Functional and phenotypic analysis of human memory $CD8^+$ T cells expressing CXCR3

Naoki Kobayashi,*,† Takaaki Kondo,* Hiroshi Takata,* Shumpei Yokota,† and Masafumi Takiguchi*,[1]

*Division of Viral Immunology, Center for AIDS Research, Kumamoto University, Japan; and †Department of Pediatrics, Yokohama City University, Japan

Abstract: Several chemokine receptors play an important role in the migration of naïve, memory, and effector T cells. Flow cytometric analyses showed that human $CD8^+$ T cells with naïve ($CD27^+CD28^+CD45RA^+$) or memory ($CD27^+CD28^{+/-}CD45RA^+$) phenotypes included a population expressing a high level of CXC chemokine receptor 3 ($CXCR3^{high}$) and one expressing a low level of it ($CXCR3^{low}$), but those with the effector phenotype ($CD27^-CD28^-CD45RA^{+/-}$) included a population that did not express CXCR3 ($CXCR3^-$) and a $CXCR3^{low}$ population. This relation between the expression level of CXCR3 and memory/effector phenotypes also applied to Epstein-Barr virus- or human cytomegalovirus-specific $CD8^+$ T cells. $CXCR3^{high}$ cells were found predominantly in CC chemokine receptor 7 $(CCR7)^+CCR5^-$ and $CCR7^-CCR5^-$ subsets of $CD8^+$ T cells with the $CD27^+CD28^+CD45RA^-$ memory phenotype, suggesting that they are memory cells with intermediate differentiation. Indeed, $CXCR3^{high}CD27^+CD28^+CD45RA^-$-$CD8^+$ T cells had the ability to produce interleukin-2 and interferon-γ. These results together indicate that the expression of CXCR3 is up-regulated on intermediately differentiated memory $CD8^+$ T cells. $CXCR3^{high}CD8^+$ T cells had a greater ability to migrate in response to CXCR3 ligands than $CXCR3^{low}$ ones. As $CXCR3^{high}$ memory $CD8^+$ T cells do not express CCR5, high expression of CXCR3 on these memory $CD8^+$ T cells might play an important role in the migration of these cells to inflammatory sites and in their differentiation. *J. Leukoc. Biol.* 80: 320–329; 2006.

Key Words: *chemokine receptor · differentiation · cytokine*

INTRODUCTION

Phenotypic classification of functional $CD8^+$ T cells, such as naïve, memory, and effector T cells, is useful in various T cell studies using mouse or human cells. In humans, the particular expression patterns of costimulatory molecules CD27 and CD28 as well as CD45RA or CD45RO are associated with naïve, memory, and effector functions of $CD8^+$ T cells [1–7]. A recent study examining the expression of CD27, CD28, and CD45RA demonstrated that $CD8^+$ T cells expressing the phenotypes of $CD27^-CD28^-CD45RA^{+/-}$ and $CD27^{low}CD28^-$-$CD45RA^{+/-}$ could be classified into effector and memory/effector cells, respectively, and naïve and memory $CD8^+$ T cells are phenotypically $CD27^+CD28^+CD45RA^+$ and $CD27^+$-$CD28^+CD45RA^-$, respectively [8].

Chemokine receptors are also useful to discriminate functional subsets among human $CD8^+$ T cells. CC chemokine receptor 7 (CCR7) is expressed on naïve $CD8^+$ T cells as well as on undifferentiated memory $CD8^+$ T cells, called central memory T cells, although it also plays an important role as a receptor for homing to secondary lymphoid tissues [1, 2, 9, 10]. CCR5 is expressed predominantly on memory and memory/effector $CD8^+$ T cells, and its expression decreases during differentiation from memory to effector $CD8^+$ T cells, i.e., $CD27^+CD28^+CD45RA^- \rightarrow CD27^{low}$-$CD28^-CD45RA^- \rightarrow CD27^-CD28^-CD45RA^-$ [1, 2, 8, 11]. A recent study demonstrated that CXC chemokine receptor 1 (CXCR1) was 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 was correlated with that of perforin [12]. CX3CR1 is another marker of effector $CD8^+$ T cells [13], and it was recently shown that CXCR4 is expressed predominantly on $CD8^+$ T cells with the naïve $CD27^+CD28^+CD45RA^+$ phenotype and that this receptor is down-regulated during differentiation from $CD8^+$ T cells with a memory phenotype to those with an effector phenotype [14].

The chemokine receptor CXCR3 is reported to be expressed on a subset of B and T cells [15–19]. CXCR3-mediated chemotaxis of these cells in response to its ligands, i.e., CXC chemokine ligand 9 (CXCL9)/monokine induced by interferon-γ (IFN-γ; Mig), CXCL10/IFN-γ-inducible protein 10 (IP-10), and the CXCL11/IFN-γ-inducible T cell chemokine (I-TAC) [20, 21]. The expression of CXCR3 on $CD4^+$ and $CD8^+$ T cells is enhanced strongly when the cells are activated [22, 23]. In human $CD4^+$ T cells, CXCR3 is expressed at high levels on T helper cell type 0 (Th0) and Th1 cells and at low

[1] Correspondence: Division of Viral Immunology, Center for AIDS Research, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, Japan. E-mail: masafumi@kumamoto-u.ac.jp

Received December 9, 2005; revised April 18, 2006; accepted April 19, 2006; doi: 10.1189/jlb.1205725.

levels on Th2 cells [24]. In contrast, the populations of human $CD8^+$ T cells expressing CXCR3 have not been studied in detail.

In the present study, we investigated the expression of CXCR3 on $CD8^+$ T cell subsets defined by three surface markers: CD27, CD28, and CD45RA. In addition, we investigated the correlation of the expression of this receptor with that of CCR7 and CCR5 and further analyzed the expression of CXCR3 on Epstein-Barr virus (EBV)-specific and human cytomegalovirus (HCMV)-specific $CD8^+$ T cells by using human leukocyte antigen (HLA)-class I-peptide tetrameric complexes (tetramers). Finally, we characterized the function of $CD8^+$ T cells highly expressing CXCR3.

MATERIALS AND METHODS

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 individuals with HLA-A*0201 or HLA-A*0206 and EBV-seropositive individuals with HLA-A*1101, respectively.

Antibodies

Anti-CD27 phycoerythrin (PE)- and allophycocyanin (APC)-Cy7-labeled monoclonal antibody (mAb); anti-CD28 fluorescein isothiocyanate (FITC)- and APC-labeled mAb; anti-CCR5 FITC-labeled mAb; anti-CCR7 PE-Cy7-labeled mAb; anti-CD19 FITC mAb; and anti-mouse-immunoglobulin (IgG) FITC and PE mAb were obtained from BD Biosciences (San Diego, CA). Anti-CXCR3 FITC- and PE-labeled mAb (49801.111 clone) and anti-CD4 APC-labeled mAb were purchased from DakoCytomation (Glostrup, Denmark). Anti-CD45RA energy-coupled dye (ECD)-labeled mAb and anti-CD3 ECD-labeled mAb were 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.

HLA-class I tetramer

HLA-class I-peptide tetrameric complexes (tetramers) were synthesized as described previously [25]. The HCMV cytolytic T lymphocyte (CTL) epitope (HCMV-1 pp65 495-503: NLVPMVATV) [26] and the EBV CTL epitope (EBNA3B 416-424: IVTDFSVIK) [27] were used for the refolding of HLA-A*0201 and HLA-A*0206 molecules and HLA-A*1101 molecules, respectively. PE-labeled streptavidin was used for generation of the tetramers (Molecular Probes).

Flow cytometric analysis

Peripheral blood mononuclear cells (PBMCs) from healthy individuals were stained with anti-CD3, anti-CD4, anti-CD8, anti-CD19, and anti-CXCR3 mAb for 30 min at 4°C and were then washed twice with phosphate-buffered saline (PBS) containing 10% newborn calf serum (NCS; PBS/10% NCS). After the cells had been washed twice with PBS/10% NCS, the percentage of $CXCR3^{high}$ and $CXCR3^{low}$ cells in $CD19^+$, $CD3^+CD4^+$, and $CD3^+CD8^+$ subpopulations was measured by using an LSR II (BD Biosciences). $CXCR3^+$ cells were divided into two subsets, i.e., $CXCR3^{high}$ (>7/8 of the maximal fluorescence intensity) and $CXCR3^{low}$ cells (≤7/8 of the maximal fluorescence intensity).

