Coregulation of CXC Chemokine Receptor and CD4 Expression on T Lymphocytes During Allogeneic Activation

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Upon activation, naïve T cells alter their migratory patterns, acquiring the ability to move through peripheral tissues as well as the general lymphoid circulation. Although the mechanisms responsible for these alterations are not well understood, changes in chemokine receptor expression may play a critical role. To investigate these changes, the expression patterns of two chemokine receptors, CXCR3 and CXCR4, were compared on CD4+ T cells following activation in the MLR. By day 9 of activation, expression of the inflammatory chemokine receptor CXCR3 was up-regulated, while expression of the homeostatic chemokine receptor CXCR4 was down-regulated. Alterations in receptor expression occurred almost exclusively on a subpopulation of T cells that expressed higher levels of CD4. These CD4high T cells demonstrated many characteristics of activated T cells and had undergone division in the MLR. By day 9 of culture, the majority of CXCR3+ and CXCR4− cells had divided and had acquired an activated/memory phenotype (CD45RA− CD45RO+ CD69+ CD25+). The levels of transcripts for both CXCR3 and CXCR4 were increased upon allo-activation. The discrepancy between levels of CXCR4 mRNA and surface protein was not due to sequestration of the receptor in intracellular compartments, as CXCR4 was not detectable intracellularly. However, intracellular CXCR3 was readily detectable. Finally, cells from allogeneic cultures demonstrated enhanced migration toward IFN-inducible T cell α chemoattractant and reduced migration toward stromal cell-derived factor-1 compared with syngeneic controls, thus suggesting that the observed switch in receptor expression may at least partly contribute to the differential patterns of migration displayed by naïve and memory T cells. The Journal of Immunology, 2001, 166: 4870–4878.

The process of mounting a primary immune response involves sequential changes in the migratory properties of naïve T cells (1–3). Resting naïve T cells traffic through the blood and enter the lymph nodes directly, through specialized high endothelial venules, consequently bypassing the peripheral tissues. However, following clonal expansion and differentiation in secondary lymphoid tissue, activated T cells must adopt additional migratory routes that allow them to pass through vascular endothelium and thereby enter peripheral tissues where target Ag is likely to be located. The molecular mechanisms responsible for this switch in migratory patterns are not well understood, but it is likely that the regulation of chemokine and chemokine receptor expression plays a critical role in addition to the regulation of adhesion molecules (2, 4).

Chemokines can be divided into two major (CC and CXC) and two minor (C and CX3C) families based on a cysteine signature motif (5). Alternatively, they can be classified as homeostatic (constitutive) or inflammatory (inducible), according to their function (4, 6). Homeostatic chemokines are expressed constitutively within lymphoid tissues and appear to be responsible for the trafficking of lymphocytes under conditions of homeostasis. On the other hand, inflammatory chemokines are specifically up-regulated at sites of inflammation and are thought to play a role in the recruitment of leukocytes to peripheral tissues in response to immunological challenge.

The chemokine receptors CXCR3 and CXCR4 are expressed on mononuclear cells, including various subsets of T lymphocytes (7–9). CXCR3 is the receptor for the inflammatory chemokine IFN-γ-inducible protein 10 kDa (IP-10); IP-10, monokine induced by IFN-γ (Mig), and IFN-inducible T cell α chemoattractant (I-TAC) (10, 11), and is expressed on the majority of circulating CD8+ T cells, but expression on peripheral blood CD4+ T cells is largely restricted to the memory population (12). A number of studies have also demonstrated preferential expression of CXCR3 on Th1 cells compared with Th2 cells (13, 14), although the extent of the association of CXCR3 with the Th1 phenotype has been questioned (15). CXCR4, the receptor for the homeostatic chemokine stromal cell-derived factor-1 (SDF-1) (16), is found on a substantial proportion of circulating CD4+ and CD8+ T cells. Expression of CXCR4 is not uniform within the T cell population, however, as higher levels of CXCR4 have been detected on naïve cells compared with memory cells (7, 9, 14, 17).

CXCR3 and CXCR4 are not the only receptors to show differential expression patterns on naïve vs memory T cells. In fact, a general trend is emerging whereby inflammatory chemokine receptors are found to be expressed on a variable proportion of memory T cells in the peripheral blood, but are virtually absent on naïve cells. On the other hand, homeostatic chemokine receptors are expressed at high levels on the majority of naïve T cells, but may show reduced expression on memory cells (4). These trends suggest that at some stage during the process of naïve T cell activation there is a switch in chemokine receptor expression from homeostatic to inflammatory. This switch would allow activated/memory

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3 Abbreviations used in this paper: IP-10, IFN-γ-inducible protein (10 kDa); Mig, monokine induced by IFN-γ; I-TAC, IFN-inducible T cell α chemoattractant; SDF-1, stromal cell-derived factor-1; DC, dendritic cell.
T cells to adopt additional migratory patterns, thereby allowing entry to the appropriate peripheral tissues (4, 18).

