Human memory CCR4+CD8+ T cell subset has the ability to produce multiple cytokines

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Abstract

The CC chemokine receptor (CCR)4 is associated with trafficking of specialized cutaneous memory type 2 T<sub>h</sub> cells in the skin. However, a CD8<sup>+</sup> T cell population expressing CCR4 still remains uncharacterized. In the present study, we investigated the expression and function of CCR4 on human CD8<sup>+</sup> T cells and characterized CCR4<sup>-</sup>CD8<sup>+</sup> human T cells. Multi-color flow cytometric analysis revealed that CCR4<sup>-</sup>CD8<sup>+</sup> T cells were predominantly found in the CD27<sup>+</sup>CD28<sup>-</sup>CD45RA<sup>-</sup> memory subset and expressed the CCR7<sup>+</sup>/CCR5<sup>-</sup> phenotype. CCR4<sup>-</sup>CD8<sup>+</sup> T cells expressed neither perforin (Per) nor granzymes (Gra) A/B, suggesting that they were more immature memory T cells than the CCR6<sup>+</sup>CD8<sup>+</sup> early effector memory T cells that express GraA and Per. CCR4<sup>-</sup>CD8<sup>+</sup> T cells effectively produced IL-4, IFN-γ, IL-2 and tumor necrosis factor-α, indicating that they are memory T cells having the ability to secrete type 1 and type 2 cytokines. These cells also showed chemotaxis activity in response to CC chemokine receptor ligand (CCL)17/thymus and activation-regulated chemokine and CCL2/macrophage-derived chemokine. These results suggest that CCR4<sup>-</sup>CD8<sup>+</sup> T cells are in an immature memory cell T subset in the differentiation pathway of human CD8<sup>+</sup> T cells and that they migrate to inflammatory sites in the skin where they are involved in cutaneous immunity.

Introduction

CD8<sup>+</sup> T cells play an important role in the eradication of viruses and tumors. When naive CD8<sup>+</sup> T cells encounter a foreign antigen in the context of MHC class 1, they divide and differentiate into large numbers of effector CD8<sup>+</sup> cells, which exert their immune function by direct cytolysis and secretion of cytokines such as IFN-γ and tumor necrosis factor (TNF)-α (1, 2). This population of cells undergoes a programmed contraction that leaves a population of memory CD8<sup>+</sup> T cells that persist after resolution of the infection. Upon the second encounter with their specific antigens, memory CD8<sup>+</sup> T cells rapidly divide, immediately exert their effector functions and give rise to a large population of secondary effectors (3).

Memory CD8<sup>+</sup> T cells are divided roughly into central memory and effector memory subsets, which possess different capacities to home to lymphoid or non-lymphoid tissues, to proliferate in response to antigens or cytokines and to perform effector functions (3–5). Phenotypic classification of memory and effector CD8<sup>+</sup> T cells has been shown to be very useful in mouse and human studies. In humans, particular expression patterns of co-stimulatory molecules CD27, CD28 and CD45RA or CD45RO are associated with naive, memory and effector functions of CD8<sup>+</sup> T cells. Van Lier and colleagues defined two CD8<sup>+</sup> memory subsets based on the expression of CD45 isoforms and CD27: CD45RO<sup>-</sup>CD27<sup>-</sup> memory cells, which lack immediate cytotoxic function, and CD45RA<sup>-</sup>CD27<sup>-</sup> effector cells, which have low proliferative capacity and high levels of perforin (Per) and cytotoxicity (6). Appay et al. (7) proposed a linear differentiation model for memory CD8<sup>+</sup> T cells, in which ‘early’ CD27<sup>+</sup>CD28<sup>-</sup> progress to ‘intermediate’ CD27<sup>-</sup>CD28<sup>-</sup> , which then become ‘late’ CD27<sup>-</sup>CD28<sup>-</sup> memory T cells. We recently reported that memory and effector CD8<sup>+</sup> T cells could be classified into CD27<sup>+</sup>CD45RA<sup>-</sup>CCR7<sup>-</sup>CCR5<sup>-</sup> central memory, CD27<sup>+</sup>CD45RA<sup>-</sup>CCR7<sup>-</sup>CCR5<sup>+</sup> and CD27<sup>+</sup>CD45RA<sup>-</sup>CCR7<sup>-</sup>CCR5<sup>-</sup>early effector memory, CD27<sup>+</sup>CD45RA<sup>-</sup>CCR7<sup>-</sup>CCR5<sup>-</sup>late effector memory and CD27<sup>-</sup>CD28<sup>-</sup>CD45RA<sup>-</sup>CCR7<sup>-</sup>CCR5<sup>-</sup> effector cells by differential expression of cell surface markers and effector molecules (8).