To investigate the CXCR3 expression in each CD27CD28CD45RA subset of total $CD8^+$ T cells, we purified $CD8^+$ T cells from PBMCs by using anti-CD8-coated magnetic beads (Miltenyi Biotec, Gladbach, Germany). The purified $CD8^+$ T cells (>98%) were stained with anti-CD27, anti-CD28, anti-CD45RA, and anti-CXCR3 mAb for 30 min at 4°C and were then washed twice with PBS/10% NCS. The percentage of $CXCR3^{high}$ and of $CXCR3^{low}$cells in each subset was measured by using a FACSCalibur (BD Biosciences).

To determine CCR7 and CCR5 expression on CXCR3 subsets of total $CD8^+$ T cells, we stained PBMCs with anti-CCR7 mAb for 30 min at room temperature (RT). After they had been washed twice with PBS/10% NCS, the cells were stained with anti-CCR5, anti-CXCR3, and anti-CD8 mAb for 30 min at 4°C.

For determination of CXCR3 expression on the CCR7CCR5 subset of each CD27CD28CD45RA subset of $CD8^+$ T cells, we stained PBMCs with anti-CCR7 mAb for 30 min at RT. After having been washed twice with PBS/10% NCS, the cells were stained with anti-CCR5, anti-CD27, anti-CD28, anti-CD45RA, and anti-CXCR3 mAb for 30 min at 4°C. The percentage of $CXCR3^{high}$ and $CXCR3^{low}$cells in each subset was measured by using the LSR II.

To clarify the expression of CXCR3 on HCMV-specific and EBV nuclear antigen 3 B (EBNA3B)-specific $CD8^+$ T cells, we incubated PBMCs with HCMV-HLA-A*0201, HCMV-HLA-A*0206, or EBNA3B-HLA-A*1101 tetramers for 30 min at 37°C. The cells were washed twice with RPMI/10% NCS and then stained with anti-CXCR3, anti-CD27, anti-CD28, anti-CD45RA, and anti-CD8 mAb for 30 min at 4°C. The cells were then washed twice again with PBS/10% NCS.

Cytokine production by $CXCR3^{high}$ or $CXCR3^{low/-}CD27^+CD28^+CD45RA^-CD8^+$ T cells

To measure cytokine production by $CXCR3^{high}$ or $CXCR3^{low/-}CD27^+CD28^+CD45RA^-CD8^+$ T cells, we purified $CD8^+$ T cells from PBMCs by using the anti-CD8-coated magnetic beads (Miltenyi Biotec). The purified $CD8^+$ T cells (>98%) were stained with anti-CD27, anti-CD28, anti-CD45RA, and anti-CXCR3 mAb, and then the $CXCR3^{high}CD27^+CD28^+CD45RA^-CD8^+$ and $CXCR3^{low/-}CD27^+CD28^+CD45RA^-CD8^+$ T cells were separated by a cell sorter (FACSAria, BD Biosciences). Sorted T cell subsets were cultured for 6 h in F-bottom 96-well plates with or without phorbol 12-myristate 13-acetate (PMA; 10 ng/ml)/ionomycin (1 μg/ml) in RPMI containing 10% fetal calf serum (FCS; R10 medium). After the first 2 h of incubation, brefeldin A (10 μg/ml) was 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 with PBS supplemented with 0.1% saponin containing 20% NCS (permeabilizing buffer). The cells were resuspended in the same buffer and then stained with anti-IFN-γ and anti-interleukin (IL)-2 mAb at RT for 30 min. Thereafter, they were washed three times in the permeabilizing buffer at 4°C. We also used PE-Cy7- and APC-labeled mouse IgG as an isotype control. The cells were finally resuspended in PBS containing 2% paraformaldehyde, and then the cytokine profile was analyzed by flow cytometry (LSR II, BD Biosciences).

Migration assay

$CD8^+$ T cells were purified from PBMCs by using the anti-CD8-coated magnetic beads (Miltenyi Biotec). The purified $CD8^+$ T cells (>98%) were stained with anti-CD3, anti-CD8, anti-CD27, and anti-CD28 mAb, and then $CD3^+CD8^+CD27^{high}CD28^-$ and $CD3^+CD8^+CD27^{low}CD28^-$ subsets were separated by a cell sorter (FACSAria, BD Biosciences). The chemotaxis of sorted $CD8^+$ T subsets was measured by using a TAXIScan holder [28] (Effector Cell Institute, Tokyo, Japan). The chemotactic assays were performed at RT in RPMI-1640 medium supplemented with 20 mM HEPES and 0.1% bovine serum albumin in the assembled KK chamber. The common space at the top end of the holes was filled with medium, and a 1-μl cell suspension (500 cells/μl) was injected via a 10-μm micro-syringe into one of the two compartments through a central hole. To adjust the position of the cells in the compartment and align them along the start line on the edge of the channel, we withdrew the medium with a 10-μl micro-syringe from the other central hole provided for the opposite compartment immediately after injection of the cells. By drawing out the medium in the common space and then more at the appropriate velocity described below, we caused the cells to begin to flow to reach the vicinity of the micro-channel. We stopped drawing medium when ~100 cells had become aligned on the edge of the channel. After alignment of the cells, a compensatory amount of medium was used for refilling to prevent migration of the contents through the channel.

A chemokine was injected in the compartment opposite that containing the cells by using a 10-μm micro-syringe. Chemokines used in the experiments were 100 μg/ml human recombinant Mig and 1 μM stromal cell-derived factor 1α (SDF-1α; PeproTech House, London, UK) as a positive control. To observe and record the migration of cells in the channel, we used a charged-coupled device camera connected to a monitor display. When the chemotaxis of sorted cells was examined, the glass plate was coated with RPMI 1640 containing 40% FCS before assembly of the KK-chamber. After 60 min, we counted the

number of sorted cells that migrated toward the middle of the channel by using a TAXIScan analyzer (Effector Cell Institute).

RESULTS

Surface expression of CXCR3 on human CD8$^+$ T cells

To analyze the expression of CXCR3 on lymphocytes, we stained PBMCs from healthy individuals with anti-CD4, anti-CD8, anti-CD3, and anti-CXCR3 mAb. A representative result of the flow cytometry analysis is shown in **Figure 1A**. Peripheral lymphocytes could be classified into three subsets (CXCR3^{high}, CXCR3^{low}, and CXCR3$^-$) by the levels of CXCR3 expression. The results from eight healthy individuals showed that ~70% of them were CXCR3$^-$ ones. Conversely, as shown in Figure 1, A and B, among T cells, CXCR3$^+$ cells were detected in much greater numbers in the CD8$^+$ T cell population (~70%) than in the CD4$^+$ one (~20%). CXCR3^{high} cells were a small part of CD4$^+$ and CD8$^+$ T cell populations.

Next, we investigated the surface expression of CXCR3 on CD27CD28CD45RA subsets of CD8$^+$ T cells to identify which of them expressed the CXCR3^{high}, CXCR3^{low}, or CXCR3$^-$ cells. A representative result is shown in **Figure 2A**. The CXCR3$^-$ populations were found predominantly in the CD27$^-$CD28$^-$CD45RA$^{+/-}$ effector subsets, which included few CXCR3^{high} cells, indicating that CXCR3 expression was down-regulated in effector CD8$^+$ T cells. The CXCR3^{high} cells were detected much more frequently in CD27^{high}CD28$^+$CD45RA$^-$ and CD27$^+$CD28$^-$CD45RA$^{+/-}$ subsets than in CD27$^+$CD28$^+$CD45RA$^+$ naïve subsets (Fig. 2A), suggesting that CXCR3 is highly expressed on the memory subset and that its surface expression is up-regulated during differentiation from naïve to memory CD8$^+$ T cells or from effector to memory CD8$^+$ T cells. We inversely analyzed the CD27CD28CD45RA expression on CXCR3^{high} CD8$^+$ T cells. Approximately 50%, 20%, and 20% of CXCR3^{high}CD8$^+$ T cells were CD27$^+$CD28$^+$CD45RA$^-$, CD27$^+$CD28$^-$CD45RA$^{+/-}$, and CD27$^+$CD28$^+$CD45RA$^+$, respectively (Fig. 2B), indicating that up-regulation of CXCR3 had already started in some of the CD8$^+$ T cells expressing the naïve phenotype.

