Switch in chemokine receptor expression upon TCR stimulation reveals novel homing potential for recently activated T cells

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When naive T lymphocytes are activated and differentiate into memory/effector cells, they down-regulate receptors for constitutive chemokines such as CXCR4 and CCR7 and acquire receptors for inflammatory chemokines such as CCR3, CCR5 and CXCR3, depending on the Th1/Th2 polarization. This switch in chemokine receptor usage leads to the acquisition of the capacity to migrate into inflamed tissues. Using RNase protection assays, staining with specific antibodies, and response to recombinant chemokines, we now show that following TCR stimulation, memory/effector T cells undergo a further and transient switch in receptor expression. CCR1, CCR2, CCR3, CCR5, CCR6 and CXCR3 are down-regulated within 6 h, while CCR7, CCR4, CCR8 and CXCR5 are up-regulated for 2 to 3 days. Up-regulation of CCR7 following TCR stimulation was observed also among resting peripheral blood T cells and required neither co-stimulation nor exogenous IL-2. On the other hand IL-2 down-regulated CXCR5, up-regulated CCR8 and facilitated the recovery of CCR3 and CCR5. Upon TCR stimulation, Th1 and Th2 cells produced comparable sets of chemokines, including RANTES, macrophage inflammatory protein-1β, I-309, IL-8 and macrophage-derived chemokine, which may modulate surface chemokine receptors and contribute to cell recruitment at sites of antigenic recognition. Altogether these results show that following TCR stimulation effector/memory T cells transiently acquire responsiveness to constitutive chemokines. As a result, T cells that are activated in tissues may either recirculate to draining lymph nodes or migrate to nearby sites of organized ectopic lymphoid tissues.

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1 Introduction

Naive T cells constitutively traffic through secondary lymphoid organs in search of antigens presented by dendritic cells (DC). Following priming, clonal expansion and differentiation, the effector memory cells that leave the lymph node have acquired new migratory capacities [1]. Early work showed that naive T cells express L-selectin which is required for extravasation from blood to lymph nodes, while effector T cells generally lack L-selectin but express receptors for E- and P-selectin which are important for migration into sites of DTH reactions [2, 3].

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Abbreviations: **SLC:** Secondary lymphoid tissue chemokine **ELC:** EBI1-ligand chemokine **DC:** Dendritic cells

There is growing evidence that chemokines play a key role in controlling leukocyte migration and positioning under physiological and inflammatory conditions [4, 5]. The set of chemokines which are produced constitutively in bone marrow, thymus and secondary lymphoid organs are denoted constitutive or immune chemokines and control leukocyte traffic under physiological conditions. By contrast, the recruitment of leukocytes at inflammatory sites is dependent on the induction by inflammatory stimuli of a different set of chemokines which are called inflammatory chemokines. Chemokine receptors are differentially expressed on naive and effector/memory T cells. Naive T cells express CCR7, a receptor for the constitutive chemokines secondary lymphoid tissue chemokine (SLC), produced by cells of the high endothelial venules and by lymphatic endothelial cells [6], and EBI1-ligand chemokine (ELC), made by interdigitating

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DC in T cell areas of the lymph nodes [7–9]. There is growing evidence that CCR7 and its ligands represent key elements for directing T lymphocytes into lymph nodes where they are primed by antigen. Indeed, paucity of lymph node T cells (*plt*) mice that lack SLC and have reduced levels of ELC are incapable of mounting T cell responses because of a failure of naive T cells and maturing DC to enter lymph nodes and spleen [10]. Naive T cells also express CXCR4, a receptor which plays a role in early hematopoiesis and vascularization, but no evidence has been yet provided that it has a function in the immune response [11–13].

