4°C for 30 min, they were washed three times in the permeabilizing buffer at 4°C. FITC- and PE-labeled mouse IgG was used as a negative control.

To determine CXCR1 expression on CXCR4$^+$ and CXCR4$^-$ subsets of total CD8$^+$ T cells, we stained PBMC with anti-CXCR1 and anti-CD8 mAb and then blocked the Fc receptors as described above. The cells were then stained with anti-CXCR4 mAb for 20 min at room temperature.

To determine CCR7 and CCR5 expression on CXCR4$^+$ and CXCR4$^-$ subsets of total CD8$^+$ T cells, we stained PBMC with anti-CCR5 and anti-CD8 mAb and then blocked the Fc receptors as described above. The cells were then stained with anti-CCR7 mAb for 30 min at room temperature. After washing twice with PBS/10% NCS, the cells were stained with anti-CXCR4 mAb for 20 min at room temperature.

To clarify the expression of CXCR4 on HCMV-specific and EBV-specific CD8$^+$ T cells, PBMC were incubated with HCMV-A*0206 or EBV-A*1101 tetramers at 37°C for 30 min. The cells were washed twice with RPMI/10% NCS and then stained with anti-CD8 and anti-CXCR4 mAb following blocking of the Fc receptor, or anti-CD27, anti-CD28, anti-CD45RA and anti-CD8 mAb were added to the cell suspension. The cells were incubated at 4°C for 30 min, and were then washed twice with PBS/10% NCS.

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