Chemokine Receptor CXCR3 Desensitization by IL-16/CD4 Signaling Is Dependent on CCR5 and Intact Membrane Cholesterol

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Chemokine Receptor CXCR3 Desensitization by IL-16/CD4 Signaling Is Dependent on CCR5 and Intact Membrane Cholesterol

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Previous work has shown that IL-16/CD4 induces desensitization of both CCR5- and CXCR4-induced migration, with no apparent effect on CCR2b or CXCR3. To investigate the functional relationship between CD4 and other chemokine receptors, we determined the effects of IL-16 interaction with CD4 on CXCR3-induced migration. In this study we demonstrate that IL-16/CD4 induced receptor desensitization of CXCR3 on primary human T cells. IL-16/CD4 stimulation does not result in surface modulation of CXCR3 or changes in CXCL10 binding affinity. This effect does require p56<sup>1ck</sup> enzymatic activity and the presence of CCR5, because desensitization is not transmitted in the absence of CCR5. Treatment of human T cells with methyl-β-cyclodextrin, a cholesterol chelator, prevented the desensitization of CXCR3 via IL-16/CD4, which was restored after reloading of cholesterol, indicating a requirement for intact cholesterol. These studies demonstrate an intimate functional relationship among CD4, CCR5, and CXCR3, in which CCR5 can act as an adaptor molecule for CD4 signaling. This process of regulating Th1 cell chemoattraction may represent a mechanism for orchestrating cell recruitment in Th1-mediated diseases. The Journal of Immunology, 2006, 176: 2337–2345.

The chemokines are a family of small proteins whose classification is based on the positioning of the first two cysteine residues. The majority of chemokines can be classified into two major groups: CXC and CC chemokines. They induce cellular responses via a family of nonexclusive, seven-transmembrane-spanning domain glycoprotein receptors that are coupled to a G protein-signaling pathway. Functionally, the chemokines participate in a wide range of bioactivities, including angiogenesis, cancer, cell development, leukocyte homeostasis (1–3), and directional recruitment or chemotaxis. Efficient and effective recruitment of immune cells from the circulation into inflamed tissue requires correct interpretation of the signals induced by a large number of chemokines and other regulatory cytokines produced either simultaneously or in sequence. The production of a large number of cytokines and chemokines at sites of inflammation suggests that regulation of receptor responsiveness is an important component of the complex regulatory system necessary to ensure that there is control over the magnitude of cellular accumulation.

It has been repeatedly described that stimulation of one chemokine receptor can affect the responsiveness to other chemokine receptors, a concept known as heterologous desensitization (4–6). Heterologous desensitization in which responses to chemokine receptors are desensitized by previous signals via nonchemokine G protein-linked receptors, such as opioid receptors, has also been described (7, 8). In addition, we have previously reported that signaling through the Ig superfamily member CD4 induced by its ligand IL-16 results in heterologous receptor cross-desensitization of the chemokine G protein-linked receptors CCR5 and CXCR4 (9, 10).

