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J Immunol 1998; 161:5111-5115; ;
<http://www.jimmunol.org/content/161/10/5111>

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Cutting Edge: Selective Up-Regulation of Chemokine Receptors CCR4 and CCR8 upon Activation of Polarized Human Type 2 Th Cells¹

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Polarized Th1 and Th2 cells differentially express adhesion molecules and chemokine receptors, endowing these cells with distinct tissue homing capabilities. Here we report that, in contrast to other chemokine receptors, the expression of CCR4 and CCR8 on Th2 cells is transiently increased following TCR and CD28 engagement. IL-4 is not required for this activation-induced up-regulation of CCR4 and CCR8. In accordance with receptor expression, the response of Th2 cells to I-309 (CCR8 ligand) and thymus- and activation-regulated chemokine (CCR4 and CCR8 ligand) is enhanced upon activation. Moreover, activated Th1 cells up-regulate CCR4 expression and functional responsiveness to thymus- and activation-regulated chemokine. Analysis of polarized subsets of CD8⁺ T cells reveals a similar pattern of chemokine receptor expression and modulation of responsiveness. Taken together, these findings suggest that an up-regulation of CCR4 and CCR8 following Ag encounter may contribute to the proper positioning of activated T cells within sites of antigenic challenge and/or specialized areas of lymphoid tissues. *The Journal of Immunology*, 1998, 161: 5111–5115.

The differentiation process of CD4⁺ T cells proceeds along two distinct pathways leading to the generation of polarized effector Th cells (1–3). IFN- γ -producing Th1 cells promote phagocyte-dependent immunity (4, 5). Th2 cells secreting IL-4 and IL-5 promote IgE production and allergic responses (6). Similar to the generation of CD4⁺ Th1 and Th2 cells, naive CD8⁺

T cells can differentiate into Th1 type 1 cytotoxic CD8⁺ T cells (Tc1)³ cells or Tc2 type cytotoxic CD8⁺ T cells (7).

A multistep process mediated by the interplay of adhesion molecules and chemokines that involves rolling, firm adhesion, and diapedesis results in the extravasation of immune effector cells within peripheral tissues (8, 9). Recent findings indicate that P- and E-selectin ligands (10) and chemokine receptors (11, 12) are differentially expressed on Th1 and Th2 cells, providing these cells with distinct tissue-homing abilities (13). Chemokines are members of a large family of small cytokines that play a key role in the leukocyte-recruitment process (14–16). We and others have reported recently that among eight CC and four CXC chemokine receptors, Th1 cells predominantly express CXCR3 and CCR5 (12, 17, 18); Th2 cells selectively express CCR3 (11), CCR4 (12, 17), and CCR8 (19). Although these findings suggest that different chemotactic signals are required for extravasation, migration, and tissue homing, the hierarchy and composition of these signals are unknown and may depend upon the chemokines present in the tissue and the chemokine receptors expressed on the invading cells.

Here, we investigated the effect of Ag receptor triggering on the expression pattern of chemokine receptors on Th1 and Th2 cells. Our data indicate that, in contrast to other chemokine receptors, the expression of CCR4 and CCR8 on Th2 cells and CCR4 on Th1 cells is markedly increased upon activation. These findings suggest a unique role for these receptors in extravasation, tissue migration, and the positioning of effector T cells.

Materials and Methods

Type 1 and type 2 lines and Th2 cell clones

Type 1 and type 2 cell lines were generated from cord blood lymphocytes and maintained in culture as described previously (20). CD4⁺ Th1 and Th2 cells and CD8⁺ Tc1 and Tc2 cells were purified by immunomagnetic negative selection using anti-CD4 or anti-CD8 mAb-coated microbeads according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). The Lolium perenne group-1-specific Th2 clone D4.11, which was obtained as described previously (12), was restimulated with PHA and irradiated PBMCs and cultured in complete medium with IL-2.