Coexpression of CXCR3, CCR7, and CCR5 on CD8$^+$ T cells

CCR7 is expressed on naïve CD8$^+$ T cells and on central memory CD8$^+$ T cells [8, 9], whereas CCR5 is expressed predominantly on a subset in memory CD8$^+$ T cells [1, 8]. To clarify the relationship between the expression of these two receptors and CXCR3, we investigated the coexpression of CXCR3 and CCR7 or CCR5 on CD8$^+$ T cells from four healthy individuals. CXCR3^{high} cells were found predominantly in CCR7$^-$ and CCR5$^-$ subsets (**Fig. 3, A** and **B**), implying that most CXCR3^{high}CD8$^+$ T cells did not express CCR7 or CCR5 on their surface. We also directly analyzed the coexpression of these three receptors on CD8$^+$ T cells from the same individual. A representative result is shown in Figure 3C. The CD8$^+$ T cells could be classified into three groups on the basis of the expression pattern of CCR7 and CCR5: CCR7$^+$CCR5$^-$, CCR7$^-$CCR5$^+$, and CCR7$^-$CCR5$^-$. The CXCR3^{low} cells were mostly in CCR7$^+$CCR5$^-$ and CCR7$^-$CCR5$^+$ subsets, whereas CXCR3^{high} was found predominantly in the CCR7$^-$CCR5$^-$ subset. The same results were found when we examined CD8$^+$ T cells from three other individuals (data not shown). We inversely analyzed the CCR5 and CCR7 expression on CXCR3^{high}CD8$^+$ T cells (Fig. 3D). These cells were predominantly CCR7$^+$CCR5$^-$ and CCR7$^-$CCR5$^-$ cells. These results suggest, together with those shown in Figure 2, that CXCR3^{high}CD8$^+$ T cells may express the CCR7$^{+/-}$CCR5$^-$CD27$^{high/low}$CD28$^+$CD45RA$^-$ phenotype, as CXCR3^{high} cells were found predominantly in the CD27$^{high/low}$CD28$^+$CD45RA$^-$ memory subset.

CXCR3^{high}CD8$^+$ T cells exist predominantly in the CCR5$^-$ subset of memory CD8$^+$ T cells

To investigate the coexpression of CXCR3, CCR5, and CCR7 on memory CD8$^+$ T cells, we analyzed PBMCs from two

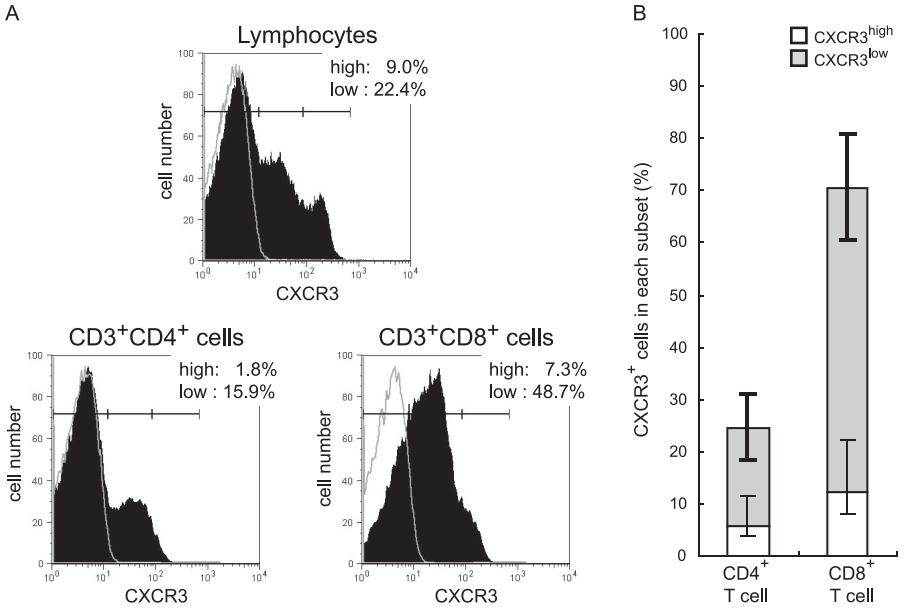

Fig. 1. Surface expression of CXCR3 on CD4$^+$ T cells and CD8$^+$ T cells. (A) PBMCs from a healthy donor, U-24, were stained with anti-CD19, anti-CD3, anti-CD4, anti-CD8, and anti-CXCR3 mAb. Lymphocytes, CD3$^+$CD4$^+$, and CD3$^+$CD8$^+$ subsets were gated, and then the surface expression of CXCR3 in each subset was analyzed by flowcytometry. (B) The frequency of CXCR3^{high} and CXCR3^{low} cells in CD4$^+$ T cells and CD8$^+$ T cell populations from eight individuals. PBMCs were isolated from eight individuals and then stained with anti-CD3, anti-CD4, anti-CD8, and anti-CXCR3 mAb. The mean percentage and SD of CXCR3^{high} and CXCR3^{low} cells in each subset are shown.