Similar to many chemokines, IL-16 has been detected at sites of inflammation in various inflammatory conditions, such as asthma, inflammatory bowel disease, rheumatoid arthritis, delayed-type hypersensitivity, multiple sclerosis, systemic lupus erythematosus, and Graves’ disease (11–17). The precise role of IL-16 in these diseases has yet to be determined; however, it has now been shown that the exogenous administration of IL-16 in a murine model of acute allergic airway inflammation results in significant attenuation of both airway hyper-reactivity and lung inflammation (18). The immunomodulatory role of IL-16 in this model may relate in part to its ability to desensitize either CCR5 and CXCR4, because both receptors have been associated with T cell recruitment in allergic inflammation (19, 20). Although both receptors are desensitized by IL-16/CD4 signals, the mechanism for desensitization is different for each receptor. Desensitization of CCR5 by CD4 requires the catalytic activity of the CD4-associated Src kinase p56<sup>1ck</sup>. Reciprocal desensitization of IL-16/CD4 chemotactic signal also occurs after CCL4 binding to CCR5 (9). However, desensitization of CXCR4 by CD4 signals is independent of p56<sup>1ck</sup> enzymatic activity, but requires the presence of the Src homology 3 domain (SH3)<sup>3</sup> on p56<sup>1ck</sup>, with subsequent activation of PI3K (10). Reciprocal CXCR4/CD4 desensitization does not occur. The differences in the mechanisms could reside in the spatial orientation of each chemokine receptor with CD4. CCR5 is constitutively associated with CD4 (21). This interaction results in a mutual synergistic effect for signaling and induction of migration (22, 23).
CXCR4 is not constitutively associated with CD4, although coligation by HIV-1 gp120 may facilitate the interaction (24). Because CCR5 is expressed predominantly on Th1 cells, the intent of this study was to determine whether another chemokine receptor expressed on Th1 cells, CXCR3, is also susceptible to IL-16/CD4-induced desensitization. The chemokine receptor, CXCR3, is present on activated T cells (both CD4 and CD8), and its three known cognate ligands, CXCL9, -10, and -11, are frequently generated at sites of inflammation, where IL-16 has been detected (25). The present studies show that IL-16/CD4 stimulation results in receptor cross-desensitization of CXCR3, as determined by inhibition of both CXCL9- and CXCL10-induced chemotaxis. Interestingly, IL-16/CD4 had no inhibitory effect on CXCL11 stimulation. Chemotactic desensitization requires the presence and enzymatic activity of p56lck. The CD4-induced effect required the presence of CCR5 and also requires intact membrane cholesterol. In addition, like CD4-CCR5 desensitization, CD4-CXCR3 desensitization was reciprocal because CXCL10 stimulation resulted in desensitization of IL-16/CD4-induced chemotaxis; however, in this circumstance CCR5 was not required for CD4 desensitization. These studies demonstrate a functional inter-receptor relationship among CD4, CCR5, and CXCR3, which provides a potential mechanism for orchestrating Th1 cell recruitment to sites of inflammation.

Materials and Methods

Media

Medium 199 (Cellgro) was supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen Life Technologies), 25 mM HEPES buffer (Invitrogen Life Technologies), and 0.4% BSA fraction V (Sigma-Aldrich). PBS was purchased from Invitrogen Life Technologies.

Mice

CCR5−/− (B6129P2-Ccnkβ5mcm/wz) and strain control (B6129PF2J) mice were purchased from The Jackson Laboratory. Only male mice, ages 5–8 wk, were used for these studies. The studies were reviewed and approved by the institutional animal care and use committee at Boston University Medical Center.

Cytokines and chemokines

Human rIL-16 was generated from the yeast, Pichia pastoris (B6129P2-Ccnkβ5mcm/wz), and purified using light microscopy to visualize the number of cells that had migrated through 8-μm fiber filters (Whatman) using a vacuum harvester. The filters were air dried at room temperature. The samples were then aspirated through GF/C microfiber filters and analyzed using Student’s t test. For most experiments, 12–17 cells/high-power field were counted for control cell migration. All samples were tested in duplicate, and the data are the mean ± SD of three or more separate experiments. Similar migration studies were performed using T cells obtained from either CCR5−/− mice or control strain mice. For these studies the murine cells were allowed to migrate for 4 h before fixation and counting.

Flow cytometric analysis

Cells were exposed to control medium or IL-16 for 30 min at 37°C at 5% CO2. Then ~0.5–1 × 106 cells were added to 3-ml culture tubes, washed once with PBS, and resuspended in 200 μl of FACS staining buffer (PBS with 1% BSA and 0.1% sodium azide) with 2 μl of isotype control or directly conjugated anti-CD4 and anti-CXCR3 Ab. Cells were incubated with Abs for 30 min on ice, washed twice, and then fixed in 2% paraformaldehyde. Cells were then transferred to 5-ml round-bottom polystyrene tubes and analyzed on a FACScan (BD Biosciences) using CellQuest software. Experiments performed in triplicate.

