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# Up-Regulation of CCR5 and CCR6 on Distinct Subpopulations of Antigen-Activated CD4$^+$ T Lymphocytes[1]

**Lisa M. Ebert and Shaun R. McColl[2]**

**Following infection, naive T cells are activated in the secondary lymphoid tissue, but then need to move to the infected tissue in the periphery to mediate their effector functions. The acquisition of inflammatory chemokine receptors, such as CCR5 and CCR6, may contribute to the efficient relocation of activated T cells to inflamed sites in the periphery. In keeping with this idea, the present study has demonstrated that CCR5 and CCR6 are up-regulated on CD4$^+$ T cells upon activation in the MLR. The observed increase in expression correlated well with the acquisition of an activated/memory phenotype and was largely (CCR5) or completely (CCR6) separated temporally from the initiation of cell division. In contrast, the regulation of two other chemokine receptors, CXCR3 and CXCR4, occurred in close parallel with the cell division process. Increased mRNA levels are likely to contribute to the enhanced surface expression of CCR5 and CCR6, but in the case of CCR6, translocation of intracellular stores of protein to the cell surface may be an additional mechanism of regulation. The up-regulation of CCR5 was more extensive than that of CCR6, as only approximately half the activated CCR5$^+$ T cells coexpressed CCR6. The increased expression of CCR5 resulted in enhanced chemotaxis toward the CCR5 ligand macrophage-inflammatory protein-1$\beta$/CCL4, but up-regulation of CCR6 did not result in altered chemotactic responsiveness to macrophage-inflammatory protein-3$\alpha$/CCL20, suggesting an alternative function for this receptor.** ***The Journal of Immunology,*** **2002, 168: 65–72.**

In response to a microbial infection in the periphery, dendritic cells (DC)[3] migrate to secondary lymphoid tissue, carrying Ag captured from the site of infection. Following interaction with these DC in secondary lymphoid tissue, activated T cells are armed with a number of potent effector mechanisms, including the capacity to produce immunoregulatory cytokines and induce apoptosis of target cells. However, these effector mechanisms generally act locally; therefore, it is essential that recently activated T cells are able to efficiently relocate to the precise site of infection in the periphery. Altered patterns of adhesion molecule expression are a prerequisite for activated T cells to pass through inflamed endothelium in peripheral tissues and provide a degree of specificity to the localization of activated T cells (1, 2). However, recent studies suggest that chemokines and their receptors are also essential for activated T cell relocation, both in the induction of firm adhesion to endothelial cells and in the directed migration of T cells once they have entered the tissue (3, 4).

Chemokines can be divided into two major (CC and CXC) and two minor (C and $CX_3C$) families, based on a cysteine signature motif (3). Alternatively, they can be classified as homeostatic (constitutive) or inflammatory (inducible) according to their function (4, 5). Homeostatic chemokines are expressed constitutively and appear to be responsible for the trafficking of lymphocytes under conditions of homeostasis. In contrast, inflammatory chemokines are specifically up-regulated at sites of inflammation and are thought to play a role in the recruitment of lymphocytes to peripheral tissues in response to immunological challenge.

CCR5 is a receptor for the inflammatory chemokines RANTES/CCL5, macrophage-inflammatory protein (MIP)-1$\alpha$/CCL3, and MIP-1$\beta$/CCL4 (6), while CCR6 is the unique receptor for the inflammatory chemokine MIP-3$\alpha$/CCL20 (7, 8). Within both the CD4$^+$ and CD8$^+$ T cell subsets, CCR5 and CCR6 are expressed primarily on memory cells, with little or no expression in the naive population (9–12). Selective expression in previously activated, rather than naive, T cells is emerging as a general phenomenon of inflammatory chemokine receptors and suggests that T cells up-regulate the expression of inflammatory chemokine receptors upon activation. We and others (13–15) have recently shown this to be the case with another inflammatory chemokine receptor, CXCR3. In contrast, stimulation of T cells with mitogens, phorbol esters, anti-CD3, or anti-CD3 plus anti-CD28 does not up-regulate the expression of CCR5 (10, 12, 16) or CCR6 (9, 10); in some studies the expression of these receptors is decreased (8, 17). However, a recent report by Riley et al. (18) revealed that the nature of the in vitro stimulation can profoundly affect the regulation of CCR5, in that costimulation through CD28 and CTLA-4 exerted paradoxical effects on CCR5 expression.

Up-regulation of CCR5 and CCR6 expression on T cell activation appears to occur in vivo, as these receptors are expressed on previously activated T cells in the blood, as discussed above, and on T cells accumulating in inflamed tissues such as synovium (13), psoriatic skin (19), and multiple sclerosis lesions (20). However, the methods of polyclonal T cell stimulation commonly used in in vitro studies do not appear to be sufficient or appropriate to induce the expression of these receptors. Accordingly, we have undertaken a detailed assessment of CCR5 and CCR6 expression on CD4$^+$ T cells using a physiologically relevant in vitro culture system, the MLR. This culture system is dependent on DC for the majority of APC function and results in the proliferation of naive as well as memory CD4$^+$ T cells. We have previously used this

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[3] Abbreviations used in this paper: DC, dendritic cell; MIP, macrophage-inflammatory protein.



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culture system to demonstrate increased CXCR3 expression and a concomitant decrease in CXCR4 expression on activated CD4$^+$ T cells (15). We now extend these findings to show that CCR5 and CCR6 are up-regulated on CD4$^+$ T cells following allogeneic activation, although the kinetics and functional outcome of receptor up-regulation differ notably from the patterns observed for CXCR3 and CXCR4.

## Materials and Methods

### *Abs and conjugates*

Anti-CD4 (clone OKT4) was a gift from Dr. R. Roy (Center de Recherche en Rheumatologie et Immunologie, Université Laval, Quebec, Canada) and isotype controls were gifts from Prof. H. Zola (Child Health Research Institute, Adelaide, Australia). Anti-CCR5 (clone 2D7) was purchased from BD PharMingen (San Diego, CA) in both an unlabeled and a PE-conjugated format, while anti-CCR6 (clone 53103.111), anti-CXCR3 (clone 49801.111), and biotinylated anti-CXCR4 (clone 44716.111) were purchased from R&D Systems (Minneapolis, MN). PE-labeled Abs to CD45RA (clone F8-11-13) and CD45RO (clone UCHL1) were obtained from Serotec (Oxford, U.K.), and PE-conjugated anti-CD25 (clone M-A251) was obtained from BD PharMingen. Secondary detection reagents (fluorescein-, PE-, and PECy5-conjugated streptavidin and PE-conjugated anti-mouse Ig) were obtained from Rockland Immunochemicals (Gilbertsville, PA). Where required, primary Abs were labeled with FITC (Sigma, Castle Hill, Australia) or aminohexanoyl-biotin-*N*-hydroxysuccinimide ester (Zymed Laboratories, South San Francisco, CA), both as previously described (21).

