Functional Expression of the Chemokine Receptor CCR5 on Virus Epitope-Specific Memory and Effector CD8$^+$ T Cells[1]

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Because the chemokine receptor CCR5 is expressed on Th1 CD4$^+$ cells, it is important to investigate the expression and function of this receptor on other T cells involved in Th1 immune responses, such as Ag-specific CD8$^+$ T cells, which to date have been only partially characterized. Therefore, we analyzed the expression and function of CCR5 on virus-specific CD8$^+$ T cells identified by HLA class I tetramers. Multicolor flow cytometry analysis demonstrated that CCR5 is expressed on memory (CD28$^+$CD45RA$^-$) and effector (CD28$^-$CD45RA$^-$ and CD28$^-$CD45RA$^+$) CD8$^+$ T cells but not on naive (CD28$^+$CD45RA$^+$) CD8$^+$ T cells. CCR5 expression was much lower on two effector CD8$^+$ T cells than on memory CD8$^+$ T cells. Analysis of CCR7 and CCR5 expression on the different types of CD8$^+$ T cells showed that memory CD8$^+$ T cells have three phenotypic subsets, CCR5$^+$CCR7$^-$, CCR5$^+$CCR7$^+$, and CCR5$^-$CCR7$^+$, while naive and effector CD8$^+$ T cells have CCR5$^-$CCR7$^+$ and CCR5$^+$CCR7$^-$ phenotypes, respectively. These results suggest the following sequence for differentiation of memory CD8$^+$ T cells: CCR5$^-$CCR7$^+$→CCR5$^+$CCR7$^+$→CCR5$^+$CCR7$^-$. CCR5$^+$CD8$^+$ T cells effectively migrated in response to RANTES, suggesting that CCR5 plays a critical role in the migration of Ag-specific effector and differentiated memory CD8$^+$ T cells to inflammatory tissues and secondary lymphoid tissues. This is in contrast to CCR7, which functions as a homing receptor in migration of naive and memory CD8$^+$ T cells to secondary lymphoid tissues. *The Journal of Immunology*, **2002, 168: 2225–2232.**

Chemokines play well-defined roles in attracting monocytes and immature dendritic cells to sites of inflammation and in directing maturing APCs to lymphatic vessels as part of the initiation of immune responses (1). The expression and function of chemokine receptors on T cells have mostly been investigated using CD4$^+$ T cells (2), while the situation on CD8$^+$ T cells is less well characterized.

CCR5 is a receptor for β-chemokines RANTES and macrophage-inflammatory protein (MIP)[3]-1α and -1β, and acts as a coreceptor for HIV-1 entry (3–5). The expression of this receptor has been well characterized in CD4$^+$ T cells: in peripheral blood CCR5 is expressed only on Th1 CD4$^+$ T cells (6), while in thymus it is not expressed on CD3$^-$ immature thymocytes but is expressed at low levels on CD3$^+$CD4$^-$CD8$^+$ and CD3$^+$CD4$^+$CD8$^-$ thymocytes (7). A few studies have examined CCR5 expression in CD8$^+$ T cells. In mice, Tc1 effector CD8$^+$ T cells, which produce type 1 cytokines, predominantly express CCR5 mRNA, while Tc2 effector CD8$^+$ T cells, which produce type 2 cytokines, express high levels of CXCR4 mRNA but not CCR5 mRNA (8–10). In humans, previous studies showed that a subset of CD8$^+$ T cells expresses CCR5 (11–14). However, the expression and function of this chemokine receptor on human CD8$^+$ T cells remain unclear.

CCR7 is another chemokine receptor and functions as a homing receptor in migration of naive and memory CD8$^+$ T cells to secondary lymphoid tissues. A role for CCR7 in CD8$^+$ T cells has been demonstrated through the identification of CCR7$^+$CD45RA$^+$ naive CD8$^+$ T cells and CCR7$^+$CD45RA$^-$ and CCR7$^-$CD45RA$^-$ memory CD8$^+$ T cells (15). Furthermore, a recent study suggested the following differentiation lineage for Ag-specific CD8$^+$ T cells: CCR7$^+$ CD45RA$^+$→CCR7$^+$ CD45RA$^-$→CCR7$^-$ CD45RA$^-$→CCR7$^-$CD45RA$^+$ (16). This study suggested that CCR7$^+$ CD45RA$^+$CD8$^+$ and CCR7$^+$CD45RA$^-$CD8$^+$ T cells are precursor memory T cells while CCR7$^-$CD45RA$^-$CD8$^+$ and CCR7$^-$ CD45RA$^+$CD8$^+$ T cells are preterminally differentiated and terminally differentiated cells, respectively.

In the present study, we examined in detail the surface expression of CCR5 and CCR7 on CD28CD45RA subpopulations in human total CD8$^+$ T cells and Ag-specific CD8$^+$ T cells. In addition, the function of CCR5 on human CD8$^+$ T cells was also analyzed. We suggest a different role for CCR5 and CCR7 in the Th1 immune response.

Materials and Methods

Abs and reagents

Anti-human CCR7 mAb (2H4), FITC-conjugated anti-human CCR5 mAb (2D7), PE- and allophycocyanin-conjugated anti-human CD28 mAb, CyChrome-conjugated anti-human CD45RA mAb, PerCP-conjugated anti-human CD8 mAb, and FITC- and biotin-conjugated rat anti-mouse IgM mAb were purchased from BD PharMingen (San Diego, CA). Allophycocyanin-conjugated anti-human CD8 mAb was purchased from Beckman Coulter (Fullerton, CA). PE-conjugated extravidin was purchased from Sigma Chemical (Irvine, CA). The anti-human CCR5 mAb (45531.111) used for the lymphocyte chemotaxis assay was purchased from DAKO (Kyoto, Japan). Recombinant human RANTES/CCL5 and MIP-1β were purchased from R&D Systems (Minneapolis, MN).

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[3] Abbreviations used in this paper: MIP, macrophage-inflammatory protein; HCMV, human CMV; HCV, hepatitis C virus; GVH, graft-vs-host; MFI, mean fluorescence intensity.

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Blood samples

PBMCs from 13 individuals with chronic HIV-1 infection were analyzed in this study. Blood samples were collected with oral informed consent from HLA-A11$^+$, HLA-A24$^+$, and/or HLA-B35$^+$ HIV-1-seropositive individuals at the International Medical Center of Japan (Tokyo, Japan). All these individuals belonged to the clinical stage of asymptomatic carrier or AIDS-related complex. PBMCs from 11 HIV-1-seronegative healthy subjects were also analyzed in this study. Two HLA-A*0201 samples were used for analysis of human CMV (HCMV)-specific CD8$^+$ T cells. Blood samples were also collected with oral informed consent.

