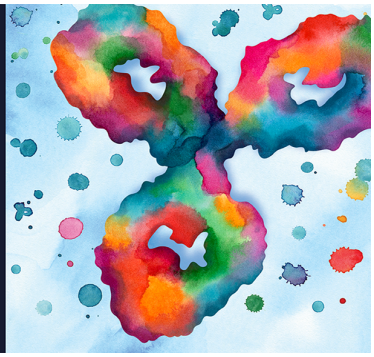

The Journal of Immunology
RESEARCH ARTICLE | JANUARY 01 1999

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J Immunol (1999) 162 (1): 186–194.
https://doi.org/10.4049/jimmunol.162.1.186

CC-Chemokine Receptor 6 Is Expressed on Diverse Memory Subsets of T Cells and Determines Responsiveness to Macrophage Inflammatory Protein 3α

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CC-chemokine receptor (CCR) 6 is the only known receptor for macrophage inflammatory protein (MIP)-3α, a CC chemokine chemotactic for lymphocytes and dendritic cells. Using anti-serum that we raised against the N-terminal residues of CCR6, we have characterized the surface expression of CCR6 on peripheral blood leukocytes and we have correlated CCR6 expression with responses to MIP-3α. We found that CCR6 was expressed only on memory T cells, including most $\alpha_4\beta_7$ memory cells and cutaneous lymphocyte-associated Ag-expressing cells, and on B cells. Accordingly, chemotaxis of T cells to MIP-3α was limited to memory cells. Moreover, calcium signals on T cells in response to MIP-3α were confined to CCR6-expressing cells, consistent with CCR6 being the only MIP-3α receptor on peripheral blood T cells. Unlike many CC chemokines, MIP-3α produced a calcium signal on freshly isolated T cells, and CCR6 expression was not increased by up to 5 days of treatment with IL-2 or by cross-linking CD3. Despite their surface expression of CCR6, freshly isolated B cells did not respond to MIP-3α. In addition to staining peripheral blood leukocytes, our anti-serum detected CCR6 on CD34$^+$ bone marrow cell-derived dendritic cells. Our data are the first to analyze surface expression of CCR6, demonstrating receptor expression on differentiated, resting memory T cells, indicating differences in receptor signaling on T cells and B cells and suggesting that CCR6 and MIP-3α may play a role in the physiology of resting memory T cells and in the interactions of memory T cells, B cells, and dendritic cells. ***The Journal of Immunology,*** **1999, 162: 186–194.**

The human chemokines now form a family of more than thirty chemotactic cytokines, whose known functional receptors, members of the seven transmembrane domain G protein-coupled receptor superfamily, number fifteen. Although the earliest studies of chemokines focused primarily on factors active on neutrophils (1) and monocytes (2), there has long been an awareness of the possible importance of chemokines in lymphocyte biology (3). This awareness has increased, related both to the discoveries of the role of the chemokine system in HIV infection (4, 5) and to the identification of an expanding group of lymphocyte-active CXC (6–10) and CC (reviewed in Ref. 11) chemokines and their receptors (9–11).

The genes for several of the novel chemokines map outside the previously described chromosomes 4 and 17 chemokine gene clusters (11, 12). A number of these chemokines are also distinguished by targeting lymphocytes to the exclusion of monocytes and neutrophils, such as the CXC chemokines IP-10 (6, 13), Mig (7), and BCA-1 (9), and the CC chemokines TARC (14), PARC/DC-CK-1 (15, 16), macrophage inflammatory protein (MIP)[3]-3β/ELC (17, 18), SLC/6-C-Kine (19, 20), and MIP-3α/Exodus/LARC (17, 21, 22). These latter CC chemokines are also of note in that several can induce calcium flux and/or chemotaxis and/or integrin-mediated adhesion in nonactivated T cells (8, 15, 16, 18–20, 22–24), consistent with a role in T cell homeostasis.

Studies of the determinants of chemokine receptor activities on lymphocytes have focused on the regulation of receptor gene and/or protein expression. Such studies have shown that some CC-chemokine receptors (CCR) require cellular activation to induce significant expression (25, 26). Recently data have emerged on the selective expression of chemokine receptors on highly differentiated Th1 and Th2 T cell subsets (27–30). The data to date on lymphocyte-active chemokines and their receptors suggest that individual receptors and their ligands will be important for particular subpopulations of lymphocytes, distinguished by states of cellular activation and pathways of differentiation.

In experiments to discover new chemokine receptors in activated T cells, we identified an orphan receptor that we named STRL22 (31). The same receptor was identified by others (32), and we and others subsequently reported that STRL22/GPR-CY4/DRY6/CKR-L3 was a receptor for the CC chemokine MIP-3α (23, 33–35), and STRL22 was renamed CCR6.

The mRNA for CCR6 was found to be expressed in lymphoid tissues, pancreas, and T and B lymphocytes (31–33, 36). Two groups reported that the CCR6 mRNA is expressed in CD34$^+$-derived dendritic cells (34, 35) and that MIP-3α is a chemotactic

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Received for publication June 24, 1998. Accepted for publication September 11, 1998.

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[3] Abbreviations used in this paper: MIP, macrophage inflammatory protein; STRL, seven transmembrane domain receptors from lymphocytes; CCR, CC-chemokine receptor; GST, glutathione *S*-transferase; PE, phycoerythrin; APC, allphycocyanin; CLA, cutaneous lymphocyte-associated Ag; SDF-1, stromal cell-derived factor 1; MCP-1, macrophage chemoattractant protein 1; CXCR, CXC-chemokine receptor.

0022-1767/99/$02.00

factor for these cells (34). The gene for CCR6 is unusual in its location on chrosome 6q27 (31), unlike the genes for CCR1-CCR5 and CCR8, which are clustered at 3p21. Similarly, CCR6 and MIP-3α are atypical among promiscuous chemokine receptors and their ligands in that thus far CCR6 is the only MIP-3α receptor identified, and MIP-3α is the sole ligand described for CCR6.

MIP-3α (17) was identified by several groups as a result of large scale sequencing projects and alternatively designated LARC (22), Exodus (21), and ckβ4 (23). MIP-3α is not very closely related to other CC chemokines, with 20–28% amino acid sequence identity with other chemokines, and, correspondingly, the MIP-3α gene is not in the major CC chemokine gene cluster at 17q11.2 but is located instead at 2q33-q37 (22). Northern blotting and analysis of cDNA libraries revealed expression of the MIP-3α gene in lung, liver, and lymphoid tissue, and in activated monocytes, endothelial cells, dendritic cells, fibroblasts, and T cells (17, 21–23). Besides its activity on dendritic cells, MIP-3α has been found to be chemotactic for freshly isolated T cells (22), to be able to inhibit colony formation by hematopoietic progenitors in vitro (21), and to be able to trigger adhesion of memory $CD4^+$ T cells to ICAM-1-coated glass (24).