Although modulation of chemokine receptor expression during T cell activation is an attractive mechanism to explain differential homing patterns of lymphocytes, and clearly there are changes to chemokine receptor expression upon T cell activation, the exact nature of these changes is in contention. While some studies have shown up-regulation of inflammatory chemokine receptors on peripheral blood T cells upon anti-CD3 or mitogen stimulation (8, 12, 19–21), others have demonstrated no change or even decreased expression upon similar treatments (7, 22, 23). The concept of a homeostatic chemokine receptor down-regulation upon TCR triggering (24, 25) is similarly challenged by considerable conflicting data (7, 12, 22, 26). The concept of a switch in chemokine receptor expression upon T cell activation is thus still awaiting rigorous proof-of-concept. Importantly, it is also not yet known what changes in receptor expression occur when Ag is presented to naive T cells by dendritic cells (DC), as occurs in a primary immune response in vivo. The aim of this study was therefore to compare the expression patterns of CXCR3 and CXCR4 on CD4+ T cells following activation in a primary MLR. This culture system is dependent on DC for the majority of APC function (27) and results in the proliferation of naive as well as memory CD4+ T cells (28).

Materials and Methods

Abs and conjugates

Abs to CD3 (OKT3) and CD4 (OKT4) were a gift from Dr. R. Roy (Center de Recherche en Rheumatologie et Immunologie, Universite Laval, Quebec, Canada). Anti-CXCR3 (clone 49801.1111) and biotinylated anti-CXCR4 (clone 4e716.111) were purchased from R&D Systems (Minneapolis, MN). Isotype controls were a gift from Prof. H. Zola (Child Health Research Institute, Adelaide, Australia). Where appropriate, Abs were labeled with FITC (Sigma Australia, Castle Hill, Australia) or aminohexanoyl-biotin-N-hydroxysuccinimide ester (Zymed, San Francisco, CA) as previously described (28a). 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) and anti-CD69 (clone FN50) were obtained from PharMingen (San Diego, CA). PE-conjugated streptavidin was obtained from Southern Biotechnology Associates (Birmingham, AL), while streptavidin conjugated to either FITC or PE-Cy5 was obtained from Rockland Immunocchemicals (Gilbertsville, PA).

PBMC isolation and MLR

Peripheral blood was collected from healthy volunteers into lithium-heparin tubes, and the erythrocytes were removed by sedimentation in 2% dextran. Mononuclear cells (PBMC) were isolated by centrifugation over Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) for 30 min, and suspended to 1.5 × 10^7 viable cells/ml in RPMI supplemented with 10 mM HEPES, 2 mM l-glutamine, antibiotics, and 10% heat-inactivated pooled human AB serum (Red Cross, Adelaide, Australia). Stimulator cells were prepared by irradiation (3000 rad), then mixed in a 1/1 ratio with nonirradiated responder cells to give either allogeneic or syngeneic cultures. Cultures were aliquoted to 200- or 2-ml volume, respectively. Following incubation at 37°C in a humidified atmosphere with 5% CO2, proliferation was quantified by the addition of 1 Ci of [3H]thymidine (Amersham, Aylesbury, U.K.) 18 h before harvest. DNA preparations were air-dried, fixed in paraformaldehyde (4% in PBS) for 20 min at 4°C, and then washed in ice-cold PBS. Cells were permeabilized by incubation in saponin buffer for 15 min at 4°C and washed, and the primary Ab (containing a one-third volume of saponin buffer) was added. Following a 1-h incubation at 4°C, the slides were washed, streptavidin-FITC was added, and the slides were incubated for an additional 1 h. Following extensive washing, slides were coverslipped with Mowiol mounting medium (Calbiochem, La Jolla, CA) containing p-phenylene-diamine (Sigma) as an anti-fade agent. Slides were viewed and photographed using an Olympus microscope and digital camera (Melville, NY).