Synthetic peptides

Sixteen CTL epitope peptides were synthesized using an automated multiple peptide synthesizer (Shimadzu Model PSSM-8; Shimadzu, Kyoto, Japan). These peptides were as follows: an HLA-A*0201-restricted HCMV epitope (HCMV-pp65 495–503 NLVPMVATV) (17), an HLA-B*3501-restricted hepatitis C virus (HCV) epitope (HCV-NS3 1359–1367 HPNIE EVAL) (18), two HLA-A*1101-restricted HIV epitopes (HIV-Pol 313–321 AIFQSSMTK, and -Nef 84–92 AVDLSHFLK) (19, 20), five HLA-A*2402-restricted HIV epitopes (HIV-Nef 138–147 RYPLTFGWCF, -Nef 138–147-2F RFPLTFGWCF, -env 584–592 RYLRDQQLL, -env 679–687 WYIKIFIMI, and -Gag 28–36 KYKLKHIVW) (21, 22), and five HLA-B*3501-restricted HIV epitopes (HIV-Pol 273–282 VPLDKD FRKY, -Nef 75–85 RPQVPLRPMTY, -Pol 448–456 IPLTEEAEL, -Pol 587–595 EPIVGAETF, and -env 77–85 DPNPQEVVL) (23) and two HLA-A*1101-restricted EBV epitopes (EBV-3B 416–424: IVTDFSVIK, and EBV-3B 399–408: AVFDRKSDAK) (24). All peptides were verified to be >90% pure by mass spectrometry and HPLC.

Production of HLA class I/peptide tetramers

HLA-A*0201, HLA-A*1101, HLA-A*2402, and HLA-B*3501/peptide tetrameric complexes were prepared as previously described (25, 26). Briefly, recombinant human β_2 microglobulin and recombinant HLA class I derivatives (COOH termini of HLA class I molecules with truncated transmembrane and cytoplasmic domains, and with a sequence containing the BirA enzymatic biotinylation site) were purified from *Escherichia coli* cells transformed with the relevant expression plasmid. Monomeric complexes were generated by in vitro refolding of human β_2 microglobulin, the appropriate HLA class I derivative, and an HIV-1-specific, EBV-specific, or HCMV-specific epitope peptide. The resultant 45-kDa complexes were separated by gel filtration using a Superdex G75 column (Amersham Pharmacia Biotech, Uppsala, Sweden) and then biotinylated enzymatically with BirA enzyme (Avidity, Denver, CO). The biotin-binding complexes were separated by gel filtration using a Superdex G75 column followed by anion exchange using a Mono Q column (Amersham Pharmacia Biotech). HLA class I/peptide tetramers were generated by mixing the monomer complexes with PE-conjugated extravidin (Sigma Chemical) or allophycocyanin-conjugated streptavidin (BD PharMingen) at a molar ratio of 4:1.

CTL clones

Three HIV-specific CTL clones for HIV-Pol 448–456, HIV-Nef 75–85, and HIV-env 679–687 (HIV-B35-SF2–24-55, HIV-B35-SF2–6-101, HIV-A24-SF2-env 679–687-3, respectively) and an HCV-specific CTL clone for HCV-NS3 1359–1367 (HCV-B35–38-20) were generated previously (18, 21, 23).

Flow cytometry analyses

Cryopreserved PBMCs from HIV-1-seropositive individuals and healthy subjects were stained with mAbs and/or tetramers in various combinations. PBMCs from HIV-1-seronegative and -seropositive individuals were stained with FITC-conjugated anti-CCR5 mAb and PerCP-conjugated anti-CD8 mAb, with those from HIV-1-seropositive individuals further stained with allophycocyanin- or PE-conjugated tetramer(s). Incubation with tetramer(s) and subsequent washing were performed at 37°C to avoid nonspecific binding to CD8$^+$ T cells, as previously described (27). For four-color flow cytometry, PBMCs isolated from healthy individuals were stained with FITC-conjugated anti-CCR5, PE-conjugated anti-CD28, CyChrome-conjugated anti-CD45RA, and allophycocyanin-conjugated anti-CD8. Similarly, after CD8$^+$ T cells were purified from PBMCs of HIV-1-seronegative and -seropositive individuals using the MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany), these cells were stained with FITC-conjugated anti-CCR5 mAb, PE-conjugated anti-CD28 mAb, CyChrome-conjugated anti-CD45RA mAb, and allophycocyanin-conjugated tetramer(s) for HCMV and HIV-1, respectively. CTL clones were stained with FITC-conjugated anti-CCR5 mAb and PerCP-conjugated anti-CD8 mAb. To investigate CCR7 expression on CD8$^+$ T cells and Ag-specific CD8$^+$ T cells, purified CD8$^+$ T cells from HCMV-seropositive healthy individuals were stained with anti-CCR7 mAb followed by FITC-conjugated rat anti-mouse IgM mAb, PE-conjugated anti-CD28 mAb, CyChrome-conjugated anti-CD45RA mAb, and allophycocyanin-conjugated tetramer for HCMV. To investigate coexpression of CCR7 and CCR5 on CD8$^+$ T cells, purified CD8$^+$ T cells from HCMV-seropositive healthy individuals were stained with anti-CCR7 mAb followed by biotin-conjugated rat anti-mouse IgM mAb and then PE-conjugated extravidin, FITC-conjugated anti-CCR5 mAb, CyChrome-conjugated anti-CD45RA mAb, and allophycocyanin-conjugated anti-CD28 mAb. Cells were washed three times with PBS supplemented with 10% FCS and then resuspended with PBS containing 2% paraformaldehyde. Samples were analyzed by FACSCalibur with CellQuest software (BD Biosciences, San Jose, CA).

CTL assay

CTL activity was measured by a standard ^{51}Cr release assay as follows. The HLA-A,-B defective cell line C1R as well as the C1R transfectants C1R-A*1101 and C1R-A*0201 (5×10^5 cells) were incubated for 60 min with 3.7 M Bq $Na_2{}^{51}CrO_4$ in saline and then washed three times with RPMI 1640 medium containing 10% newborn calf serum. Labeled target cells (2×10^3/well) were added into U-bottom 96-well microtiter plates with 10 μM HCMV (HCMV-pp65 495–503) or two EBV (EBV-3B 416–424 and EBV 3B 399–408) peptides. After incubation for 1 h, CD8$^+$ T cells purified from PBMC of EBV-seropositive, HLA-A11$^+$ individuals by anti-CD8 mAb-coated beads or CD28$^-$CD45RA$^-$CD8$^+$ and CD28$^-$CD45RA$^+$CD8$^+$ T cells purified from PBMC of HCMV-seropositive, HLA-A2$^+$ individual by a cell sorter (FACSVantage SE; BD Biosciences) were added at various effector (tetramer$^+$CD8$^+$ T cells):target ratios. The mixtures were incubated for 6 h at 37°C and then the supernatants were collected and analyzed with a gamma counter. Spontaneous ^{51}Cr release was determined by measuring the cpm in supernatants from wells containing only target cells (cpm spn). Maximum ^{51}Cr release was determined by measuring the cpm in supernatants from wells containing target cells in the presence of 2.5% Triton X-100 (cpm max). Specific lysis = ((cpm exp − cpm spn)/(cpm max − cpm spn)) × 100, where cpm exp is the cpm in supernatants of wells containing both target and effector cells.