To investigate the roles of CCR6 and MIP-3α in lymphocyte biology, we have raised anti-serum against the human CCR6, and we report here the first studies of surface expression of the receptor, correlating CCR6 expression on peripheral blood cells with responses to MIP-3α. Our data demonstrate that CCR6 is expressed on diverse subsets of highly differentiated, resting memory T cells as well as B cells and dendritic cells, suggesting that CCR6 and MIP-3α may be important for coordinating interactions among these cells, particularly as part of the memory response.

Materials and Methods

Raising anti-sera against CCR6

Rabbit anti-sera were raised against the N-terminal domain of CCR6 using a glutathione *S*-transferase (GST) fusion protein as immunogen. A DNA fragment encoding the first 46 residues of CCR6 and containing *Bam*HI and *Eco*RI sites at the 5′ and 3′ ends, respectively, was synthesized using a CCR6 cDNA clone (31), a standard PCR, and the primers 5′-CGCG GATCCCGACAATGAGCGGGGAATCAATG and 5′-CCGGAATTC CGGCCTGGAGAACTGCCTGACCT. After digestion with *Bam*HI and *Eco*RI, the fragment was inserted into the multiple-cloning site of the pGEX-5X-3 vector (Pharmacia Biotech, Piscataway, NJ), placing the sequences encoding the N-terminal of CCR6 3′ to those encoding the GST of *Schistosoma japonicum*. The recombinant plasmid was used to transform the BL21 strain of *Escherichia coli* (Novagen, Madsion, WI). Following growth of bacterial cultures, induction using isopropyl β-D-thiogalactoside (IPTG), and lysis of cells with lysozyme digestion and sonication in the presence of PMSF and EDTA, the GST/CCR6 fusion protein was purified using Glutathione Sepharose 4B according to the manufacturer's protocol (Pharmacia Biotech) with analysis for size and purity by SDS-PAGE. Rabbits 5145 and 5146 were immunized at multiple sites s.c. with ~200 μg of fusion protein in CFA followed by boosts using 100 μg of protein in IFA. Although both rabbits produced Abs against CCR6, all studies shown below used anti-serum 5146.

Cell culture and leukocyte preparation

Lines of HEK 293 cells transfected with pCEP4 alone or pCEP4-encoding CCR6 or the orphan receptor STRL33 were cultured as described previously (23, 36). Elutriated lymphocytes or buffy coats were collected from normal donors by the Department of Transfusion Medicine at the National Institutes of Health and PBMC were purified by Ficoll-Paque (Pharmacia Biotech). For staining of cells in whole blood, samples were collected from normal donors after informed consent under a clinical research protocol approved by the National Institute of Allergy and Infectious Diseases. For preparation of dendritic cells, bone marrow was aspirated from the iliac crest of a normal donor after informed consent under a human use protocol approved by the National Cancer Institute. $CD34^+$ cells were isolated and dendritic cells were derived as described (37, 38) except that at 6 days cells were transferred to serum-substituted medium containing 10 ng/ml granulocyte-macrophage-CSF, 10 ng/ml TNF-α, and 1 ng/ml IL-4 (37) and cells were harvested at 12 days. To activate T lymphocytes, PBMC were cultured in RPMI 1640 (Life Technologies, Gaithersburg, MD) plus 10% FBS (Life Technologies) either in the presence of 5 μg/ml of anti-CD3 Ab (OKT3, OrthoBiotech, Raritan, NJ) for 3 days or in the presence of 400 U/ml of human rIL-2 (Hoffmann-La Roche, Nutley, NJ) for 5 days.

Flow cytometry

The mAbs used and their suppliers were: FITC-conjugated Ab to HLA-DR, phycoerythrin (PE)-conjugated Abs to CD19 or HLA-DR, Cy-5-conjugated Ab to CD14, and allphycocyanin (APC)-conjugated Abs to CD4 or CD8 from Caltag Laboratory (Burlingame, CA); PE-conjugated Ab to CD56 from Coulter (Miami, FL); Cy-5-conjugated Abs to CD4 and CD19 from Sigma (St. Louis, MO) and Life Technologies, respectively; FITC-conjugated Ab to cutaneous lymphocyte-associated Ag (CLA) (HECA 452) (39) kindly provided by L. J. Picker; FITC-conjugated mAb to $\alpha_4\beta_7$ (40) kindly provided by A. Lazarovits and LeukoSite (Cambridge, MA); FITC-conjugated Abs to CD8, CD14, and CD16, PE-conjugated Abs to CD5, CD4, CD8, CD1a, CD45R0, and CD26, and Cy-5-conjugated Ab to HLA-DR from PharMingen (San Diego, CA).

To detect surface expression of CCR6 on transfected cells and leukocytes, indirect immunostaining and flow cytometry were performed. For staining transfected cells, 10^6 HEK 293 cells were resuspended in 100 μl of PBS containing 1% FBS and 10 mM HEPES and were incubated with preimmune or immune serum to CCR6 (1:10 dilution) for 1 h at room temperature, washed three times, and stained with FITC-conjugated, affinity purified $F(ab')_2$ goat anti-rabbit IgG (Caltag Laboratory) for 15 min at room temperature. After three washes, cells were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA). For staining PBMC, cells were first incubated with the Fab of anti-FcγRII mAb IV.3 (Medarex, Annandale, NJ) followed by preimmune or immune serum to CCR6 at room temperature for 1 h, washed three times, and stained for 15 min at room temperature with FITC- or PE-conjugated affinity-purified $F(ab')_2$ goat anti-rabbit IgG plus two additional mAbs to leukocyte Ag conjugated with either PE or FITC and Cy-5. After washing, cells were analyzed for immunofluorescence using either a FACScan or FACScalibur flow cytometer (Becton Dickinson). For four-color immunofluorescent analysis, an additional leukocyte marker conjugated with APC was added.