RNA extraction and RT-PCR

Cell pellets from day 9 allogeneic or syngeneic cultures were resuspended in TRIzol (Life Technologies, Melbourne, Australia), and RNA was extracted according to the manufacturer’s recommendations. RNA (5 μg) was treated with DNase I (Promega, Madison, WI) according to the manufacturer’s instructions, and 2 μg was used in first-strand cDNA synthesis, priming with oligo-(dT)15 (Promega) and using the SuperScript II Pre-amplification system (Life Technologies). PCR was performed using AmpliTaq Gold (Perkin-Elmer) following the manufacturer’s instructions. PCR cycling conditions were 95°C for 10 min, 95°C for 30 s, 55°C 1 min, and 72°C 1 min, with steps 2–4 repeated 30 times. Preliminary experiments were conducted at a range of cycle numbers to ensure that the PCR was in the linear phase for each of the cDNAs amplified. Primer sequences were as follows: CXCR3, 5'-ACC TAG CTT CAG ACA GC-3' and 5'-CAT AGC AGT AGG ACC TGA CC-3'; CXCR4, 5'-CCA CCA TCT ACT CCA TCA TC-3' and 5'-GGT TCA GAC AAC AGT GGA AG-3'; GADPH, 5'-TCC TTG GAC GGC ATG TAG GCC AT-3' and 5'-TGA TCA CAT CAA GGT GGT GAA G-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 GADPH PCR product amplified from the same template.

Chemosatosis assay

Cells were resuspended to 5 × 10^6 viable cells/ml in RPMI containing 0.5% BSA (RPMI-BSA). Synthetic SDF-1 and I-TAC (30) (a gift from Dr. I. Clark-Lewis, Biomedical Research Center, University of British Columbia, Vancouver, Canada) were diluted to 100 or 500 ng/ml, respectively, in RPMI-BSA, and 600 μl was added to the lower chambers of a Transwell plate (6.5 mm diameter filter, 5 μm pore size; Corning, Corning, NY).
After addition of 100 μl 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. The total number of cells in the lower chamber was quantified by counting on a hemocytometer, 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 (MI), calculated by dividing the percent migration value obtained in the presence of chemokine by the percent migration value for negative controls.

Statistical analysis
Statistical analyses were performed using a two-tailed Student t test. p < 0.05 was considered significant. Data in the text are presented as the mean ± SEM.

Results
CXCR3 is up-regulated during allogeneic activation, while CXCR4 is down-regulated

Chemokine receptor expression on CD4+ T cells was quantified by flow cytometry for allogeneic and syngeneic (control) MLR cultures. By day 9 of incubation, the inflammatory chemokine receptor CXCR3 was expressed on significantly more CD4+ T cells in allogeneic cultures compared with syngeneic controls (Fig. 1, A and B; p = 0.0046). In contrast, the homeostatic chemokine receptor CXCR4 was notably down-regulated on CD4+ T cells following a 9-day incubation under allogeneic conditions, compared with culture under syngeneic conditions (Fig. 1, C and D). It was not possible to perform a t test on the CXCR4 data, as the SE could not be calculated for the syngeneic data due to the uniformity of values (100% in every experiment).

The time-course analysis presented in Fig. 2 reveals that the percentage of CD4+ T cells expressing CXCR3 was significantly increased by the end of culture (Fig. 2A; 57.9 ± 3.4% positive on day 12 compared with 34.6 ± 3.4% on day 0; p = 0.013), while no significant changes were noted in the syngeneic controls (p = 0.188). The expression of CXCR4, however, showed more complex kinetics (Fig. 2B). On day 0, 75.8 ± 10.2% of CD4+ T cells were positive for this receptor, but in both allogeneic and syngeneic cultures, this figure rose to 100% after 3 days. This nonspecific up-regulation of CXCR4 upon introduction to culture has been documented previously (24, 25), but the reason for this phenomenon is at present unknown. Following allogeneic activation, however, CXCR4 was down-regulated to 73.8 ± 1.8% cells positive on day 12, while at the same time point in syngeneic cultures, CD4+ T cells remained 100% positive.

The observed changes in chemokine receptor expression occurred after the initiation of cell division. The most notable changes to receptor expression occurred between days 9 and 12 (Fig. 2A and B), while proliferation peaked on day 6 and had largely declined by day 9 (Fig. 2C). This observation suggests that changes in the expression of CXCR3 and CXCR4 are unlikely to play a significant role in the initial steps of T cell activation in the secondary lymphoid organs. In support of this hypothesis, a CXCR4 receptor antagonist (31) failed to have any effect on proliferation (not shown).

Changes in chemokine receptor expression occur on a subpopulation of T cells expressing elevated levels of CD4

Changes in chemokine receptor expression occurred almost exclusively on a subset of T cells that expressed higher levels of CD4

FIGURE 1. Expression of CXCR3 and CXCR4 on CD4+ T cells in allogeneic and syngeneic cultures. PMBC were purified from two unrelated donors and cultured under syngeneic (A and C) or allogeneic (B and D) conditions. On day 9 of culture, cells were labeled with anti-CD4 and anti-CXCR3 (A and B) or anti-CXCR4 (C and D) and analyzed by flow cytometry. Shown are density plots representative of at least six experiments. FL1-H, Fluorescence.