### *PBMC isolation and MLR*

Mononuclear cells were prepared from healthy volunteers, and MLR cultures were set up as previously described (15).

### *CFSE labeling*

PBMC were suspended to $2 \times 10^7$ cells/ml in PBS containing 0.1% human AB serum, and CFSE (Molecular Probes, Eugene, OR) was added to a final concentration of 10 $\mu$M. After incubation at 37°C for 10 min, the staining reaction was quenched by the addition of a large volume of complete medium for 5 min, followed by two washes in the same medium.

### *Immunofluorescence staining and flow cytometric analysis*

Cells were suspended to $4 \times 10^6$ viable cells/ml in PBS containing 2% pooled human AB serum and 0.04% sodium azide (staining buffer). FcRs were blocked by incubation for 30 min at room temperature with 50 $\mu$g/million cells human IgG (Sigma), then 50 $\mu$l of blocked cells were aliquoted into round-bottom tubes. For most experiments, cells were mixed with saturating concentrations of the unlabeled primary Ab, incubated for 30 min at room temperature, and washed once with 3 ml of staining buffer before addition of biotin- or PE-conjugated anti-mouse detection Ab. Following a 30-min incubation on ice, cells were washed, and free binding sites on the detection Ab were blocked by incubation with 20 $\mu$g/tube mouse gammaglobulin (Rockland Immunochemicals) for 20 min at room temperature. Fluorescein- and PE-conjugated primary Abs as well as PECy5-conjugated streptavidin if required were then added and incubated for 30 min on ice. Cells were washed with 3 ml of staining buffer followed by 3 ml of serum-free staining buffer and fixed in 200 $\mu$l of paraformaldehyde (1% in PBS; BDH Laboratory Supplies, Poole, U.K.). A slightly different approach was adopted when using CXCR3 and CXCR4 Abs, which were directly biotinylated. Cells were mixed with all of the required primary Abs, incubated at room temperature for 30 min, washed, incubated with PECy5-conjugated streptavidin, then washed and fixed as described above. Labeled cells were analyzed on a FACScan and data were analyzed using CellQuest 3.1 software (BD Biosciences, Mountain View, CA). For all analyses, lymphocytes were gated using forward and side angle light scatter characteristics, and background staining observed using isotype-matched control Abs was subtracted from all percentages calculated. In some experiments ethidium monoazide staining (10 $\mu$l of a 50 $\mu$g/ml solution/million cells, incubated under fluorescent light for 15 min, followed by washing in staining buffer) was used to determine that viability within the lymphocyte gate was at least 98% (data not shown).

### *Detection of intracellular chemokine receptor protein by flow cytometry*

Preparation of permeabilized and nonpermeabilized cells was conducted as previously described (15), and the treated cells were processed for flow cytometry as usual, except that cells were maintained in staining buffer containing 0.02% saponin to ensure that complete membrane permeabilization was maintained throughout.

### *Cytospin preparation and immunofluorescence microscopy*

Cells were spun onto glass slides, and immunofluorescence staining was performed as previously described (15).

### *RNA extraction and RT-PCR*

RNA purification from day 9 cell pellets and RT-PCR were conducted as previously described (15). The following primers were used in this study: GAPDH, 5′-TCCTTGGAGGCCATGTAGGCCAT-3′ and 5′-TGATGACATCAAGAAGGTGGTGAAG-3′; CCR5, 5′-TGACATCTACCTGCTCAACC-3′ and 5′-CCTGTGCCTCTTCTTCTCAT-3′; and CCR6, 5′-TCATCTGCCTTGTTGTGTGG-3′ and 5′-TGTCGTTATCTGCGGTCTCA-3′. PCR products were resolved on 2% agarose gels, stained with SYBR-gold (Molecular Probes) and visualized/analyzed using a Molecular Imager FX (Bio-Rad, Hercules, CA). The band intensity values for each receptor were expressed as a ratio relative to band intensity for the GAPDH PCR product amplified from the same template.

### *Chemotaxis assay*

Cells were suspended to $5 \times 10^6$ viable cells/ml in RPMI 1640 containing 0.5% BSA (RPMI-BSA). Synthetic MIP-1$\beta$/CCL4 and MIP-3$\alpha$/CCL20 (kind gifts of Prof. I. Clark-Lewis, Biomedical Research Center, University of British Columbia, Vancouver, Canada) were diluted to 200 ng/ml or 1 $\mu$g/ml, respectively, in RPMI-BSA, and 600 $\mu$l was added to the lower chambers of a Transwell plate (6.5-mm diameter filter, 5-$\mu$m pore size; Corning Glass, Corning, NY). After adding 100 $\mu$l of cells to the upper chambers, the assay was incubated for 3 h at 37°C, and cells were collected from the lower chamber after extensive washing of the filter underside. In most experiments the total number of cells in the lower chamber was quantified by duplicate hemocytometer counts, and this value was divided by the number of input cells to calculate the percent migration. To correct for any variations in spontaneous migration, the data are expressed as the migration index, calculated by dividing the percentage of migration obtained in the presence of chemokine by the percentage of migration for negative controls. For the dose-response studies using MIP-3$\alpha$/CCL20, cells were first fluorescently labeled by incubation with calcein (Molecular Probes; 40 nM final concentration in RPMI-BSA) for 30 min at 37°C, followed by three washes in RPMI-BSA. Labeled cells were used in Transwell assays as described above, and then the cells in the lower chamber were quantified by transferring them to a 96-well microtiter tray and measuring fluorescent emission on the Molecular Imager FX. Following quantification, cells were washed in staining buffer and incubated with biotin-conjugated anti-CD4 for 30 min on ice. After washing with 3 ml of staining buffer, PE-conjugated streptavidin was added and incubated for 30 min on ice, and the cells were washed, fixed, and analyzed by flow cytometry as described above. The percentage of migration of CD4$^+$ T cells was calculated by multiplying each fluorescence value by the percentage of cells positive for CD4 in that sample, then inserting the values into the following formula: $(Y - Y_{min})/Y_{max})$, where $Y_{min}$ is the value obtained in the absence of chemokine, $Y_{max}$ is the value obtained for 100 $\mu$l of cells added directly to the lower chamber of the Transwell, and $Y$ is the relevant experimental value.

