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An Abortive Ligand-Induced Activation of CCR1-Mediated Downstream Signaling Event and a Deficiency of CCR5 Expression Are Associated with the Hyporesponsiveness of Human Naive CD4^+ T Cells to CCL3 and CCL5

Katsuaki Sato, Hiroshi Kawasaki, Chikao Morimoto, Naohide Yamashima, and Takami Matsuyama

Human memory CD4^+ T cells respond better to inflammatory CCLs/CC chemokines, CCL3 and CCL5, than naive CD4^+ T cells. We analyzed the regulatory mechanism underlying this difference. Memory and naive CD4^+ T cells expressed similarly high levels of CCR1; however, CCR5 was only expressed in memory CD4^+ T cells at low levels. Experiments using mAbs to block chemokine receptors revealed that CCR1 functioned as a major receptor for the binding of CCL5 in memory and naive CD4^+ T cells as well as the ligand-induced chemotaxis in memory CD4^+ T cells. Stimulation of memory CD4^+ T cells with CCL5 activated protein tyrosine kinase-dependent cascades, which were significantly blocked by anti-CCR1 mAb, whereas this stimulation failed to induce these events in naive CD4^+ T cells. Intracellular expressions of regulator of G protein signaling 3 and 4 were only detected in naive CD4^+ T cells. Pretreatment of cell membrane fractions from memory and naive CD4^+ T cells with GTP-γS inhibited CCL5 binding, indicating the involvement of G proteins in the interaction of CCL5 and its receptor(s). In contrast, CCL5 enhanced the GTP binding to Ga<sub>q</sub> and Ga<sub>qγ</sub> in memory CD4^+ T cells, but not in naive CD4^+ T cells. Thus, a failure of the ligand-induced activation of CCR1-mediated downstream signaling event as well as a deficiency of CCR5 expression may be involved in the hyporesponsiveness of naive CD4^+ T cells to CCL3 and CCL5.

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Materials and Methods

Preparation and culture of T cells, their subsets, and monocytes

CD4^+ CD45RO^+ T cells and CD4^+ CD45RA^+ T cells were purified from PBMCs as described previously (16). Purity (>97% CD3^+ CD4^+ CD45RA^+ cells and >97% CD3^+ CD4^+ CD45RA^+ cells) was tested by FACS analysis. In some experiments, cells were untreated or treated with various concentrations (0.01–1 μg/ml) of blocking mAbs to CCR1 (clone 141-2; Ref. 17), CCR3 (17), CCR5 (BD Pharmingen, San Diego, CA), CXCR4 (BD Pharmingen), or control IgG (clLG; Sigma-Aldrich, St. Louis, MO) for 30 min at 4°C, and then used for subsequent experiments. For preparation of monocytes, cells were negatively selected with mAbs to CD3, CD19, and CD56 (all from BD Pharmingen) in combination with...
anti-mouse IgG mAb-conjugated immunomagnetic beads (Dynal Biotech, Oslo, Norway). The purity of monocytes was >98% by FACS analysis with anti-CD14-FITC (BD Biosciences, Mountain View, CA). CCR1-expressing transfectants were established by the transfection of cDNA for PCR-amplified CCR1 cDNA into mouse preB cell lymphoma B300-19 (parental cells) (17), and maintained in RPMI 1640 (Sigma-Aldrich) supplemented with antibiotic-antimycotic (Life Technologies, Rockville, MD) and 10% heat inactivated FCS (Life Technologies).

Flow cytometry

Cells were analyzed as described previously (16–18), using anti-CD3-PE, anti-CD4-PE, anti-CD8-PE, anti-CD28-PE, anti-CD45RA-PE, anti-CD45RO-PE, anti-CCR5-FITC, anti-CCR6-FITC, anti-CCR4-FITC, anti-CD44-FITC (all from BD PharMingen) (18) for 3 min at 37°C.