CCR7 and CXCR4 are down-regulated following T cell priming and differentiation to memory/effector T cells. The latter, however, express a variety of receptors for inflammatory chemokines, depending on the type of polarization (reviewed in [14]). CCR3, the eotaxin-specific receptor, is expressed on Th2 cells [15, 16], while CXCR3, the receptor for monokine induced by IFN-γ (Mig) and interferon-inducible protein (IP)-10, is expressed at higher levels on Th1 than on Th2 cells [17, 18]. CCR4, the receptor for thymus and activation-regulated chemokine (TARC) and macrophage-derived chemokine (MDC), is expressed on Th2 but also on cells that do not produce IL-4 such as semi-naive cells [17, 18]. CCR5, a receptor for RANTES, macrophage inflammatory protein (MIP)-1α and MIP-1β, is expressed preferentially on recently activated T cells and Th1 clones [18–20]. Finally, CCR1 and CCR2 are expressed on both Th1 and Th2 cells [18]. It has been proposed that the distinctive set of chemokine receptors expressed endows effector T cells with the capacity to migrate to particular inflammatory sites depending on which chemokines are elaborated and to co-localize with different types of inflammatory cells with which they share receptors [14].

There have been reports indicating that chemokine receptor expression can be modulated by T cell activation. When Th2 cells are triggered, CCR3 is down-regulated [15], while CCR4 and CCR8 are up-regulated [21]. In addition CXCR5, a receptor involved in B lymphocyte homing and germinal center formation [22], is up-regulated following T cell stimulation [23], especially if T cells are co-stimulated through OX-40/OX-40L interaction [24]. In addition, IL-2 has been reported to selectively up-regulate the expression and function of chemokine receptors such as CCR1, CCR2 and CCR5 [25].

Here we present a systematic analysis of chemokine receptor regulation in memory/effector T cells after TCR stimulation in the presence or absence of IL-2. We show that upon TCR triggering, memory/effector T cells rapidly down-regulate CCR1, CCR2, CCR3, CCR5, CCR6 and CXCR3, *i.e.* the receptors for inflammatory chemokines. At the same time they up-regulate CCR8, CXCR5 and, most importantly, CCR7. This transient shift in chemokine receptor expression may be responsible for: 1) recirculation of recently activated T cells from tissues to lymph nodes, 2) encounter with activated B cells and 3) the organization of chronic inflammatory reactions.

2 Results

2.1 The pattern of chemokine receptors expressed by memory/effector T cells is rapidly and transiently switched upon TCR stimulation

Polyclonal T cell lines were analyzed for chemokine receptor expression using an RNase protection assay before and at different times after restimulation with plate-bound anti-CD3 in the presence or absence of exogenous IL-2. The cell line shown in Fig. 1 contained Th1, Th2 and Th0 cells (24 %, 25 % and 36 %, respectively) and consequently expressed CCR1, CCR2, CCR3, CCR4, CCR5, CXCR3 and CXCR4. As early as 6 h after stimulation with anti-CD3, a complete loss of CCR1, CCR2, CCR3 and CCR5 mRNA was observed. In the absence of exogenous IL-2, these mRNA remained down-regulated for up to 72 h, while, in its presence, they recovered after approximately 24 h. CXCR3 was also down-regulated, although to a lower extent and with slower kinetics.

CCR7, CCR8 and CXCR5 were up-regulated following TCR stimulation. CCR7 mRNA was absent before stimulation, sharply increased at 6 h and remained at a constant level for at least 3 days both in the presence and absence of IL-2. CCR8 was also absent before stimulation, was transiently up-regulated in the absence, and more stably up-regulated in the presence of IL-2. Low but stable levels of CXCR5 mRNA were induced in the absence of IL-2, while in its presence the up-regulation was only transient. Finally, CCR4 and CXCR4 mRNA levels were not affected significantly upon T cell stimulation.

Altogether these results demonstrate a complex and coordinated regulation of chemokine receptor mRNA levels. On the one hand, receptors for inflammatory chemokines are rapidly lost following T cell stimulation and recover in the presence of IL-2. On the other hand, CCR7, CXCR5 and CCR8 are up-regulated by TCR stimulation, with IL-2 playing an either neutral, inhibitory, or enhancing effect.