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Received for publication August 6, 1998. Accepted for publication September 2, 1998.

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¹ This work was partially funded by Consiglio Nazionale delle Ricerche Biotechnology Grant 95-95.

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³ Abbreviations used in this paper: Tc, cytotoxic CD8⁺ T cell; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; AM, acetoxymethyl ester; IP-10, IFN-inducible protein-10; TARC, thymus and activation-regulated chemokine.

Northern blot analysis

For Northern blots, total RNA was extracted from polarized CD4⁺ or CD8⁺ T cells by TRIzol (Life Technologies, Grand Island, NY). For activation experiments, purified CD4⁺ Th1 and Th2 cells were either left untreated or incubated for 16–24 h on anti-CD3 (Tr66 mAb)-coated plates with 1 μ g/ml of anti-CD28 mAb (PharMingen, San Diego, CA). The Th2 clone D4.11 was either left untreated or incubated for 24 h on anti-CD3-coated plates with 1 μ g/ml of anti-CD28 mAb. TCR triggering was terminated by washing and culturing the cells in complete medium with IL-2. Untreated and TCR-stimulated cells were lysed at various times after the termination of TCR-triggering. Total RNA was extracted, and equal amounts of RNA (10 μ g/lane) were fractionated on a 1% agarose-formaldehyde gel. The specific mRNAs were detected by the hybridization of nylon membranes (NorthernMax Kit, Ambion, Austin, TX) with ³²P-labeled DNA probes for human CCR3, CCR4, CCR5, CCR8, and CXCR3 according to the manufacturer's instructions (NorthernMax Kit, Ambion). The filters were exposed to X-OMAT AR film (Eastman Kodak, Rochester, NY) between double intensifying screens (DuPont, San Diego, CA) at -70°C. The extent of the hybridization was quantified by densitometric analysis with the entry level image system (Immagini e computer, Milan, Italy), and the values were normalized using the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) signal as a reference.

Analysis of intracellular calcium mobilization

Type 1 and type 2 polarized T cells were either left untreated or activated as described above. Next, cells were washed and rested by incubation in complete medium with IL-2 for 12 h. Fluo-3/acetoxymethyl ester (AM) loading (21) was performed by incubating the cells (5 \times 10⁶/ml) in buffer A (HBSS with 10 mM HEPES) with 2 μ M fluo-3/AM (Molecular Probes, Eugene, OR) at 37°C for 30 min. The incubation was prolonged by 30 min after the addition of an equal volume of buffer B (HBSS with 10 mM HEPES and 5% FCS). Cells were washed twice in buffer B and stained with quantum red-conjugated anti-CD4 or anti-CD8 Abs (Sigma, St. Louis, MO). Cells were washed, resuspended at 2 \times 10⁶/ml, and analyzed by FACS. Emissions at 525 and 613 nm were measured on a log scale before and after stimulation with the chemokines (IFN-inducible protein-10 (IP-10), eotaxin, I-309, and thymus- and activation-regulated chemokine (TARC) were purchased from R&D Systems, Minneapolis, MN). Analysis was restricted to CD4⁺ or CD8⁺ T cells by gating on FL3⁺ cells, with an acquisition of 3000 events.