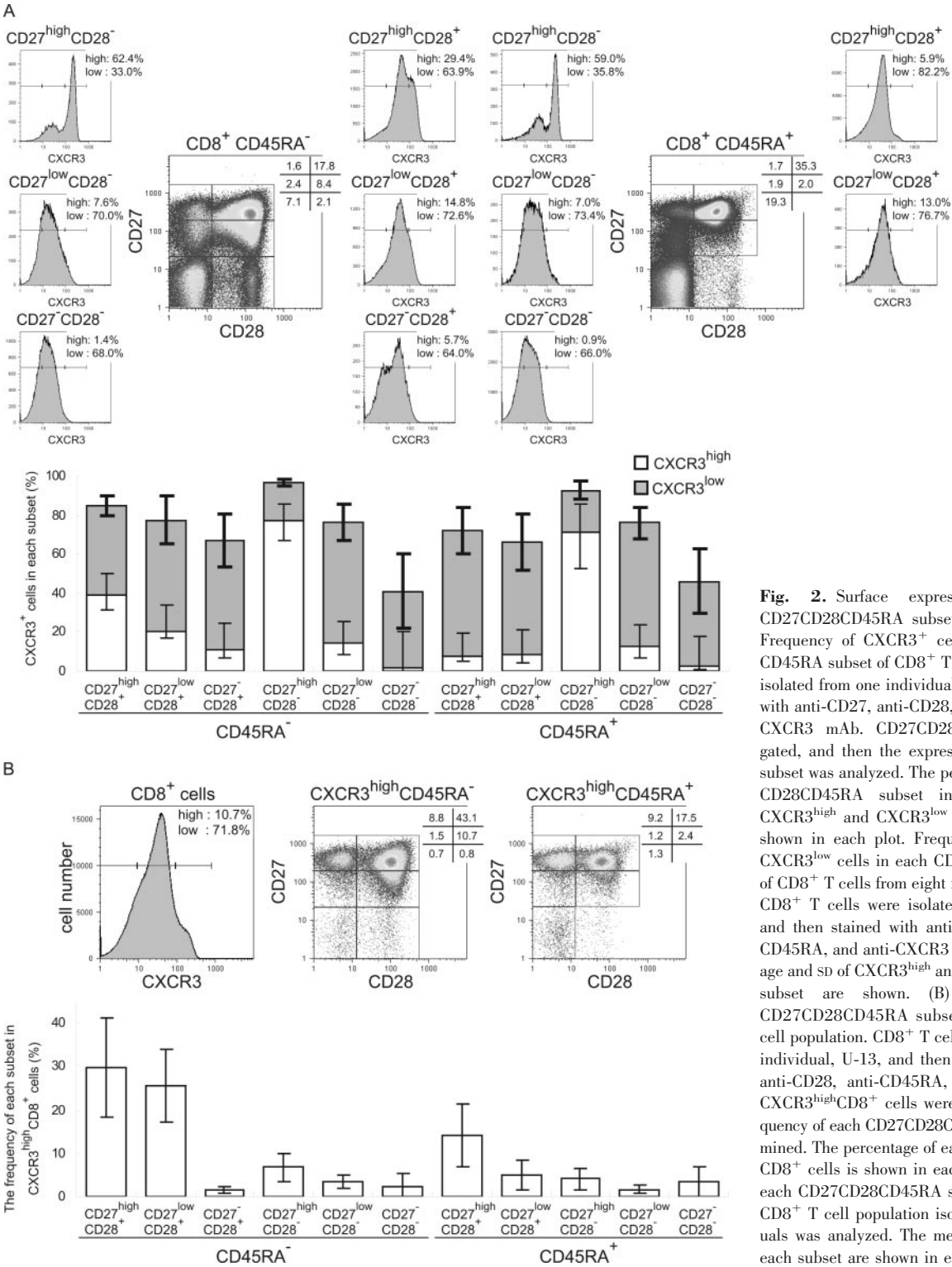

Fig. 2. Surface expression of CXCR3 on CD27CD28CD45RA subsets of $CD8^+$ T cells. (A) Frequency of $CXCR3^+$ cells in each CD27CD28-CD45RA subset of $CD8^+$ T cells. $CD8^+$ T cells were isolated from one individual, U-13, and then stained with anti-CD27, anti-CD28, anti-CD45RA, and anti-CXCR3 mAb. CD27CD28CD45RA subsets were gated, and then the expression of CXCR3 on each subset was analyzed. The percentages of each CD27-CD28CD45RA subset in $CD8^+$ cells and of $CXCR3^{high}$ and $CXCR3^{low}$ cells in each subset are shown in each plot. Frequency of $CXCR3^{high}$ and $CXCR3^{low}$ cells in each CD27CD28CD45RA subset of $CD8^+$ T cells from eight individuals was analyzed. $CD8^+$ T cells were isolated from eight individuals and then stained with anti-CD27, anti-CD28, anti-CD45RA, and anti-CXCR3 mAb. The mean percentage and SD of $CXCR3^{high}$ and $CXCR3^{low}$ cells in each subset are shown. (B) Frequency of each CD27CD28CD45RA subset in $CXCR3^{high}CD8^+$ T cell population. $CD8^+$ T cells were isolated from one individual, U-13, and then stained with anti-CD27, anti-CD28, anti-CD45RA, and anti-CXCR3 mAb. $CXCR3^{high}CD8^+$ cells were gated, and then the frequency of each CD27CD28CD45RA subset was determined. The percentage of each subset in $CXCR3^{high}$-$CD8^+$ cells is shown in each plot. The frequency of each CD27CD28CD45RA subset in the $CXCR3^{high}$-$CD8^+$ T cell population isolated from eight individuals was analyzed. The mean percentage and SD of each subset are shown in each plot.

individuals by seven-color flow cytometric analysis with anti-CXCR3, anti-CCR5, anti-CCR7, anti-CD45RA, anti-CD27, anti-CD28, and anti-CD8 mAb (**Fig. 4**). $CCR7^+CCR5^-$ and $CCR7^-CCR5^-$ subsets of memory $CD8^+$ T cells with the $CD27^{high}CD28^+CD45RA^-$ phenotype were mostly $CXCR3^{high}$ cells, whereas the $CCR7^-CCR5^+$ subset was mostly $CXCR3^{low}$ cells. Similar findings were found for the $CD27^{low}CD28^+$-$CD45RA^-$ memory subset, which contains more mature $CD8^+$ T cells than the $CD27^{high}CD28^+CD45RA^-$ memory subset. $CCR7^+CD27^+CD28^+CD45RA^-CD8^+$ T cells are known to be central memory cells [8]. Thus, $CXCR3^{high}CD8^+$ T cells were found in the central memory cell population ($CCR7^+$-$CCR5^-CD27^+CD28^+CD45RA^-CD8^+$ T cells). They were also found in the $CCR7^-CD27^+CD28^+CD45RA^-CD8^+$ T cell one. Similar results were obtained from experiments using PBMCs from two additional individuals (data not shown). These findings together suggest that the $CCR7^-CCR5^-$ subset of $CD27^+CD28^+CD45RA^-CD8^+$ T cells may be less-mature

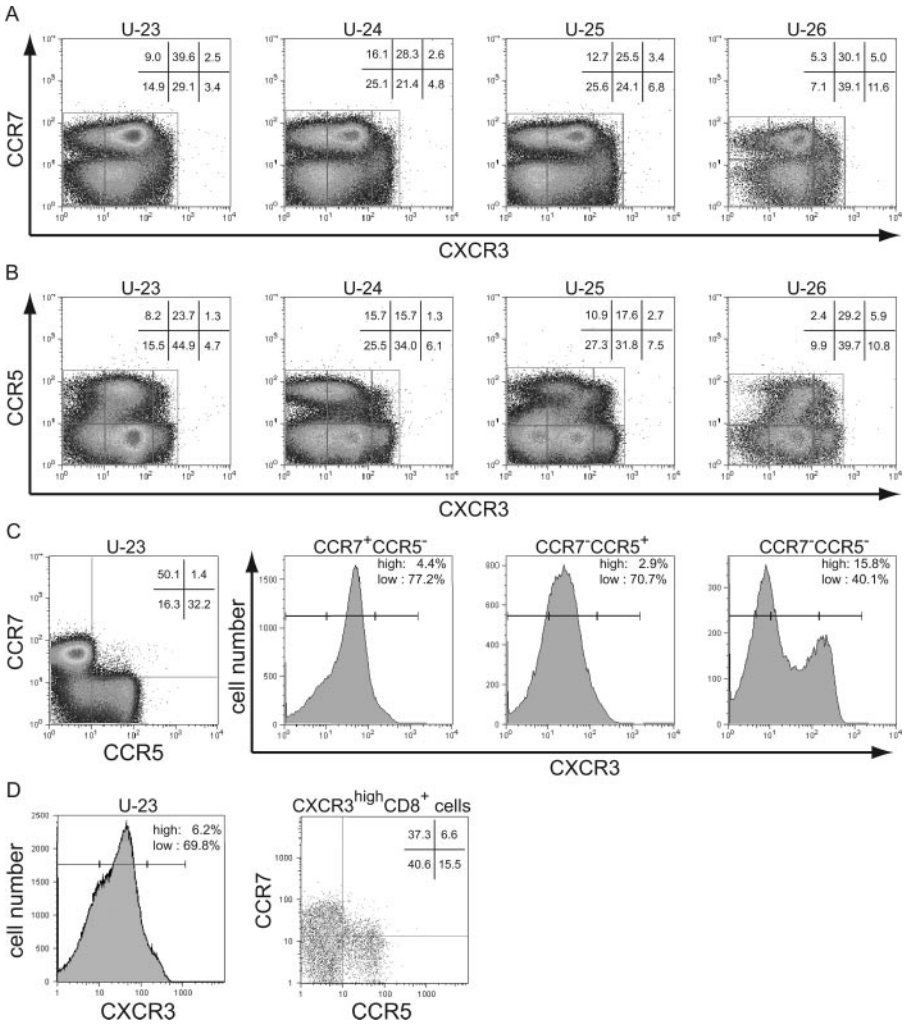

Fig. 3. Surface expression of CCR5, CCR7, and CXCR3 on $CD8^+$ T cells. (A) PBMCs from four individuals were stained with anti-CD8, anti-CCR7, and anti-CXCR3 mAb or with mouse IgG mAb as a negative control. The $CD8^+$ subset was gated and then analyzed for the expression of CXCR3 and CCR7. The percentage of each CCR7CXCR3 subset in the $CD8^+$ cell population is shown in each plot. (B) PBMCs from four individuals were stained with anti-CD8, anti-CCR5, and anti-CXCR3 mAb or with mouse IgG mAb as a negative control. The $CD8^+$ subset was gated and then analyzed for the expression of CXCR3 and CCR5. The percentage of each CCR5CXCR3 subset in the $CD8^+$ cell population is shown in each plot. (C) PBMCs from U-23 were stained with anti-CD8, anti-CCR7, anti-CCR5, and anti-CXCR3 mAb or with mouse IgG mAb as a negative control. The $CD8^+$ subset was gated and then analyzed for the expression of CXCR3 on CCR7CCR5 subsets. The percentage of $CXCR3^{high}$ and $CXCR3^{low}$ subsets in each subset is shown in each plot.

memory cells than the $CCR7^-CCR5^+$ subset of $CD27^+CD28^+$-$CD45RA^-CD8^+$ T cells and that the expression of CXCR3 is up-regulated in relatively immature memory $CD8^+$ T cells.