CCR6 expression on NK cells, monocytes, eosinophils, and neutrophils was analyzed by whole blood staining. For staining of NK cells and monocytes, 100 μl of whole blood was preincubated with anti-human FcγRII and anti-human FcγRIII for 30 min at 4°C followed by preimmune serum or immune serum to CCR6 at 4°C for 1 h. Cells were washed with buffer containing PBS/0.2% BSA/0.1%NaN_3 and stained for the NK cell marker CD56, or the monocyte marker CD14, plus FITC- or PE-conjugated affinity-purified $F(ab')_2$ goat anti-rabbit IgG. After a 30-min incubation at 4°C, the cells were washed, and the RBC were lysed using 1× FACS lysing solution (Becton Dickinson) for 12 min at room temperature. After additional washing, cells were resuspended in 250 μl of buffer and analyzed for immunofluorescence using a FACScalibur flow cytometer (Becton Dickinson).

For staining eosinophils and neutrophils, 100 μl of whole blood was incubated with anti-CD16-FITC for 30 min at 4°C followed by preimmune or immune serum to CCR6 at 4°C for 1 h. After washing, cells were stained with PE-conjugated affinity-purified $F(ab')_2$ goat anti-rabbit IgG for 30 min at 4°C and lysed with 1× FACS lysing buffer as described above. Eosinophils were distinguished from neutrophils based on side scatter and their failing to stain with anti-CD16.

Dendritic cells were preincubated with anti-human FcγRII for 15 min at 4°C followed by preimmune serum or immune serum to CCR6 at 4°C for 45 min, followed by FITC-conjugated affinity-purified $F(ab')_2$ goat anti-rabbit IgG plus anti-CD1a-PE and anti-HLA-DR-Cy-5 or anti-CD14-Cy-5 and anti-HLA-DR-PE.

Assaying calcium flux

Calcium flux in lymphocytes was measured using multiparameter FACS analysis as will be described in detail elsewhere (R. L. Rabin, M. Park, F. Liao, Ruth Swofford, D. Stephany, and J. M. Farber, manuscript in preparation). Briefly, PBMC were resuspended in HBSS containing 1%FBS and 10 mM HEPES and loaded with 10 μM Indo-1, AM plus 300 μg/ml pluronic acid (Molecular Probes, Eugene, Oregon) at 30°C for 45 min with constant shaking. Dynabeads M-450 CD14 (Dynal, Lake Success, NY) were added during the loading with dye to remove monocytes. Cells were washed three times, and stained with fluorophore-conjugated Abs to T cell markers including anti-CD8 FITC, anti-CD45RO PE, and anti-CD4 Cy-5. Calcium flux in T cell subsets was detected using a FACSVantage (Becton Dickinson) dual-laser flow cytometer with a Time Zero injection module

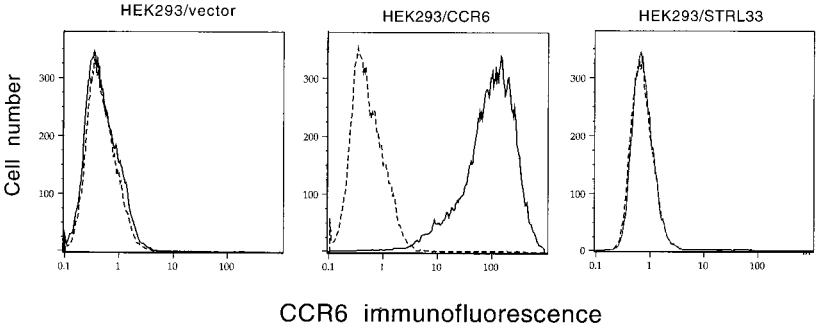

FIGURE 1. Anti-serum raised against N-terminal residues of CCR6 stains CCR6-transfected cells. 10^6 HEK 293 cells were incubated with preimmune serum (dashed lines) or anti-CCR6 immune serum (solid lines), then stained with FITC-conjugated $F(ab')_2$ goat anti-rabbit IgG and analyzed on a FACScan flow cytometer. Results are shown for cell lines derived after transfection with the pCEP4 vector alone (*left*), pCEP4 encoding CCR6 (*center*), or pCEP4 encoding the orphan receptor STRL33 (*right*).

(Cytek, Fremont, CA), and data were analyzed using the Multitime (Phoenix, AZ) software. MIP-3α was kindly provided by Human Genome Sciences (Rockville, MD), and stromal cell-derived factor 1 (SDF-1) was purchased from PeproTech (Rocky Hill, NJ).

Assaying chemotaxis

In vitro chemotaxis assays were performed using the 96-well ChemoTx #106–5 microplate (Neuro Probe, Gaithersburg, MD) according to the manufacturer's protocol. PBMC were resuspended in prewarmed RPMI 1640 containing 1% FBS and 10 mM HEPES at 2×10^6 cells/ml. Chemokines were prewarmed in the above buffer at concentrations of 1 μg/ml for MIP-3α and 1.5 μg/ml for SDF-1. The plate was incubated at 37°C in 5% CO_2/95% air for 3.5 h. Following incubation, cells on top of the filter were removed and cold 0.5 mM EDTA in Dulbecco's phosphate buffered salt solution was added for 20 min at 4°C before centrifugation to dislodge any cells on the filter's underside. The migrated cells in the bottom wells were collected, counted, and stained for flow cytometry.

Northern blot analysis

Total RNA from PBMC cultured with or without IL-2 (400 U/ml) as described above was prepared using Trizol reagent (Life Technologies) according to the supplier's protocol. Then, 30 μg of total RNA were loaded onto a 1.2% agarose-formaldehyde gel, transferred to nitrocellulose, and hybridized with CCR6 ^{32}P-labeled probe prepared from a fragment of the CCR6 gene as described (31). The blot's final wash was in 0.1× SSC, 0.1% SDS at 50°C.

Results

Anti-serum raised against N-terminal residues of CCR6 stains CCR6 on PBL

A DNA fragment encoding the N-terminal 46 residues of human CCR6 was ligated to sequences encoding GST from *Schistosoma japonicum* to create a sequence encoding a GST fusion protein with CCR6 residues at its carboxyl terminus. Anti-sera were raised by immunizing with fusion protein produced in bacteria and purified by affinity chromatography with Glutathione Sepharose (not shown). Cell staining using anti-serum 5146 is shown in Fig. 1. Neither immune nor preimmune serum stained a control-transfected HEK 293 cell line or an HEK 293 cell line expressing the