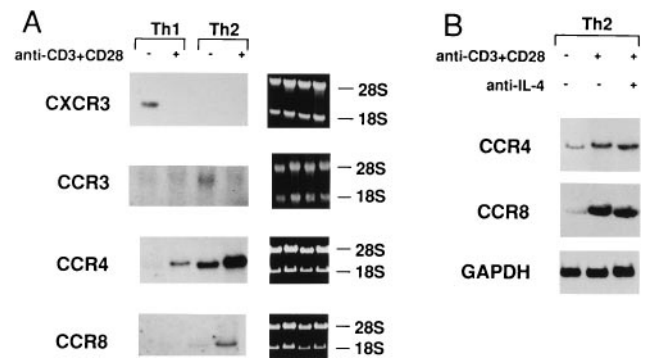


FIGURE 1. Activation-dependent regulation of chemokine receptor expression in Th1 and Th2 cells. *A*, Th1 and Th2 cells were either left untreated or cultured on anti-CD3 mAb-coated plates with soluble anti-CD28 mAb. Cells were harvested, and total RNA was extracted. A total of 10 μ g of total RNA was subsequently used in Northern blot analysis. Filters were hybridized with ³²P-labeled probes for CXCR3, CCR3, CCR4, and CCR8. Equivalent loading of RNA was verified by ethidium bromide staining of 18S and 28S ribosomal RNA. *B*, Th2 cells were either left untreated or cultured on anti-CD3 mAb-coated plates with soluble anti-CD28 mAb in the presence or absence of anti-IL-4 mAb (500 ng/ml). A total of 10 μ g of total RNA was subsequently used in Northern blot analysis. Filters were hybridized with ³²P-labeled probes for CCR4, CCR8, and GAPDH.

Results and Discussion

To study the regulation of chemokine receptor expression on Th cells, we generated T cell lines from leukocytes isolated from cord blood as described previously (20). The Th phenotype of CD4⁺ and CD8⁺ T cells was determined by restimulation and intracellular staining for IFN- γ and IL-4 production (data not shown). Purified CD4⁺ Th1 or Th2 cells were either left in culture with

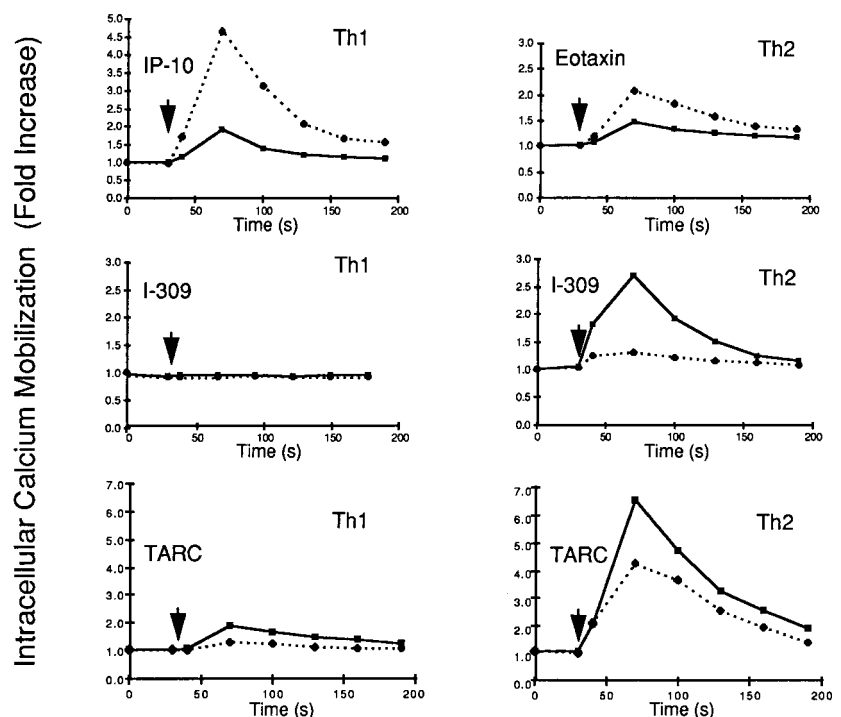


FIGURE 2. Chemokine-induced intracellular Ca²⁺ mobilization in resting and activated Th1 and Th2 cells. Th1 and Th2 cells were either left untreated or activated as described in Fig. 1. Cells that had been loaded with fluo-3/AM and stained with quantum red-conjugated anti-CD4 Abs were analyzed by flow cytometry by gating on CD4⁺ cells. Resting (dashed lines) and activated (solid lines) Th1 and Th2 cells were stimulated with IP-10 (50 ng/ml), eotaxin (400 ng/ml), I-309 (200 ng/ml), and TARC (10 ng/ml). The time of the addition of the chemokines is indicated by the arrow. The response is expressed as the fold increase of mean fluorescence intensity at time 0 for emissions at 525 nm. One representative experiment of five is shown.