Chemokine receptors are also useful to discriminate naive, memory and effector subsets in the human CD8<sup>+</sup> T cell population. For example, CCR7 is expressed on naive and memory CD8<sup>+</sup> T cells and acts as a receptor for homing of these cells to secondary lymphoid tissues (3, 4). CCR5 is predominantly expressed on memory CD8<sup>+</sup> T cells, and its expression decreases during differentiation from memory to
effect T cells: CD27<sup>+</sup>CD8<sup>-</sup>CD45RA<sup>-</sup> → CD27<sup>-</sup>CD8<sup>-</sup>CD45RA<sup>-</sup> → CD27<sup>-</sup>CD28<sup>-</sup>CD45RA<sup>-</sup> (9, 10). A recent study demonstrated that CXC chemokine receptor (CXCR)1 is expressed on effector and effector memory CD8<sup>-</sup> T cells with phenotypes of CD27<sup>-</sup>CD28<sup>+</sup>CD45RA<sup>-</sup> and CD27<sup>-</sup>CD28<sup>-</sup>CD45RA<sup>-</sup><sup>-</sup>, respectively. CXCR4 is predominantly expressed on naive CD27<sup>-</sup>CD28<sup>-</sup>CD45RA<sup>-</sup> CD8<sup>-</sup> T cells, and its expression decreases as the cells differentiate from naive to memory and effector CD8<sup>-</sup> T cells (11, 12). CXCR3 is mainly expressed on almost naive and memory CD8<sup>-</sup> T cells, especially on those of CCR7<sup>+</sup>-CCR5<sup>-</sup>CD27<sup>-</sup>CD28<sup>-</sup>CD45RA<sup>-</sup>- memory subsets (13). In addition, another study demonstrated that CCR6 is expressed on human effector memory CD8<sup>-</sup> T cells with the CD27<sup>-</sup>CD28<sup>-</sup>CD45RA<sup>-</sup> phenotype (14).

CCR4 is a chemokine receptor for thymus and activation-regulated chemokine (TARC) and macrophage-derived chemokine (MDC) (15, 16). It has been proposed to be a preferential marker for T<sub>p</sub>2 cells, and thus, CCR4 and its ligands may function as regulators of T<sub>p</sub>2 cells. CCR4 is selectively expressed on the majority of peripheral memory T<sub>p</sub>2 cells and antigen presenting cells that produce TARC and MDC (17). Its expression is increased in most skin-homing T<sub>p</sub>2 cells that express cutaneous lymphocyte-associated antigen (CLA) (18, 19). In addition to CD4<sup>+</sup> T cells, CD8<sup>-</sup> T cells also express CCR4 in atopic dermatitis (AD), psoriasis and adult T cell leukemia/lymphoma (19–22). In psoriasis patients, the frequencies of peripheral CD4<sup>+</sup> and CD8<sup>-</sup> T cells expressing CCR4 are significantly higher than those in healthy donors, and the expression of CCR4 in epidermal T cells is even higher than that in peripheral ones (20). These observations suggest an important role for CCR4<sup>+</sup>CD8<sup>-</sup> T cells in the pathogenesis of cutaneous diseases. However, the phenotype and function of human CD8<sup>-</sup> T cells expressing CCR4 have not been clearly defined.

In the present study, we investigated the expression and function of CCR4 on human CD8<sup>-</sup> T cells and characterized CCR4<sup>+</sup>CD8<sup>-</sup> T cells. We here show that CCR4 was expressed on a part of the memory cell population and that CCR4<sup>+</sup>CD8<sup>-</sup> memory T cells had the ability to produce multiple cytokines including IL-4 and to migrate in response to CC chemokine receptor ligand (CCL)17/TARC and CCL22/MDC.

**Materials and methods**

**Blood samples**

Blood samples were taken from healthy adult individuals. For analysis of EBV-specific and human cytomegalovirus (HCMV)-specific CD8<sup>-</sup> T cells, samples were obtained from EBV-seropositive individuals with HLA-A*1101 and those 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**

Antibodies were obtained from BD Biosciences (San Diego, CA, USA) except for the following ones: FITC-labeled anti-CD3 mAb was purchased from Dako Cytomation (Glostrup, Denmark). Energy-coupled dye (ECD)-labeled anti-CD3, ECD-labeled anti-CD45RA and ECD-labeled anti-CD28 mAbs were obtained from Immunotech (Marseille, France). Cascade Blue-labeled/Cascade Yellow-labeled anti-CD8 mAbs were prepared by conjugating anti-CD8 mAb with Cascade Blue and Cascade Yellow (Molecular Probes, Eugene, OR, USA), respectively.