FIGURE 2. Time course of chemokine receptor expression and proliferation. 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 plus anti-CXCR3 (A) or anti-CXCR4 (B) and analyzed by flow cytometry. The percentage of double-positive cells was calculated as a proportion of total CD4+ cells. Alternatively, 1 μCi of tritium-labeled thymidine was added 18 h before harvest, and incorporation was determined by scintillation counting (C). Values represent the mean ± SEM (n = 3–5).
than the syngeneic controls (see Fig. 1). CD4\textsuperscript{high} T cells were first detectable on day 6 of culture and greatly increased in number between days 6 and 9 (Fig. 3A). Furthermore, following extended culture for up to 24 days many T cells still expressed elevated levels of CD4. CD4\textsuperscript{high} T cells demonstrated many characteristics of activated T cells. On day 9 the majority expressed the memory marker CD45RO and low or undetectable levels of the naive T cell marker CD45RA (Table I). The activation markers CD25 and CD69 were also expressed on a large proportion of CD4\textsuperscript{high} T cells, with approximately 95% of this population staining positively for CD25 and approximately 50% staining positively for CD69. Forward angle light scatter measurements also indicated that CD4\textsuperscript{high} T cells were larger than CD4\textsuperscript{normal} T cells, another characteristic of activated T cells. Further, the majority of CD4\textsuperscript{high} cells identified on day 9 had undergone division in the MLR, as assessed by dilution of the intracellular fluorescent dye CFSE. Co-expression of CD3 confirmed that CD4\textsuperscript{high} cells were indeed T lymphocytes rather than DC or monocytes, which are also known to express CD4, but do not express CD3 (not shown).

CXCR3 was expressed on 80.8 ± 4.9% of CD4\textsuperscript{high} T cells, while only 30.8 ± 3.5% of CD4\textsuperscript{normal} T cells expressed this receptor (Fig. 3B; \(p < 0.0001\)), a figure almost identical with that in the syngeneic control. Similarly, CXCR4 was expressed on only 28.5 ± 7.5% of the CD4\textsuperscript{high} population, while 88.7 ± 1.3% of cells within the CD4\textsuperscript{normal} population were positive for this receptor (\(p < 0.0001\)). Thus, CD4\textsuperscript{high} cells, which have many characteristics of activated T cells, generally have a CXCR3\textsuperscript{1}CXCR4\textsuperscript{2} phenotype, while CD4\textsuperscript{normal} cells, which appear to be in a resting state, do not have altered expression of these chemokine receptors compared with syngeneic controls.

CXCR3\textsuperscript{+} and CXCR4\textsuperscript{–} CD4\textsuperscript{+} T cells have undergone division in the MLR and have an activated/memory phenotype

The CXCR3\textsuperscript{+} and CXCR4\textsuperscript{–} cells on day 9 were characterized in terms of coexpression of memory and activation markers. CD45RA was used as a marker of naive cells, CD45RO as a marker of memory cells, and CD25 and CD69 as markers of acute activation. Fig. 4A shows that the percentage of CD4\textsuperscript{+} T cells expressing CD45RA decreased significantly in allogeneic cultures over the 9-day incubation period (\(p = 0.0001\)), with a corresponding increase in CD45RO expression (Fig. 4B; \(p = 0.016\)), indicating the transition of a subpopulation of CD4\textsuperscript{+} T cells from naive to memory status. No notable changes in the expression of these markers were observed in syngeneic cultures. The two activation markers investigated, CD25 and CD69, were both up-regulated on a significant proportion of CD4\textsuperscript{+} T cells in allogeneic cultures (\(p = 0.0009\) and \(p = 0.0004\)), but showed no notable changes in syngeneic cultures. It was interesting to note that CD69 was expressed on a smaller proportion of allo-activated CD4\textsuperscript{+} T cells than was CD25. This observation suggests that CD69, a very early activation marker, may have been lost from some of the activated T cells at this relatively late time point, in keeping with the highly unstable nature of its mRNA (32).