18 primers, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 5 mM MgCl₂, 10 mM dithiothreitol, 10 U RNase inhibitor, 50 U of Superscript II reverse transcriptase, and 2 U RNase H according to the manufacturer’s instructions. Subsequently, amplification of each cDNA (1 μl) was performed with a SuperTag Premix kit (Sawady Technology, Tokyo, Japan) using specific primers as follows: CCR1 (19), 5′-TCCTCAGGAAGGCTGAGATGGACG-3′ and 5′-CAACGGGAAAGGGGACCATTTAACC-3′; CCR5 (19), 5′-GTTGGAACAAATGTTGATTAT-3′ and 5′-CATGTGCAACACTCTGATG-3′; CXCR4 (19), 5′-CTGAGAAGCGTGACGACGAACTGACG-3′ and 5′-GATAGTGGTGCTCTCTTGGCT-3′; TCR (20), 5′-CCAGGGTCGCTGTTGTGAGGCACT-3′ and 5′-GCTCTACCCCAGGCCTCGGC-3′; CD4 (21), 5′-GCTGACGAGTAGAATGTTCC-3′ and 5′-ATGTTGAGAGTGTTAGGCG-3′. Specific primers for β-actin (Toyobo, Osaka, Japan) were also used for amplification. To activate DNA polymerase, preheating (95°C for 5 min) was performed. The reaction mixture was subjected to 30 cycles of PCR with the following conditions: CCR1, CXCR4, TCR, CD14, and β-actin, 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min; CCR5, 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Following these procedures, a final extension (72°C for 3 min) was performed. The expected sizes of PCR products for CCR1, CCR5, CXCR4, TCR, CD14, or β-actin was 440, 1117, 810, 386, 535, or 645 bp, respectively. Contamination with genomic DNA was routinely checked by omitting the Superscript II during reverse transcription. Amplification without cDNA was also carried out to assess later contamination of the PCR products. The PCR products were analyzed by electrophoresis through 2% agarose gel and visualized under UV light after ethidium bromide staining.

Western blotting and immune complex kinase assay

Whole cell binding assay

The binding of [125I]-labeled CCL5 to target cells was assayed as reported previously (22, 23). In brief, cells (10⁶/sample) were resuspended in 200 μl of binding medium (RPMI 1640/1% BSA) and incubated for 1 h at room temperature with [125I]-labeled CCL5 (~0.1 nM; specific radioactivity = 2200 Ci/mmol; NEN Life Science Products, Boston, MA) in the presence of an excess of competitive unlabeled (cold) CCL5 (10 nM). Cells were then spun (12,000 rpm for 1 min) through an 800-μl cushion of 10% (w/v) sucrose in PBS. The pellet was dried and then measured with an automatic gamma counter (ARC-380; Aloka, Tokyo, Japan).

Membrane binding assay

Cell membranes were prepared by lysis of cells (4 × 10⁶) in a lysis buffer (10 mM HEPES (pH 7.5), 3 mM MgCl₂, 2 mM EDTA, 40 μg/ml PMSF, 10 μg/ml leupeptin, 2 μg/ml pepstatin A, and 2 μg/ml aprotinin). After homogenization and sonication, they were centrifuged at 1,000 × g for 10 min, and the supernatant was transferred into Beckman tubes and ultracentrifuged at 150,000 × g for 30 min at 4°C. Membrane binding assay was performed as reported previously (24). In brief, cell membranes were preincubated with various concentrations (10⁻⁷-10⁻⁵ M) of GTP-γS (Sigma-Aldrich) for 30 min at 37°C. Subsequently, cell membranes were incubated in a 96-well plate with [125I]-labeled CCL5 (~0.1 nM) in the presence of an excess of competitive unlabeled (cold) CCL5 (10 nM) in a total volume of 100 μl of binding buffer (50 mM HEPES (pH 7.2), 5 mM MgCl₂, 1 mM CaCl₂, 0.5% BSA, 0.002% sodium azide, and protease inhibitors). Following an incubation for 90 min at 24°C, the membranes were centrifuged at 2500 rpm for 10 min. The supernatant was decanted, 100 μl of binding buffer (4°C) containing 0.5 M NaCl was added, and the membranes were transferred to a microtube. Following two additional rinses, they were transferred to scintillation vials, and then measured with an automatic gamma counter.
FIGURE 1. Chemokine receptor expression in memory and naïve CD4\(^+\) T cells. A, Cells were stained with anti-CCR1 mAb (clone 141-2 or clone 53504.11) or isotype-matched mAbs, and their cell surface expressions were analyzed by FACS. Data are represented by histograms in which cells were stained with the stated mAbs (thick lines) or isotype-matched mAbs (thin lines). B, Cells were stained with the mAbs to CD3 plus CD14, anti-CCR1 mAb (clone 141-2), or isotype-matched mAbs, and their cell surface expressions were analyzed by FACS. Data are represented by dot plots (left) and histograms (right) in which cells were stained with the stated mAbs (thick lines) or isotype-matched mAbs (thin lines). C and D, Cells were stained with the stated mAbs or isotype-matched mAbs, and their cell surface expressions were analyzed by FACS. Data are represented by dot plots (C) and histograms (D) in which cells were stained with the stated mAbs (thick lines) or isotype-matched mAbs (thin lines). E, RNA was extracted from monocytes, memory and naïve CD4\(^+\) T cells obtained from same individual used in C and D, and the mRNA expressions of CCR1, CCR5, and CXCR4 were evaluated by semiquantitative RT-PCR. PCR products for CCR1 (440 bp), CCR5 (1117 bp), CXCR4 (810 bp), TCR (386 bp), CD14 (535 bp), and β-actin (645 bp) are shown. The results of RT-PCR for β-actin demonstrate the loading of equal amounts of DNA on the gel.
cells (Ref. 16 and Fig. 1A). In addition, our anti-CCR1 mAb (MFI = 216) showed a higher staining of CCR1 in CCR1-expressing transfectants than the previously established anti-CCR1 mAb (clone 53504.111; MFI = 134) (Fig. 1A).