**HLA-class I tetramer**

HLA-class I–peptide tetrameric complexes (tetramers) were synthesized as previously described (23). EBV CTL epitopes [EBV nuclear antigen 3B (EBNA3B) 416–424; IVTDFSVK (24)] and HCMV CTL epitope [HCMV-1 pp65 495-503: NLVPVMVAT (25)] were used for the refolding of HLA-A*1101 and HLA-A*0201 molecules, respectively. PE-labeled streptavidin was used for generation of the tetramers (Molecular Probes).

**Flow cytometric analysis**

PBMC from healthy individuals were stained with anti-CCR4 mAbs for 30 min at room temperature (RT). After having been washed with PBS containing 10% newborn calf serum (PBS/10% NCS), the cells were stained with anti-CD8 and anti-CD3 and anti-CD8 mAbs for 30 min at 4°C and then washed twice with PBS/10% NCS. The percentage of CCR4<sup>+</sup> cells in the CD3<sup>+</sup>CD8<sup>-</sup> sub-population was measured by using a FACS-Calibur<sup>TM</sup> flow cytometer (BD Biosciences). The percentage of CCR4<sup>+</sup> cells was determined by following the isotype control for negative control in all experiments.

To investigate CCR4 expression in each CD27CD28CD45RA subset in the total CD8<sup>-</sup> T cell population, we stained PBMC with anti-CCR4 mAbs for 30 min at RT. Subsequently, they were washed with PBS/10% NCS and stained with anti-CD27, anti-CD8, anti-CD45RA and anti-CD8 mAbs for 30 min at 4°C followed by two washes with PBS/10% NCS. PE-labeled mouse IgG was used as negative control. The percentage of CCR4<sup>+</sup> cells in each subset was measured by using an LSR II flow cytometer (BD Biosciences).

To determine CCR7, CCR6 and CCR5 expression on the CCR4<sup>+</sup>CD8<sup>-</sup> T cell population, we stained PBMC with anti-CCR7, anti-CCR6, anti-CCR5 and anti-CCR4 mAbs for 30 min at RT. After the cells had been washed with PBS/10% NCS, they were then stained with anti-CD8 mAb for 30 min at 4°C. FITC-, PE-, PE Cy7- and allophycocyanin (APC)-labeled mouse IgGs were used as negative controls.

For determination of CCR4 expression in the CCR7CCR5 subset in each CD27CD28CD45RA subset, PBMC were stained with anti-CCR7, anti-CCR5 and anti-CCR4 mAbs for 30 min at RT. They were subsequently washed with PBS/10% NCS and then incubated with anti-CD27, anti-CD8, anti-CD45RA and anti-CD8 mAbs for 30 min at 4°C. Stained cells were analyzed by use of the LSR II flow cytometer. FITC-, PE- and PE Cy7-labeled mouse IgGs were used as negative controls.

To examine the intracellular expression of Per, granzyme (Gra) A and GraB in CCR4<sup>+</sup>, CCR7<sup>+</sup> and CCR6<sup>+</sup> subsets of CD27<sup>high</sup>/lowCD28<sup>-</sup>CD45RA<sup>-</sup> CD8<sup>-</sup> T cells, first we stained PBMC with anti-CD8, anti-CD27, anti-CD45RA and anti-CCR4 mAbs. Subsequently, the cells were fixed with 4% PFA at 4°C for 20 min and then permeabilized at
4°C for 10 min with PBS containing 0.1% saponin and 20% NCS (permeabilizing buffer) and thereafter washed with the permeabilizing buffer. After having been stained with anti-Per and anti-GraA mAbs or anti-GraA and anti-GraB mAbs at 4°C for 30 min, they were finally washed three times in the permeabilizing buffer at 4°C: FITC-, PE-, PE Cy7- and APC-labeled mouse IgGs were used as negative controls.

To clarify the expression of CCR4 on EBV- and HCMV-specific CD8+ T cells, we incubated PBMC with EBV-A*1101 and HCMV-A*0201 tetramers for 30 min at 37°C. The cells were washed twice with RPMI/10% NCS and then stained with anti-CCR5 and anti-CCR4 mAbs for 30 min at RT. After having been washed with PBS/10% NCS, the cells were stained anti-CD27, anti-CD28, anti-CD45RA and anti-CD8 mAbs for 30 min at 4°C. The percentage of CCR4+ cells in each subset was measured by using the FACSaria™.