### *Statistical analyses*

Statistical analyses were performed using a two-tailed Student's $t$ test; $p < 0.05$ was considered significant. Data in the text are presented as the mean ± SEM.

## Results

### *CCR5 and CCR6 are up-regulated on activated CD4$^+$ T cells*

Chemokine receptor expression on CD4$^+$ T cells was quantified by flow cytometry for allogeneic and syngeneic (control) MLR cultures over a 12-day period (Fig. 1). CD4$^+$ T cells in syngeneic cultures did not undergo any notable changes in CCR5 expression over the time course, although a modest, steady decline in CCR6 expression was evident. In contrast, culture under allogeneic conditions resulted in a marked increase in the percentage of CD4$^+$ T cells expressing CCR5 or CCR6 over the 9- to 12-day period. The percentage of CD4$^+$ T cells expressing CCR5 was significantly greater in allogeneic compared with syngeneic cultures at 9 days

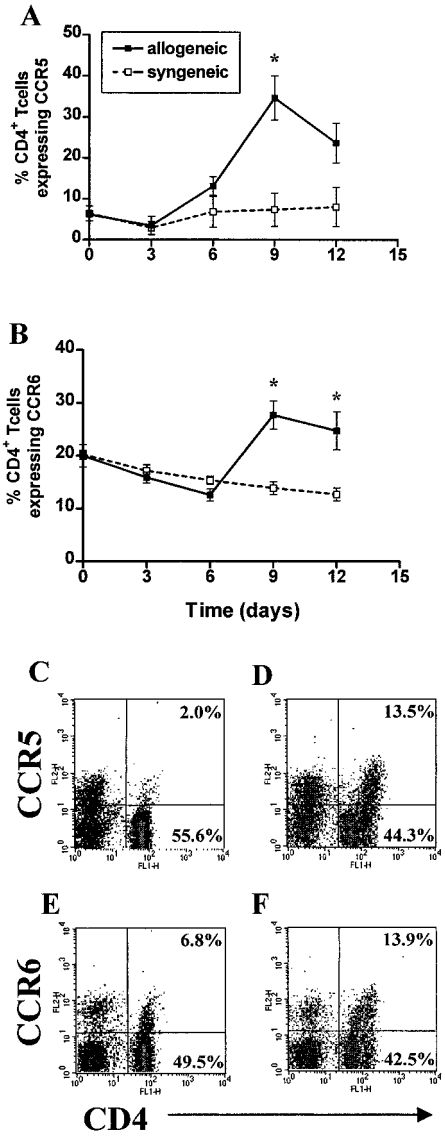


**FIGURE 1.** Time course of chemokine receptor expression in allogeneic and syngeneic cultures. PMBC were purified from two unrelated donors and cultured under syngeneic or allogeneic conditions. Immediately after preparation of cultures (day 0) or at the time points indicated cells were labeled with anti-CD4 and either anti-CCR5 (*A*, *C*, and *D*) or anti-CCR6 (*B*, *E*, and *F*), and analyzed by flow cytometry. *A* and *B*, The percentage of double-positive cells was calculated as a proportion of the total $CD4^+$ cells and plotted as a function of time for allogeneic (solid line) and syngeneic (dashed line) cultures. Values are the mean ± SEM ($n = 4$). A statistically significant difference between allogeneic and syngeneic samples at a given time point is indicated by an asterisk. *C–F*, Representative density plots comparing the expression of CCR5 and CCR6 in syngeneic (*C* and *E*) and allogeneic (*D* and *F*) cultures on day 9.

($p < 0.05$), while a significant difference in the percentage of $CD4^+$ T cells expressing CCR6 was detected at both 9 and 12 days ($p < 0.01$ and $p < 0.05$, respectively). Of interest, $CD8^+$ T cells from the same cultures did not undergo any significant change in the expression of either CCR5 or CCR6, even though CFSE studies indicated that proliferation of $CD8^+$ T cells did occur in the MLR (data not shown).

The up-regulation of CCR5 and CCR6 in allogeneic compared with syngeneic cultures at 9 days is illustrated by representative flow cytometric density plots in Fig. 1, *C–F*. It can be seen that the $CD4^+$ T cell population from allogeneic cultures not only contains more $CCR5^+$ and $CCR6^+$ cells, but that the level of expression of these receptors, particularly CCR5, is also higher, as evidenced by a shift in fluorescence intensity. Furthermore, we have previously shown that $CD4^+$ T cells activated in the MLR are characterized by increased expression of CD4, and that the $CD4^{high}$ phenotype correlates well with cellular activation and division (15). Accordingly, these plots demonstrate that the up-regulation of CCR5 and CCR6 is restricted to the $CD4^{high}$ population.

Table I. *Coregulation of chemokine receptors and memory/activation markers on $CD4^+$ T cells*[a]

| | Percentage Coexpressing | | |
|---|---|---|---|
| | CD45RA | CD45RO | CD25 |
| CCR5 | 14.6 ± 2.8 | 91.3 ± 5.2 | 90.3 ± 1.1 |
| CCR6 | 19.1 ± 4.8 | 93.7 ± 3.2 | 80.1 ± 1.6 |

[a] PMBC were purified from two unrelated donors and mixed to generate an allogeneic MLR. On day 9 of culture, cells were labeled with anti-CD4 and the other Abs as indicated and analyzed by flow cytometry. $CD4^+$ T cells were electronically gated, and the percentage of cells double-positive for the chemokine receptor and the memory/activation marker was determined as a proportion of total chemokine receptor-positive cells (mean ± SEM; $n = 4$).