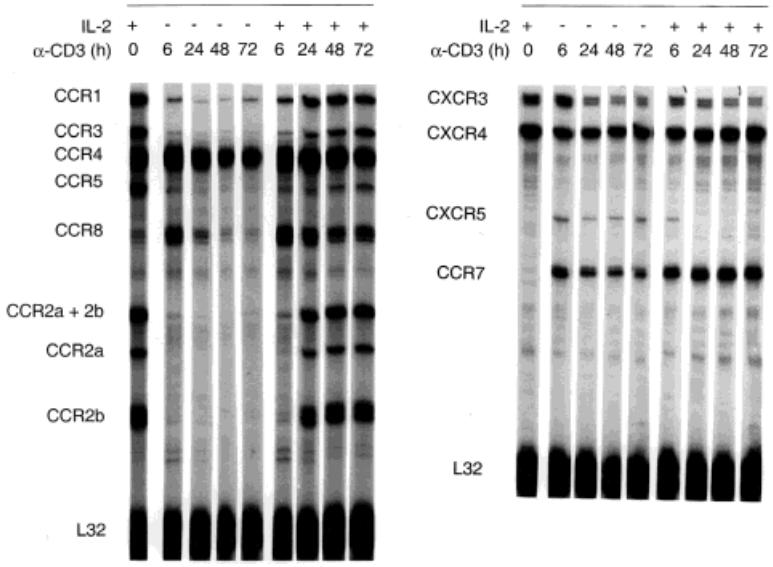

Figure 1. Chemokine receptor mRNA levels are modulated by TCR triggering and IL-2 in a polyclonal T cell line. A polyclonal T cell line containing cells with Th2 (25 %), Th1 (24 %) and Th0 (36 %) phenotype was stimulated with plate-bound anti-CD3 antibodies in the absence or presence of exogenous IL-2. Chemokine receptor mRNA levels were measured by RNase protection assays at different hours after stimulation. The up-regulation of CCR8 was restricted to Th2 cells as demonstrated in a separate experiment with *in vitro* polarized Th1 and Th2 cell lines (not shown).

2.2 Up-regulation of surface CCR7 and down-regulation of CCR2, CCR3, CCR5, CCR6 and CXCR3 following TCR stimulation of memory/effector T cells

The expression of chemokine receptors on Th1 and Th2 polarized cell lines was analyzed using a panel of monoclonal antibodies before and after restimulation. CCR2, CCR3, CCR5, CCR6, CXCR3 and CXCR4 were down-regulated 24 h after TCR stimulation (Fig. 2, note the differential expression of CCR3 and CXCR3 and CCR6 on Th2 and Th1 cells, respectively). At the same time CCR7 was markedly up-regulated in both lines. CCR7 peaked at 24 h and was lost or down-regulated by 96 h (Fig. 2C).

In all cases where the comparison was possible there was a good correlation between chemokine receptor mRNA and protein levels, the only exception being CXCR4, which was expressed at the mRNA level but was hardly detectable on the cell surface. This discrepancy may be due to CXCR4 down-regulation through a PKC responsive motif [26].

Some T cells present in peripheral blood lack CCR7 and express the CD45RA$^-$ memory phenotype. When these cells were sorted and stimulated with anti-CD3 and anti-CD28, they up-regulated CCR7 with maximum levels achieved at 48 h (Fig. 3). Addition of exogenous IL-2 did not affect CCR7 up-regulation. Importantly, stimulation with anti-CD3 alone without co-stimulation was sufficient to induce the same extent of CCR7 up-regulation (data not shown).

2.3 Switch in responsiveness to chemokines following TCR triggering

The function of chemokine receptors expressed by a polyclonal T cell line before and after anti-CD3 stimulation was tested by measuring calcium mobilization in response to recombinant chemokines (Fig. 4). The response to MIP-1α and monocyte chemoattractant protein (MCP)-3, which was present in resting T cells, was lost 24 h after activation, while the response to MCP-1 was markedly decreased. These findings are consistent with loss of CCR1, CCR2, CCR3 and CCR5. Responsiveness to stromal cell-derived factor (SDF)-1 and MDC was not affected by TCR stimulation, consistent with steady-state levels of these receptors. Most strikingly, the response to ELC, which was absent before, was markedly induced following TCR triggering, indicating that recently activated T cells possess a functional CCR7. Altogether these results demonstrate a switch in responsiveness to chemokines consistent with changes at the mRNA and protein levels.