Surface expression of CXCR3 on EBV-specific and HCMV-specific $CD8^+$ T cells

In healthy individuals, most EBV-specific $CD8^+$ T cells express the $CD27^+CD28^+CD45RA^-$ memory phenotype, whereas HCMV-specific $CD8^+$ T cells have the $CD27^-CD28^-$-$CD45RA^{+/-}$ effector or $CD27^{low}CD28^-CD45RA^{+/-}$ memory/effector phenotype [1]. Therefore, we hypothesized that some of the former cells would express high levels of CXCR3 and the latter, only low levels or none at all. So we examined the CXCR3 expression on EBNA3B-specific and HCMV-specific $CD8^+$ T cells by using HLA-A*1101 and HLA-A*0201 or HLA-A*0206 tetramers, respectively. PBMCs from HLA-$A*0201^+$, HLA-$A*0206^+$, or HLA-$A*1101^+$ healthy individuals were stained with the combination of anti-CD8 and anti-CXCR3 mAb as well as the tetramer or with that of anti-CD8, anti-CD27, anti-CD28, and anti-CD45RA mAb as well as the tetramer. All EBV-specific $CD8^+$ T cells, which were mostly of the $CD27^+CD28^+CD45RA^-$ phenotype, were contained in $CXCR3^{high}$ and $CXCR3^{low}$ subsets, although the number in the latter subset was approximately twice that in the former (**Fig. 5A**). Conversely, HCMV-specific $CD8^+$ T cells, which have the $CD27^-CD28^-CD45RA^{+/-}$ and $CD27^{low}CD28^{+/-}$-$CD45RA^{+/-}$ phenotypes, were in $CXCR3^{low}$ and $CXCR3^-$ subsets (Fig. 5B). These findings were confirmed in EBNA3B-specific and HCMV-specific $CD8^+$ T cells from five healthy individuals. The results from these individuals showed that ~45% of EBNA3B-specific $CD8^+$ T cells were $CXCR3^{high}$ cells, whereas they were hardly detected in HCMV-specific $CD8^+$ T cells (Fig. 5C). These results show that in antigen-specific $CD8^+$ T cells, $CXCR3^{high}$ cells exist predominantly in the population of cells expressing the $CD27^+CD28^+$-$CD45RA^-$ memory phenotype and that $CXCR3^-$ cells are only present in the population expressing the effector phenotype. EBNA3B-specific $CXCR3^{high}CD8^+$ T cells included $Perforin^{low}$ and $Perforin^-$ populations ($Perforin^{low}$: 48.7±0.4%; $Perforin^-$: 47.7±1.3%), whereas $CXCR3^{low}$ and $CXCR3^-$ cells were predominantly $Perforin^{low}$ ($Perforin^{low}$: 84.9±9.7% and 83.2±5.3%, respectively; data not shown). These findings also indicate that EBNA3B-specific memory $CD8^+$ T cells include two populations, i.e., $CXCR3^{high}$ immature and $CXCR3^{low}$ mature memory cells.

Ability of $CXCR3^{high}$ and $CXCR3^{low/-}$ memory subsets of $CD8^+$ T cells to produce cytokines

To investigate the difference in functional ability between $CXCR3^{high}$ and $CXCR3^{low/-}$ memory $CD8^+$ T cells, we sorted

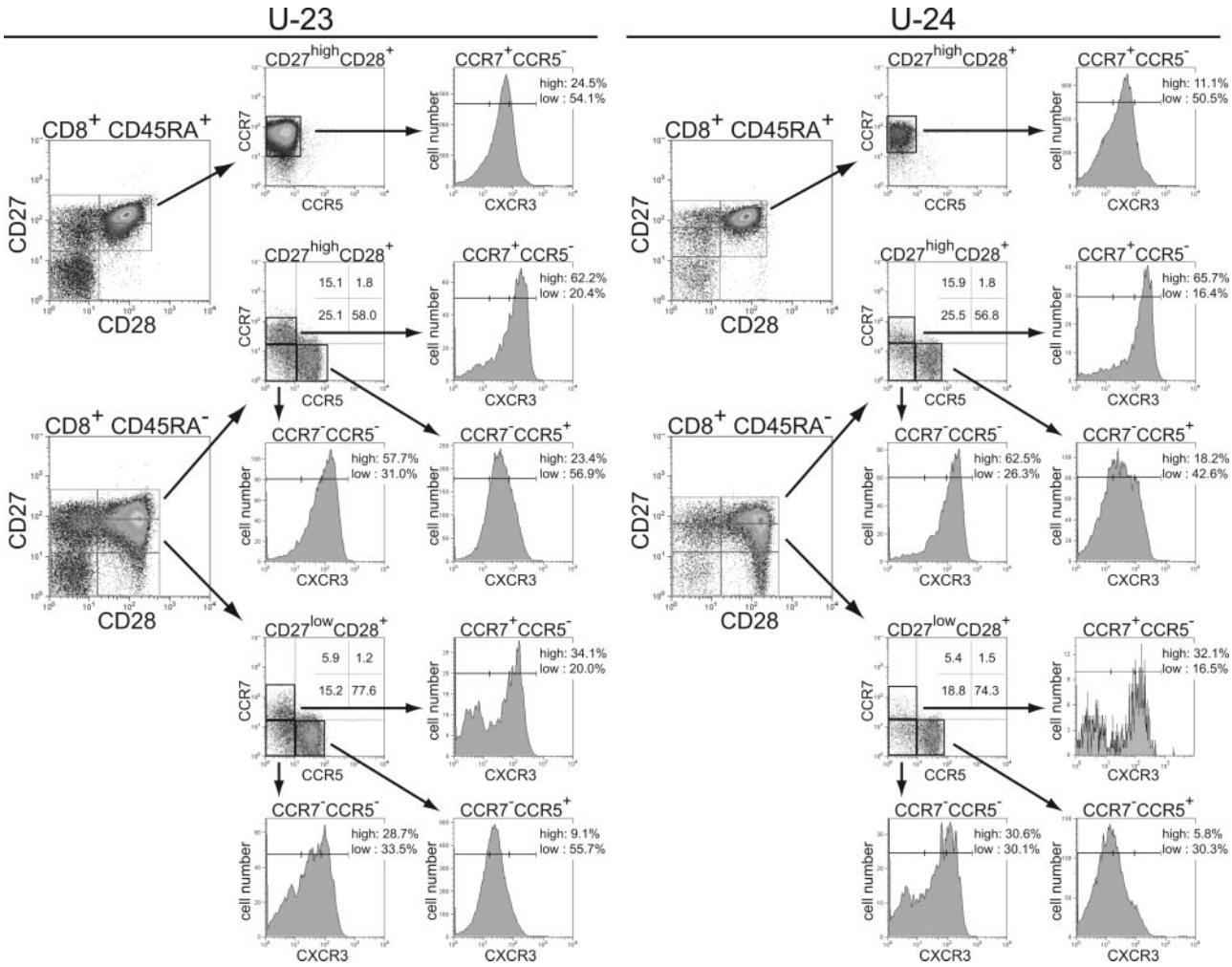

Fig. 4. Surface expression of CXCR3 on CCR5CCR7CD27CD28CD45RA subsets of $CD8^+$ T cells. PBMCs from two individuals, U-23 and U-24, were stained with anti-CD8, anti-CD45RA, anti-CD27, anti-CD28, anti-CCR7, anti-CCR5, and anti-CXCR3 mAb or with mouse IgG mAb as a negative control. The CCR7CCR5CD27CD28CD45RA subsets of the $CD8^+$ cell population were gated, and then each was analyzed for the expression of CXCR3. The percentage of each CCR7CCR5 subset in the $CD27^{high/low}CD28^+CD45RA^{-/+}CD8^+$ T cells and of $CXCR3^{high}$ and $CXCR3^{low}$ cells in each subset is shown in each plot.