### *Expression of CCR5 and CCR6 is coordinated with the acquisition of an activated/memory phenotype*

More detailed flow cytometric analysis at 9 days revealed that $CD4^+$ T cells expressing CCR5 or CCR6 were generally characterized by an activated/memory phenotype (Fig. 2 and Table I). $CD4^+$ T cells that expressed CCR5 or CCR6 were mostly positive for CD45RO and negative for CD45RA, a phenotype typical of memory T cells. Furthermore, most of these $CCR5^+$ and $CCR6^+$ cells also coexpressed the activation marker CD25. Expression of an additional activation marker, CD69, showed a less complete correlation with CCR5 and CCR6 expression (data not shown), possibly due to decreased expression of this early activation marker at a relatively late time point, such as day 9.

### *CCR5 and CCR6 define distinct subpopulations of $CD4^+$ T cells* following activation in the MLR

To determine whether CCR5 and CCR6 were expressed on the same or distinct subpopulations of activated $CD4^+$ T cells, cells from day 9 allogeneic MLR cultures were colabeled with Abs to

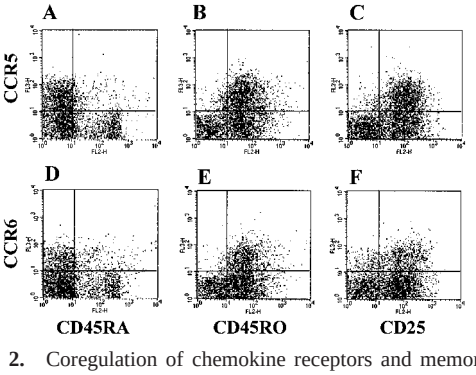


**FIGURE 2.** Coregulation of chemokine receptors and memory/activation markers on $CD4^+$ T cells. PMBC were purified from two unrelated donors and mixed to generate an allogeneic MLR. On day 9 of culture, cells were labeled with anti-CD4 and either anti-CCR5 (*A–C*) or anti-CCR6 (*D–F*) and anti-CD45RA (*A* and *D*), anti-CD45RO (*B* and *E*), or anti-CD25 (*C* and *F*). The cells were then analyzed by flow cytometry. $CD4^+$ T cells were electronically gated, and staining for the remaining markers was displayed on bivariate density plots. Data shown are representative of at least four experiments.

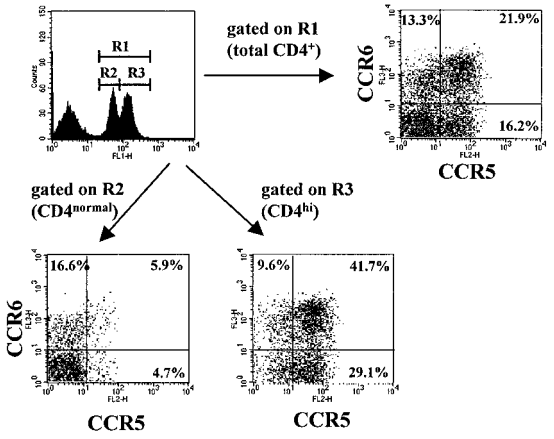


**FIGURE 3.** CCR5 and CCR6 define distinct subpopulations of CD4$^+$ T cells following allogeneic activation. PMBC were purified from two unrelated donors and mixed to generate an allogeneic MLR. On day 9 of culture cells were labeled with anti-CD4, anti-CCR5, and anti-CCR6 and analyzed by flow cytometry. CD4$^+$, CD4$^{normal}$, or CD4$^{high}$ T cells were electronically gated as indicated, and staining for CCR5 and CCR6 is displayed on bivariate density plots. Data shown are representative of four experiments.

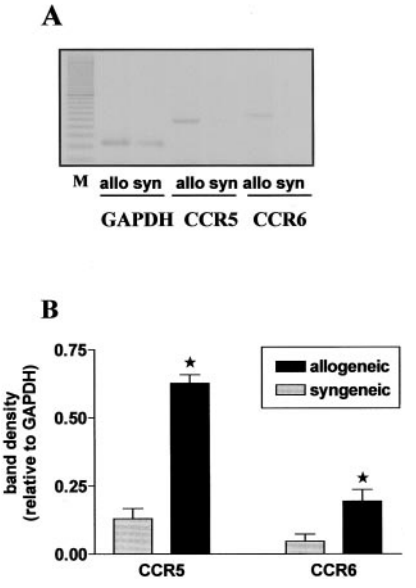


**FIGURE 4.** Up-regulation of CCR5 and CCR6 mRNA in allogeneic MLR. PMBC were purified from two unrelated donors and cultured under syngeneic or allogeneic conditions for 9 days. RNA was extracted from cell pellets, reverse transcribed, and used in PCR reactions with CCR5-, CCR6-, or GAPDH-specific primers. A representative agarose gel is shown in *A*, while pooled data from three experiments are shown in *B*, presented as the ratio of band intensity relative to GAPDH. ∗, Statistical significance at $p < 0.05$.

CD4, CCR5, and CCR6. As illustrated in Fig. 3, four distinct subpopulations could be identified within the total CD4$^+$ population on the basis of CCR5 and CCR6 expression. The double-negative (CCR5$^-$CCR6$^-$) population was the largest (see total CD4$^+$, R1 in Fig. 3); however, by separating the total CD4$^+$ population into resting (CD4$^{normal}$, R2) and activated (CD4$^{high}$, R3) subpopulations, it was observed that most of this double-negative population resided in the CD4$^{normal}$ subpopulation, indicating that the double-negative cells were mostly resting T cells that had not been activated in the MLR. The activated (CD4$^{high}$) T cell population contained a small number of double-negative cells and CCR6 single-positive cells and larger populations of CCR5 single-positive and double-positive (CCR5$^+$CCR6$^+$) cells.

### *Mechanisms of CCR5 and CCR6 up-regulation in the allogeneic MLR*

To determine whether the increased expression of CCR5 and CCR6 was due to increased levels of mRNA for the receptors, RT-PCR analysis was performed on cell pellets collected from allogeneic and syngeneic cultures after a 9-day incubation period. As illustrated in Fig. 4, the levels of mRNA for both CCR5 and CCR6 were significantly higher in allogeneic compared with syngeneic cultures ($p < 0.05$ for both), suggesting that enhanced transcription of the CCR5 and CCR6 genes resulted in the increased levels of surface protein detected by flow cytometry.