Lymphocyte chemotaxis assay

The lymphocyte chemotaxis assay was performed using 96-well microchemotaxis chambers (NeuroProbe, Gaithersburg, MD) as previously described (28). The MACS magnetic cell separation system was used to isolate CD8$^+$ T cells from fresh PBMCs taken from healthy individuals. More than 98% of the purified cells were CD8$^+$ T cells. The purified CD8$^+$ T cells (5×10^5 cells/well) were placed over the filter (5-μm pore diameter) in the upper wells of the chamber. Recombinant human RANTES/CCL5 and an anti-human CCR5 mAb were diluted with RPMI 1640 and then applied to either the upper or lower wells of the chamber. After incubation at 37°C for 2 h, cells remaining above the filter (i.e., cells that did not migrate) were removed by washing with PBS containing 2 mM EDTA and wiping. Cells that had migrated to the lower well of the chamber and below the filter were collected by centrifugation. These cells were counted using a hemocytometer and then stained with PE-conjugated anti-CD28 mAb and CyChrome-conjugated anti-CD45RA mAb for flow cytometry analysis. The percentage of migrated cells with each CD28/CD45RA phenotype was determined as follows: the total number of cells with each phenotype added to the upper well was calculated from total number of cells added to the upper well and the percentage of each phenotype determined by FACS analysis. The migrated cell number of each phenotype was calculated from both the total number of cells migrated to the lower well and the percentage of each phenotype. The percentage of the migrated cells with each phenotype = (migrated cell number with each phenotype/total cell number added to the upper wells with each phenotype) × 100.

Results

Expression of CCR5 on HIV-1-specific CD8$^+$ T cells

We used flow cytometry to investigate the surface expression of CCR5 on total CD8$^+$ T cells in PBMCs isolated from 11 healthy individuals. A significant number of total CD8$^+$ T cells expressed CCR5 (Fig. 1*A*). Expression varied among individuals, with the per-

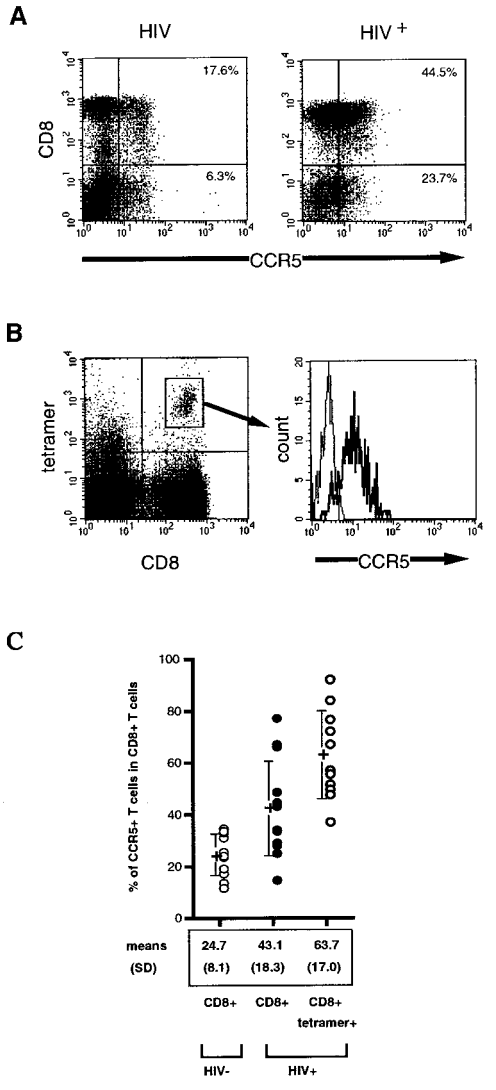

FIGURE 1. CCR5 expression on total $CD8^+$ T cells and HIV-1-specific $CD8^+$ T cells from HIV-1-seronegative healthy and HIV-1-seropositive individuals. *A*, The surface expression of CCR5 on $CD8^+$ T cells from an HIV-1-seronegative healthy individual (HIV^-) and an HIV-1-seropositive individual (HIV^+) was examined by flow cytometry following staining of PBMCs isolated from these individuals with anti-CD8 mAb and anti-CCR5 mAb. The number in each *upper right quadrant* and *lower right quadrant* is the percentage of CCR5-positive cells in total $CD8^+$ or total $CD8^-$ cells, respectively. *B*, PBMCs isolated from an HIV-1-seropositive individual (KI-003) were costained with three HLA-B*3501 tetramers (HIV-Pol 273–282, -Pol 587–595, and -env 77–85), anti-CD8 mAb, and anti-CCR5 mAb and analyzed by flow cytometry. A total of 0.83% of total $CD8^+$ T cells were tetramer positive. $Tetramer^+CD8^+$ T cells were analyzed for expression of CCR5. The mean fluorescence intensity (MFI) of $tetramer^+CD8^+$ T cells stained with and without anti-CCR5 mAb was 10.2 and 2.4, respectively. *C*, PBMCs from 11 HIV-1-seronegative healthy and 13 HIV-1-seropositive individuals were costained with HLA-A*1101, HLA-A*2402, and/or HLA-B*3501 tetramers, anti-CD8 mAb, and anti-CCR5 mAb and then analyzed by flow cytometry. The percentage of $CCR5^+CD8^+$ cells in $CD8^+$ T cells from HIV-1-seronegative healthy individuals (HIV^-) and HIV-1-seropositive individuals (HIV^+) was determined. The percentage of $CCR5^+$ in HIV-1-specific $CD8^+$ T cells from HIV-1-seropositive individuals ($CD8^+tetramer^+$) was determined by gating $tetramer^+CD8^+$ T cells. The numbers under the plots are the mean percentage and SD of $CCR5^+$ cells in each population.

centage of $CCR5^+CD8^+$ T cells in the total $CD8^+$ T cell population ranging from 11.9 to 34.9% (mean, 24.7 ± 8.1%; Fig. 1*C*). These results indicate that a subset of $CD8^+$ T cells expresses CCR5.

To determine whether Ag-specific $CD8^+$ T cells express CCR5, we investigated the surface expression of this receptor on $CD8^+$ T cells in PBMCs isolated from 13 HIV-1-seropositive individuals. Compared with HIV-1-seronegative healthy individuals, the number of $CCR5^+CD8^+$ T cells was increased in HIV-1-seropositive individuals (Fig. 1, *A* and *C*), with the percentage of these cells in the total $CD8^+$ T cell population ranging from 15.1 to 77.3% (mean, 43.1 ± 18.3%; Fig. 1*C*). As the number of HIV-1-specific $CD8^+$ T cells is thought to increase in HIV-1-infected individuals, these results suggest that most HIV-1-specific $CD8^+$ T cells express CCR5. Therefore, we investigated the surface expression of CCR5 on HIV-1-specific $CD8^+$ T cells in PBMCs isolated from HIV-1-seropositive individuals using HLA-A*2402, HLA-A*1101, and/or HLA-B*3501 tetramers. Results from a representative individual (KI-003) are shown in Fig. 1*B*. HIV-1-specific $CD8^+$ T cells were detected by the three HLA-B*3501 tetramers in PBMCs from KI-003 (Fig. 1*B*, *left panel*). These $tetramer^+CD8^+$ T cells expressed CCR5 (Fig. 1*B*, *right panel*). Analysis of PBMCs from 11 HIV-1-infected individuals showed that the majority of HIV-1-specific $CD8^+$ T cells expressed CCR5 (mean, 63.7 ± 17.0%; Fig. 1*C*). CCR5 expression was similarly observed on HCMV-specific $CD8^+$ T cells (data not shown). CCR5 was also expressed on HIV-1-specific and HCV-specific $CD8^+$ CTL clones (Fig. 2), further confirming that Ag-specific $CD8^+$ T cells express CCR5.