IL-2 or cultured on anti-CD3 mAb-coated plates with soluble anti-CD28 mAb. Subsequently, the cells were lysed, and total RNA was extracted and analyzed for CXCR3, CCR3, CCR4, and CCR8 mRNA expression. In agreement with recent reports (11, 12, 18, 19), CXCR3 is preferentially expressed on Th1 cells, whereas CCR3, CCR4, and CCR8 are preferentially expressed on Th2 cells (Fig. 1A). Stimulation with anti-CD3 and anti-CD28 Abs down-regulates the mRNA expression of CXCR3 in Th1 cells and CCR3 in Th2 cells (Fig. 1A). In contrast, CCR4 and, to a greater extent, CCR8 transcripts are up-regulated in TCR-stimulated Th2 cells (Fig. 1A). In addition, CCR4 mRNA becomes detectable in TCR-stimulated Th1 cells (Fig. 1A).

Since CCR4 and CCR8 are expressed on Th2 cells that have been generated *in vitro* in the presence of IL-4, it is possible that the IL-4 produced upon restimulation of Th2 cells is responsible for the increased expression of CCR4 and CCR8. Therefore, we performed the stimulation in the presence of anti-IL-4 blocking Abs. This experiment shows that blocking IL-4 does not inhibit the up-regulation of CCR4 and CCR8 upon activation of Th2 cells (Fig. 1B). Moreover, the addition of exogenous IL-4 or supernatants obtained from activated Th2 cells does not result in an up-regulation of CCR4 and/or CCR8 in Th1 cells or a further enhancement of these receptors in Th2 cells (data not shown).

To test the functional relevance of these observations, we explored chemokine responsiveness by measuring intracellular calcium mobilization following activation of Th1 and Th2 cells. Consistent with receptor expression, Th1 cells preferentially respond to IP-10 (CXCR3 ligand (22)), whereas Th2 cells selectively respond to eotaxin (CCR3 ligand (23)), I-309 (CCR8 ligand (24)), and TARC (CCR4 and CCR8 ligand (25, 26)) (Fig. 2). Stimulation

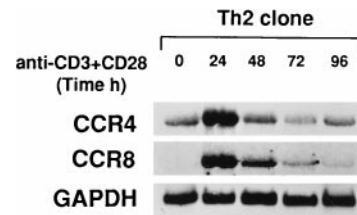


FIGURE 3. Transient up-regulation of CCR4 and CCR8 expression upon activation of a Th2 clone. The Th2 clone D4.11 was either left untreated or activated as described in Fig. 1. A fraction of the cells was lysed immediately (24 h). The remaining cells were washed, cultured, and harvested at 48, 72, and 96 h; total RNA was extracted. A total of 10 μ g of total RNA was subsequently used for Northern blot analysis. Filters were hybridized with 32 P-labeled probes for CCR4, CCR8, and GAPDH.

with anti-CD3 and anti-CD28 Abs results in diminished responses of Th1 cells to IP-10 (Fig. 2, upper left panel) and of Th2 cells to eotaxin (Fig. 2, upper right panel). In contrast, the response to TARC and, more markedly, to I-309 is increased upon activation of Th2 cells (Fig. 2, middle and lower right panels), and the response to TARC becomes detectable in activated Th1 cells (Fig. 2, lower left panel).