Of the CXCR3\textsuperscript{–} CD4\textsuperscript{+} cells, the majority expressed CD45RO and CD25, a large proportion expressed CD69, but few expressed CD45RA (85.7 ± 3.4, 82.3 ± 5.0, 42.9 ± 4.5, and 17.9 ± 1.0% positive, respectively; Fig. 5, A–D), a phenotype consistent with recent activation. Similarly, CD4\textsuperscript{+} T cells that had lost CXCR4 were predominantly CD45RO\textsuperscript{–} CD45RA\textsuperscript{–} (86.3 ± 3.9 and 6.0 ± levels of CD4 were electronically gated, and the percentages of cells expressing the relevant chemokine receptor were determined. Values represent the mean ± SEM (\(n = 7\)). FL1-H, Fluorescence.
0.7% positive, respectively; Fig. 5, E and F), and demonstrated up-regulated expression of the activation markers CD25 and CD69 (98.6 ± 0.4 and 56.5 ± 11.5% positive, respectively; Fig. 5, G and H). Further, the majority of cells that had divided in the MLR, as demonstrated by a reduction in CFSE fluorescence intensity, were CXCR3+ and CXCR4- (92.9 ± 2.4 and 88.4 ± 4.0% of divided cells were CXCR3+ and CXCR4-; respectively; Fig. 5, I and J). Thus, the observed changes in chemokine receptor expression mostly occurred in conjunction with cell division and the acquisition of an activated/memory phenotype.

**CXCR3 and CXCR4 mRNA levels are altered during allogeneic activation**

To determine whether the altered patterns of CXCR3 and CXCR4 expression observed following activation were due to altered 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 (Fig. 6). In keeping with the increased expression of CXCR3 on the cell surface in allogeneic cultures, the expression of CXCR3 transcripts was also significantly elevated in allogeneic cultures compared with syngeneic controls ($p = 0.03$). The down-regulation of CXCR4 following allogeneic activation was not, however, mirrored by a corresponding decrease in CXCR4 mRNA. In fact, the levels of CXCR4 message were consistently and significantly higher in allogeneic compared with syngeneic samples ($p = 0.03$).

**FIGURE 4.** Changes in memory/activation markers observed 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 after 9 days in culture, cells were labeled with anti-CD4 plus anti-CD45RA/anti-CD45RO/anti-CD25/anti-CD69 and analyzed by flow cytometry. Subpopulations of T cells expressing high or low concentrations of CD4 were electronically gated, and the forward light scatter characteristics, percentages of cells expressing activation markers, and the percentages of cells showing a reduction in CFSE fluorescence were determined. Values represent mean ± SEM ($n = 3$).

**FIGURE 5.** Memory/activation marker expression and division status of CD4+ T cells expressing CXCR3 and not CXCR4. 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 plus either anti-CXCR3 (A–D) or anti-CXCR4 (E–H) and anti-CD45RA (A and E), anti-CD45RO (B and F), anti-CD25 (C and G), or anti-CD69 (D and H) and were analyzed by flow cytometry. CD4+ cells were electronically gated, and staining for the remaining markers was displayed on bivariate density plots. Alternatively, cells were labeled before initiation of culture with CFSE, cultured for 9 days, stained with anti-CXCR3/anti-CXCR4, and analyzed by flow cytometry (I and J). Data shown are representative of at least three experiments. FL1-H, Fluorescence.

| Phenotype | CD4 Expression |
|-----------|----------------|
|           | High | Low |
| Size (forward light scatter) | Large (high FSC) | Small (low FSC) |
| % of cells divided | 93.8 ± 3.7 | 24.8 ± 13.7 |
| % expressing CD45RA (naive) | 4.5 ± 1.7 | 56.1 ± 3.1 |
| % expressing CD45RO (memory) | 86.8 ± 4.6 | 37.9 ± 3.7 |
| % expressing CD25 (activated) | 95.4 ± 0.7 | 28.2 ± 2.5 |
| % expressing CD69 (activated) | 50.6 ± 9.1 | 6.0 ± 0.7 |

* PMBC were purified from two unrelated donors, mixed to generate an allogeneic MLR and either labeled with CFSE or left untreated. On day 9 of culture, cells were labeled with anti-CD4 and (where appropriate) anti-CD45RA/anti-CD45RO/anti-CD25/anti-CD69 and analyzed by flow cytometry. Subpopulations of T cells expressing high or low concentrations of CD4 were electronically gated, and the forward light scatter characteristics, percentages of cells expressing activation markers, and the percentages of cells showing a reduction in CFSE fluorescence were determined. Values represent mean ± SEM ($n = 3$).
CXCR3, but not CXCR4, is stored intracellularly in CD4\(^+\) T cells

The amounts of CXCR3 and CXCR4 protein on the cell surface are likely to reflect not only mRNA levels, but also the amount and nature of receptor trafficking between the cell surface and intracellular compartments. We therefore sought to determine whether these receptors were present intracellularly in CD4\(^+\) T cells and, if so, whether the levels of intracellular protein differed between cells cultured under allogeneic and syngeneic conditions. To address this issue, 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 (29).