CXCL10 binding

Binding assays were conducted as previously described (9, 10). Briefly, 3 × 106 human T cells were cultured in the presence of IL-16 (250–1000 pg) or cold CXCL10 (250–1000 pg) for 30 min at room temperature before the addition of 125I-labeled CXCL10 (IFN-inducible protein-10; 814TBq/mmol; New England Nuclear) was added to each sample for 120 min at room temperature. The samples were then aspirated through GF/C microfiber filters (Whatman) using a vacuum harvester. The filters were air dried.
and counted by a gamma counter. Nonspecific radioactivity present with 10- to 1000-fold excess of cold CXCL10 was subtracted from total bound counts for each dose and used to calculate specifically bound counts. The percent inhibition of CXCL10 binding induced by IL-16 was calculated by subtracting counts from IL-16-treated cells from specific counts, divided by specific binding counts, times 100%.

Methyl-β-cyclodextrin (MCD) and saponin-treated human T cells

T cells were pretreated with 1 μM MCD (Sigma-Aldrich) for 30 min at 37°C in 5% CO2 for 30 min, as previously described (27). MCD was dissolved in PBS. After 30 min, IL-16 was added to half the cells, and they were incubated for an additional 30 min at 37°C in 5% CO2. The cells were then washed and resuspended at a concentration of 6–8 × 10^6 cells/ml to perform the migration assays as previously described, using IL-16 (10^{-10}M), CXCL10 (50 ng/ml), CCL4 (50 ng/ml), or control medium.

Cholesterol reloading of MCD

Cholesterol reloading of MCD was performed using a modified protocol from Nguyen and Taub (27). In brief, T cells were pretreated with 1 μM MCD loaded with either 300 μM cholesterol (5-cholesten-3β-ol) or the oxidized form, 4-cholesten-3-one (both from Sigma-Aldrich), for 2 h. The MCD-cholesterol solutions were prepared in PBS by constant vortexing at 25°C for 6–8 h, then filtered through a 0.22-μm pore size filter unit. T cells (10 × 10^6) were exposed to MCD-cholesterol for 30 min at 37°C. Removal of MCD-cholesterol was accomplished by at least 10 washings with PBS. Some of the cells were then stimulated with IL-16 (10^{-10}M) for 30 min, as described above, before washing again in PBS and then were used in the chemotaxis assay with CXCL10 as the chemoattractant.

Lipid raft isolation from human T cells

Lipid raft fractions were generated using a method previously described by Nguyen et al. (28). Briefly, human T cells (1 × 10^7) were treated, or not, with MCD as described above before lysing for 30 min at 4°C in 500 μl of 1% Triton X-100 in TKM buffer (50 mM Tris-HCl (pH 7.4), 25 mM KCl, 5 mM MgCl2, and 1 mM EDTA). Lysates were centrifuged at 8000 g for 10 min at 4°C to remove nuclei and debris. Extracts were then adjusted to 40% sucrose in TKM buffer and loaded into SW41 tubes. The extracts were overlaid with 6 ml of 38% sucrose-TKM, followed by 4.5 ml of 5% sucrose-TKM. Tubes were centrifuged at 100,000 × g for 18 h at 4°C. Eleven 1-ml fractions were collected from the bottom of the tube and stored at −20°C. SDS-PAGE and Western blotting, using anti-CCR5 and anti-CXCR3 Abs, were performed to visualize receptor distribution.

Results

**CD4-CXCR3 reciprocal desensitization**

To determine whether IL-16/CD4 signaling could affect CXCR3-induced migration, human T lymphocytes were incubated with human rIL-16 for 30 min before conducting the chemotaxis assay with the CXCR3-specific chemokines CXCL9 (monokine induced by IFN-β), CXCL10 (IFN-inducible protein-10), or CXCL11 (IFN-inducible T cell α chemoattractant). As stimulation with IL-16 for 30 min could increase cell motility, baseline migration was established using cells pretreated with IL-16 without subsequent chemokine stimulation. As shown in Fig. 1, cells pretreated with IL-16 were completely unresponsive to either CXCL9 or CXCL10 stimulation (1–100 ng/ml). Interestingly, IL-16-treated cells responded normally to CXCL11 stimulation, suggesting a different mechanism for CXCL11-induced migration that is not affected by CD4 signaling. For the remaining experiments, designed to determine the mechanism for desensitization, results were identical for both CXCL9 and CXCL10 stimulation. Because the magnitude for desensitization was slightly greater for CXCL10, these data will be shown to represent findings for both chemokines.