However, the expression of CCR5 and CCR6 on the cell surface is likely to reflect not only mRNA levels, but also the level and nature of receptor trafficking between the cell surface and intracellular compartments. Our previous studies with this system revealed intracellular stores of CXCR3 protein that may serve as an additional regulatory mechanism contributing to the up-regulation of this receptor (15). To determine the cellular distribution of CCR5 and CCR6, cells cultured for 9 days were processed for flow cytometric analysis of chemokine receptor expression with or without prior permeabilization to allow detection of intracellular as well as extracellular receptors. The level of staining for CCR5 was identical in permeabilized and nonpermeabilized CD4$^+$ T cells regardless of whether the cells were taken from syngeneic or allogeneic cultures, indicating that CCR5 protein is not found intracellularly under either culture condition (Fig. 5, *A* and *B*). The lack of intracellular CCR5 was confirmed by immunofluorescence microscopy (Fig. 5*C*), which demonstrated that CCR5 staining was primarily restricted to the cell surface despite the inclusion of a permeabilization step before labeling. Because lymphocytes have only a small amount of cytoplasm, it was possible that low level intracellular staining may be mistaken for surface staining. However, the patterns of CCR5 staining were virtually identical with those observed when nonpermeabilized cells were labeled with anti-CD4 (data not shown), suggesting that CCR5 is, in fact, localized exclusively on the cell surface.

In contrast to the results for CCR5, permeabilization of CD4$^+$ T cells from syngeneic or allogeneic cultures resulted in an increased number of cells staining positively for CCR6 ($p < 0.05$ for both), suggesting that this receptor is stored intracellularly or is undergoing continual recycling between the cytoplasmic and cell surface compartments. In both allogeneic and syngeneic cultures, ~50% of the CD4$^+$ T cells stained positively for CCR6 following permeabilization, suggesting that a subpopulation of cells constitutively expresses CCR6 intracellularly in the absence of surface expression, and that following alloactivation some, but not all, of these cells begin expressing CCR6 on the surface. The intracellular localization of CCR6 protein was confirmed by immunofluorescence microscopy, as illustrated in Fig. 5*C*. In both syngeneic and allogeneic cultures a punctate distribution of protein was evident throughout the cell. Surprisingly, surface staining around the circumference of the cells was not obvious. However, many of the pockets of staining appear to be localized close to the cell surface, suggesting that extracellular CCR6 protein may be arranged in distinct foci on the cell membrane.

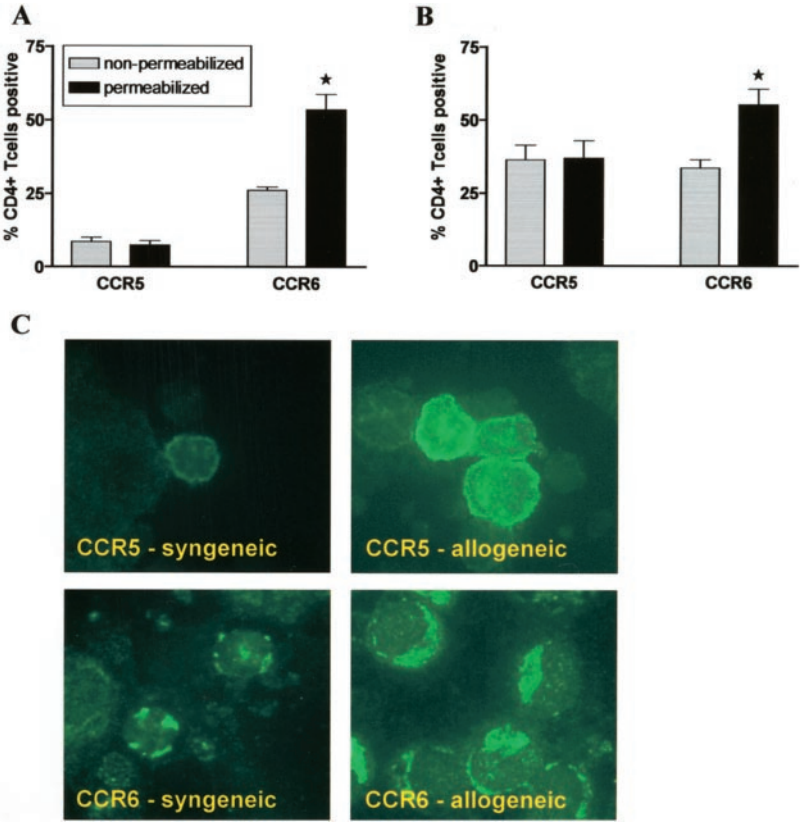


**FIGURE 5.** Detection of intracellular chemokine receptor protein. PMBC were purified from two unrelated donors and cultured under syngeneic or allogeneic conditions for 9 days. *A* and *B*, Cells were collected from syngeneic (*A*) or allogeneic (*B*) cultures, fixed in paraformaldehyde, then either permeabilized or left untreated. Permeabilized and nonpermeabilized cells were labeled with anti-CD4 and either anti-CCR5 or anti-CCR6 and analyzed by flow cytometry, and the percentage of double-positive cells was calculated as a proportion of total $CD4^+$ cells. Data are presented as the mean ± SEM ($n = 4–8$). *C*, Cells were spun onto microscope slides, fixed, permeabilized, stained with anti-CCR5 or anti-CCR6 as indicated, and subjected to fluorescent microscopic analysis. Images shown are representative of at least four experiments. ∗, Statistical significance at $p < 0.05$.

*In contrast to CXCR3 and CXCR4, the regulation of CCR5 and CCR6 expression is more dependent on time in culture than cell division status*

Some programs of T cell gene expression, such as the up-regulation of cytokine genes, are intimately dependent on the initiation and progression of cell division that occur in response to TCR triggering and costimulation (22). To determine the relationship between the up-regulation of CCR5 and CCR6 and cell division, studies using the intracellular tracker dye CFSE were undertaken. Cells were labeled with the dye before culture, then analyzed by flow cytometry 6 or 9 days later. Cell division is associated with a progressive 2-fold loss in CFSE fluorescence intensity (23), thus enabling the expression patterns of chemokine receptors on $CD4^+$ T cells that have undergone a defined number of cell divisions to be compared.