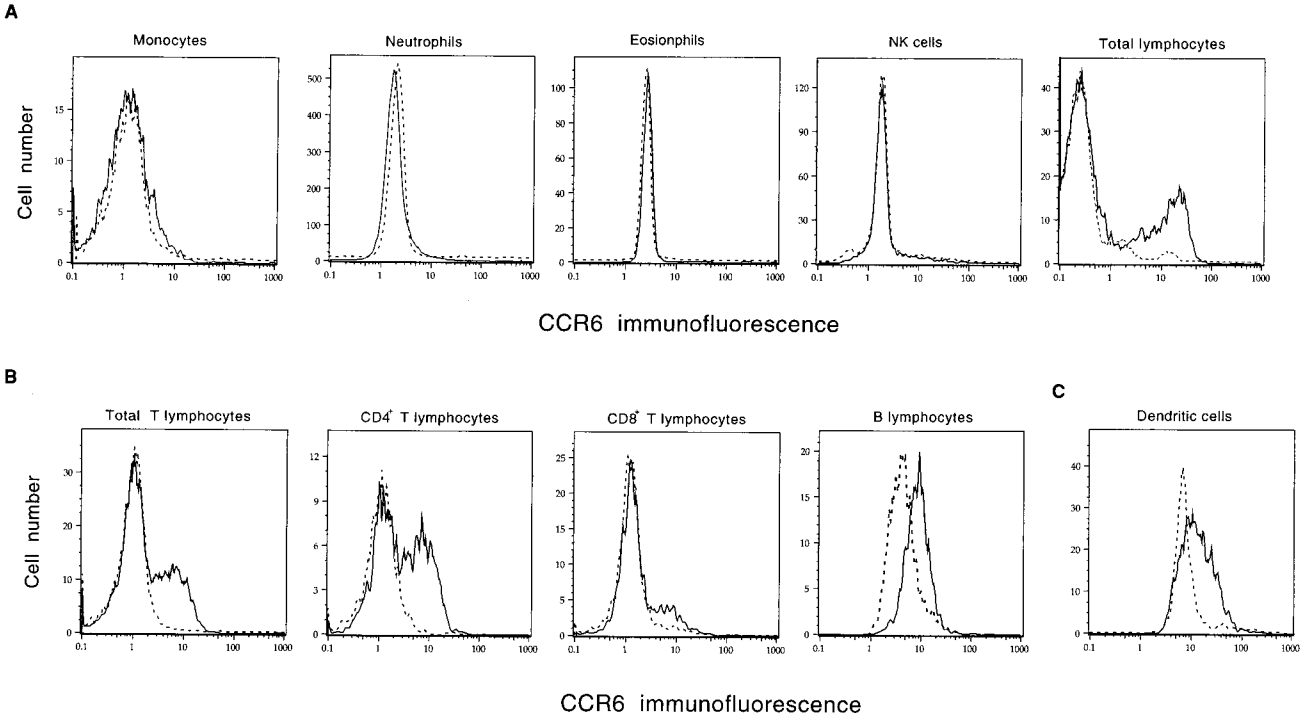

FIGURE 2. CCR6 is expressed on lymphocytes and dendritic cells. Cells were incubated with preimmune serum (dashed lines) or anti-CCR6 immune serum (solid lines), and stained with secondary Abs as in Fig. 1. *A*, Staining of peripheral blood leukocytes. Gating on individual populations of cells was done using the scatter profile and Abs against CD14, CD16, and CD56. Three donors gave similar results and data from one donor are shown. *B*, Staining of PBL. Gating on individual populations of cells was done using the scatter profile and Abs against CD5, CD4, CD8, and CD19. Multiple donors gave similar results and data from one donor are shown. *C*, Staining of $CD34^+$ progenitor cell-derived dendritic cells. Gaiting on dendritic cells was done using the scatter profile and Abs against CD1a and HLA-DR.