**Cytokine production by CCR4+ and CCR7+ subsets of CD45RA−/CD8+ T cells**

To measure cytokine production by CCR4+ and CCR7+ subsets of CD45RA−/CD8+ T cells, we purified CD8+ T cells from PBMC by using anti-CD8-coated magnetic beads (Miltenyi Biotec, Gladbach, Germany). The purified CD8+ T cells (>98%) were stained with anti-CD45RA, anti-CD8 and either anti-CCR4 or anti-CCR7 mAbs, and then CCR4+/CD45RA−/CD8+ and CCR7+/CD45RA−/CD8+ T cells were separated by a cell sorter (FACSaria; BD Biosciences, San Jose, CA, USA). The sorted T cell subsets were cultured for 6 h in F-bottom 96-well plates with or without phorbol myristate acetate (PMA) (10 ng ml−1)/ionomycin (1 μg ml−1) in RPMI containing 10% FCS (R10 medium) in the presence of brefeldin A (10 μg ml−1). The cells were fixed with 4% PFA at 4°C for 20 min and then permeabilized at 4°C for 20 min. Next, they were re-suspended in the same buffer and stained with anti-IFN-γ, anti-IL-2, TNF-α and IL-4 mAb at RT for 30 min. Thereafter, they were washed three times in the permeabilizing buffer at 4°C. We also used FITC-, PE-, PE Cy7- and APC-labeled mouse IgGs for isotype controls. The cells were finally re-suspended in PBS containing 2% PFA, and the cytokine profile was analyzed by using a FACSaria.

**Migration assay**

The chemotaxis was measured by a TAXIScan holder (Effector Cell Institute, Tokyo, Japan) as previously described (26). Chemokines used in this study were 100 μg ml−1 of human recombinant TARC, 50 μg ml−1 of human recombinant MDC and 100 μg ml−1 stem cell factor (SDF)-1α (PeproTech House, London, UK). A glass plate was coated with RPMI/40% FCS before assembly of the TAXIScan chamber. After 180 min, we counted the number of sorted cells that had migrated toward the middle of the channel by using a TAXIScan analyzer (Effector Cell Institute).

**Results**

**Functional expression of CCR4 on human memory CD8+ T cells**

We first investigated the surface expression of CCR4 on CD8+ T cells from 15 healthy individuals by using anti-CD8, anti-CD3 and anti-CCR4 mAbs. A representative result of flow cytometric analysis is shown in the left panel of Fig. 1(A). The expression of CCR4 varied among the 15 individuals, ranging from 0.6 to 4.8% (mean ± SD, 2.3 ± 1.5%; Fig. 1A) of the total CD8+ T cell population. These results suggest that a limited number of CD8+ T cells expressed CCR4 in a given CD8+ T cell subset.

A previous study showed that CD8+ T cells could be classified into naive cells, memory cells, effector memory cells and effector cells based on the expression patterns of three cell surface markers, i.e. CD27, CD28 and CD45RA (6, 7, 9). To identify populations expressing CCR4, we investigated the surface expression of CCR4 in each CD27+CD28+CD45RA− subset of CD8+ T cells. PBMC were isolated from seven healthy donors and then analyzed by flow cytometry after staining with anti-CCR4, anti-CD8, anti-CD45RA, anti-CD27 and anti-CD28 mAbs. A representative result is shown in Fig. 1(B). Almost all the CCR4+ cells were found in the CD27+CD28+CD45RA− and CD27−CD28+CD45RA− subsets. This result was confirmed by analysis of the other six individuals (Fig. 1C). Taken together, these results show that CCR4 was expressed in the memory subsets. We next analyzed the CD27+CD28+CD45RA+ expression on CCR4+CD8+ T cells. Approximately 60% of the CCR4+CD8+ T cells were included in the CD27+CD28+CD45RA− subset and another 20% in each of the CD27+CD28−CD45RA− and CD27−CD28+CD45RA− subsets (data not shown), supporting our idea that CCR4 was expressed in the memory subsets.