CCR5 expression changes during $CD8^+$ T cell differentiation

A previous study showed that $CD27^+CD45RA^+CD8^+$ T cells have neither perforin nor cytolytic activity while $CD27^+CD45RA^-CD8^+$, $CD27^-CD45RA^-CD8^+$, and $CD27^-CD45RA^+CD8^+$ T cells have

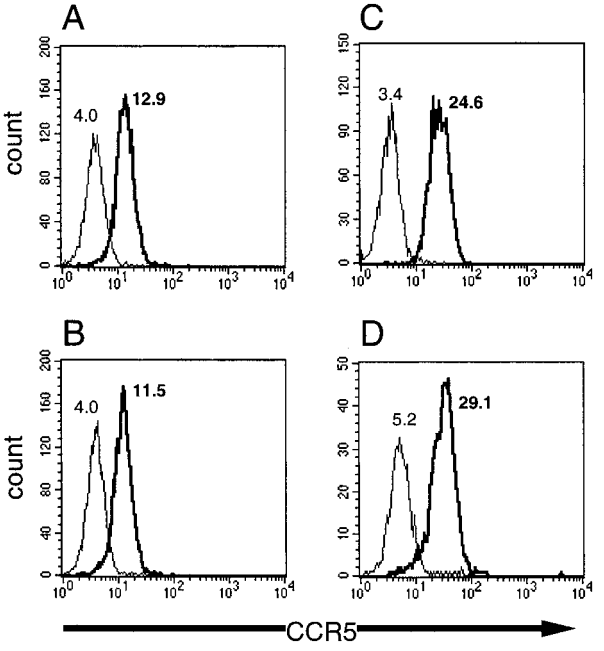

FIGURE 2. CCR5 expression on HIV-1-specific and HCV-specific $CD8^+$ T cell clones. Three HIV-1-specific CTL clones, HIV-B35-SF2–24-55 (Pol 448–456-specific and HLA-B35-restricted) (*A*), HIV-B35-SF2–6-101 (Nef 75–85-specific and HLA-B35-restricted) (*B*), and HIV-A24-SF2-env 679–687-3 (env 679–687-specific and HLA-A24-restricted) (*C*), and an HCV-specific CTL clone, HCV-B35–38-20 (NS3 1359–1367-specific and HLA-B35-restricted) (*D*) were costained with anti-CD8 mAb and anti-CCR5 mAb and analyzed by flow cytometry. $CD8^+$ T cells were analyzed for expression of CCR5. The numbers show the MFI of $CD8^+$ T cells stained with (bold text) or without (normal text) anti-CCR5 mAb.

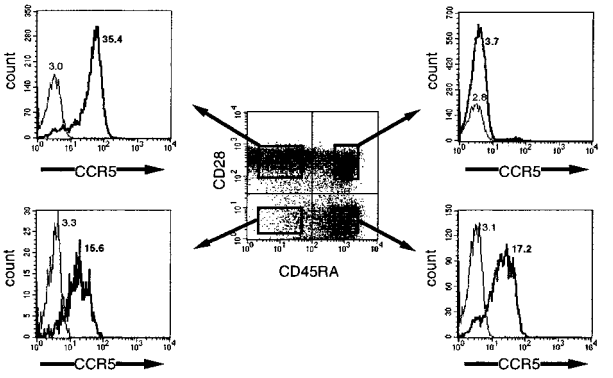

FIGURE 3. CCR5 expression on CD8$^+$ T cells with different CD28/CD45RA phenotypes from an HIV-1-seronegative healthy individual. PBMCs from an HIV-1-seronegative healthy individual (U-7) were costained with anti-CD8, anti-CD28, anti-CD45RA, and anti-CCR5 mAb and analyzed by flow cytometry. CD8$^+$ T cells were first analyzed for CD28 and CD45RA expression, and then CCR5 expression on each CD28/CD45RA phenotype (CD28$^+$CD45RA$^+$, CD28$^+$CD45RA$^-$, CD28$^-$CD45RA$^-$, and CD28$^-$CD45RA$^+$ cells) was measured. The numbers show the MFI for cells of each CD28/CD45RA phenotype stained with (bold text) or without (normal text) anti-CCR5 mAb.

a low, medium, and high level of perforin, respectively, and have cytolytic activity in proportion to perforin expression (29). This study also showed that the latter three types of CD8$^+$ T cells can effectively produce cytokines. Similarly, our previous study of CD28, CD45RA, and perforin expression in CD8$^+$ T cells showed that CD28$^+$CD45RA$^+$CD8$^+$ T cells have no perforin while CD28$^+$CD45RA$^-$CD8$^+$, CD28$^-$CD45RA$^-$CD8$^+$, and CD28$^-$CD45RA$^+$CD8$^+$ T cells have a low, medium, and high level of perforin, respectively (25). Therefore, we investigated the cytolytic activity of these CD28CD45RA subsets. HCMV-specific CD28$^-$CD45RA$^-$CD8$^+$ and CD28$^-$CD45RA$^+$CD8$^+$ T cells effectively lysed target cells pulsed with HCMV epitope peptide while EBV-specific CD28$^+$CD45RA$^-$CD8$^+$ T cells failed to kill target cells pulsed with EBV epitope peptide (data not shown). Thus, naive, memory, and effector CD8$^+$ T cells can be discriminated by their different surface expression of CD28(CD27) and CD45RA (CD28$^+$(CD27$^+$)CD45RA$^+$, naive cells; CD28$^+$(CD27$^+$)CD45RA$^-$, memory cells; CD28$^-$(CD27$^-$)CD45RA$^-$, memory/effector cells; and CD28$^-$(CD27$^-$)CD45RA$^+$, effector cells) (25, 29, 30).

The observation that the level of CCR5 expression on CD8$^+$ T cells varied among individuals suggests that CCR5 may be expressed at different levels on naive, memory, and/or effector CD8$^+$ T cells. Therefore, we examined CCR5 expression in these four CD8$^+$ T cell populations. PBMCs from one healthy individual were analyzed by four-color flow cytometry analysis using four mAbs (anti-CD8, anti-CD28, anti-CD45RA, and anti-CCR5 mAbs). CD28$^-$CD45RA$^-$CD8$^+$ and CD28$^-$CD45RA$^+$CD8$^+$ T cells expressed moderate levels of CCR5 while CD28$^+$CD45RA$^-$CD8$^+$ T cells expressed high levels of CCR5. In contrast, CD28$^+$CD45RA$^+$CD8$^+$ T cells did not express CCR5 (Fig. 3). The same results were observed in CD8$^+$ T cells from eight HIV-1-seronegative healthy individuals (Table I).