$CXCR3^{high}$ and $CXCR3^{low/-}$ subsets of $CD27^+CD28^+CD45RA^-$-$CD8^+$ T cells and then measured IL-2 and IFN-γ production of these sorted cells after having stimulated them with PMA and ionomycin (**Fig. 6**). The purities of the sorted $CXCR3^{high}$ and $CXCR3^{low}$ cells were 96.0% and 98.7%, respectively. The $CXCR3^{high}$ cells produced IL-2 and IFN-γ, suggesting that the $CXCR3^{high}$ cells were memory $CD8^+$ T cells possessing the ability to produce both cytokines. Conversely, the $CXCR3^{low/-}$ cells contained four populations producing IL-2, IFN-γ, both of them, or neither of them, indicating that at least four functionally different subpopulations exist in the $CXCR3^{low/-}$ cell population. The naïve $CD8^+$ T cell subset included both cells producing only IL-2 and those producing neither IL-2 nor IFN-γ, whereas the effector subset included both cells producing only IFN-γ and those producing neither IL-2 nor IFN-γ. These results together suggest that the $CXCR3^{low/-}$ cells producing only IL-2 were immature memory cells and that the $CXCR3^{low/-}$ cells producing only IFN-γ were effector cells. The cells that failed to produce either cytokine may have been immature memory cells or well-differentiated, mature effector cells. These results were confirmed in an experiment using the cells from a different individual (data not shown). Thus, $CXCR3^{high}CD27^+CD28^+CD45RA^-CD8^+$ T cells were a functionally homogenous population.

Different chemotactic responses between $CXCR3^{high}$ and $CXCR3^{low}$ cells

To investigate the functional differences between $CXCR3^{high}$ and $CXCR3^{low}$ $CD8^+$ T subsets, we measured the chemotactic responses of $CD27^{high}CD28^-$ $CD8^+$ T subsets mostly comprising $CXCR3^{high}$ cells and $CD27^{low}CD28^-$ $CD8^+$ T subsets predominantly including $CXCR3^{low}$ cells. $CD27^{high}CD28^-$-$CD8^+$ and $CD27^{low}CD28^-CD8^+$ T cells were sorted from PBMCs of a healthy individual, and then the migration of the sorted cells induced by the CXCR3 ligand Mig or the CXCR4 ligand SDF-1 was measured by using a TAXIScan holder (**Fig. 7**). As a result, 65.7% and 20.0% of $CD27^{high}CD28^-CD8^+$ T cells were $CXCR3^{high}$ and $CXCR3^{low}$ cells, respectively; whereas, 5.3% and 55.1% of $CD27^{low}CD28^-CD8^+$ T cells were $CXCR3^{high}$ and $CXCR3^{low}$ cells, respectively. The chemotactic response of $CD8^+$ T cells with the $CD27^{high}CD28^-$ phenotype to Mig was approximately fourfold higher than that

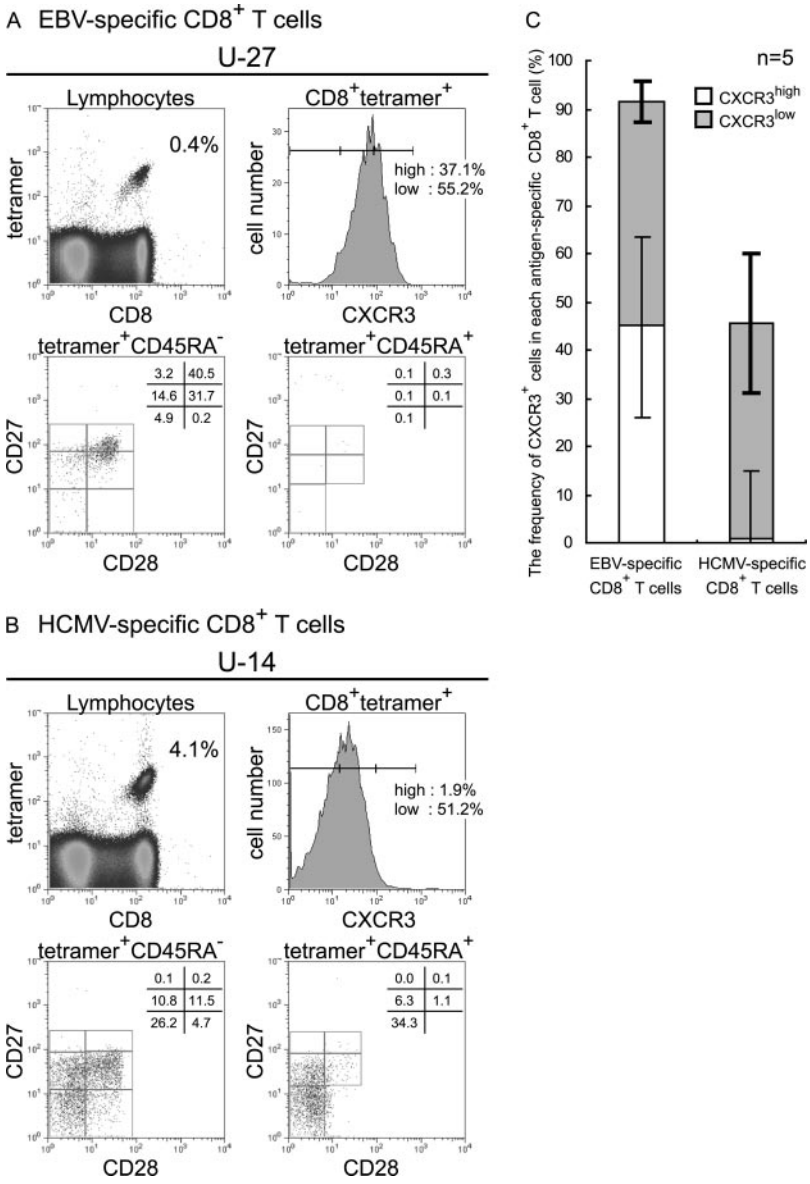

Fig. 5. Surface expression of CXCR3 on EBV-specific and HCMV-specific $CD8^+$ T cells. (A) Surface expression of CXCR3 on EBNA3B-specific $CD8^+$ T cells. PBMCs from an individual, U-27, with HLA-A*1101 were stained with anti-CD8, anti-CD27, anti-CD28, anti-CD45RA, and anti-CXCR3 mAb and the HLA-A*1101 tetramer carrying EBNA3B. Total $CD8^+$ cells (open histograms) and $CD8^+$tetramer$^+$ cells (filled histograms) were gated and then analyzed for their respective expression of CXCR3. The percentage of tetramer$^+$ subsets among $CD8^+$ T cells is shown in the figure. The expression of CD27, CD28, and CD45RA on $CD8^+$ tetramer$^+$ cells from each individual is shown in each plot. (B) Surface expression of CXCR3 on HCMV-specific $CD8^+$ T cells. PBMCs from an individual, U-14, with HLA-A*0201 were stained with anti-CD8, anti-CD27, anti-CD28, anti-CD45RA, and anti-CXCR3 mAb and the HLA-A*0201 tetramer. Total $CD8^+$ cells (open histograms) and $CD8^+$tetramer$^+$ cells (filled histograms) were gated and then analyzed for their expression of CXCR3. The percentage of tetramer$^+$ subsets among $CD8^+$ T cells is shown in the figure. The expression of CD27, CD28, and CD45RA on the $CD8^+$tetramer$^+$ cells from each individual is shown in each plot. (C) The frequency of $CXCR3^{high}$ and $CXCR3^{low}$ cells in EBNA3B-specific and HCMV-specific $CD8^+$ T cells from each of five individuals, respectively, was analyzed. The percentages of $CXCR3^{high}$ and $CXCR3^{low}$ cells in each antigen-specific $CD8^+$ cell are shown in each plot. The mean percentage and SD of $CXCR3^{high}$ and $CXCR3^{low}$ cells in each subset are shown.

of $CD8^+$ T cells with the $CD27^{low}CD28^-$ phenotype. A previous study demonstrated no difference in CXCR4 expression between $CD27^{high}CD28^-CD8^+$ and $CD27^{low}CD28^-CD8^+$ T cells [14]. Indeed, there was no difference between the chemotactic response of two cell populations to SDF-1. The above results were confirmed in an experiment using cells from a different individual (data not shown). These findings indicate that $CXCR3^{high}CD8^+$ T subsets had a strong ability to migrate in response to CXCR3 ligands.