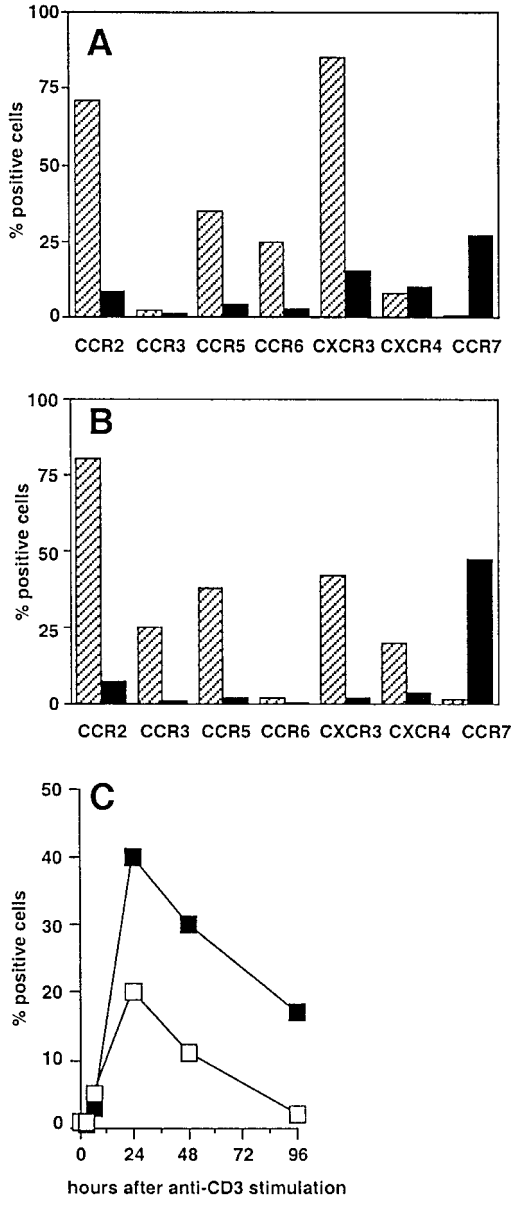

Figure 2. T cell activation results in up-regulation of surface CCR7 and down-regulation of CCR2, CCR3, CCR5, CCR6 and CXCR3. T cells from a Th1 (A) or Th2 line (B) were left untreated or stimulated for 24 h with plate-bound anti-CD3 antibody (clone T3.4, mouse IgA). Unstimulated cells (hatched bars) and stimulated cells (filled bars) were stained with mAb specific for seven chemokine receptors. (C) Kinetics of CCR7 up-regulation in Th1 (□) and Th2 (■) cell lines stimulated with anti-CD3 antibody (clone TR66, mouse IgG1). One representative experiment out of seven is shown.

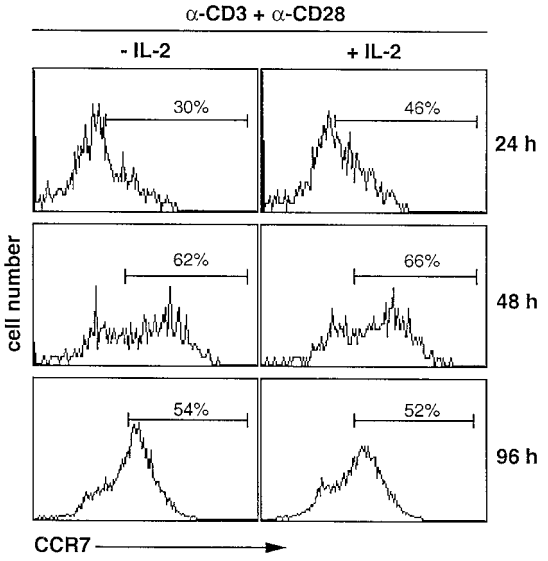

Figure 3. CCR7$^-$ peripheral blood T cells up-regulate CCR7 upon activation. CCR7$^-$ CD4$^+$ CD45RA$^-$ T lymphocytes were isolated by cell sorting from PBMC and analyzed at different times after stimulation with plate-bound anti-CD3 and anti-CD28 antibodies in the absence or presence of exogenous IL-2. Stimulation with anti-CD3 alone resulted in a comparable up-regulation of CCR7. The percent of positive cells, as compared to isotype-matched control antibody, is indicated. One representative experiment out of four is shown.