We also investigated CCR5 expression in the four CD8$^+$ T cell populations using Ag-specific CD8$^+$ T cells isolated from HIV-1-seropositive individuals and HCMV-seropositive individuals. CD8$^+$ T cells were isolated from PBMCs using anti-CD8 mAb-coated immunobeads, costained with tetramers, anti-CD28 mAb, anti-CD45RA mAb, and anti-CCR5 mAb, and analyzed by flow cytometry. These analyses showed that in HIV-1- and HCMV-specific T cells, CD28$^+$CD45RA$^-$CD8$^+$ T cells express high levels of CCR5 while CD28$^-$CD45RA$^-$CD8$^+$ and CD28$^-$CD45RA$^+$CD8$^+$ T cells express lower levels of CCR5 (Fig. 4). Thus, in both HIV-1-seropositive and HCMV-seropositive individuals, CCR5 expression increases during differentiation of naive CD8$^+$ T cells to memory CD8$^+$ T cells and then decreases during differentiation to effector CD8$^+$ T cells.

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

Recent studies demonstrated that CCR7 is expressed on naive (CD27$^+$CD45RA$^+$) and memory (CD27$^+$CD45RA$^-$) CD8$^+$ T cells (30). We analyzed CCR7 expression on the different CD8$^+$ T cell populations from three healthy individuals. Results from a representative individual are shown in Fig. 5*A*. CD28$^+$CD45RA$^+$CD8$^+$ T cells and ~50% of CD28$^+$CD45RA$^-$CD8$^+$ T cells express CCR7 while CD28$^-$CD45RA$^-$CD8$^+$ and CD28$^-$CD45RA$^+$CD8$^+$ T cells did not express CCR7. Thus, we confirmed that naive and memory CD8$^+$ T cells express CCR7. To clarify whether Ag-specific memory CD8$^+$ T cells express CCR7, we analyzed CCR7 expression on HCMV-specific CD8$^+$ T cells isolated from two HCMV-seropositive individuals. These results showed that in HCMV-specific CD8$^+$ T cells, CD28$^-$CD45RA$^-$CD8$^+$ and CD28$^-$CD45RA$^+$CD8$^+$ T cells did not express CCR7 while a small population of CD28$^+$CD45RA$^-$CD8$^+$ T cells did express CCR7 (Fig. 5*B*), suggesting that HCMV-specific memory CD8$^+$ T cells may be well differentiated.

A large percentage of memory T cells expressed CCR5 while only ~50% expressed CCR7, suggesting that the memory CD8$^+$ T cell population has at least two different subsets. Therefore, we further analyzed expression of CCR5 and CCR7 on CD28$^+$CD45RA$^-$CD8$^+$ T cells. Results from three individuals demonstrated the existence of three subsets of CD28$^+$CD45RA$^-$ memory CD8$^+$ T cells: CCR5$^+$CCR7$^-$, CCR5$^-$CCR7$^+$, and CCR5$^+$CCR7$^+$ (Fig. 6). The expression level of CCR5 in the CCR5$^+$CCR7$^-$ subset was higher than that in the CCR5$^+$CCR7$^+$ subset, while the expression level of CCR7 in the CCR5$^-$CCR7$^+$ subset was higher than that in the CCR5$^+$CCR7$^+$ subset. CCR5 was expressed on CD28$^-$CD45RA$^-$CD8$^+$ T cells but not on CD28$^+$CD45RA$^+$CD8$^+$ T cells, while CCR7 was expressed on CD28$^+$CD45RA$^+$CD8$^+$ T cells but not on CD28$^-$CD45RA$^-$CD8$^+$ T cells. These findings strongly suggest the following differential lineage in CD28$^+$CD45RA$^-$ memory CD8$^+$ T cells: CCR5$^-$CCR7$^+$→CCR5$^+$CCR7$^+$→CCR5$^+$CCR7$^-$.

Table I. *CCR5 expression on CD8$^+$ T cells with different CD28/CD45RA phenotypes in PBMCs isolated from eight healthy individuals*

	CD28$^+$CD45RA$^+$	CD28$^+$CD45RA$^-$	CD28$^-$CD45RA$^-$	CD28$^-$CD45RA$^+$
Percentage of CCR5$^+$ T cells[a]	3.6 ± 2.6	82.6 ± 7.5	53.2 ± 20.8	42.4 ± 22.1
MFI[b]	3.1 ± 0.3	28.6 ± 9.0	9.5 ± 3.7	7.7 ± 4.4

[a] Data (mean ± SD) were calculated from the percentage of CCR5$^+$ T cells in total CD8$^+$ T cells in PBMCs isolated from eight HIV-1-seronegative, healthy individuals.

[b] MFI (mean ± SD) for CCR5 expression in CD8$^+$ T cells with each phenotype from eight HIV-1-seronegative, healthy individuals.