**EBV-specific CD8+ T cells have the CD27+CD28+CD45RA− memory phenotype and fail to kill target cells, whereas HCMV-specific CD8+ T cells have the CD27−CD28−CD45RA−/− effector phenotype or CD27lowCD28−CD45RA+/− effector memory phenotype and the ability to kill the target cells (9, 10).** Our previous study showed that EBV- and HCMV-specific CD8+ T cells in the peripheral blood expressed CCR5, but neither CCR7 nor CCR6 (14). It implied that EBV-specific CD8+ T cells would express CCR4 and HCMV-specific ones would not. Therefore, we next analyzed the expression of CCR4 on EBV- and HCMV-specific CD8+ T cells. The representative result was shown in Fig. 1(D). EBNA3B- and HCMV-specific CD8+ T cells did not express CCR4, whereas these subsets predominantly express CCR5. We also investigated the CCR4 expression on EBV-specific CD27+CD28+CD45RA−/− CD8+ T cells and HCMV-specific CD27−CD28−CD45RA−/− CD8+ T cells or CD27low−CD28−CD45RA−/− CD8+ T cells by eight-color flow cytometry. Each subset of EBV- and HCMV-specific CD8+ T cells did not express CCR4 (data not shown). These results indicate that EBV-specific CD8+ T cells have CCR4−CCR5+CCR6−/CCR7+CD27+CD28+CD45RA− memory phenotype.

CCR7 and CCR5 are predominantly expressed on naive and central memory CD8+ T cells and on effector memory ones, respectively (9, 10), whereas CCR6 was shown to be expressed on early effector memory CD8+ T cells (14). To examine the correlation between the expression of CCR4 and that of the other three receptors, we next investigated the co-expression of CCR4 and CCR7, CCR6 or CCR5 on CD8+ T cells from six healthy donors. A representative result of flow cytometric analysis is shown in Fig. 2(A). The result subjects showed that 25.0 ± 9.8 and 40.6 ± 9.6% of the
Fig. 1. Surface expression of CCR4 on CD8+ T cells. (A) Surface expression of CCR4 on human CD8+ T cells. PBMC isolated from a healthy donor, U-16, were stained with PE-labeled anti-CCR4, ECD-labeled anti-CD3 and FITC-labeled anti-CD8 mAbs and then analyzed by flow cytometry. The CD3+CD8+ T cells were analyzed for surface expression of CCR4. Variation of CCR4 expression on the CD3+CD8+ T cells in 15 healthy individuals is noted. The mean percentage and standard deviation (SD) of CCR4+ cells among the CD3+CD8+ T cell population are
Function of human CCR4+CD8+ T cells

CCR4+CD8+ T cell subset expressed CCR5 and CCR7, respectively, and that a small population of CCR4+CD8+ T cells expressed CCR6 (6.3 ± 2.8%; data not shown). These results demonstrate that CCR4+CD8+ T cells expressed CCR7 or CCR5 but not CCR6.

![Graph](image1)

**Fig. 2.** Co-expression of CCR5, CCR7, CCR6 and CCR4 on CD8+ T cells and CD27high/lowCD8+CD45RA−CD8+ T cells. (A) Co-expression of CCR4 and CCR5, CCR6 or CCR7 on CD8+ T cells. PBMC from individual U-16 were stained with APC-labeled and Cascade Blue-labeled anti-CD8, PE-labeled anti-CCR4, FITC-labeled anti-CCR5, Alexa Fluor®647-labeled anti-CCR6 and PE Cy7-labeled anti-CCR7 mAbs or with mouse IgG mAb as a negative control. The CCR6 subset was gated and then analyzed for the expression of CCR4 and CCR5, CCR6 or CCR7. The percentage of each CCR4CCR5, CCR4CCR6 or CCR4CCR7 subset in the CD8+ T cell population is shown in each plot. (B) Surface expression of CCR4 on CCR5CCR7 subsets of CD27high/lowCD8+CD45RA−CD8+ T cells. PBMC from individual U-27 were stained with Cascade Blue-labeled anti-CD8, ECD-labeled anti-CD45RA, APC Cy7-labeled anti-CD27, APC-labeled anti-CD28, PE Cy7-labeled anti-CCR7, FITC-labeled anti-CCR6 and PE-labeled anti-CCR4 mAbs (gray) or mouse IgG mAb as negative control (bold line). The CCR7CCR5 subsets of the CD27high/lowCD8+CD45RA−CD8+ T cells were gated and then analyzed for the expression of CCR4 on each of them. The percentages of each CCR7CCR5 subset in the CD27high/lowCD8+CD45RA−CD8+ T cell populations and of CCR4 subset from a representative individual are shown in each plot.