2.4 Comparable sets of chemokines are produced by activated Th1 and Th2 cells

Chemokine mRNA levels were measured by semi-quantitative reverse transcription (RT)-PCR before and 4 h after stimulation with anti-CD3 (Table 1). TCR stimulation induced high levels of RANTES, MIP-1α, MIP-1β, I-309, IL-8 and MDC, while Mig and IP-10 mRNA were present at lower levels. TARC, SDF-1, ELC, SLC, eotaxin, MCP-1 and MCP-2 were not produced at this time point. The same pattern of chemokine mRNA was found in Th1 and Th2 cell lines.

3 Discussion

We have shown that upon TCR stimulation memory/effector T cells transiently switch chemokine receptor expression, thus acquiring new homing potential (summarized in Fig. 5). There are two distinct aspects in this change. First, receptors for inflammatory chemokines, which are differentially expressed on Th1 and Th2 cells, are rapidly down-regulated at the mRNA and protein level. Second, receptors for constitutive chemokines are transiently up-regulated.

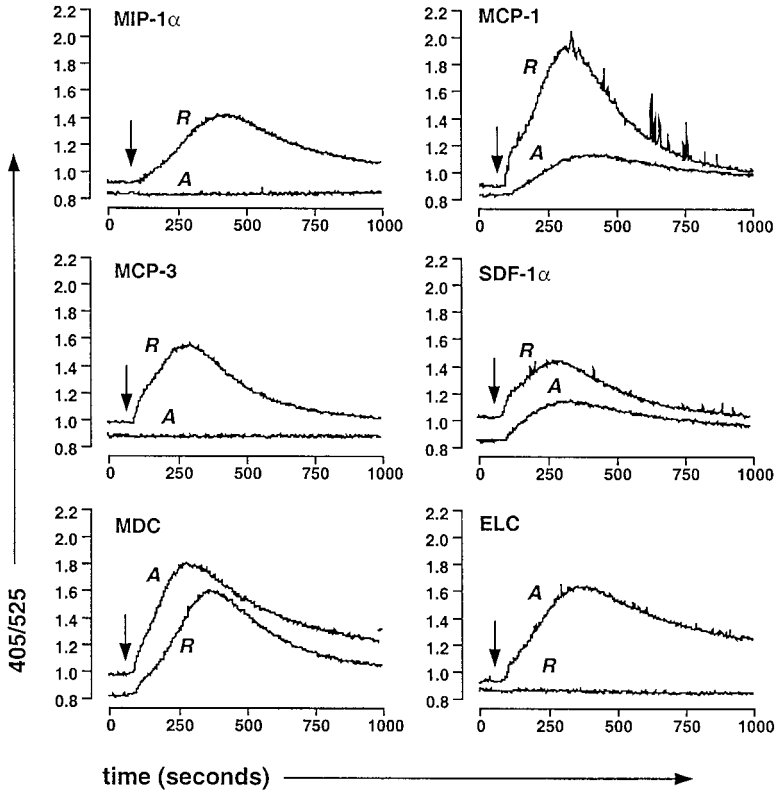

Figure 4. Changes in responsiveness to chemokines after TCR triggering. A Th2 cell line (same as in Fig. 2B) was activated for 24 h with plastic-bound anti-CD3 antibody or left untreated. The cells were loaded with Indo-1 and intracellular Ca^{2+} mobilization in response to different chemokines was measured on a fluorescence spectrophotometer. The arrows indicate the addition of the chemokines (all used at 500 ng/ml). *R* indicates resting unstimulated T cells; *A* indicates T cells 24 h after TCR stimulation. The specificity of the chemokines is as follows: MIP-1α (CCR1 and CCR5); MCP-1 (CCR2); MCP-3 (CCR3 and CCR2); MDC (CCR4); SDF-1α (CXCR4) and ELC (CCR7).