DISCUSSION

Previous studies demonstrated that a subset of $CD8^+$ and $CD4^+$ T cells expresses CXCR3 [16]. However, these studies did not address the level of CXCR3 on the cells. We here showed that $CXCR3^{high}$ cells were found in a small subset of $CD8^+$ and $CD4^+$ T cells. $CXCR3^{high}$ cells were detected predominantly in $CD8^+$ T cells carrying the $CD27^+CD28^+$-$CD45RA^-$ or $CD27^+CD28^-CD45RA^{+/-}$ phenotype, whereas they were rarely detected in $CD8^+$ T cells carrying the $CD27^+$-$CD28^+CD45RA^+$ naive phenotype. Conversely, the analysis of CD27CD28CD45RA expression on $CXCR3^{high}CD8^+$ T cells (Fig. 2B) revealed that ~60% and 15% of $CXCR3^{high}CD8^+$ T cells expressed $CD27^+CD28^+CD45RA^-$ memory and $CD27^+$-$CD28^+CD45RA^+$ naïve phenotypes, respectively, suggesting that the up-regulation of CXCR3 expression starts in some of the immature $CD8^+$ T cells carrying the $CD27^+CD28^+$-$CD45RA^+$ naïve phenotype. The findings that these $CD27^+$-$CD28^+CD45RA^+CD8^+$ T cells expressed CCR7 but not CCR5 (Fig. 4) support the idea that they are a part of the naïve cell population, although the possibility that they are central memory cells cannot be excluded. In the $CD8^+$ T cells expressing the $CD27^+CD28^+CD45RA^-$ memory phenotype, $CXCR3^{high}$ cells were found predominantly in $CCR7^+CCR5^-$ and $CCR7^-CCR5^-$ subsets. As the $CCR7^+CCR5^-$ subset of $CD8^+$ T cells carrying the $CD27^+CD28^+CD45RA^-$ phenotype is central memory cells, the $CCR7^-CCR5^-$ subset of $CD8^+$ T cells carrying this phenotype would seem to be more immature than the $CCR7^-CCR5^+$ subset. These findings im-

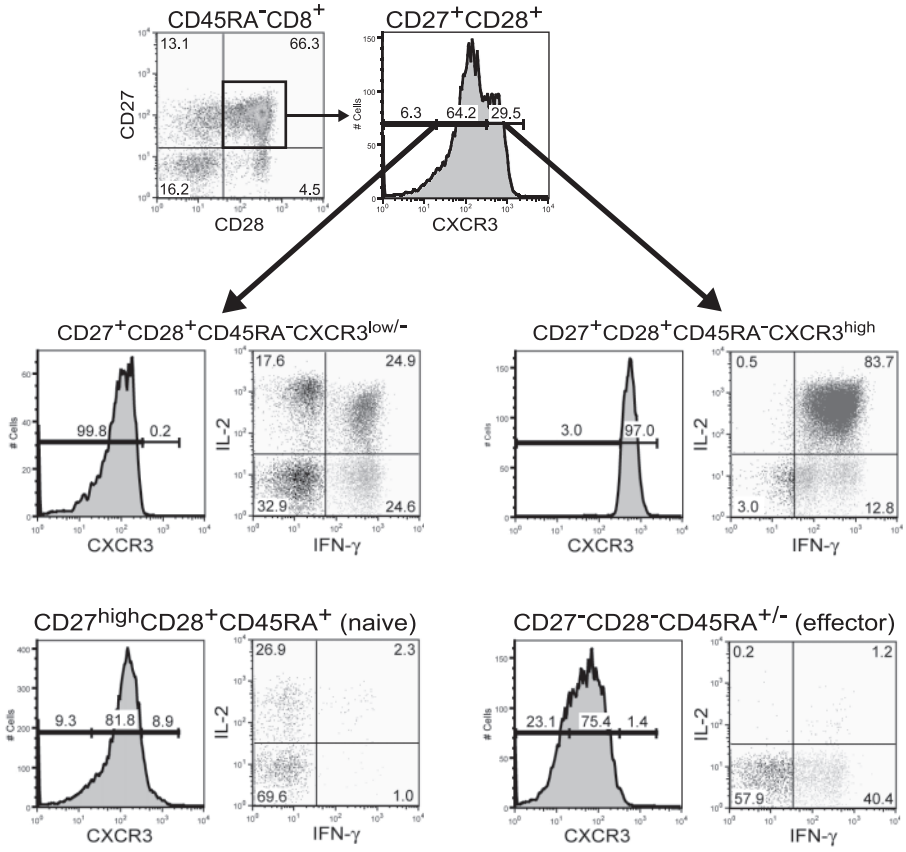

Fig. 6. Cytokine production by CXCR3^{high} and CXCR3^{low} memory subsets of CD8$^+$ T cells. The potential for cytokine production by CXCR3^{high} and CXCR 3$^{low/-}$ memory CD8$^+$ T cells was investigated by measuring IFN-γ and IL-2 levels of these cells stimulated with PMA and ionomycin. CD8$^+$ T cells were isolated from healthy individual U-13 and stained with anti-CD27, anti-CD28, anti-CD45RA, and anti-CXCR3 mAb. CD27$^+$CD28$^+$-CD45RA$^-$CXCR3^{high}, CD27$^+$CD28$^+$CD45RA$^-$-CXCR3$^{low/-}$, CD27^{high}CD28$^+$CD45RA$^+$, and CD27$^-$CD28$^-$CD8$^+$ T cells were then isolated by using a cell sorter. The sorted CD8$^+$ T cells were cultured for 6 h in F-bottom 96-well plates in R10 medium containing PMA (10 ng/ml) and ionomycin (1 μg/ml). IFN-γ- and IL-2-producing cells were analyzed by flow cytometry. Sorting purities of CD27$^+$CD28$^+$CD45RA$^-$CXCR3^{high}, CD27$^+$CD28$^+$-CD45RA$^-$CXCR3$^{low/-}$, CD27^{high}CD28$^+$CD45RA$^+$ naïve, and CD27$^-$CD28$^-$CD8$^+$ effector T cells were 96.0%, 98.7%, 98.3%, and 99.0%, respectively.

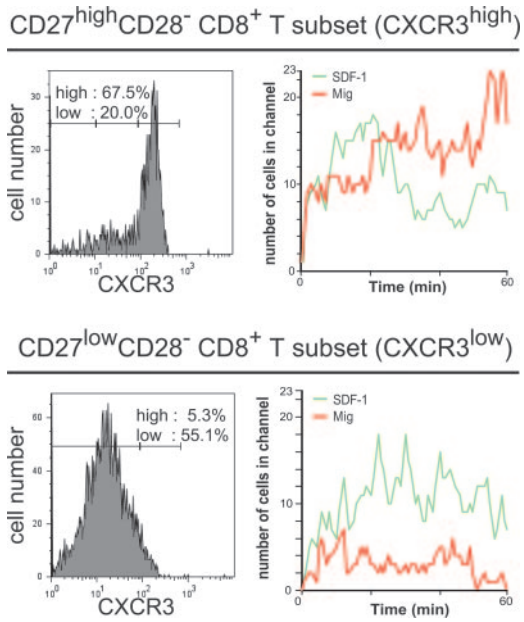

Fig. 7. Higher chemotactic response of CXCR3^{high}CD8$^+$ T cells to CXCR3 ligand Mig than that of CXCR3^{low}CD8$^+$ T cells to it. CD27^{high}CD28$^-$ and CD27^{low}CD28$^-$ subsets of CD8$^+$ T cells were separated by using a cell sorter. CXCR3 expression on both sorted subsets was confirmed simultaneously by staining with anti-CXCR3 mAb. The chemotactic responses of CD27^{high}-CD28$^-$ and CD27^{low}CD28$^-$ subsets were analyzed by using the TAXIScan (Effector Cell Institute). The migration was induced by 100 μg/ml Mig (red line) and SDF-1 (green line), as a positive control is also shown.

ply that the up-regulation of CXCR3 starts in some of the naïve cells or central memory cells expressing the naïve CD27CD28CD45RA phenotype and then expands in central memory cells and relatively immature memory cells (Fig. 8).