As shown in Fig. 6*A*, the percentage of cells positive for CCR5 on day 6 increased progressively over the first two rounds of cell division (the difference between populations that had divided zero and two times was statistically significant; $p < 0.005$), but then remained relatively stable over subsequent division cycles. Even

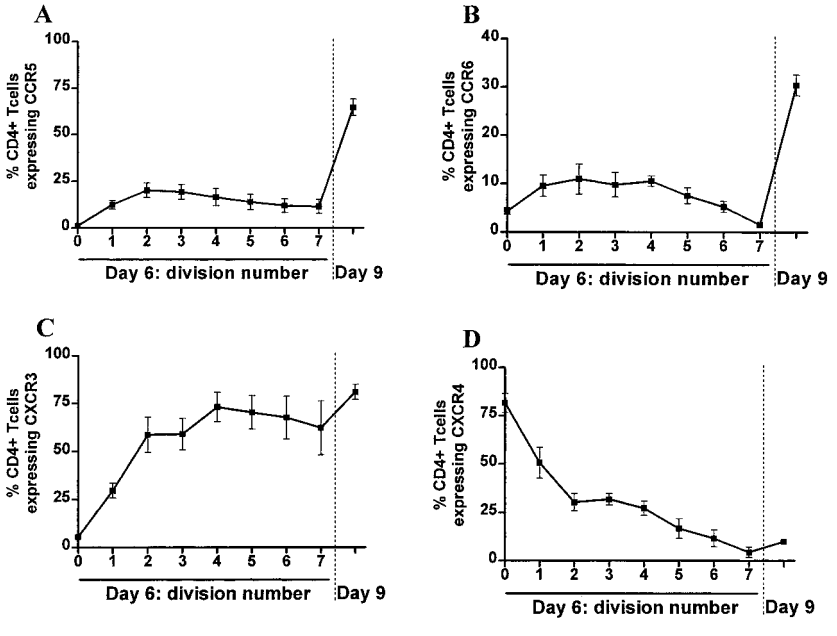


**FIGURE 6.** Division and time dependence of chemokine receptor regulation. PMBC were purified from two unrelated donors, mixed to generate an allogeneic MLR, then labeled with CFSE and cultured for 6 or 9 days. Following culture, cells were labeled with anti-CD4 and either anti-CCR5 or anti-CCR6 and analyzed by flow cytometry. On day 6 the intensity of CFSE fluorescence was used to designate cells as undivided or as having undergone the indicated number of cell divisions. On day 9 it was not possible to accurately discriminate the number of cell division cycles; accordingly, all divided cells were considered as a single population. Data represent the percentage of $CD4^+$ T cells expressing CCR5 (*A*), CCR6 (*B*), CXCR3 (*C*), or CXCR4 (*D*) within each population (mean ± SEM; $n = 4–6$).

after seven or more rounds of cell division, there were no further increases in CCR5 expression on day 6. However, after another 3 days in culture, a striking up-regulation of CCR5 was observed in the divided population ($p < 0.001$ compared with the undivided population; note that it was not possible to accurately discriminate the number of cell division cycles at 9 days; accordingly, all divided cells were considered as a single population). These observations suggest that there are two distinct phases to CCR5 up-regulation: one that occurs immediately following the initiation of cell division and another that occurs many days after the T cell activation process is initiated.

In contrast to the patterns observed for CCR5, no up-regulation of CCR6 was detectable on CD4$^+$ T cells on day 6, even after at least seven rounds of cell division (the difference between, for example, divisions 0 and 2 was not statistically significant; $p > 0.05$; Fig. 6*B*). However, by day 9 the percentage of divided cells staining positively for CCR6 had increased significantly ($p < 0.005$ compared with the undivided population), indicating that CCR6 up-regulation is highly dependent on the time that has elapsed since cellular activation and occurs as a late activation event, temporally distinct from the cell division process.

These results suggest that, unlike the regulation of the cytokine-producing phenotype, the regulation of chemokine receptor expression is partially (CCR5) or completely (CCR6) dependent on the time that has elapsed since the initial TCR-triggering event. To determine whether this phenomenon applied to the regulation of other chemokine receptors, similar studies were performed using Abs to CXCR3 and CXCR4. Remarkably, the up-regulation of CXCR3 (Fig. 6*C*) and the down-regulation of CXCR4 (Fig. 6*D*) occurred in close parallel with the cell division process on day 6, and no further changes in receptor expression were noted on day 9.

### *Allogeneic activation results in increased migration toward CCR5, but not CCR6, ligands*

The most apparent reason for increased CCR5 and CCR6 expression on activated T cells would be to enable migration along gradients of inflammatory chemokines in peripheral tissues. To test whether the up-regulation of CCR5 and CCR6 expression enabled activated T cells to migrate more efficiently toward the relevant chemokine ligands, cells from day 9 allogeneic and syngeneic cultures were subject to Transwell chemotaxis assays. As shown in Fig. 7*A*, migration toward the CCR5 ligand MIP-1$\beta$/CCL4 was virtually undetectable in syngeneic cultures, but increased significantly following allogeneic activation ($p < 0.005$). In marked contrast, cells from allogeneic and syngeneic cultures displayed an almost identical migratory response to the CCR6 ligand MIP-3$\alpha$/CCL20 ($p > 0.05$). To confirm this negative result with respect to MIP-3$\alpha$/CCL20, more detailed chemotaxis studies were undertaken. A highly sensitive, fluorescent-based assay was used to test migration at a range of MIP-3$\alpha$/CCL20 concentrations, and the cells were labeled with anti-CD4 before and after migration, such that the specific response of CD4$^+$ T cells could be assessed in the absence of other potentially contaminating lymphocyte populations (Fig. 7*B*). In agreement with the data presented in Fig. 7*A*, these studies confirmed the lack of enhanced migration toward MIP-3$\alpha$/CCL20 in allogeneic compared with syngeneic cultures. At the lowest concentration of MIP-3/$\alpha$CCL20 tested, there appeared to be slightly enhanced chemotaxis in the allogeneic population compared with that in the syngeneic controls, but this difference was not statistically significant ($p > 0.05$).