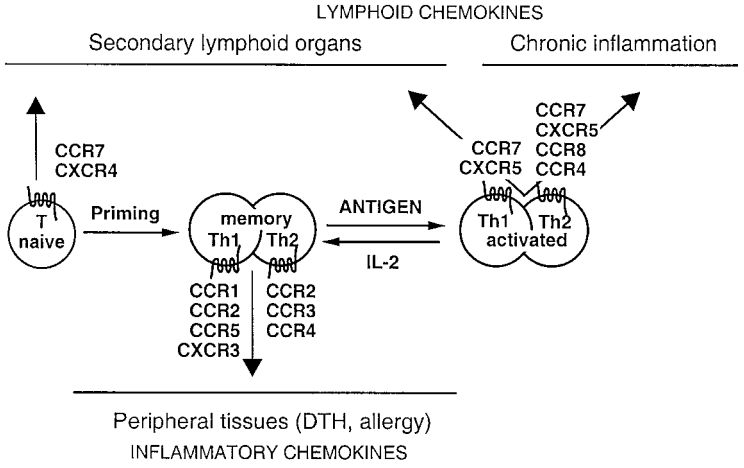

Figure 5. Developmental and activation-induced control of chemokine receptor expression in T cells.

Table 1. Chemokine mRNA expression following activation of Th1 and Th2 cell lines[a)]

	Th1-R	Th1-A	Th2-R	Th2-A
RANTES	++	+++	++	+++
MIP-1α	–	++	–	++
MIP-1β	+	++++	+	++++
I-309	+	++++	+	++++
IL-8	+	++++	+	++++
IP-10	–	+	–	+
Mig	+	+	+	+
MDC	–	+++	–	+++
TARC	–	–	–	–
SDF-1	–	–	–	–
ELC	–	–	–	–
SLC	–	–	–	–
Eotaxin	–	–	–	–
MCP-1	–	–	–	–
MCP-2	–	–	–	–

a) Th1 or Th2 cells were left untreated (R) or were activated with plate-bound anti-CD3 antibody (A) and mRNA was extracted after 4 h. Different dilutions of cDNA were amplified for 30 cycles and scored as follows. +: positivity at 1/10; ++: 1/50; +++: 1/250; ++++: 1/1250. Abbreviations of chemokines: RANTES, regulated on activation of normal T cell expressed and secreted; MIP, macrophage inflammatory protein; IP-10, interferon-inducible protein 10; Mig, monokine induced by IFN-γ; MDC, macrophage-derived chemokine; TARC, thymus and activation-regulated chemokine; ELC, EBI1-ligand chemokine; SLC, secondary lymphoid tissue chemokine; MCP, monocyte chemoattractant protein.

In anti-CD3-stimulated T cells the loss of CCR1, CCR2, CCR3 and CCR5 is due to a rapid down-regulation of the corresponding mRNA levels, which can be detected as early as 6 h following stimulation. This is different from what is observed in maturing DC, where the same receptors are down-regulated from the cell surface by autocrine production of the cognate chemokines, while the mRNA levels persist for at least 40 h [9].

Although the regulation in T cells appears to be at the mRNA level, we cannot exclude the possibility that chemokine production by activated T cells may contribute to down-regulating the cognate receptors from the cell surface at early time points as has been shown for DC. The loss of responsiveness to inflammatory chemokines induced by antigenic stimulation may result in a migratory arrest for activated T cells and may allow these cells to sense new chemotactic gradients and thus redirect their traffic.

While both CCR3 and CCR4 are expressed by resting Th2 cells and are consequently expected to play a role in their recruitment from blood to inflamed tissues, only CCR3 is down-regulated following TCR stimulation, whereas CCR4 is not affected ([15, 21] and this study). The different behavior of CCR3 and CCR4 following T cell activation may explain the difficulty that some authors have reported in detecting CCR3 on Th2 cells. This difficulty may actually relate to the state (resting or activated) of the cells analyzed.