Since the experiments described above were performed on polarized T cell lines obtained by a single cycle of stimulation, we asked whether a T cell clone that had been subjected to repeated cycles of stimulation would exhibit a similar pattern of CCR4 and CCR8 regulation. TCR and CD28 triggering of a Th2 clone results in a marked up-regulation of CCR4 and CCR8 (Fig. 3). In addition, following termination of TCR triggering, the expression of CCR4

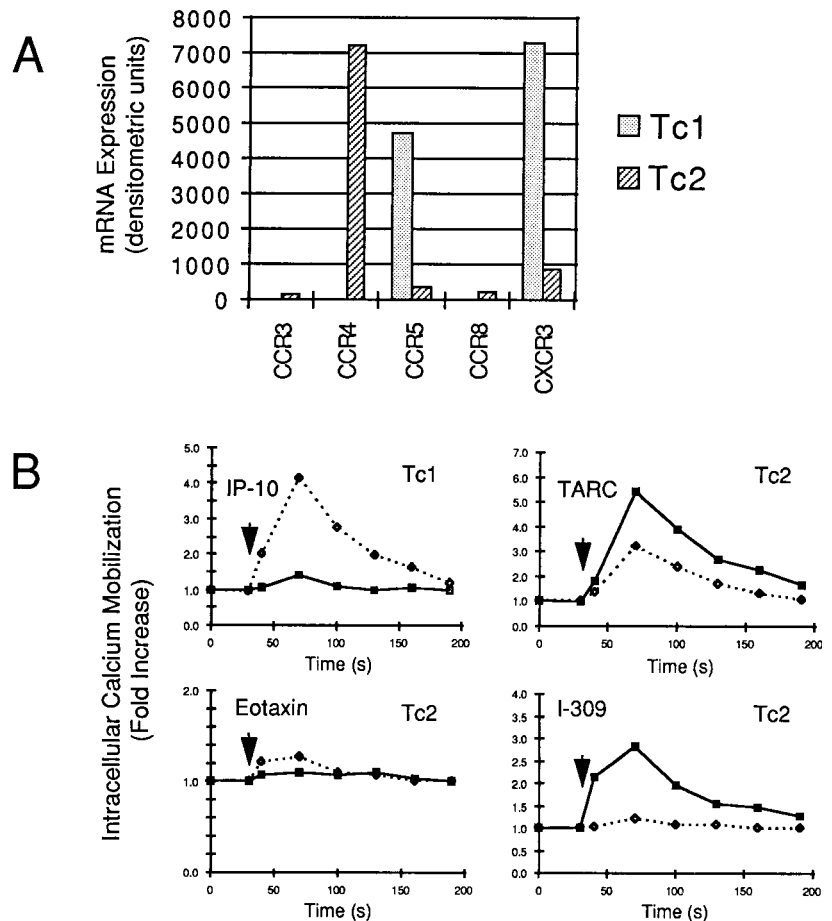


FIGURE 4. Chemokine receptor expression and modulation of chemokine responsiveness in Tc1 and Tc2 cells. **A**, A total of 10 μ g of total RNA from Tc1 and Tc2 cells was used for Northern blot analysis. Filters were hybridized with 32 P-labeled probes for CXCR3, CCR3, CCR4, CCR5, and CCR8. A normalized densitometric analysis of a representative experiment is shown. **B**, Tc1 and Tc2 cells were either left untreated or activated, loaded with fluo-3/AM, stained with quantum red-conjugated anti-CD8 Abs, and analyzed by flow cytometry by gating on CD8⁺ cells. Resting (dashed lines) and activated (solid lines) Tc1 and Tc2 cells were stimulated with IP-10 (50 ng/ml), eotaxin (400 ng/ml), I-309 (200 ng/ml), and TARC (10 ng/ml). The time of the addition of the chemokines is indicated by the arrow. The response is expressed as the fold increase of mean fluorescence intensity at time 0 for emissions at 525 nm. One representative experiment of three is shown.

and CCR8 returns to basal levels within 72 h (Fig. 3). Similar findings were observed with cord blood-derived Th2 cells (data not shown). These data indicate that Th2 cells transiently up-regulate CCR4 and CCR8 upon activation via the TCR. Collectively, our findings suggest that TCR-mediated activation of Th cells results in a transient alteration of the pattern of chemokine receptor expression.