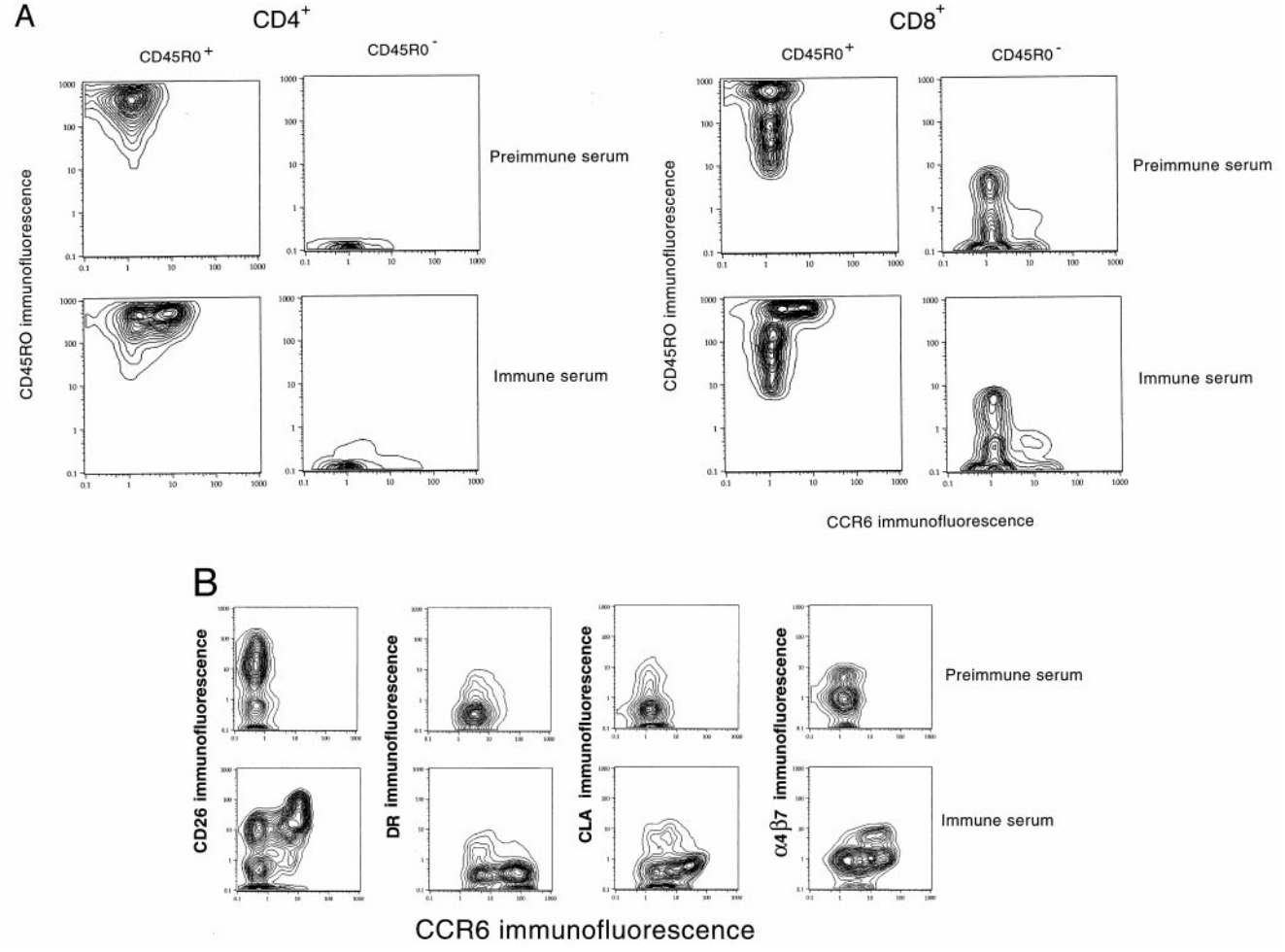

FIGURE 3. CCR6 is expressed on diverse subsets of memory T cells. *A*, CCR6 expression is limited to memory T cells. Four-color stainings of lymphocytes with preimmune serum or anti-CCR6 immune serum followed by FITC-conjugated $F(ab')_2$ goat anti-rabbit IgG Abs and mAbs against CD4, CD8, and CD45R0 are shown as two-dimensional contour plots. Three donors gave similar results and data from one donor are shown. *B*, Expression on diverse memory subsets. Cells were stained with preimmune serum or anti-CCR6 immune serum followed by either FITC-conjugated (for the CD26 panels) or PE-conjugated (for all other panels) $F(ab')_2$ goat anti-rabbit IgG Abs plus anti-CD45R0-Cy-5, anti-CD4-APC, and, as noted, anti-CD26-PE, anti-HLA-DR-FITC, anti-CLA-FITC, or anti-$\alpha_4\beta_7$-FITC. Displays are limited to the $CD4^+CD45R0^+$ cells. Three donors were analyzed for each marker and results from representative donors are shown.

orphan receptor, STRL33 (36). Immune, but not preimmune, serum stained the HEK 293 cells expressing CCR6. Several transfected HEK 293 cell lines expressing different levels of CCR6 mRNA were stained with anti-serum 5146, and fluorescent intensities correlated with levels of CCR6 mRNA (not shown). Using the immune serum to stain peripheral blood leukocytes revealed that among the major leukocyte populations CCR6 expression was limited to T cells and B cells (Fig. 2). The anti-serum stained ~15–40% of $CD4^+$ T cells and 0–14% of $CD8^+$ T cells, depending on the donor. B cells stained as a single normally distributed population. We also detected CCR6 on ~25% of dendritic cells derived by culturing $CD34^+$ bone marrow progenitors for 12 days with appropriate cytokines.

CCR6 expression on T cells is limited to memory cells

Additional analysis of T cell subsets revealed that for both $CD4^+$ and $CD8^+$ cells, expression of CCR6 was limited to the $CD45R0^+$ subset (Fig. 3) and, in particular, the CD45R0 bright cells. Accordingly, CCR6 was coexpressed with other markers prominent on memory cells such as CD95 and CD28, although among the $CD4^+$ memory cells, a higher percentage of $CD28^{low}$ cells were $CCR6^+$ than was true for the $CD28^{high}$ cells (not shown). We did not detect significant numbers of $CD25^+$ and $CD69^+$ PBL, so that all the $CCR6^+$ cells we identified were $CD25^-$ and $CD69^-$ (not shown). In Fig. 3*B*, HLA-DR^+ cells are $CCR6^-$. Although this pattern was not found in all donors, in no case did we see a positive correlation between expression of HLA-DR and CCR6. In contrast, levels of CCR6 and CD26 were positively correlated. Moreover, almost all the $\alpha_4\beta_7^+$ memory cells and many of the CLA^+ cells were $CCR6^+$.

T cell activation does not increase expression of CCR6

Although we found CCR6 expressed on memory cells, we had no indication from analysis of the freshly isolated PBL that CCR6 expression correlated with cellular activation per se. We investigated the relationship between CCR6 expression and cellular activation further by treating cells in vitro with IL-2 for 5 days or with Ab against CD3 for 3 days. These were similar to protocols that we or others (25, 33) or we (R.L.R. and J.M.F., unpublished observations) have used to enhance chemokine receptor expression. As shown in Fig. 4, *A* and *B*, neither 5 days of treatment with IL-2 nor 3 days of treatment with the anti-CD3 Ab OKT3 increased expression of CCR6 on T cells. Because of a report that CCR6 mRNA levels were increased in T cells by treatment for 5 days with IL-2 (33), we analyzed mRNA as well and found no