With regard to CXCR4 staining in allogeneic cultures, permeabilized and nonpermeabilized CD4\(^+\) T cells showed identical levels of staining in terms of both the percentage of positive cells (Fig. 7A) and the intensity of staining (data not shown). CXCR4 staining of CD4\(^+\) T cells from syngeneic cultures was also identical in permeabilized and nonpermeabilized samples. These results indicate that CXCR4 protein is localized exclusively to the surface of the cells under both allogeneic and syngeneic conditions. This conclusion is further supported by the immunofluorescence microscopy images presented in Fig. 7B, which demonstrate that despite the inclusion of a permeabilization step, staining for CXCR4 was restricted to the cell membrane in both allogeneic and syngeneic cultures. Thus, it appears unlikely that the observed down-regulation of CXCR4 upon alloactivation was due to internalization of surface receptor.

With regard to CXCR3 expression on allogeneic cells, the percentage of CD4\(^+\) T cells stained was significantly higher in permeabilized compared with nonpermeabilized samples, indicating that CXCR3 protein was present intracellularly (Fig. 7A; \(p = 0.002\)). Similarly, in syngeneic cultures, the percentage of CD4\(^+\) T cells positive for CXCR3 was significantly greater following permeabilization (\(p < 0.0001\)). The presence of intracellular stores of CXCR3 was confirmed by immunofluorescence microscopy of permeabilized cells. For both allogeneic and syngeneic samples, CXCR3 was shown to have a punctate distribution throughout the cell in addition to surface staining (Fig. 7B).

Notably, when the cells were permeabilized, the percentage of cells positive for CXCR3 was virtually identical between allogeneic and syngeneic cultures (Fig. 7A). This is in sharp contrast to the results obtained using nonpermeabilized cells, which demonstrate (as observed previously in Figs. 1 and 2) that a significantly higher percentage of CD4\(^+\) T cells expressed CXCR3 when cultured under allogeneic conditions compared with syngeneic controls. These results suggest that a large proportion of resting CD4\(^+\) T cells express CXCR3 intracellularly in the absence of detectable surface expression, and that following allogeneic activation some (but not all) of these cells begin expressing CXCR3 on the surface.

**FIGURE 6.** Up-regulation of CXCR3 and CXCR4 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 CXCR3-, CXCR4-, or GAPDH-specific primers. A representative agarose gel is shown in A, and pooled data from three independent experiments are shown in B; data are presented as the ratio of band intensity relative to GAPDH. *, \(p < 0.05\). M, 100-bp marker.

**FIGURE 7.** Detection of intracellular chemokine receptor protein. PMBC were purified from two unrelated donors and cultured under syngeneic or allogeneic conditions for 9 days. A, Cells were collected, fixed in paraformaldehyde, then either permeabilized or left untreated. Permeabilized and nonpermeabilized cells were labeled with anti-CD4 plus either anti-CXCR3 or anti-CXCR4 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). B, Cells were spun onto microscope slides, fixed, permeabilized, stained with anti-CXCR3 or anti-CXCR4, and viewed under UV light. Images shown are representative of four independent experiments.
biological significance of these changes in receptor expression, we tested the ability of cells from allogeneic and syngeneic day 9 cultures to migrate toward I-TAC, a CXCR3 ligand, and SDF-1, a CXCR4 ligand (Fig. 8). The mean migration index for I-TAC was significantly higher in allogeneic than syngeneic cultures (3.6 ± 0.4 for allogeneic cells compared with 2.5 ± 0.2 for syngeneic cultures; \( p = 0.017 \)). Conversely, migration in response to SDF-1 was significantly reduced in allogeneic cultures compared with syngeneic controls (3.3 ± 0.2 migration for allogeneic cells compared with 5.2 ± 0.3 migration for syngeneic cells; \( p = 0.0005 \)). These results indicate that regulation of chemokine receptor expression in response to allogeneic stimulation can alter the responsiveness of T cells to inflammatory vs homeostatic chemokines.