CCR4+CD8+ T cells expressed the CD27+CD28+CD45RA− memory phenotype, and some of these CCR4+ cells expressed CCR7 or CCR5. CCR7+CD8+ T cells are naive T cells and central memory T cells. CCR5+CD8+ T cells are predominantly found in the effector memory T cell population (9, 10). To better understand the differentiation state of CCR4+CD8+ T cells in the CD27+CD28+CD45RA− memory subset, we analyzed the PBMC from five healthy individuals by seven-color flow cytometric analysis with anti-CCR4, anti-CCR5, anti-CCR7, anti-CD27, anti-CD28, anti-CD45RA and anti-CD8 mAbs. A representative result is shown in Fig. 2(B). The results from five subjects showed that 45.2 ± 10.6 and 25.6 ± 13.0% of CCR7CCR5− and CCR7CCR5− cells, respectively, expressed CCR4 in the CD27+CD28+CD45RA−CD8+ T cell subset. In contrast, a small population of the CCR7CCR5+CD27+CD28+CD45RA−CD8+ T cell subset expressed CCR4 (4.5 ± 2.5%; data not shown). Similar results were obtained for the CD27highCD28+CD45RA− memory CD8+ T cell population, which contains fewer mature CD8+ T cells than the CD27highCD28+CD45RA− memory subset. These findings confirm that CCR4 was expressed on CD8+ T cells with the CCR5−CD27+CD28+CD45RA− phenotype and suggest the expression of CCR4 on a part of central memory and effector memory T cell populations.

CCR4 is a specific receptor for TARC/CCL17 and MDC/CCL22 produced by macrophages and dendritic cells (DCs). Therefore, we examined the chemotaxic response of CCR5−CD27+CD28+CD45RA−CD8+ T cells to TARC, MDC, and SDF-1 by using a TAXIScan holder. Both CCR5− and CCR5+ cells in the CD27+CD28+CD45RA−CD8+ T cell population were sorted from the PBMC of healthy individuals and examined for their migration activities in response to TARC, MDC and SDF-1 (Fig. 3). Since both populations expressed CCR4, SDF-1 was used as a positive control. Of the sorted CCR5−CD27+CD28+CD45RA−CD8+ T cells, 20.3% expressed CCR4, whereas only 5.2% of the sorted CCR5+CD27−CD28+CD45RA−CD8+ T cells expressed it. TARC, MDC and SDF-1 induced the chemotaxis of the CCR5−CD27+CD28+CD45RA−CD8+ T cells. In contrast, the CCR5+CD27+CD28+CD45RA−CD8+ T cells responded to SDF-1 but not to TARC or MDC. These findings indicate that CCR4− memory CD8+ T cells expressing the CCR5−CD27+CD28+CD45RA−CD8+ phenotype had chemotactic activity in response to CCR4 ligands, indicating that CCR4 functions as a chemokine receptor on these cells.

**Expression of Per and GraA in CCR4+, CCR7+ or CCR6+CD8+ T cells**

Central memory, early effector memory and late effector memory CD8+ T cells express the phenotypes of presented in the figure. (B) and (C) PBMC were isolated from seven healthy donors and then stained with Cascade Blue-labeled anti-CD8, APC Cy7-labeled anti-CD27, FITC-labeled anti-CD28, ECD-labeled anti-CD45RA and PE-labeled anti-CCR4 mAbs. Each CD27+CD28+CD45RA− subset was gated and then analyzed for the expression of CCR4 on it. The percentage of CCR4+ cells in each subset from individual U-16 is shown in each plot. The percentage and SD of CCR4+ cells in each subset from seven individuals are shown in (C). (D) Surface expression of CCR4 on EBV- and HCMV-specific CD8+ T cells. PBMC from healthy donor having HLA-A*1101 or HLA-A*0201 were stained with anti-CD8, anti-CD45RA, anti-CD27, anti-CD28, anti-CCR4 and anti-CD45RA mAbs as well as with HLA-A*1101-restricted EBNA3B 416-specific or with the HLA-A*0201-restricted pp65 495-specific tetramer. We used the mouse IgG mAb as a negative control. The percentage of tetramer+ subsets in the CD8+ T cell population is shown in the figure. The CD8+ tetramer+ and total CD8+ subset were gated and then analyzed for the expression of CCR4 and CCR5. The percentage of CCR4+ cells in each subset is shown in each plot.
Per−GraA−GraB−, Per−lowGraA+GraB− and PerlowGraA+GraB+, respectively (8). To determine the functional differences among CCR4+, CCR7- and CCR6-expressing CD27+CD28+CD45RA−CD8+ T cells, we investigated Per and GraA expression in each subset from a single individual. In the CD27+CD28+CD45RA− population, 66.1% of the CCR4+ cells and 75.7% of the CCR7+ cells expressed neither Per nor GraA. In contrast, 83.7% of the CCR6+ subset expressed GraA and a low level of Per (Fig. 4A). Similar results were also found for the CD27+CD28+CD45RA− memory population. We further analyzed GraB expression and found that the CCR4+ subset of CD27+CD28+CD45RA−CD8+ T cells did not express GraB (data not shown). These results were confirmed with an experiment using the PBMC from two other individuals (Fig. 4B). These observations indicate that CCR4+CD8+ and CCR7+CD8+ T cells were less differentiated than CCR6+ ones.