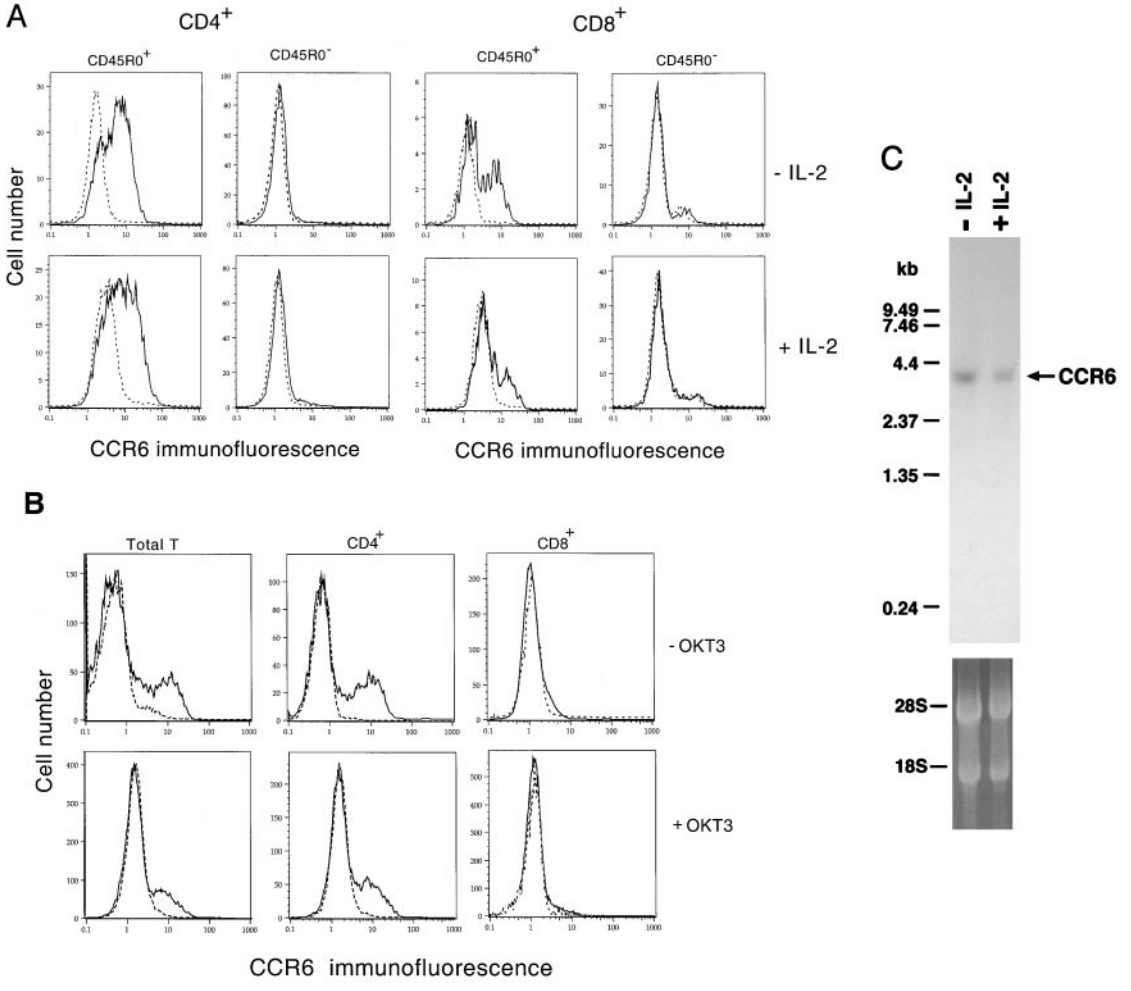

FIGURE 4. Expression of CCR6 on populations of T cells is not increased by cellular activation. *A*, Expression of surface protein is not increased by activation. PBMC were cultured for 5 days with or without human rIL-2 and were then stained with preimmune serum (dashed lines) or anti-CCR6 immune serum (solid lines) followed by FITC-conjugated $F(ab')_2$ goat anti-rabbit IgG, and Abs against CD4, CD8, and CD45R0. Five donors gave similar results and data from one donor are shown. *B*, PBMC were cultured for 3 days with or without Ab against CD3 (OKT3), and T cells were analyzed with Abs to CD5, CD4, and CD8. Multiple donors gave similar results and data from one donor are shown. *C*, Expression of CCR6 mRNA is not increased by IL-2 treatment. RNA was prepared from PBMC treated with or without IL-2 as described in *A* and analyzed by Northern blot using a CCR6 probe. Ethidium bromide staining of the gel before transfer demonstrated equal loading of intact RNA, as shown. The autoradiogram is from a 5 day exposure with an intensifying screen. The CCR6 mRNA, RNA size markers, and the 18S and 28S rRNAs are indicated.

increase with IL-2 treatment (Fig. 4*C*), consistent with the results on receptor expression. IL-2-treated cells showed significant expression of CD25, indicating effective activation (not shown).

Like CCR6, responses to MIP-3α are limited to memory cells

Analysis of responses of PBL to MIP-3α using the flow cytometer revealed a MIP-3α-induced rise in T cell intracytoplasmic calcium. In contrast, no calcium flux was seen in B cells (not shown). Responses of T cell subsets to MIP-3α, as shown in Fig. 5, paralleled the subset expression of CCR6. Fig. 5*A* demonstrates that calcium flux after exposure to MIP-3α is limited to the $CD45R0^+$ T cells, with a higher percentage of $CD4^+$ cells than $CD8^+$ cells responding. Fig. 5*B* shows the results of chemotaxis experiments with MIP-3α and, for comparison, with SDF-1, using a modified Boyden chamber assay and freshly isolated PBL. As anticipated, SDF-1 was a potent chemoattractant for PBL, with ~30% of input $CD4^+$ and $CD8^+$ cells moving to lower wells containing 1.5 μg/ml SDF-1. The $CD4^+$ cells migrating were both $CD45R0^+$ and $CD45R0^-$ so that the ratio of $CD45R0^+$ to $CD45R0^-$ cells in the migrated cells was equal to that in the input cells. For MIP-3α, ~20% of input $CD4^+$ cells and 13% of input $CD8^+$ cells migrated, and, in contrast to the response to SDF-1, only $CD45R0^+$ cells migrated so that the ratio of $CD45R0^+$ to $CD45R0^-$ cells in the migrated cells was ~10-fold greater than in the input cells.

Response to MIP-3α is confined to CCR6-expressing cells

To address more directly the correspondence between the populations of cells expressing CCR6 and those responding to MIP-3α, we used the flow cytometer to analyze calcium flux in T cells stained for CCR6. As shown in Fig. 6, all the MIP-3α-responding cells were found among those staining for CCR6. These findings are consistent with the supposition that CCR6 is the only receptor for MIP-3α on peripheral blood T cells.

Discussion

Although the activity of chemokines as chemotactic factors for lymphocytes has been recognized for some time, there is now increased interest in this area with the continuing identification of novel lymphocyte-active chemokines and their receptors (reviewed in Ref. 11). Our current report is the first to analyze the