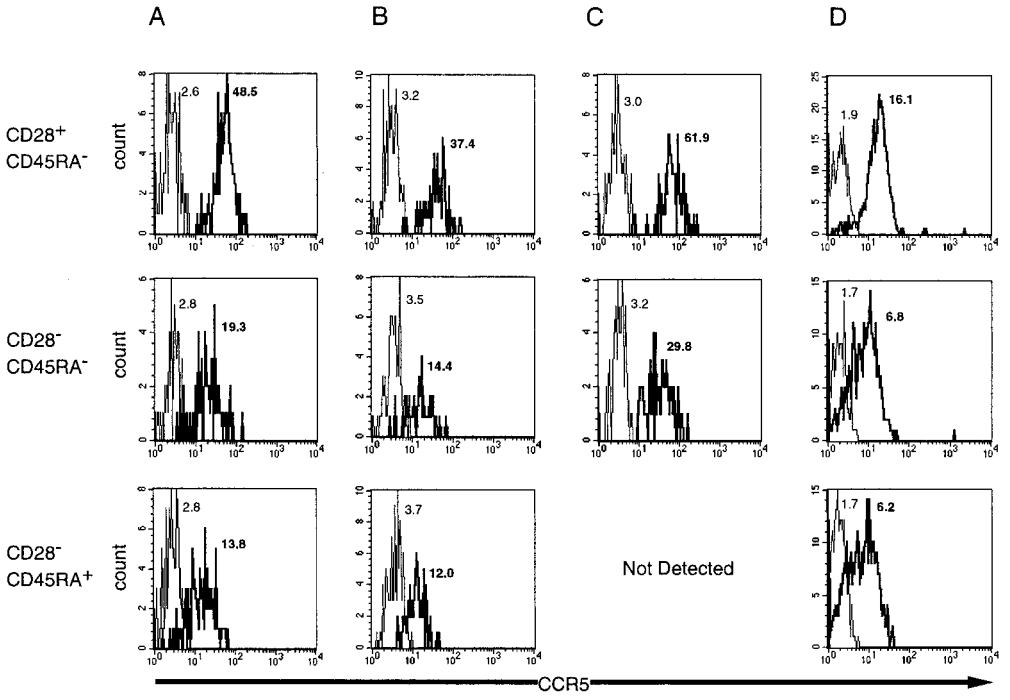

FIGURE 4. CCR5 expression on HIV-1-specific and HCMV-specific $CD8^+$ T cells with each CD28/CD45RA phenotype. $CD8^+$ T cells were purified from three HIV-1-seropositive individuals (*A*, KI-003; *B*, KI-005; and *C*, KI-011) and an HCMV-seropositive healthy individual (*D*). The purified cells (>98% pure) were costained with tetramers (HLA-A*0201, HLA-A*1101, HLA-A*2402, and/or HLA-B*3501 tetramers), anti-CD28 mAb, anti-CD45RA mAb, and anti-CCR5 mAb. $Tetramer^+CD8^+$ T cells were analyzed for CD28/CD45RA expression and then CCR5 expression in three CD28 and CD45RA phenotypes ($CD28^+CD45RA^-$, $CD28^-CD45RA^-$, and $CD28^-CD45RA^+$) was measured. The numbers show the MFI for cells of each CD28/CD45RA phenotype stained with (bold text) or without (normal text) anti-CCR5 mAb.

Effective migration of memory and effector $CD8^+$ T cells

Migration of virus-specific memory and effector $CD8^+$ T cells to regions of viral replication seems to be one of the most important events in virus eradication. As CCR5 is a receptor for the β-chemokines RANTES, MIP-1α, and MIP-1β, $CCR5^+CD8^+$ T cells could be expected to actively migrate to inflammatory regions where these chemokines are produced. To clarify the function of CCR5 in $CD8^+$ T cells, we investigated RANTES-mediated migration of $CCR5^+CD8^+$ T cells. $CD8^+$ T cells were isolated from PBMCs of a healthy individual and then added to the upper wells of a 96-well microchemotaxis chamber. The CD28/CD45RA phenotype of cells that migrated to the bottom well of the chambers was determined by staining with anti-CD28 mAb and anti-CD45RA mAb followed by flow cytometry. An effect of RANTES on $CD8^+$ T cell migration was observed for $CD28^+CD45RA^-$, $CD28^-CD45RA^-$, and $CD28^-CD45RA^+$ populations in a dose-dependent fashion (Fig. 7*A*). This effect was stronger on the $CD28^-CD45RA^+$ population than on the other two populations. These results were confirmed by three independent experiments. The effect of RANTES on these three populations was abrogated by an anti-CCR5 mAb (Fig. 7*B*). These three populations did not express CCR1, another receptor for RANTES (data not shown). MIP-1β had a similar effect on these three populations (data not shown). MIP-1β also had a weak effect on the $CD28^+CD45RA^+$ population (data not shown), suggesting that this population may express another receptor for MIP-1β. These results together suggest that memory and effector $CD8^+$ T cells can actively migrate in response to the β-chemokines RANTES and MIP-1β.

Discussion

A previous study, which investigated the functions (cytolytic activity, cytokine production, and perforin expression) of $CD8^+$ T cell CD27CD45RA subsets, suggested that $CD27^+CD45RA^+CD8^+$ T cells are naive cells, while $CD27^+CD45RA^-CD8^+$, $CD27^-CD45RA^-CD8^+$, and $CD27^-CD45RA^+CD8^+$ T cells are memory, memory/effector, and effector cells, respectively (29). This study as well as our recent study (25), which investigated perforin expression and cytolytic activity of CD28CD45RA subsets, showed the same correlation between CD28CD45RA phenotypes and functional classifications (naive, memory, memory/effector, and effector cells). In addition, we have found that $CD28^+CD45RA^-CD8^+$, $CD28^-CD45RA^-CD8^+$, and $CD28^-CD45RA^+CD8^+$ T cells have the capacity to produce cytokines (our unpublished observation). Thus, because CD28 and CD45RA are useful molecules for discriminating naive, memory, memory/effector, and effector $CD8^+$ T cells, we used these markers to investigate the expression of CCR5 and CCR7 on naive, memory, memory/effector, and effector $CD8^+$ T cells.

Expression of CCR5 on $CD8^+$ T cells is thought to be important in Th1 immune responses associated with β-chemokines. Though previous studies showed that some $CD8^+$ T cell populations express CCR5 (11–14), in this study we demonstrated that CCR5 expression is restricted to $CD28^+CD45RA^-CD8^+$, $CD28^-CD45RA^-CD8^+$, and $CD28^-CD45RA^+CD8^+$ T cell populations, i.e., memory and effector $CD8^+$ T cells but not naive $CD8^+$ T cells express CCR5. $CD8^+$ T cells have been shown to express the chemokine receptor CCR7 (15). A previous study suggested the following differentiation lineage for Ag-specific $CD8^+$ T cells: $CCR7^+CD45RA^+ \rightarrow CCR7^+CD45RA^- \rightarrow CCR7^-CD45RA^- \rightarrow CCR7^-CD45RA^+$ (16). Furthermore, a recent study showed that CCR7 is expressed on $CD27^+CD45RA^+CD8^+$ and $CD27^+CD45RA^-CD8^+$ but not on $CD27^-CD45RA^+CD8^+$ T cells (31), suggesting that CCR7 is expressed only on naive and memory $CD8^+$ T cells. The present study