We tested the expression level of CCR1 (for CCL3 and CCL5) in CD4⁺ T cells (total CD4⁺ T cells) by flow cytometry with our anti-CCR1 mAb using monocytes for comparison because the expression level of CCR1 in PB CD4⁺ T cells was controversially reported (9, 10, 16). Figure 1B shows that the expression level of CCR1 in total CD4⁺ T cells (MFI = 34) was lower than that in monocytes (MFI = 58).

A series of studies have shown that CCL3 and CCL5 selectively attract a subset of memory T cells (5, 7, 9, 16). To elucidate the molecular mechanism underlying the responsiveness of CD45RO⁺ CD4⁺ T cells and CD45RA⁺ CD4⁺ T cells (naive phenotype) to CCL3 and CCL5, these subsets were isolated from PB CD4⁺ T cells (Fig. 1C), and the expressions of CCR1, CCR3 (for CCL5 and CCL11/eotaxin), CCR5 (for CCL3 and CCL5), and CXCR4 (for CXCL12/stromal cell-derived factor (SDF)-1α) were examined (Fig. 1D). Flow cytometric analysis revealed that memory and naive CD4⁺ T cells highly expressed CCR1 at similar levels, whereas the expression level of CXCR4 was higher in naive CD4⁺ T cells than in memory CD4⁺ T cells. Furthermore, CCR5 was only expressed in memory CD4⁺ T cells at low levels. We also observed that there was little or no expression of CCR3 on the cell surface of either cell type.

We also examined the transcriptional expressions of CCR1, CCR5, and CXCR4 in memory and naive CD4⁺ T cells by semi-quantitative RT-PCR analysis (Fig. 1E). The transcriptional expression level of CCR1 in memory CD4⁺ T cells was similar to that in naive CD4⁺ T cells, and these expression levels were lower than that in monocytes. Furthermore, the expression level of CCR5 transcript was significantly higher in memory CD4⁺ T cells than in naive CD4⁺ T cells, and this expression level was similar to that in monocytes. In addition, the transcriptional expression of CXCR4 was higher in naive CD4⁺ T cells than in monocytes and memory CD4⁺ T cells, and monocytes showed a higher expression of CXCR4 transcript than memory CD4⁺ T cells. We also observed that CD14 transcript, but not TCR transcript, was not detected in the preparations of memory and naive CD4⁺ T cells, whereas CD14 transcript was exclusively detected in the preparations of monocytes (Fig. 1E). These results exclude the possibility that the CCR1 mRNA of monocytes is the source for the PCR results with these T cells.

To address the specific binding of anti-CCR1 mAb with CCR1 expressed on the cell surface in memory and naive CD4⁺ T cells, we examined the blocking effect of CCL5 on the binding of anti-CCR1 mAb to the cells. Pretreatment of memory and naive CD4⁺
T cells with CCL5, but not CCL12, significantly impaired the binding of our anti-CCR1 mAb to the cells (Fig. 2A). These results indicate that our anti-CCR1 mAb specifically recognized CCR1 expressed on the cell surface in memory and naive CD4+ T cells.