Previous studies have demonstrated that, in addition to CD4⁺ T cells, type 1 and type 2 populations of CD8⁺ T cells (Tc1 and Tc2 cells) can also be generated in vitro and isolated in vivo in immunopathologic conditions (7, 27, 28). Thus, we have extended our analysis of chemokine receptors to polarized Tc1 and Tc2 cells generated from cord blood T cells. An analysis of the mRNA expression of several chemokine receptors revealed that, similar to CD4⁺ Th cells, Tc1 cells preferentially express CXCR3 and CCR5 (Fig. 4A); Tc2 cells selectively express CCR3, CCR4, and CCR8 (Fig. 4A). To verify the modulation of chemokine responsiveness upon activation of polarized CD8⁺ T cells, TCR- and CD28-stimulated Tc1 and Tc2 cells were assayed for their ability to mobilize calcium in response to various chemokines. The overall pattern of calcium mobilization in response to chemokines is concordant with the expression profile of chemokine receptors in Tc1 and Tc2 cells and is similar to Th1 and Th2 cells (Fig. 4B and data not shown). The response of Tc1 cells to IP-10 is greatly reduced following TCR-mediated activation (Fig. 4B). Activation of Tc2 cells results in a diminished response to eotaxin, whereas the response to TARC and I-309 is increased (Fig. 4B). Taken together, these data indicate that the pattern and regulation of chemokine receptor expression of polarized subsets of CD8⁺ T cells may overlap that of CD4⁺ T cells.

Recent studies have reported that TCR-mediated activation of T cells results in a down-modulation of CCR1, CCR2, CCR3, CCR5, and CXCR3 expression (11, 18, 29, 30). Our study reveals that, in contrast to other receptors, CCR4 and, to a greater extent, CCR8 are up-regulated upon TCR-mediated activation of Th2 cells. CXCR4 and CCR7 have been reported to be up-regulated upon activation of T cells (31, 32). However, recent work established that TCR triggering results in a down-modulation of CXCR4 that is subsequently reinduced by the autocrine action of secreted IL-4 (33). Thus, CCR7 is the only other receptor to be up-regulated upon TCR triggering (31). Interestingly, the CCR7 ligands, secondary lymphoid-tissue chemokine, and EB11 ligand chemokine have been proposed to control the initial entry of naive T cells and the subsequent encounter of activated T and B cells in secondary lymphoid tissues (34, 35). Notably, CCR7 is also up-regulated upon activation of both Th1 and Th2 cells, suggesting a potential contribution to their migration patterns (A.I. and D.D., unpublished observations). Given their mode of regulation, CCR4 and CCR8 may play a role similar to CCR7 in focusing activated T cells to certain areas of lymphoid tissues. However, in contrast to CCR7, CCR4 and particularly CCR8 are selectively expressed on polarized effector Th2 cells, suggesting a unique function in localizing and positioning activated Th2 cells. It is noteworthy that CCR4 and CCR8 share many common features. Their sequence is highly homologous (36, 37); they are both highly expressed on thymocytes, basophils, and Th2 cells (12, 19, 24, 36); and both are receptors for TARC (26). Collectively, these data suggest that CCR4 and CCR8 may play a critical role in localizing activated Th2 cells within sites of antigenic challenge and/or to specialized areas of lymphoid tissues.

Great attention has recently been placed on the possibility of distinguishing Th1 vs Th2 cells on the basis of the differential expression of chemokine receptors. Our findings suggest that CCR8, which is highly expressed upon TCR-mediated activation,

may be a very useful and selective marker for the in situ identification of recently activated Th2 cells.

Acknowledgments

We thank M. Napolitano for the CCR8 probe, P. Panina-Bordignon for T cell clones and discussions, and P. Vigano for cord blood samples. We also thank H. Hess and L. Adorini for their critical reading of the manuscript.

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