**Discussion**

The MLR provides a powerful in vitro tool for studying naïve T cell activation, as it involves cell-cell interactions between DC and resting peripheral blood T cells. The mechanism of T cell activation is therefore biologically relevant, yet polyclonal in nature and consequently easily measurable. Using this system we have demonstrated specific up-regulation of CXCR3, an inflammatory chemokine receptor, and simultaneous down-regulation of CXCR4, a homeostatic chemokine receptor. The observation that only a subpopulation of T cells modulated the expression of CXCR3 and CXCR4 was expected, as only a proportion of T cells in the MLR will recognize foreign MHC and thus be activated. Further, the fact that these receptors were not regulated similarly on all CD4+ T cells in the culture provides strong evidence that the observed changes were not mediated solely by a soluble factor, such as a cytokine or chemokine, but required Ag-induced cellular activation.

The up-regulation of CXCR3 observed in the MLR is in keeping with previous reports of CXCR3 up-regulation following CD3 cross-linking (8, 12), suggesting that control of this receptor is directly linked to stimulation of the TCR complex, rather than dependent on the nature of the Ag or costimulatory signals provided by the APC. Of interest, however, is a report of short-term CXCR3 down-regulation upon anti-CD3 stimulation. This effect was observed over a 2- to 8-h period, suggesting that expression of CXCR3 may initially drop in the early stages of T cell activation, then increase later in the activation process (23). With regard to CXCR4, previous studies have demonstrated differing expression patterns of this receptor following peripheral blood T cell activation using anti-CD3 or PHA, with expression being increased (7, 22), decreased (24, 26), or remaining relatively unchanged (12). The present study provides evidence, using a biologically relevant model that simulates the primary immune response, that CXCR4 is down-regulated on naïve T cells following activation. A possible explanation for the conflicting results described is the observation that CXCR4 surface expression is increased in culture without stimulation. Previous studies that demonstrated up-regulation of CXCR4 in response to PHA or anti-CD3/anti-CD28 treatments did not include parallel cultures without stimulation (7, 22), thus raising the possibility that the increased expression observed was not stimulus specific. In support of the findings of the present study, Abbal and colleagues used purified allergen extracts to activate Ag-specific memory Th2 cells from the peripheral blood of allergic individuals and demonstrated significant down-regulation of CXCR4 on the surface of T cells (25). Considering that the MLR has been shown previously to be a Th1-type response (33), the combined results of the present study and that of Abbal et al. (25) suggest that the down-regulation of CXCR4 in response to Ag-induced activation occurs similarly in naïve and memory populations and in both Th1 and Th2 environments.

The up-regulation of CD4 upon allogeneic stimulation was unexpected, and to the best of our knowledge this phenomenon has not been previously documented on human T cells. However, up-regulation of CD4 on murine T cells has recently been observed following activation in vitro and in vivo (34), and the down-regulation of CD4 on human T cells in response to phorbol ester treatment is well documented (35, 36), suggesting that the levels of CD4 on peripheral blood T cells are not necessarily constant. The biological significance of CD4 up-regulation is not clear at present. One possibility is that it may represent a mechanism to maintain a constant density of CD4 molecules on the cell surface, as cell size increases with activation. However, it is not clear what advantage this would provide, as T cell blasts are unlikely to require CD4/CD8 class II MHC interactions at this late stage. The observation that high levels of CD4 were maintained for at least 24 days suggests that the CD4high phenotype may be maintained permanently, as the cell returns to a resting, memory phenotype. This raises the possibility that the higher level of CD4 expression may allow for a lower triggering threshold upon subsequent stimulation of memory T cells. Further study is clearly warranted to determine the biological significance of our observation. Moreover, modulation of CD4 could have significant implications for HIV infection, as CD4 is the primary receptor for cellular entry of HIV-1 (37).

The observed increase in CXCR3 mRNA levels in allo-activated cells is in keeping with the up-regulation of this receptor on the cell surface and suggests that transcription of the CXCR3 gene is linked to the T cell activation process. In previous studies CCR5, another inflammatory chemokine receptor, has similarly been shown to be up-regulated at the mRNA level following T cell activation (38). In contrast to CCR5, however, the present study has also identified intracellular stores of CXCR3 protein in CD4+ T cells, suggesting the presence of additional regulatory mechanisms. While the percentage of nonpermeabilized CD4+ T cells staining positively for CXCR3 was significantly greater for allogeneic than syngeneic cultures, the percentage staining positively for CXCR3 after permeabilization was almost identical for the two culture conditions. This suggests that the traffic of CXCR3 protein between the cell surface and intracellular compartments is tightly regulated, such that in resting T cells the majority of CXCR3 protein is prevented from reaching the cell surface or is internalized almost immediately after reaching the cell surface. Thus, an increase in the proportion of CXCR3 protein that reaches the cell surface could account for the observed up-regulation of this receptor on the cell surface, although the conspicuous increase in the level of CXCR3 mRNA is also likely to contribute significantly to the increased surface expression. Regulation mechanisms involving receptor internalization and recycling have been extensively

**FIGURE 8.** Chemotactic responsiveness of cells from MLR cultures to I-TAC and SDF-1. 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 I-TAC, SDF-1, or diluent alone in the lower chamber. The absolute number of migrated cells was determined by duplicate hemocytometer counts, and the migration index was calculated, as described in Materials and Methods. Values represent the mean ± SEM (\( n = 6 \)). * \( p < 0.05 \).
described for CXCR4 (9, 24, 39–41); however, to the best of our knowledge, these are the first data to suggest that similar mechanisms may exist for CXCR3.