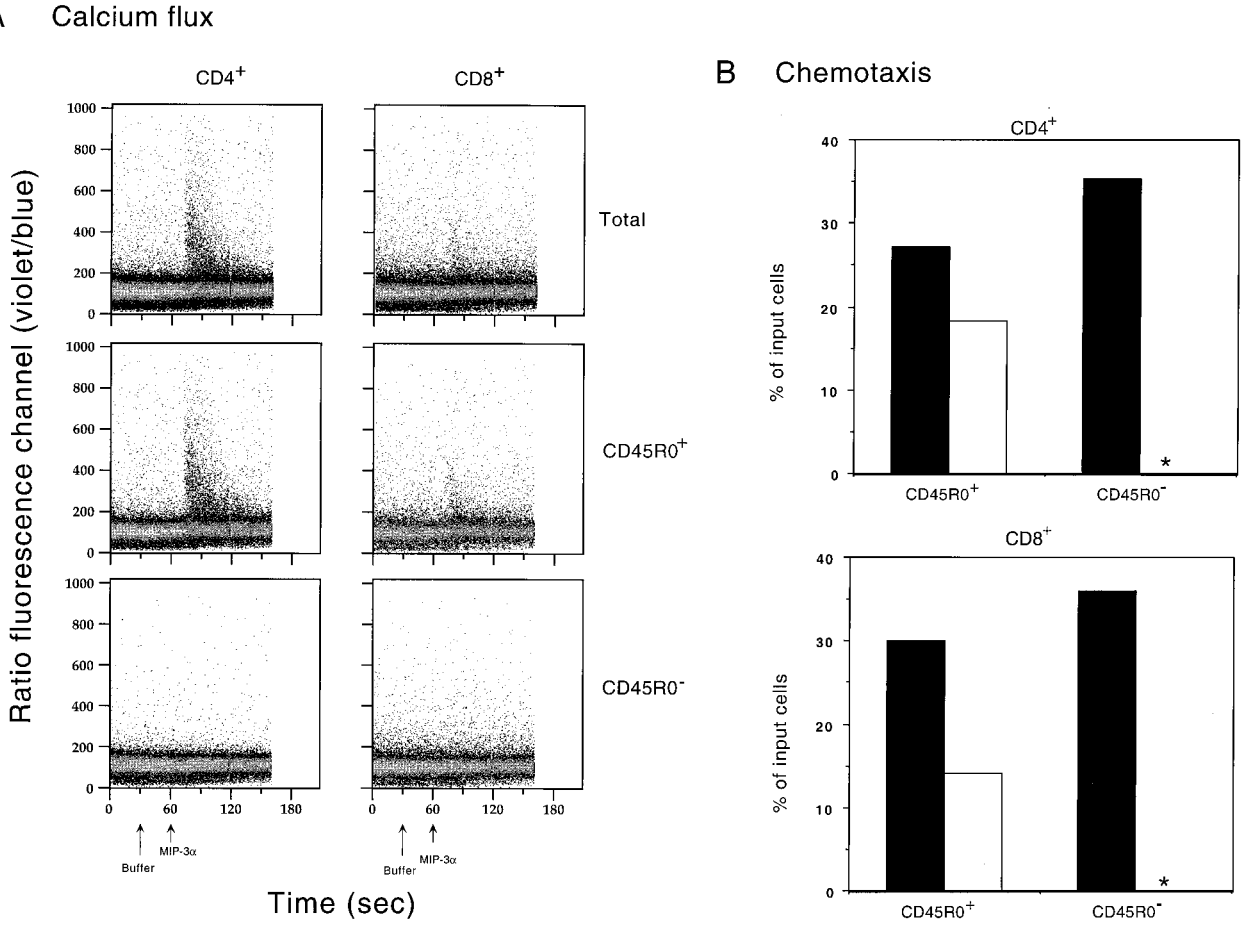

FIGURE 5. MIP-3α specifically targets memory T cells. *A*, Calcium flux. PBL were loaded with the fluorescent calcium probe Indo-1 acetoxymethyl ester, and stained with mAbs against CD4, CD8, and CD45R0. Scattergrams show the ratio of fluorescence in lymphocytes recorded continuously and displayed as a function of time with additions of buffer at 30 s or MIP-3α at 60 s as indicated. Two donors gave similar results, and data from one donor are shown. *B*, Chemotaxis. Chemotaxis of PBL was measured using a modified Boyden chamber assay. Migrated cells from all wells were pooled for analysis. Input cells and migrated cells were stained with Abs against CD4, CD8, and CD45R0. Bars show percentages of input cells in each subset that migrated over 3.5 h to lower chambers containing 1 μg/ml MIP-3α (open) or 1.5 μg/ml SDF-1 (solid), corrected for percentages of cells migrating in the absence of chemokines. Asterisks indicate bars of 0 value. Two donors gave similar results, and data from one donor are shown.

expression of the CCR6 protein, a receptor for the MIP-3α chemokine. Among T cell subsets, we found expression of CCR6 on both $CD4^+$ and $CD8^+$ cells, but a higher percentage of the former stained positive as compared with the latter. Of particular note, CCR6 expression, as well as calcium flux and chemotaxis in response to MIP-3α, were all limited to the $CD45R0^+$ population, i.e., memory T cells (41). Furthermore, by analyzing the calcium response to MIP-3α with freshly isolated PBL that had been stained for CCR6, we found that all the MIP-3α-responding cells were in the CCR6-positive population. These results are consistent with the supposition that, at least among T cells, there are no receptors for MIP-3α other than CCR6, strengthening the data to date on the dedicated relationship between this ligand and receptor. Together these data establish that MIP-3α targets exclusively memory cells and that this is based on a subset-selective expression of CCR6.

We have shown that CCR6 is expressed on $CD26^+$ cells. CD26 is a marker with high expression on memory cells (reviewed in Ref. 42). The chemokine receptors CCR2 and CCR5 are expressed primarily on CD26 bright cells (26, 43), and it is the CD26 bright cells that show transendothelial migration both in the absence of added chemokine (44) and in response to MCP-1 and some other CC chemokines (26, 45, 46). With the addition of our data on CCR6, CD26 bright cells are now known to show the highest expression of at least three CCR, suggesting that these cells may be a memory population poised to migrate to inflammatory sites.

Despite the overlap in the patterns of expression for CCR6, CCR5, and CCR2, our data and those of others suggest that CCR6 plays a distinct role. In our previous work on CCR6, we noted that MIP-3α had the somewhat unusual property among CC chemokines of being able to generate a calcium signal not only in activated T cells but also in freshly isolated PBL (23), and here we show expression of CCR6, as well as activity of MIP-3α for both calcium flux and chemotaxis, on freshly isolated T cells. These findings are consistent with those of others for chemotaxis to MIP-3α (22) and with the report that MIP-3α stimulated the adhesion of freshly isolated lymphocytes to ICAM-1-coated glass (24). Of equal importance, we did not find any correlation between HLA-DR or other activation markers and CCR6 on PBL, nor did we see an increase in CCR6 surface expression, CCR6 gene expression (Fig. 4), or MIP-3α signaling (F.L., R.L.R., and J.M.F., unpublished observations) by activation of T cells in vitro. In fact, in vitro activation tended to decrease the proportion of $CCR6^+$ cells, similar to what has been reported recently for CCR6 expression on dendritic cells exposed to activating agents (47). This is in contrast to what others, as well as ourselves, have observed for CCR2 and