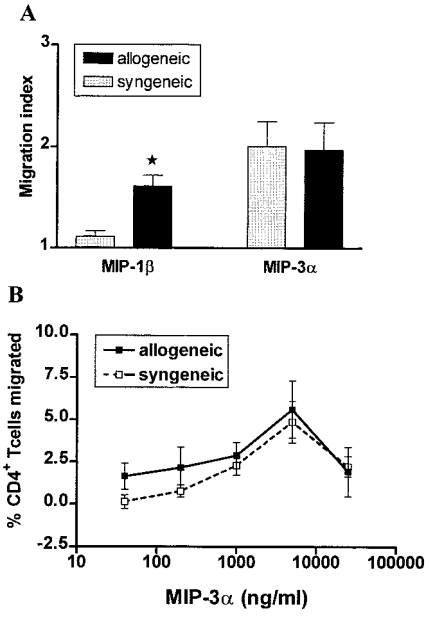


**FIGURE 7.** Chemotactic responsiveness of cells from MLR cultures to MIP-1$\beta$/CCL4 and MIP-3$\alpha$/CCL20. PMBC were purified from two unrelated donors and cultured under syngeneic or allogeneic conditions. On day 9 of culture cells were collected and subject to Transwell chemotaxis assays using MIP-1$\beta$/CCL4, MIP-3$\alpha$/CCL20, or diluent alone in the lower chamber. *A*, The absolute number of migrated cells was determined by duplicate hemocytometer counts, and the migration index was calculated, as described in *Materials and Methods*. *B*, The percent migration was determined fluorometrically, as described in *Materials and Methods*, and the migrated cells were labeled with anti-CD4 and subjected to flow cytometric analysis to determine the percentage of CD4$^+$ T cells migrated. Values are the mean ± SEM ($n = 4–6$). ∗, Statistical significance at $p < 0.05$.

## Discussion

Like many other receptors for inflammatory chemokines, CCR5 and CCR6 are expressed on circulating memory T cells and long-term activated T cell clones, but are absent on naive T cells (9–12, 14, 17). This distinction suggests that CCR5 and CCR6 are acquired by T cells during the activation process, a concept that is supported by early observations of CCR5 expression on clusters of cells in lymph nodes that are morphologically consistent with T cell blasts (11). However, direct evidence of the acquisition of CCR5 or CCR6 during the T cell activation process has been difficult to obtain, given that previous studies have failed to observe significant up-regulation of these receptors in response to T cell activation with mitogenic lectins (8, 12), anti-CD3 (9, 14), or anti-CD3 plus anti-CD28 (10, 24).

In the present study, we have documented significant and consistent up-regulation of CCR5 and CCR6 during the allogeneic activation of primary T cells. It is likely that the activation of T cells by APC, as occurs in the MLR, provides T cells with various signals in addition to stimulation through the TCR that are required for the induction of novel patterns of chemokine receptor expression. If these signals are absent or inappropriate, as may be the case with lectin, anti-CD3, or anti-CD3 plus anti-CD28 stimulation, up-regulation will not be observed. In support of this concept, HIV infection studies have demonstrated that the infection of CD4$^+$ T cells with R5 virus isolates is increased following activation by APC in vitro or in vivo, but is decreased following in vitro stimulation with anti-CD3 plus anti-CD28 (18). These findings suggest that the up-regulation of CCR5, which is required for the entry of R5 viruses, only occurs following T cell interaction with APC and not in response to APC-independent in vitro stimulation.

The signals required for up-regulation of CCR5 and CCR6 could include physiologically relevant costimulatory pathways and immunomodulatory cytokines. In this regard it has been demonstrated that costimulation through CD28 down-regulates CCR5 expression, whereas simultaneous costimulation through a combination of CD28 and CTLA-4 results in CCR5 up-regulation (18). Cytokines also clearly play a critical role in the regulation of CCR5 and CCR6 expression. Although T cell activation using anti-CD3 alone does not have a significant effect on CCR5 or CCR6 expression (9, 14), the combination of anti-CD3 plus IL-2 induces a dramatic up-regulation of CCR5 expression levels (11, 13, 24). Moreover, IL-2 alone is sufficient to up-regulate the expression of CCR5 (11, 12, 17, 24), although the ability of IL-2 to up-regulate CCR6 is unclear (8, 9, 17). Thus, it is possible that the lack of up-regulation of CCR5, and possibly CCR6, in response to anti-CD3 treatment is due to insufficient production of IL-2 in the cultures. Notably, IL-15, a cytokine with properties similar to those of IL-2, has also been shown to up-regulate CCR5 and CCR6 expression (17, 25).

In the present study, the expression of CCR5 and CCR6 on $CD4^+$ T cells activated in the MLR showed a good correlation with the acquisition of an activated/memory phenotype. However, the inverse was not necessarily true, in that a proportion of activated/memory cells remained negative for CCR5 and, in particular, CCR6. Colabeling activated $CD4^+$ T cells for both CCR5 and CCR6 confirmed that CCR5 was expressed on a greater percentage of cells and revealed that three distinct subpopulations could be identified on the basis of CCR5 and CCR6 expression: a small population of $CCR6^+CCR5^-$ cells and much larger populations of $CCR5^+CCR6^-$ and $CCR5^+CCR6^+$ cells. Interestingly, another inflammatory chemokine receptor, CXCR6, shows almost perfect coexpression with CCR5, but only around half of the $CXCR6^+$ T cells coexpressed CCR6 (17), providing further evidence that the expression of CCR6 in the memory population is more restricted than that of other inflammatory chemokine receptors. It is not clear at this stage whether the groups of activated T cells defined by CCR5 and CCR6 expression represent functionally distinct subpopulations or simply reflect the heterogeneity of the T cell response. Future studies aimed at isolating each subpopulation by cell sorting may allow the populations to be compared in terms of, for example, their trafficking properties in vivo or their cytokine-producing phenotype.

In keeping with the increased surface expression of CCR5 and CCR6, the level of mRNA for each receptor was higher in allogeneic compared with syngeneic cultures. However, intracellular stores of CCR6, but not CCR5, were detected in $CD4^+$ T cells, suggesting the existence of more complex regulatory mechanisms. Thus, whether the increased surface expression of CCR6 is due to translocation of preformed receptor protein from the cytoplasm or to enhanced de novo protein production resulting from increased transcription is not clear at this stage. However, it is interesting to note that only ~50% of $CD4^+$ T cells expressed CCR6 intracellularly, and that this was not altered upon allogeneic activation. Therefore, a possible scenario is that only a proportion of $CD4^+$ T cells are able to produce CCR6, and that these cells do so constitutively and store the protein intracellularly. Upon activation, transcription of the CCR6 gene is increased, saturating the mechanisms that maintain CCR6 inside the cell and enabling expression on the cell surface. This increased level of transcription would presumably be maintained as the cells return to a resting state, thereby enabling memory T cells to continue expressing the receptor on the surface. This scenario is in keeping with the observation that only ~50% of the activated $CD4^+$ T cells that up-regulated CCR5 also up-regulated CCR6.