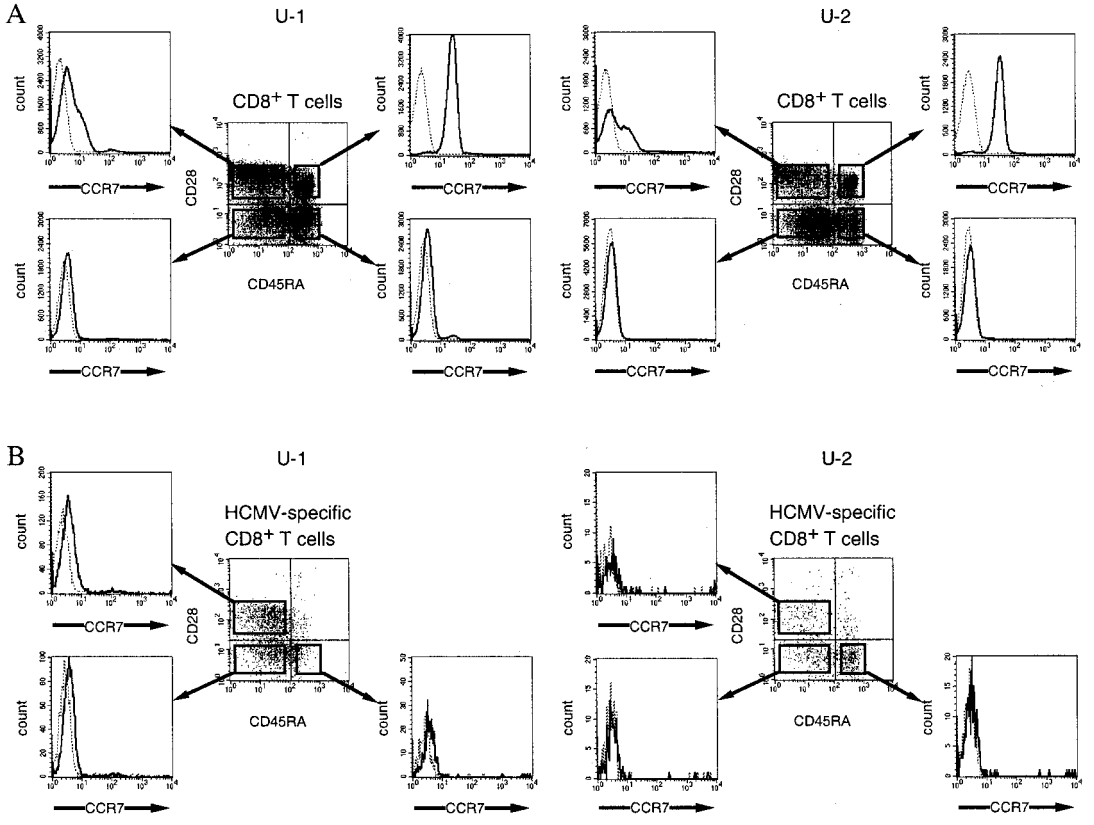

FIGURE 5. Expression of CCR7 on total CD8$^+$ T cells and virus-specific CD8$^+$ T cells with different CD28/CD45RA phenotypes. *A*, PBMCs purified from healthy individuals (U-1 and U-2) were costained with anti-CD8, anti-CD28, anti-CD45RA, and anti-CCR7 mAb and analyzed by flow cytometry. CD8$^+$ T cells were first analyzed for CD28 and CD45RA expression, and then CCR7 expression on each CD28/CD45RA phenotype (CD28$^+$CD45RA$^+$, CD28$^+$CD45RA$^-$, CD28$^-$CD45RA$^-$, and CD28$^-$CD45RA$^+$ cells) was measured. *B*, CD8$^+$ T cells were purified from HCMV-seropositive HLA-A*0201$^+$ healthy individuals (U-1 and U-2). The purified cells (>98% pure) were costained with HCMV tetramer, anti-CD28 mAb, anti-CD45RA mAb, and anti-CCR7 mAb. Tetramer$^+$CD8$^+$ T cells were analyzed for CD28 and CD45RA expression and then CCR7 expression in three CD28/CD45RA phenotypes (CD28$^+$CD45RA$^-$, CD28$^-$CD45RA$^-$, and CD28$^-$CD45RA$^+$) was measured.

also demonstrated that all CD28$^+$CD45RA$^+$CD8$^+$ T cells and approximately half of CD28$^+$CD45RA$^-$CD8$^+$ T cells express CCR7 while CD28$^-$CD45RA$^-$CD8$^+$ T cells and CD28$^-$CD45RA$^+$CD8$^+$ T cells do not. Analysis of CCR5 and CCR7 coexpression demonstrated three CD28$^+$CD45RA$^-$ memory CD8$^+$ T cell subsets, CCR5$^+$CCR7$^-$, CCR5$^-$CCR7$^+$, and CCR5$^+$CCR7$^+$. Naive and effector CD8$^+$ T cells are CCR5$^-$CCR7$^+$ and CCR5$^+$CCR7$^-$, respectively. These results indicate the following differential lineage of

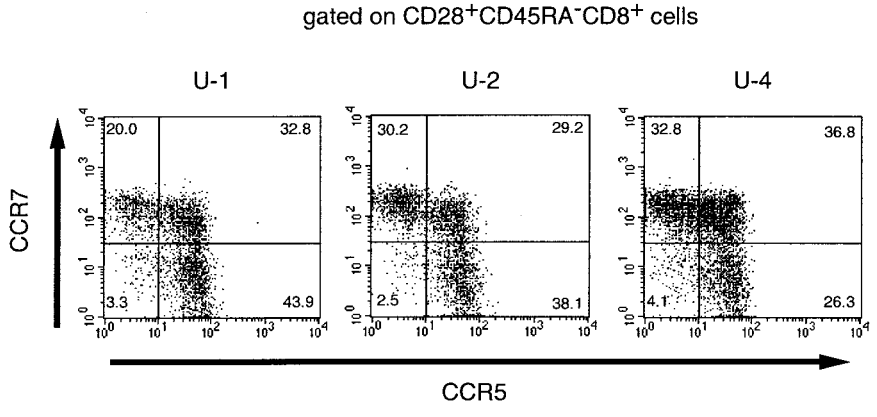

FIGURE 6. Three memory CD8$^+$ T cell subsets have different CCR5/CCR7 phenotypes. CD8$^+$ T cells were purified from three healthy individuals (U-1, U-2, and U-4). The purified cells (>98% pure) were analyzed for CD28 and CD45RA expression and then CCR5 and CCR7 expressions in four CD28/CD45RA subsets (CD28$^+$CD45RA$^+$, CD28$^+$CD45RA$^-$, CD28$^-$CD45RA$^-$, and CD28$^-$CD45RA$^+$) were measured. The numbers show the percentages of cells in each quadrant. MFI for CCR5 and CCR7 is as follows. Individual U-1: CCR5$^+$CCR7$^+$subset, CCR5/31.9, CCR7/87.9; CCR5$^-$CCR7$^+$subset, CCR5/3.7, CCR7/138.8; CCR5$^-$CCR7$^-$subset, CCR5/4.7, CCR7/9.8; CCR5$^+$CCR7$^-$subset, CCR5/44.1, CCR7/5.6. Individual U-2: CCR5$^+$CCR7$^+$subset, CCR5/29.0, CCR7/92.9; CCR5$^-$CCR7$^+$subset, CCR5/3.5, CCR7/159.1; CCR5$^-$CCR7$^-$subset, CCR5/5.0, CCR7/9.7; CCR5$^+$CCR7$^-$subset, CCR5/41.8, CCR7/5.1. Individual U-4: CCR5$^+$CCR7$^+$subset, CCR5/25.3, CCR7/109.0; CCR5$^-$CCR7$^+$subset, CCR5/3.5, CCR7/139.1; CCR5$^-$CCR7$^-$subset, CCR5/4.6, CCR7/8.1; CCR5$^+$CCR7$^-$subset, CCR5/33.6, CCR7/5.3.