We further examined the effect of the mAbs blocking CCRs and CXCR on the specific binding of memory and naive CD4+ T cells to 125I-labeled CCL5 (Fig. 2B). Anti-CCR1 mAb, but not mAb to CCR3 and CXCR4, significantly inhibited specific binding of 125I-labeled CCL5 to memory and naive CD4+ T cells as well as CD4+ T cells, while anti-CCR5 mAb partly suppressed this binding to memory CD4+ T cells and total CD4+ T cells, but not to naive CD4+ T cells. Furthermore, the extent of the binding of 125I-labeled CCL5 to anti-CCR5 mAb-treated memory CD4+ T cells was similar to that of naive CD4+ T cells. These results indicate that CCR1 acts as a major functional receptor for CCL5 in memory and naive CD4+ T cells.

Chemokine responsiveness of memory and naive CD4+ T cells

To address the features of chemokine responsiveness in memory and naive CD4+ T cells, we examined the chemotactic migratory responses of these cells to CCL3, CCL5, or CXCL12. Fig. 3A shows that memory CD4+ T cells vigorously responded to CCL3 and CCL5, whereas these chemokines caused little response of naive CD4+ T cells. On the other hand, CXCL12 caused a greater attraction of naive CD4+ T cells than memory CD4+ T cells.

To elucidate the role of CCRs in CCL5-induced chemotaxis of memory and naive CD4+ T cells, we examined the effect of mAbs to block CCR1, CCR3, CCR5, and CXCR4 on the chemotaxis of these subsets to CCL5 and CXCL12 (Fig. 3B). The chemotactic migratory responses of memory CD4+ T cells as well as total CD4+ T cells to CCL5 were significantly inhibited by anti-CCR1 mAb, while anti-CCR5 mAb caused only a slight suppression of these responses. In addition, a combination of mAbs to CCR1 and CCR5 completely inhibited their responses (data not shown). In contrast, mAbs to CCR3 and CXCR4 did not suppress CCL5-induced chemotaxis. Similar results were observed for the effect of these mAbs on CCL3-induced chemotaxis of memory CD4+ T cells as well as total CD4+ T cells (data not shown). We also observed that anti-CXCR4 mAb significantly inhibited CXCL12-induced chemotaxis of memory and naive CD4+ T cells as well as total CD4+ T cells. These results indicate that CCR1 plays a crucial role in the chemotaxis of memory CD4+ T cells to CCL3 and CCL5.

Different chemokine-induced signaling events in memory and naive CD4+ T cells

The engagement of chemokine receptors by their respective chemokines increases the tyrosine phosphorylation of targeted intracellular proteins in various cell types, and these intracellular events appear to be crucial to the chemotactic migratory responses of these cells (26–31). CCL5 induced tyrosine phosphorylation of various intracellular proteins in total CD4+ T cells, and the pattern of this event was distinct from that of cells stimulated with CXCL12 or mAbs to CD3 and CD28 (Fig. 4A). Furthermore, mAbs to CCR1 and CCR5, but not mAbs to CCR3 and CXCR4, inhibited CCL5-induced tyrosine phosphorylation, and anti-CCR1 mAb caused a greater suppression than anti-CCR5 mAb in total CD4+ T cells (Fig. 4B). Similar results were observed for the effect of these mAbs on the CCL3-induced tyrosine phosphorylation of total CD4+ T cells (data not shown). We also observed that anti-CXCR4 mAb completely inhibited CXCL12-induced tyrosine phosphorylation in total CD4+ T cells (Fig. 4B).

The results suggest that a simple absence of CCR5 may not be the reason for the hyporesponsiveness of naive CD4+ T cells to CCL5, because CCR1 played a crucial role in the ligand binding and the ligand-induced chemotaxis (Figs. 1–3). To clarify the molecular mechanism responsible for the distinct difference in responsiveness to CCL5 between memory and naive CD4+ T cells, we examined the potential involvement of tyrosine phosphorylation events in the chemokine responsiveness of these cell types (Fig. 4D). Stimulation with CCL5 induced tyrosine phosphorylation of various intracellular proteins in memory CD4+ T cells but not in naive CD4+ T cells. In contrast, stimulation with CXCL12 or a combination of mAbs to CD3 and CD28 caused a more potent tyrosine phosphorylation of several intracellular proteins in naive CD4+ T cells than in memory CD4+ T cells.