The time-course studies presented in Fig. 1 suggested that the up-regulation of CCR5 and CCR6 expression was a late event, peaking around days 9–12. This was confirmed by the CFSE studies. On day 6, up-regulation of these receptors was either minimal (CCR5) or undetectable (CCR6) regardless of the number of rounds of division the cells had undergone. In contrast, a striking up-regulation of both CCR5 and CCR6 was detectable on the divided population on day 9, a time at which cellular proliferation is markedly reduced (15). This observation suggests that the up-regulation of CCR6 and, to a lesser extent, CCR5 is only initiated after a defined period of time has elapsed since activation and initiation of cell division. Despite the temporal distinction, however, cell division is clearly a prerequisite for up-regulated expression of CCR5 and CCR6, as the undivided population did not alter the expression of these receptors.

Delaying chemokine receptor up-regulation until after T cells have completed the cell division process may be a mechanism to prevent activated T cells leaving the secondary lymphoid tissue until they have undergone proliferation and differentiation in an appropriate microenvironment. However, such a mechanism is clearly not a universal theme in chemokine biology, as both CXCR3 and CXCR4 were subject to changes in expression that began immediately upon initiation of division and progressed in coordination with subsequent divisions. Additionally, there was a small increase in the expression of CCR5, but not CCR6, after the first two rounds of cell division on day 6. It is possible that chemokine receptors that are modulated immediately upon cellular activation play a role distinct from those that are modulated several days after initiation of cell division. One possibility is that these early receptors are required for altered microenvironmental positioning of dividing T cells within the secondary lymphoid tissue, for example, to provide B cell help. Of relevance, the CXCR3 ligands Mig/CXCL9, IP-10/CXCL10, and I-TAC/CXCL11 are expressed in the lymph nodes of rats during experimental autoimmune encephalomyelitis,[4] and ligands for CCR5 have also been identified in the draining lymph node during elicitation of contact hypersensitivity (26). The expression of these chemokines and others (27) in lymph nodes under inflammatory conditions suggests a functional role within secondary lymphoid tissue.

The most apparent reason for increased expression of CCR5 and CCR6 on activated T cells is to enable these cells to begin sensing inflammatory chemokine gradients produced in peripheral tissues in response to infection and thereby move specifically to the appropriate site. In keeping with this hypothesis, allogeneic activation resulted in activated T cells being able to respond chemotactically to the CCR5-specific ligand MIP-1$\beta$/CCL4. In contrast, $CD4^+$ T cells from syngeneic cultures were already able to respond to MIP-3$\alpha$/CCL20, presumably due to the expression of CCR6 on a reasonable proportion of T cells in the starting population, and allogeneic activation did not increase the chemotactic response further.

The lack of increased migration toward MIP-3$\alpha$/CCL20 following allogeneic activation suggests that the up-regulation of CCR6 plays an alternative function. While a number of possibilities exist, integrin activation is a likely candidate. MIP-3$\alpha$/CCL20 induces the adhesion of memory T cells to ICAM (28), and moreover, a recent study by Fitzhugh et al. (29) suggests that the interaction

[4] S. R. McColl, S. Mahalingham, M. Staykova, L. A. Tylaska, K. E. Fisher, C. A. Strick, R. P. Gladue, K. S. Neote, and D. O. Willenborg. Cloning and characterization of rat I-TAC/CXCL11 and other CXCR3 ligands in experimental autoimmune encephalomyelitis in the rat. *Submitted for publication.*

between MIP-3$\alpha$/CCL20 and CCR6 is a critical factor in the ability of memory T cells to firmly adhere to endothelial cells, a prerequisite for extravasation. Alternatively, it is conceivable that signaling through MIP-3$\alpha$ is required for the induction of alternative patterns of protein synthesis that are required for activated T cell function, such as the initiation of distinct cytokine secretion pathways or the expression of altered cell surface molecules. Finally, the possibility remains that the purpose of CCR6 up-regulation is to mediate chemotaxis toward MIP-3$\alpha$, but that a further signal, such as that provided by a cytokine, is required to couple the newly acquired receptor to the appropriate intracellular signaling cascades. In this context it is interesting to note that while CCR6 is expressed at readily detectable levels by freshly isolated B cells, no chemotactic or calcium flux response to MIP-3$\alpha$ is observed (9).

Together, the present study and our previous study of chemokine receptor expression in the MLR (15) demonstrate that four chemokine receptors are regulated on CD4$^+$ T cells in response to allogeneic activation. However, there are notable differences in the kinetics and functional outcome of regulation, the proportion of activated cells that modulate the receptors, and the molecular mechanisms by which changes in surface expression are achieved. Based on the proportion of divided cells expressing each receptor, there appears to be a hierarchy in the extent of receptor regulation, with CXCR3 and CXCR4 being modulated on the majority of divided cells (81 and 91% respectively), CCR5 on ~65% of the divided population, and CCR6 on ~30% of divided cells. Two of the receptors, CXCR3 and CXCR4, are modulated in close parallel with cell division, while in the case of CCR5 and CCR6, division and receptor regulation are temporally distinct. The up-regulation of CCR5, CCR6, and CXCR3 is associated with an increase in mRNA levels, while, in contrast, the down-regulation of CXCR4 protein is not mirrored by a corresponding decrease in CXCR4 transcripts. CXCR3 and CCR6, but not the other two receptors, are stored intracellularly, although even here there are differences; the percentage of CD4$^+$ T cells expressing intracellular CXCR3 is nearly 100%, while only around half that express CCR6 intracellularly. Finally, while the regulation of CCR5, CXCR3, and CXCR4 resulted in altered chemotactic responsiveness toward the respective ligands, the up-regulation of CCR6 did not result in enhanced migration toward MIP-3$\alpha$/CCL20. Thus, although activated T cells express a number of different chemokine receptors, the variability observed with regard to their expression and function suggests that each may play a unique biological role and its expression be fine-tuned by distinct mechanisms.

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