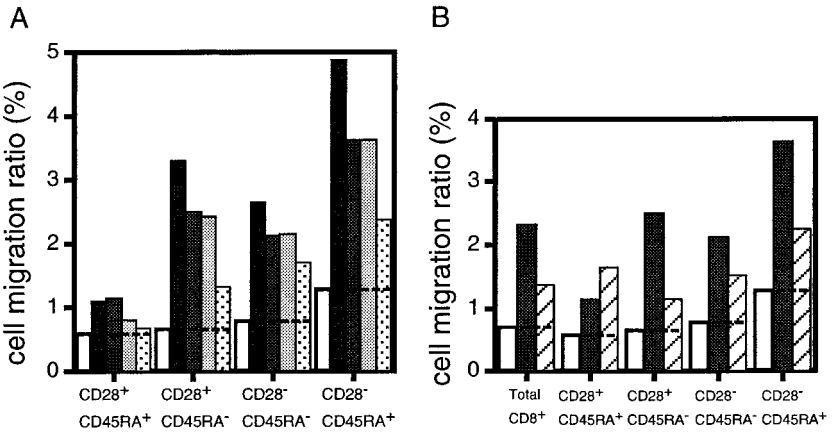

FIGURE 7. Migration of $CD8^+$ T cells with different CD28/CD45RA phenotypes in response to β-chemokines. $CD8^+$ T cells were purified from fresh PBMCs isolated from an HIV-1-seronegative healthy individual. The purified cells (>98% pure) were subjected to a lymphocyte chemotaxis assay to investigate their migration in response to β-chemokines. *A*, The percentage of $CD8^+$ T cells that migrated from the upper to lower well was determined for $CD8^+$ T cells of the different CD28/CD45RA phenotypes. Various concentrations of human RANTES/CCL5 were added to the lower wells (250, 100, 33.3, or 11.1 ng/ml, dotted bars, from *left* to *right*). Control wells contained no RANTES/CCL5 (open bar). *B*, Effect of an anti-CCR5 mAb on migration of $CD8^+$ T cells. The percentage of migrated cells was determined as described above. A total of 100 ng/ml human RANTES/CCL5 was added to the lower well (dotted bar and hatched bar). Control wells contained no RANTES/CCL5 (open bar). A total of 10 μg/ml anti-CCR5 mAb was applied into both the upper and lower wells (hatched bar).

these subsets in $CD28^+CD45RA^-CD8^+$ memory T cells: $CCR5^-CCR7^+ \rightarrow CCR5^+CCR7^+ \rightarrow CCR5^+CCR7^-$. From these results, we speculate the differentiation lineage of $CD8^+$ T cells shown in Fig. 8.

A previous study (16) suggested the following lineage differentiation pattern for Ag-specific $CD8^+$ T cells: $CCR7^+CD45RA^+ \rightarrow CCR7^+CD45RA^- \rightarrow CCR7^-CD45RA^- \rightarrow CCR7^-CD45RA^+$. This is consistent with the results in the present study except that we did not observe a $CCR7^+CD45RA^+$ subset in virus-specific $CD8^+$ T cells. However, this does not exclude the possibility that a $CCR7^+CD45RA^+$ subset might be a minor population of memory $CD8^+$ T cells. HCMV-specific $CD8^+$ T cells mostly expressed CCR5, although a small number expressed CCR7. These results strongly suggest that the majority of HCMV-specific memory $CD8^+$ T cells have a $CCR5^+CCR7^-$ phenotype. Because most HCMV-specific $CD8^+$ T cells are $CD28^-CD45RA^-CD8^+$ and $CD28^-CD45RA^+CD8^+$ T cells, these findings together support the idea that HCMV-specific $CD8^+$ T cells are well-differentiated $CD8^+$ T cells (32).

A previous study showed that $CD8^+CD4^-$ thymocytes express a low level of CCR5 (7). This study together with the present study suggests that CCR5 expression is suppressed during differentiation of $CD8^+$ thymocyte to naive $CD8^+$ T cells and to resting memory ($CCR5^-CCR7^+$) $CD8^+$ T cells in the periphery, and then induced after differentiation to $CCR5^+CCR7^+$ memory $CD8^+$ T cells. The molecular mechanism of CCR5 expression during differentiation is unknown. Further studies will address the role of CCR5 in the early stages of Ag-specific $CD8^+$ T cell differentiation.

Chemotaxis assays showed that $CCR5^+CD8^+$ T cells migrate in response to RANTES and MIP-1β, suggesting that CCR5 expressed on the surface of $CD8^+$ T cells physiologically functions as a receptor for β-chemokines. Interestingly, although CCR5 expression on $CD28^-CD45RA^+CD8^+$ T cells was much lower than on $CD28^+CD45RA^-CD8^+$ T cells and slightly lower than on $CD28^-CD45RA^-CD8^+$ T cells, the chemotactic activity of these β-chemokines was highest for $CD28^-CD45RA^+$ T cells. The mechanism of the different effect of the β-chemokines on the different $CD8^+$ T cell populations is unknown. Several explanations for this may be proposed. One is that $CD28^-CD45RA^+CD8^+$ T cells express an unknown chemokine receptor for RANTES and MIP-1β. Another is that these cells have stronger systems to convert signals via CCR5 to chemotaxis function than the other $CD8^+$ T cell populations. Further studies are expected to clarify the mechanisms of β-chemokine effect on the different $CD8^+$ T cell populations. The stronger chemotactic response on effector $CD28^-CD45RA^+CD8^+$ T cells, which express high levels of perforin, may be important for eradication of virus-infected cells and cancer cells.

A recent study of graft-vs-host (GVH) disease in mice showed that administration of anti-CCR5 mAb blocks the disease (33). This strongly suggests that alloreactive $CD8^+$ T cells associated

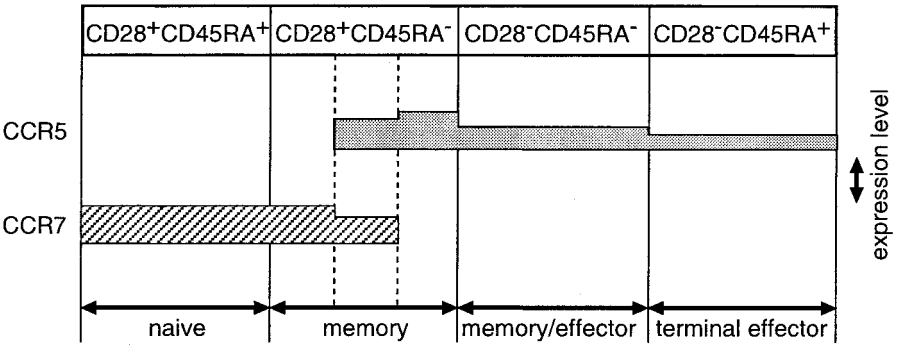

FIGURE 8. Expression of CCR5 and CCR7 on naive, memory, and effector $CD8^+$ T cells

with GVH disease express CCR5 and that the effect of β-chemokines on these cells is critical for the onset of GVH disease. Blocking the interaction between β-chemokines and CCR5 with β-chemokine analogs may be effective for prevention of GVH diseases. Because HIV-1-specific CD8$^+$ T cells with memory, memory/effector, or effector phenotype express CCR5, it is likely that CCR5 plays a role in the migration of memory and effector CD8$^+$ T cells to inflammatory tissues where virus replicates. Thus, β-chemokines for CCR5 may be important factors in the pathogenesis of GVH disease and in eradication of virus-infected cells.

In the present study we demonstrated functional expression of CCR5 on Ag-specific memory and effector CD8$^+$ T cells. Th1 CD4$^+$ T cells also express CCR5 (6), indicating that this receptor is expressed on T cells that are involved in the Th1 immune response. β-chemokines for CCR5 may attract Th1 CD4$^+$ T cells as well as memory and effector CD8$^+$ T cells to sites of inflammation. Further studies of CCR5 on CD8$^+$ T cells will help clarify the role of CCR5$^+$CD8$^+$ T cells in immune responses.

Acknowledgments

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