**Different capability of CCR4+CD45RA− and CCR7+CD45RA− memory CD8+ T cells to produce cytokines**

We found similar expression patterns of Per and GraA in CD27+CD28+CD45RA−CD8+ T cells expressing CCR4 or CCR7. To determine the difference in functional ability in the CCR4+ and CCR7+ subsets of CD8+ T cells, we sorted the CCR4+ and CCR7+ subsets and then measured the amount of IFN-γ, IL-2 and TNF-α secretion after PMA and ionomycin stimulation. Data from individual U-34 are shown in Fig. 5(A). Of the CCR4+ cells, 34.1, 49.5, 55.4 and 54.3% produced IFN-γ, IL-2, TNF-α and IL-4, respectively. CCR4+CD45RA−CD8+ T cells producing IL-2, TNF-α or IL-4 were greater in number than CCR7+ ones producing these cytokines. Similar results were found in the analysis of four individuals, though a significant difference in TNF-α and IL-4 production was found between the CCR4+ and CCR7+ subsets (Fig. 5B).

To examine the combinations of cytokines produced by CCR4+CD45RA−CD8+ cells, we analyzed the production of four cytokines by U-26 PBMC by using seven-color flow cytometry with anti-CD45RA, anti-IFN-γ, anti-IL-2, anti-TNF-α, anti-IL-4, anti-CD8 and anti-CCR4 or anti-CCR7 mAbs. More than 50% of the CCR4+CD45RA−CD8+ cells produced three or four cytokines: 30.3, 10.4 and 17.4% of these subsets and then measured the amount of IFN-γ, IL-2 and TNF-α secretion after PMA and ionomycin stimulation. Data from individual U-34 are shown in Fig. 5(A). Of the CCR4+ cells, 34.1, 49.5, 55.4 and 54.3% produced IFN-γ, IL-2, TNF-α and IL-4, respectively. CCR4+CD45RA−CD8+ T cells producing IL-2, TNF-α or IL-4 were greater in number than CCR7+ ones producing these cytokines. Similar results were found in the analysis of four individuals, though a significant difference in TNF-α and IL-4 production was found between the CCR4+ and CCR7+ subsets (Fig. 5B).
CCR7^+CD45RA^+CD8^+ T cells (Fig. 5C and D). The same results were found in a different experiment using memory T cells from other individuals (data not shown). These data indicate that CCR4^+CD8^+ cells were memory subsets having the ability to produce multiple cytokines including type 2 ones.

**Discussion**

Phenotypic classification of human CD8^+ T cells is necessary to investigate the differentiation of human memory CD8^+ T cells and to analyze cell-mediated immunity in various diseases (6–8). Previous studies revealed that human memory CD8^+ T cells can be characterized by the
CD27*CD28*CD45RA− phenotype (7, 8, 10), though early study showed that surface expression of CCR7 discriminates central memory (CCR7*CD45RA−) from effector memory (CCR7−CD45RA−) subsets (4). Although these surface markers are useful tools for the phenotypic classification of the human memory CD8+ T cells, there seems to be no clear consensus regarding the pathway of central/effector memory T cell differentiation. Studies using ex vivo phenotypic analysis of human CD8+ T cells in viral infections (7–9) suggest that the CCR7*CD27*CD28*CD45RA− subset includes...
heterogeneous sub-populations. A recent study showed that CCR6 is a cell surface marker for early effector memory CD8+ T cells (14). In the present study, we demonstrated that CCR4 was predominantly expressed on CCR7+CCR5+ and CCR7+CCR5− subsets of CD27+CD8+CD45RA- memory CD8+ T cells, but not on CCR6+ early effector memory subsets. Thus, CCR4 can be a useful marker to identify detailed subsets of CD8+ memory T cells.

The CCR4+CD8+ and CCR7+CD8+ T cells did not express Per, GraA or Grab, whereas CCR6+CD8+ T cells expressed a high level of GraA and a low one of Per. The data on these effector molecules indicate that CCR4+CD8+ T cells are less differentiated than CCR6+CD8+ memory T cells, but we could not discriminate the difference between CCR4+CD8+ and CCR7+CD8+ T cells. However, analysis of cytokine production showed that the CCR4+ memory CD8+ T cells were polynuclear memory ones and produced more TNF-α and IL-4 than CCR7+CD8+ T cells. A previous study demonstrated that CCR6+ memory CD8+ T cells effectively produced IFN-γ and TNF-α (14). These findings together indicate that CCR4+CD8+ T cells are a more less differentiated than CCR7+ central memory ones and less differentiated than CCR6+ early effector memory ones.