To examine the difference in the activation of protein tyrosine kinases (PTKs) between memory and naive CD4+ T cells following stimulation with various stimuli, we tested their tyrosine phosphorylation level and kinase activity of p60src. Stimulation with CCL5 or CXCL12 caused a tyrosine phosphorylation of p60src, and levels of activation were slightly lower than those in cells stimulated with mAb to CD3 and CD28 in the total CD4+ T cell population (Fig. 4C). Furthermore, CCL5 induced activation of...
p60Src in memory CD4+ T cells but not in naive CD4+ T cells (Fig. 4E). On the other hand, the level of the activation of p60Src was higher in naive CD4+ T cells than in memory CD4+ T cells following stimulation with CXCL12 or a combination of mAbs to CD3 and CD28 (Fig. 4E).

To address the CCL5-induced activation status of other PTKs including ZAP-70, p125Fak, and Pyk2, which play crucial roles in ligand-induced chemotaxis of T cells (27–30), we examined their tyrosine phosphorylation and kinase activities of ERK2, SAPK/JNK, and p38 MAPK in memory and naive CD4+ T cells. We also observed that tyrosine phosphorylation of CCR1 in memory CD4+ T cells but only a slight activation in naive CD4+ T cells following stimulation with CCL5 (Fig. 4E).

We further examined the level of tyrosine phosphorylation and kinase activities of a family of mitogen-activated protein kinase (MAPK) ERK2, JNK/SAPK, and p38MAPK in memory and naive CD4+ T cells following stimulation with CCL5 (Fig. 5E-G). Stimulation of memory CD4+ T cells with CCL5 increased tyrosine phosphorylation and kinase activities of ERK2, SAPK/JNK, and p38MAPK compared to unstimulated cells. In contrast, engagement by CCL5 resulted in a weaker activation of these MAPKs in naive CD4+ T cells than in memory CD4+ T cells.

Expressions of Gαi, complex and RGS proteins in memory and naive CD4+ T cells

To address the role of CCR1 in PTK-dependent cascades, we examined the tyrosine phosphorylation of CCR1 in memory and naive CD4+ T cells following stimulation with CCL5. Fig. 6A shows that ligation by CCL5 induced tyrosine phosphorylation of CCR1 in memory CD4+ T cells, whereas little tyrosine phosphorylation was observed in naive CD4+ T cells.

Chemokine receptors constitutively couple with various sets of heterotrimeric G protein subunits, including Gα and Gβγ complex, and ligation by the appropriate chemokines induces a dissociation of these subunits from GPCRs (29–32). Subsequently, the Gα subunit acts as a GTPase to regulate various downstream signaling cascades while the Gβγ complex mediates Ras and Rac1-dependent MAPK activation via a PTK-dependent pathway (26, 27, 31–34). CCR1 reportedly coupled with Gαi, or Gαq, as well as various sets of Gα, complex (34), while CXCR4 was associated with Gαs and Gβγ complex (35). To establish if there was any difference in the expression of the Gα subunit and Gβγ complex between memory and naive CD4+ T cells, we examined the intracellular expression levels of Gαi, Gαq, and Gβγ complex. Fig. 6B shows that there was little or no difference in the expression levels between the cell types.

Proteins of the RGS family act as GTPase-activating proteins to accelerate GTP hydrolysis by the Gα subunit, leading to a negative
regulation of GPCR-mediated signaling events (36–39). Furthermore, accumulating results indicate that a family of RGS proteins were involved in the impairment of ligand-induced chemotaxis in certain cell types (37–40). Therefore, we examined the intracellular expression levels of RGS1, RGS3, and RGS4 in memory and naive CD4+ T cells. Fig. 6B shows that RGS3 and RGS4 were only expressed in naive CD4+ T cells, whereas RGS1 was undetected in either cell type.

**Effect of CCL5 binding on GTP-GDP exchange in memory and naive CD4+ T cells**

To address the involvement of G proteins in the binding of CCL5 to memory and naive CD4+ T cells, we examined the effect of GTP-γ-S on the binding of 125I-labeled CCL5 to cell membrane fractions (Fig. 7A). Preincubation with GTP-γ-S suppressed the binding of 125I-labeled CCL5 to the cell membrane fractions obtained from memory and naive CD4+ T cells in a dose-dependent manner. These results indicate that CCR1- and/or CCR5-associated G proteins regulate the binding of CCL5 to memory and naive CD4+ T cells.