The up-regulation of CXCR4 transcripts in allogeneic compared with syngeneic cultures was unexpected, considering the down-regulation of CXCR4 protein on the cell surface. Of note, the detection of abundant CXCR4 transcripts in the absence of CXCR4 surface protein has been observed previously (18). In this study the discrepancy was suggested to be due to internalization of surface CXCR4 protein through a protein kinase C-responsive motif. Indeed, a number of previous studies have detected a rapid internalization of CXCR4 upon activation of T cells with various stimuli, including phorbol esters and mitogens (9, 24, 39). In the MLR culture system, however, we have demonstrated the absence of intracellular CXCR4 in CD4+ T cells after 9 days of allogeneic activation. The lack of intracellular CXCR4 during the time at which CXCR4 down-regulation peaks suggests that CXCR4 internalization is not a major mechanism of receptor down-regulation in this system. The mechanism accounting for the observed decrease in CXCR4 surface protein therefore remains unclear. It is possible, although unlikely, that the increase in CXCR4 transcription is coupled with a decrease in translation of the message or an increased rate of protein turnover, thereby resulting in lower levels of surface protein. The regulation of CXCR4 protein and mRNA is clearly a complex system and requires further study to fully elucidate the regulatory mechanisms that operate following T cell stimulation.

It is conceivable that down-regulation of CXCR4 on the cell surface is required to release activated T cells from secondary lymphoid tissues such as spleen and tonsil, where SDF-1 transcripts are expressed (42–44), while concurrent up-regulation of CXCR3 allows directed relocation of T cells to peripheral sites of inflammation, characterized by the expression of Mig, IP-10, and I-TAC (45). This hypothesis is supported by our observation of enhanced migration toward I-TAC and reduced migration toward SDF-1 upon allogeneic stimulation. The fact that changes in receptor expression were shown to occur after proliferation had been initiated may provide a mechanism to prevent T cells leaving the secondary lymphoid tissue before they have undergone clonal expansion in an appropriate microenvironment. The temporal distinction between receptor regulation and proliferation also provides further evidence that the primary purpose of chemokine receptor switching is to allow for altered migratory patterns following division and differentiation; the observed changes would be expected to occur much earlier if these receptors were involved in the initial steps of T cell activation. Nevertheless, it is important to note that RANTES has been shown to induce T cell proliferation and up-regulation of the IL-2R (46), suggesting that chemokine receptors other than those investigated here may indeed be involved in the proliferation phase of T cell activation.

It will be of considerable interest to determine the longer term patterns of chemokine receptor expression upon return to a resting state. The results of previous studies imply that CXCR3 expression is retained upon return to a resting state, as the majority of memory CD4+ T cells in peripheral blood express this receptor (12). Furthermore, the detection of CXCR4 on up to half of circulating memory CD4+ T cells (17) suggests that expression of this receptor is restored to some degree upon return to a resting state. It is important to note that the present study has examined the regulation of chemokine receptors on peripheral blood CD4+ T cells, a cell population that contains a large proportion of Ag-inexperienced cells in a resting state. Quite different results have been obtained using polyclonal T cell lines, which are populations of previously activated, effector/memory cells. These studies have shown that inflammatory chemokine receptors are rapidly lost upon TCR triggering, while the homeostatic chemokine receptor CCR7 is transiently up-regulated (18).

In conclusion, the present study provides direct evidence for a switch in chemokine receptor expression on CD4+ T cells, from homeostatic to inflammatory, following activation by DC in a model of a primary immune response. Further, this switch was accompanied by cell division, up-regulation of CD4, acquisition of an activated/memory phenotype, and altered migratory capabilities. The observed coordinated regulation of chemokine receptors and activation markers, including CD4, suggests that the regulation of these molecules may involve common intracellular signaling pathways. Identification of the molecular signals that regulate chemokine receptor expression following Ag presentation and determining how these signals are integrated with the T cell activation and differentiation processes will be the subject of further study.

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