Of particular relevances is the up-regulation of CCR7, which leads to responsiveness of recently activated T cells to SLC and ELC. CCR7 up-regulation was also observed in resting T cells isolated from peripheral blood, was sustained for up to 96 h and was not affected by co-stimulation or IL-2. Thus the requirement for up-regulation of CCR7 can be easily met by stimulation of T cells in peripheral tissues, even by non-professional APC. CCR7 up-regulation may account for two phenomena. First, CCR7 may allow recently activated T cells to enter the afferent lymphatics and home to the lymph nodes in response to SLC and ELC. This mechanism may explain the observation that activated T cells are present in the afferent lymph [27]. Alternatively, up-regulation of CCR7 may drive recently activated T cells towards sites of chronic inflammation, where ELC might be produced by mature DC [28]. This mechanism may explain the accumulation of chronically stimulated T cells, such as EBV-specific CTL, into joints of patients with rheumatoid arthritis [29] and the organization of ectopic lymphoid tissue [30]. CXCR5 has been shown to be up-regulated following T cell stimulation [23] and is likely to play an important role in driving recently activated T cells to B cell areas where the cognate chemokine B cell-attracting chemokine (BCA)-1 is produced [31, 32]. The up-regulation of CXCR5 observed in this study was modest, possibly due to selective expression on a small subset of cells. Furthermore, it was inhibited by exogenous IL-2. It has been demonstrated that stimulation via OX40/OX40L is required for optimal CXCR5 expression [24]. The up-regulation of CCR8 on activated Th2 cells has been previously reported and we have confirmed these findings [21]. Its consequence, however, is unclear. Production of the cognate chemokine I-309 by activated T cells ([33] and this study) might represent an autocrine mechanism to keep the herd of responding cells together.

Altogether our results show that in T lymphocytes chemokine receptor transcription is regulated both developmentally and by TCR signals. CCR7, the key regulatory receptor for T cell priming, is constitutively expressed by naive T cells, lost upon differentiation to memory/effector cells, and transiently re-acquired following TCR stimulation to be lost again within 2–3 days. In contrast, CCR3 and CCR5, two receptors for inflammatory chemokines, are expressed only in memory/effector T cells and transiently down-regulated by TCR signals. IL-2 adds further to this regulation by differentially affecting the expression of some, but not other receptors. There are aspects of chemokine receptor gene regulation that should be addressed to explain the extraordinary flexibility observed which is required to regulate T lymphocyte traffic.

4 Materials and methods

4.1 Media and reagents

The medium used throughout was RPMI 1640 supplemented with 2 mM L-glutamine, 1 % nonessential amino acids, 1 % pyruvate, 50 μg/ml kanamycin (Gibco, Grand Island, NY), 5×10^{-5} M 2-ME (Merck, Darmstadt, Germany) and 5 % human serum (Swiss Red Cross, Bern, Switzerland) or 10 % FCS (Hyclone Laboratories, Inc., Logan, UT). Human recombinant IL-2 and IL-4 were produced in our laboratory by PCR cloning and expression in the myeloma expression system. IL-12 was obtained from Hoffmann La Roche, Nutley, NJ. Neutralizing antibodies to IL-12 were purchased from R&D Systems, Inc., Minneapolis, MN, and antibodies to IL-4 from PharMingen, San Diego, CA. MIP-1α, MCP-1, MCP-3, SDF-1α, MDC and ELC were purchased from Peprotech (London, GB).

4.2 Cells and T cell lines

Cord blood or adult peripheral blood samples were separated by the standard Ficoll-Paque method. *In vitro* polarized polyclonal Th1 and Th2 cell lines were obtained and maintained as described [18]. Resting peripheral blood T cells or T cell lines were stimulated with plate-bound anti-CD3 (clone TR66, IgG1 [34], or clone T3.4, IgA, kindly provided by Dr. E. Roosnek) alone or in combination with anti-CD28 antibody (clone CD28.6, kindly provided by Dr. D. Olive) for different times in the absence or presence of 100 U/ml rIL-2.