We also examined the effect of CCL5 or CXCL12 on GTP-GDP exchange in cell membrane fractions obtained from memory and naive CD4+ T cells (Fig. 7B). CCL5 enhanced the GTP binding to Go and Gq in memory CD4+ T cells, whereas this stimulation failed to induce this event in naive CD4+ T cells. In contrast, CXCL12 enhanced the GTP binding to Go, but not Gq, in both cell types.

**Discussion**

The findings reported in this work suggest that the hyporesponsiveness of naive CD4+ T cells to CCL3 and CCL5 involves a failure of ligand-induced activation of CCR1-mediated downstream signaling event as well as a deficiency of CCR5 expression.

There are conflicting reports about the expression of CCR1 in PB naive CD4+ T cells (9, 10, 16), although similar results for the chemotaxis of these subsets to CCL3 and CCL5 were obtained. Consistent with previous reports (9, 16), a similar expression level of CCR1 was observed in PB memory and naive CD4+ T cells. In contrast, unlike PB naive T cells, cord blood naive CD4+ T cells did not express CCR1 (16). This discrepancy might be due to the cell preparation and the binding affinity of anti-CCR1 mAb used in the experiments.

Analysis of the responsiveness of memory CD4+ T cells to CCL5, with respect to cell surface expression levels, chemotaxis,
and ligand binding, indicates that CCR1 and CCR5 play a role in these events ~80 and 20% of the time, respectively. Indeed, CCL3 and CCL5 exhibit more potent binding affinities to CCR1 than CCR5 by ~20-fold (22). Collectively, the deficiency of CCR5 expression is not the main reason for the hyporesponsiveness of naive CD4 T cells to CCL3 and CCL5. Thus, our results suggest that some other molecular mechanism involving CCR1-mediated signaling event accounts for the inability of naive CD4 T cells to respond to these inflammatory CCLs.

Stimulation with CCL5, CXCL12, or a combination of mAbs to CD3 and CD28 induced distinct patterns of tyrosine phosphorylation of intracellular proteins in total T cells and memory CD4 T cells, suggesting that specific PTK-dependent cascades are activated via the respective receptors, although several components may be shared. In contrast, the blocking experiments with mAbs to CCR1 and CCR5 show that CCR1 and CCR5 caused the different patterns and degree of tyrosine phosphorylation events in total T cells following stimulation with CCL5. Therefore, the ligation of CCR1 and CCR5 may activate the respective specific PTK-dependent cascade, although the precise difference in their downstream signaling event is still unclear because CCR1 and CCR5 share the ligands in total T cells and memory CD4 T cells (1-3).

FIGURE 6. Expressions of $G_{\alpha_i}$, complex and RGS proteins in memory and naive CD4 T cells. A, Memory and naive CD4 T cells were unstimulated or stimulated with CCL5. Immunoprecipitates obtained with Ab to CCR1 were analyzed by Western blotting with anti-pTyr mAb (phosphoproteins) and CCR1 to ensure that similar amounts of protein were present in each sample. B, Intracellular expression levels of $G_{\alpha_i}$, $G_{\beta_i}$, $G_{\gamma}$, and RGS proteins in memory and naive CD4 T cells of two individuals were analyzed by Western blotting with the stated Abs. The results of Western blotting for actin demonstrate the loading of equal amounts of samples. The results are representative of 10 experiments.

FIGURE 7. Effect of CCL5 binding on GTP-GDP exchange in memory and naive CD4 T cells. A, Effect of GTP-$\gamma$S on the binding of $^{125}$I-labeled CCL5 to cell membrane fractions was determined by preincubating cell membrane fractions with the indicated concentrations of GTP-$\gamma$S ($10^{-7}$–$10^{-3}$ nM) followed by incubation with $^{125}$I-labeled CCL5 (~0.1 nM) in the presence of an excess of unlabeled CCL5 (10 nM). B, Cell membrane fractions obtained from memory and naive CD4 T cells were stimulated with CCL5 or CXCL12 and assayed for their GTP-GDP exchange. * Value of $p < 0.001$ (compared with clgG-activated cell membrane by Student’s paired $t$ test). The results are representative of 10 experiments.
because CCR1 is the only receptor for CCL3 and CCL5 in this subset.