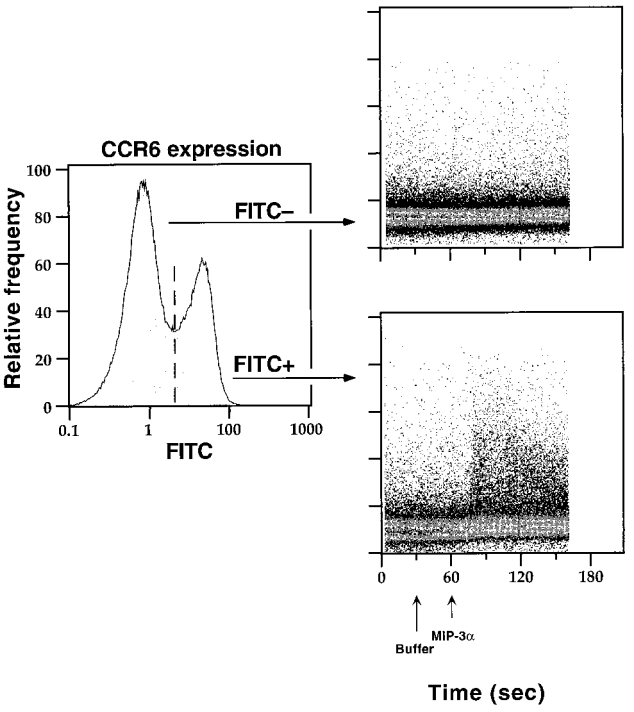

FIGURE 6. All T cells responding to MIP-3α stain for CCR6. PBL were loaded with the fluorescent calcium probe Indo-1 acetoxymethyl ester and stained with preimmune (not shown) and anti-CCR6 immune serum followed by FITC-conjugated F(ab′)$_2$ goat anti-rabbit IgG. Cells to the right of the vertical dashed line were considered CCR6$^+$. Calcium flux data are displayed in scattergrams as in Fig. 5, and separate scattergrams are shown for CCR6$^+$ and CCR6$^-$ cells.

CCR5, namely an increase in receptor expression and in chemotactic responses to the relevant ligands with activation (25, 26, 43) (R. L. Rabin, M. Park, F. Liao, R. Swofford, D. Stephany, and J. M. Farber, unpublished observations). Taken together, the data suggest that CCR2, CCR5, and likely CCR1 (25) and CXCR3 (13) as well, function primarily on activated T cells, while CCR6 functions on resting memory cells.

In the early phases of an inflammatory response, both tissue sites and lymph node recruit CD4$^+$ memory cells (48) and the recruitment into lymph node may be particularly important for a recall response, given the low frequency of memory cells that persist within the node in the absence of re-exposure to Ag (49, 50). Because the other lymphocyte receptors for proinflammatory chemokines function best after cellular activation, a mechanism is needed for the recruitment of resting cells to sites where activation can occur. Our data suggest that MIP-3α and CCR6 may have a role in this first stage of inflammation by recruiting memory cells to sites where they can encounter Ag, leading in turn to cellular activation, including enhanced expression and function of chemokine receptors that are important for trafficking of lymphoblasts to tissue as the immune response expands. Consistent with this, the MIP-3α gene is dramatically induced in endothelial cells by TNF (21), an early mediator of innate immunity, and has also been found to be expressed in lymph node and appendix (17, 21).

Our data on the failure of T cell activation to up-regulate CCR6 expression is contrary to the effects of IL-2 reported by Baba et al. (33). While we have no ready explanation for the discrepancy, our findings are consistent with those reported by Greaves et al., who reported down-regulation of CCR6 mRNA in PBMC activated with anti-CD3 and PMA (35). However, we do not think it is likely that T cell activation is incompatible with expression of CCR6, because some HLA-DR$^+$ PBL were CCR6$^+$ and many tumor infiltrating lymphocyte lines grown in IL-2 express CCR6 (36) and respond to MIP-3α (R.L.R. and J.M.F., unpublished observations).

It is of interest that almost all $\alpha_4\beta_7^+$ memory cells and many CLA$^+$ cells express CCR6. These represent memory subsets that home to skin and mucosal sites, respectively (reviewed in Refs. 51 and 52). CLA is up-regulated by IL-12 (53), and consistent with these findings, E-selectin, which binds CLA (54), mediates recruitment of Th1 cells (55). In contrast, β_7 is induced by TGFβ, a signature cytokine of mucosa-associated lymphoid tissue (56). So CLA and $\alpha_4\beta_7$ identify mutually exclusive T cell subsets (57) that have been activated and differentiated in separate anatomic compartments and cytokine milieus and will home appropriately. Therefore, our data suggest that CCR6 is induced on T cells that were activated in very diverse environments, and that production of MIP-3α on stimulated endothelium and/or other cells would be expected to act through CCR6 to recruit these highly differentiated, resting memory cells to sites of inflammation in both skin and mucosa.

In addition to expression on T cells, we also found CCR6 on peripheral blood B cells, consistent with data from our laboratory (36) and others (33), but contrary to one laboratory's findings (35) on the expression of the CCR6 mRNA. However, in contrast to T cells we saw no reproducible functional effects of MIP-3α on freshly isolated B cells either in calcium or chemotaxis assays (F.L. and J.M.F., unpublished observations). This suggests that factors in addition to CCR6 expression may control B cell signaling to MIP-3α, and we are investigating how B cell activation and differentiation may influence CCR6 responses. It is also possible that there are as yet undiscovered ligands for CCR6 that may be able to activate the receptor on B cells, analogous to the recent report that suggests that, unlike other CCR1 ligands, the chemokine leukotactin can activate CCR1 on human neutrophils (58).

Previous reports had identified CCR6 mRNA expressed in dendritic cells derived from CD34$^+$ progenitors (34, 35), and our findings using the anti-CCR6 serum documented expression of the receptor on many of these cells. A recent report demonstrates that expression of CCR6 mRNA is down-regulated on immature CD34$^+$ cell-derived dendritic cells by activating and differentiating agents, and these authors have suggested that CCR6 is important in recruiting immature dendritic cells to inflammatory sites (47). Because immature dendritic cells are efficient at Ag capture and activation specifically of memory and effector T cells (59), MIP-3α may, by activating CCR6, bring these cells together at inflammatory sites. While we have not shown responses by B cells to MIP-3α, receptor expression on these cells raises the possibility that CCR6 may be involved in aggregating, and perhaps in communication among memory T cells, dendritic cells and B cells—three critical cellular elements that contact and cooperate with each other as part of an immune response (60).

Acknowledgments

We thank Brent Kreider, Gianni Garotta and others at Human Genome Sciences for providing MIP-3α/ckβ4, Gretchen Schwartz for providing dendritic cells, Louis Picker for the Ab to CLA, Andrew Lazarovits and LeukoSite for the Ab to $\alpha_4\beta_7$, Ramya Gopinath for advice on whole-blood staining, and Meta Snyder for help with the manuscript.

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