4.3 FCM analysis

Cell staining was performed using mAb followed by FITC- or PE-conjugated affinity-purified, isotype-specific goat anti-mouse or goat anti-rat antibodies (Southern Biotechnology Associates, Inc., Birmingham, AL). The following antibodies were used: anti-CCR1 (2D4, mouse IgG1), anti-CCR5 (2D7, mouse IgG2a) anti-CCR6 (11A9, mouse IgG1), anti-CXCR3 (1C6, mouse IgG1) (all produced at LeukoSite, Cambridge, MA), anti-CXCR4 (12G5, IgG2a, PharMingen), anti-CD4 (13B8.2, IgG1) anti-CD45RA (ALB11, IgG1, Immunotech, Marseille, France), anti-CCR7 (rat IgG2; R. Burgstahler, manuscript in preparation). The samples were analyzed on a FACSCalibur® (Becton Dickinson, Mountain View, CA) using propidium iodide to exclude dead cells.

4.4 Ca^{2+} flux measurement

T cells were loaded with Indo-1 (Sigma Chemicals, Co.) and their response to various concentrations of chemokines was analyzed on a Hitachi F-4500 fluorescence spectrophotometer (Tokyo, Japan) [35].

4.5 RNase protection assay

Total RNA was extracted from T cells using RNAzol B (Tel-Test, Inc., Friendswood, TX). Multi-probe template sets hCR5 (containing DNA templates for CCR1, CCR3, CCR4, CCR5, TER1/CCR8, CCR2a + b, CCR2a, CCR2b, L32, GAPDH) and hCR6 (containing DNA template for CXCR1, CXCR2, CXCR3, CXCR4, BLR-1, BLR-2/CCR7, V28, L32, GAPDH) were purchased from PharMingen. The DNA templates were used to synthesize the [α^{32}P]UTP (3000 Ci/mmol, 10 mCi/ml, Amersham Life Science, Amersham, GB) labeled probes in the presence of a GACU pool using a T7 RNA-polymerase (Promega, Madison, WI). Hybridization with 5–15 μg of each target RNA was performed overnight followed by digestion with RNase A (Boehringer-Mannheim, Rotkreuz, Switzerland) and T1 (Calbiochem, La Jolla, CA) according to the PharMingen standard protocol. The samples were treated with proteinase K (Boehringer-Mannheim) / SDS mixture and then extracted with chloropan and precipitated in the presence of ammonium acetate. The samples were loaded on an acrylamide-urea sequencing gel next to labeled DNA molecular weight markers and to the labeled probes, and run at 50 W with 0.5 × TBE. The gel was adsorbed to filter paper, dried under vacuum and exposed on Kodak X-AR film with intensifying screens at –70 °C.

4.6 RT-PCR

Single-strand cDNA was synthesized from 2 μg total RNA using avian myeloblastosis virus reverse transcriptase (Promega). Primers used for PCR were: hMIP-1β: 5'-TACCATGAAGCTCTGCGTGACT-3' and 5'-ATTAAGAGAAGGGACAGGAACT-3' (annealing temperature 56 °C), amplifying a 397-bp product; hI-309: 5'-GCCCAAGCCAGACCAGAAGACA-3' and 5'-AAGCAGGGCAGAAGGAATGGTG (annealing temperature 65 °C), amplifying a 403-bp product; hIL-8: 5'-ACCGGAAGGAACCATCTCACTG-3' and 5'-GCATCTG-

GCAACCCTACAACA-3' (annealing temperature 65 °C), amplifying a 445-bp product; hIP-10: 5'-TGAAAAAGAAGG-GTGAGAAGAGAT-3' and 5'-GATAAACCCCAAAGCAGAAA-GATT-3' (annealing temperature 64 °C), amplifying a 451-bp product; hMig: 5'-GTCAGCCAAAAGAAAAAGCAAAAG-3' and 5'-TCAACTGGTGGGTGGTAGAAGAAC-3' (annealing temperature 64 °C), amplifying a 333-bp product. The primers and conditions for amplification of the other chemokines were as described in Sallusto et al. [9]. cDNA were amplified by PCR using the following conditions: 30 s at 94 °C, 20 s at 56, 60 or 64 °C (as specified for each primer pair) and 30 s at 72 °C. PCR products were resolved on a 1.5 % agarose gel containing ethidium bromide.

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