Uncoupling of GPCRs with G proteins is thought to prevent their high-affinity ligand binding (24). We showed that pretreatment of the cell membrane fractions obtained from memory and naive CD4+ T cells with GTPγS inhibited their ligand binding. Therefore, the high-affinity ligand binding of CCR1 and/or CCR5 in both cell types may involve their coupling with G proteins. In contrast, CCL5 induced GTP-GDP exchange in memory CD4+ T cells, whereas this stimulation failed to induce this event in naive CD4+ T cells. These phenomena imply that the ligation of CCR1 by CCL5 may not induce GTP-GDP exchange in Gxi in naive CD4+ T cells, although these G protein subunits may bind the CCL5-CCR1 complex. In contrast, mutation of GPCRs and certain stimulations led to the retention of a high ligand binding affinity of GPCRs but abolished their downstream signaling events, although the precise mechanism remains unclear (41–43).

Therefore, our findings involving GTP-GDP exchange in Gxi subunits and the ligand binding affinity of CCR1 in naive CD4+ T cells may be a novel regulatory mechanism for GPCR. Collectively, our findings suggest that a failure of CCR1 to activate PTK-dependent cascade may involve the deficiency in the ligand-induced GTP-GDP exchange in Gxi.

The intracellular expressions of RGS3 and RGS4 were only detected in naive CD4+ T cells. It has been previously reported that the expression of RGS1, RGS3, and RGS4, but not RGS2, suppressed the chemotactic response of certain transfectants to FMLP, C5a, CXCL8/IL-8, and CCL2/monocyte chemotactrant protein-1, while the expression of RGS3 and RGS4 inhibited the CCL3-induced CCR1-mediated chemotaxis of these cell types (37). In contrast, p60Src and Pyk2 link GPCR with various downstream PTK-dependent cascades (26, 27, 30, 31). In addition, Yan et al. (39) have previously reported that RGS4 inhibited Gxi-mediated activation of MAPKs in certain transfectants. However, the role of RGS3 and RGS4 in the defective CCL5-induced CCR1-mediated signaling events in naive CD4+ T cells remains unknown because Gxi proteins are not thought to affect GTP-GDP exchange (36–39); the failure of ligand-induced GTP-GDP exchange in Gxi subunits may play a crucial role in these events. These phenomena imply that RGS3 and RGS4 would not contribute to the failure of CCR1 to activate Gxi subunit-dependent signaling events in naive CD4+ T cells.

Naive CD4+ T cells showed greater surface expression level of CXCR4 and response to CXCL12 than memory CD4+ T cells. In addition, CXCL12-induced GTP binding to Gi in naive CD4+ T cells was higher than in memory CD4+ T cells. Moratz et al. (38) have previously reported that the impairment of CXCL12-induced CXCR4-mediated migratory responsiveness in germinal center B cells involved a constitutive expression of RGS1, whereas CXCL12 attracted CXCR4-expressed naive and memory B cells lacking the expression of RGS1. We showed that memory CD4+ T cells as well as naive CD4+ T cells did not exhibit the intracellular expression of RGS1. Therefore, the difference in the response to CXCL12 between memory and naive CD4+ T cells may be correlated with the cell surface expression level of CXCR4- and CXCL12-induced GTP-GDP exchange in Gi. However, Reif et al. (40) have recently reported that a short isoform of RGS3 (sRGS3) as well as RGS1 are effective inhibitors of Gi-dependent response to CXCL12 in murine B cell transfectants. The discrepancy in the role of RGS3 between human CD4+ T cells and the murine B cell line in the CXCL12-induced response via CXCR4 remains unclear; this might be due to the isoform variances or the species differences.

In summary, our findings suggest that the failure of ligand-induced activation of CCR1-mediated downstream signaling events as well as the deficiency of CCR5 expression are associated with the hyporesponsiveness of human naive CD4+ T cells to CCL3 and CCL5. We (16) and others (9) have previously reported that CCR2, CCR6, and CXCX3 were only expressed on memory CD4+ T cells, and their expression patterns were associated with the respective chemokine responsiveness. Thus, the different chemotactic properties of memory and naive CD4+ T cells may be explained by chemokine receptor expression levels as well as their abilities to activate downstream signaling events. Aberrant trafficking properties of T cells are suggested to be involved in the initiation and persistence of immunopathological diseases (1–3, 44). Furthermore, chemokines and their receptor system are thought to be potential target molecules for therapeutic intervention to prevent these diseases (1–3, 44). Thus, the molecular manipulation of chemokine receptor-mediated signaling events may be a novel approach to the prevention and therapy of immunopathological diseases.

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References