CD8$^+$ T cells producing IFN-γ but not IL-2 were found among the CD8$^+$ T cells carrying the effector phenotype but not among those with the naïve phenotype (Fig. 5). Previous studies demonstrated that CD8$^+$ T cells displaying the effector phenotype, CD45RA$^+$CCR7$^-$ or CD45RA$^+$CD27$^-$, produce IFN-γ but not IL-2 [29, 30], indicating that CD8$^+$ T cells having the ability to produce IFN-γ but not IL-2 are effector CD8$^+$ T cells. In contrast, CD8$^+$ T cells producing IL-2 but not IFN-γ were found among those with the naive phenotype but not in those showing the effector one (Fig. 5). This finding indicates that CD8$^+$ T cells having the ability to produce IL-2 but not IFN-γ are immature cells. Therefore, it is likely that CD8$^+$ T cells that can produce IFN-γ and IL-2 are more mature cells than those that are able to produce IL-2 but not IFN-γ. The CXCR3$^{low/-}$CD8$^+$ T cells carrying the CD27$^+$-CD28$^+$CD45RA$^-$ phenotype included four populations, i.e., those producing IL-2, IFN-γ, both, and neither (Fig. 5). Thus, the population of CXCR3$^{low/-}$CD8$^+$ T cells carrying the memory phenotype includes CD8$^+$ T cells at different stages of differentiation. Conversely, CXCR3^{high}CD8$^+$ T cells carrying the CD27$^+$CD28$^+$CD45RA$^-$ phenotype included only cells that produced IL-2 and IFN-γ. Thus, in memory CD8$^+$ T cells, the up-regulation of CXCR3 expression is found predominantly in those cells possessing the ability to produce IL-2 and IFN-γ. It would be interesting to know whether CXCR3^{high}CD8$^+$ T cells carrying the CD27$^+$CD28$^+$CD45RA$^+$ naive phenotype

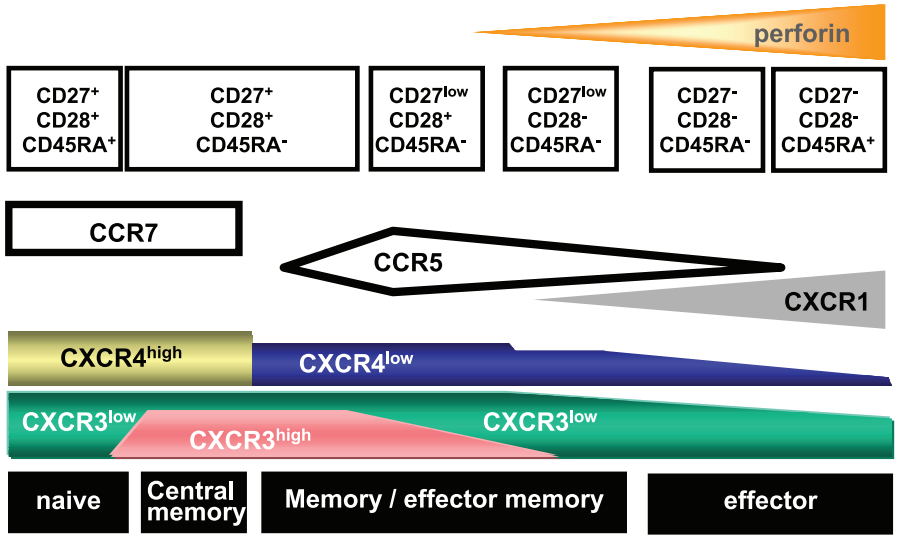

Fig. 8. Summary of the expression of CXCR3 and other chemokine receptors on human peripheral $CD8^+$ T cells. The expression of CXCR4 and CXCR1 on $CD8^+$ T cell subsets was studied previously [12, 14], and perforin expression was reported to occur in effector $CD8^+$ T cell subsets [8].

can produce IL-2 or not, because if these cells can do so, the up-regulation of CXCR3 would be an event in restricted populations such as naïve T cells having the ability to produce IL-2 and memory T cells possessing the ability to produce IL-2 and IFN-γ. However, this experiment using purified $CXCR3^{high}$-$CD8^+$ T cells with the $CD27^+CD28^+CD45RA^+$ naive phenotype is difficult to do, as these cells are rarely detected in the $CD27^+CD28^+CD45RA^+$ subset. A previous study showed that $CD45RA^-CCR7^+$ central memory $CD8^+$ T cells have the ability to produce IL-2 but not IFN-γ [9]. In contrast, our result showed that $CXCR3^{high}CD8^+$ T cells carrying the $CD27^+$-$CD28^+CD45RA^-$ memory phenotype, in which most $CCR7^+$-$CCR5^-$ central memory cells are included, could produce IL-2 and IFN-γ. This discrepancy may be explained by the difference in stimulation methods between the present study and previous studies.

Earlier studies indicated that most EBV-specific $CD8^+$ T cells in healthy individuals carry the $CD27^+CD28^+CD45RA^-$ memory phenotype and express CCR5 but not CCR7 [1]. Conversely, we showed that 45% of EBNA3B-specific $CD8^+$ T cells was $CXCR3^{high}$. Thus, EBNA3B-specific $CD8^+$ T cells in healthy individuals seem to have at least two different populations, $CXCR3^{high}CCR5^{-/+}CCR7^-$ and $CXCR3^{low}CCR5^+$-$CCR7^-$. We speculated that the former are less-matured memory cells than the latter, as $CXCR3^{high}$ cells are mostly found in the $CCR7^+CCR5^-$ and $CCR7^-CCR5^-$ subsets of $CD27^+$-$CD28^+CD45RA^-$ memory $CD8^+$ T cells. Functional studies about these subsets of EBV-specific $CD8^+$ T cells may be expected to clarify the classification of human memory $CD8^+$ T cells and their relationships during differentiation.

The physiological importance of high expression of CXCR3 on memory $CD8^+$ T cells is unknown. IFN-γ-induced chemokines such as Mig, IP-10, and I-TAC are ligands for CXCR3 and can be produced by various cells including macrophages, neutrophils, and bronchial epithelial cells [31–33]. As the expression of these chemokines is induced by IFN-γ, they are to be produced mostly at sites of inflammation. A recent study found an increase in Ca^{2+} flux in response to a low level of IP-10 in Th1 $CD4^+$ T cells, which expressed CXCR3 at high levels, but not in Th2 $CD4^+$ T cells, which expressed CXCR3 at low levels, thus indicating that the level of the chemokine receptor expression determines the sensitivity to CXCR3 ligands [24]. The expression level of CXCR3 on $CXCR3^{high}$ cells was approximately tenfold higher than that on $CXCR3^{low}$ cells. The present study demonstrates that the migration capacity of $CXCR3^+$ cells was dependent on the expression level of CXCR3 on these cells, suggesting that $CXCR3^{high}$ memory T cells might migrate more actively to inflammatory sites in response to CXCR3 ligands than $CXCR3^{low}$ T cells. As $CXCR3^{high}$ memory $CD8^+$ T cells do not express CCR5, the migration of these cells by the ligands for CCR5 is not expected. Therefore, it is suggested that high expression of CXCR3 is an important event in migration of these memory $CD8^+$ T cells to sites of inflammation. In addition, as Th1 cells expressing a high level of CXCR3 are thought to actively migrate to inflammatory sites in response to CXCR3 ligands, they may help memory $CD8^+$ T cells to differentiate into effector $CD8^+$ T cells in these sites. Especially, the help of $CD4^+$ Th cells may be necessary for $CCR7^+CXCR3^{high}$ immature memory $CD8^+$ T cells to differentiate to effector cells. These $CXCR3^{high}CD8^+$ and $CD4^+$ T cells together may induce T cell proliferation by stimulation with cytokines such as IL-2 from these cells and may be involved in the suppression of viral replication by producing effector $CD8^+$ T cells and various cytokines after their migration to the inflammatory sites.

In summary, the present study revealed that CXCR3 was expressed predominantly on a part of $CD8^+$ T cell populations with naïve and memory phenotypes, especially on $CCR7^+$-$CCR5^-$ and $CCR7^-CCR5^-$ subsets of $CD27^+CD28^+CD45RA^-$ memory $CD8^+$ T cells but that its expression was reduced during differentiation to $CD8^+$ T cells having the effector phenotype. It also showed that $CXCR3^{high}$ memory cells, which were found in $CCR7^+CCR5^-$ and $CCR7^-CCR5^-$ subsets of $CD27^+CD28^+CD45RA^-$ memory $CD8^+$ T cells, had the ability to produce IL-2 and IFN-γ and that the migration capacity of $CXCR3^+$ cells was dependent on the expression level of CXCR3 on these cells. Thus, up-regulation of CXCR3 affords a considerable advantage to memory $CD8^+$ T cells for the migration to sites of inflammation.

ACKNOWLEDGMENTS

This research was supported by a grant-in aid for scientific research from the Ministry of Health, Labor, and Welfare of the Government of Japan and by a Grant-in Aid for Scientific Research from the Ministry of Education, Science, Sport and Culture (17047033), the government of Japan. The authors thank Sachiko Sakai and Keiko Akiyama for secretarial assistance. N. K. and T. K. contributed equally to this work.

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