Previous studies showed that CCR4+CCR7+CD8+ T cells have the ability to produce IL-4 but not IFN-γ and possess no cytotoxic activity (27, 28). These T cells were classified as type 2 CD8+ T cells. In the present study, we showed that ~40% of the CCR4+CD8+ T cells secreted IL-4 and that the majority of these T cells consisted of three subsets producing four (IFN-γ/L-2/TNF-α/L-4) or three (IL-2/TNF-α/L-4 or IFN-γ/L-2/TNF-α) cytokines. These results indicate that the human CCR4+CD8+ cell population consisted of three functional CD8+ T cells: type 1 CD8+ T cells producing IFN-γ, IL-2 and TNF-α, type 2 CD8+ T cells secreting IL-2, TNF-α and IL-4, and polyfunctional CD8+ T cells producing IFN-γ, IL-2, TNF-α and IL-4. Judging from a previous finding that CCR4+CCR7+CD8+ T cells have the ability to produce IL-4 but not IFN-γ (27, 28), we hypothesize that the type 2 CD8+ T cells may express the CCR4+CCR7+ phenotype.

Earlier studies demonstrated that EBV-specific CD8+ T cells carry the memory phenotype (10, 29) but that HCMV-specific CD8+ T cells express both effector memory and effector phenotypes (30). Therefore, we speculate that EBV-specific CD8+ T cells expressed CCR7, CCR6 or CCR4. Our previous study showed that EBV-specific CD8+ T cells in the peripheral blood expressed neither CCR7 nor CCR6 (14). In the present study, we found no expression of CCR4 on EBV-specific CD8+ T cells in the peripheral blood (Fig. 1D). These results together indicate that the EBV antigen-specific CD8+ T cells were not only central memory CD8+ T cells but also early effector memory CD8+ T cells.

Our present study demonstrated the effective migration of CCR4+ memory CD8+ T cells in response to TARC and production of IFN-γ and TNF-α in these cells. CCR4 and its ligand TARC are involved in lymphocyte-endothelial interactions during lymphocyte recruitment to normal and inflamed cutaneous sites (18, 31). A previous study using the human HaCaT cell line as a model for human keratinocytes showed that these cells produced TARC in response to IFN-γ or TNF-α (32). An in vivo study using NC/Nga mice as a model for human AD confirmed that TARC was produced mainly by the keratinocytes in the basal layer of the skin, whereas MDC was produced by dermal DCs (33). In extrinsic AD patients, most of CLA+CD8+ T cells express CCR4 (19). These results suggest the possibility that CCR4+CD8+ T cells can migrate into the secondary lymphoid organs, and they mature after interacting with DCs expressing CCR4 ligand. These mature CCR4+CD8+ T cells may migrate into inflamed skin and secrete IFN-γ and TNF-α, which cytokines then induce TARC and MDC production by the keratinocytes and dermal DCs. Finally, these TARC and MDC ligands enhance the migration of a number of Tc2 cells to the site of inflammation. Thus, we assume that CCR4+ memory CD8+ T cells would play an important role in the pathogenesis of AD.

In summary, we showed that CCR4+CD8+ T cells had the characteristics of memory subsets secreting type 1 and type 2 cytokines but not expressing effector molecules and that the expression of CCR4 was down-regulated during the differentiation of human memory CD8+ T cells. The expression pattern of this receptor, together with that of other chemokine receptors, is shown in Fig. 6. It is useful for functional classification of human memory T cells. The classification of memory CD8+ T cell subsets using the expression pattern of these chemokine receptors will help to clarify the differentiation and maturation pathway of human memory T cells.

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Function of human CCR4+CD8+ T cells

Abbreviations

AD atopic dermatitis
APC aliphycocyanin
CCL CC chemokine receptor ligand
CCR CC chemokine receptor
CLA cutaneous lymphocyte-associated antigen
CXCR CXC chemokine receptor
DC dendritic cell
ECD energy-coupled dye
EBNA3B EBV nuclear antigen 3B
Gra granzyme
HCMV human cytomegalovirus
MDC macrophage-derived chemokine
NCS newborn calf serum
Per perforin
PMA phorbol myristate acetate
RT room temperature
SDF stem cell factor
TARC thymus and activation-regulated chemokine
TNF tumor necrosis factor

References