A time course for exposure demonstrated that IL-16 stimulation required ∼10 min to induce desensitization of CXCL10 (Fig. 2A). This time course is consistent with previous studies that found that IL-16 could desensitize CCR5-induced migration after 10-min exposure (9). The inhibitory effect was transient, however, because cells exposed to IL-16 for 15 min, followed by an 8-h incubation, demonstrated a statistically similar responsiveness to CXCL10 stimulation as untreated cells (Fig. 2B). The time course for desensitization of CXCL9 was similar to that observed for CXCL10 (data not shown). There was no effect of IL-16 pretreatment for all times tested on CXCL11 stimulation (data not shown). For this reason, CXCL11 was not included in subsequent studies.

**FIGURE 1.** Dose response and time course for CXCR3 desensitization. Purified human CD4+ T cells were incubated with IL-16 before washing and stimulated for migration using CXCL9, CXCL10, and CXCL11. The dose response to the chemokines with (dashed lines) or without (solid lines) 15-min pretreatment with IL-16 (10^{-9}M) is shown. The data are presented as a percentage of control cell migration, determined by IL-16-pretreated cells, but stimulated for migration using medium alone (designated 100%). CXCL9 (A), CXCL10 (B), or CXCL11 (C) alone designates migration of cells not pretreated with IL-16, but stimulated with the chemokines (50 ng/ml) for migration. *, Migration that is statistically different from that with CXCL10 alone (p < 0.05).
To determine whether IL-16-induced chemotaxis via CD4 is reciprocally desensitized by CXCL10, T cells were stimulated with CXCL10 for up to 60 min before washing, followed by stimulation with IL-16 (10^{-9} M). As shown in Fig. 3A, an inhibitory effect was detected in CXCL10-stimulated cells, demonstrating a reciprocal interaction between CD4 and CXCR3. Maximal inhibition was seen after 15–30 min of CXCL10 stimulation (Fig. 3B), suggesting that the mechanism for receptor cross-desensitization was different for CXCR3 compared with CD4. A similar effect and time course were detected for CXCL9 treatment (data not shown). The desensitization effect of IL-16 on CXCR3 is relatively selective, because we have previously reported no cross-desensitization of other chemokine receptors, such as CCL2/CCR2 and CCL11/CCR3 (9, 10).

Effect of IL-16 on CXCR3 receptor expression

To investigate the mechanism by which IL-16 affected CXCR3 signaling, receptor expression was first assessed. T cells were stimulated with IL-16 (10^{-9} M) for up to 1 h, at which time the cells were dual stained with fluorescently conjugated anti-CXCR3 and anti-CD4 mAbs. Using identical conditions for which IL-16 completely blocked migration toward CXCL10, there was no evidence of loss of CXCR3 surface expression (Fig. 4A).

Because surface expression of CXCR3 may not necessarily reflect changes in ligand-receptor interactions, the ability of IL-16 to directly alter CXCL10 binding to CXCR3 was investigated. Human T cells were incubated in the presence or the absence of IL-16 before the addition of radiolabeled [125I]CXCL10. As shown in Fig. 4B, the addition of IL-16 had no effect on the binding of CXCL10. This finding suggested that the inhibitory effect of IL-16 was mediated through CD4 signaling rather than by altering CXCR3 expression, decreasing the binding affinity for CXCL10, or sterically competing with CXCL10 binding to CXCR3. CXCR9 binding was similarly unaffected by IL-16 pretreatment (data not shown).

Requirement of p56\textsuperscript{Lck} enzymatic activity for desensitization of CXCR3

IL-16 binding to CD4 activates p56\textsuperscript{Lck} and the PI3K pathway (29). We have previously reported that IL-16-induced chemokine receptor desensitization was mediated through the enzymatic activity of p56\textsuperscript{Lck} for CCR5; however, desensitization of CXCR4 was independent of p56\textsuperscript{Lck} enzymatic activity, but required the activation of PI3K (9, 10). To further define the mechanism by which IL-16 blocked CXCR3-induced migration, human T cells were treated with either herbimycin A, a Src kinase inhibitor, or wortmannin, a PI3K inhibitor, before IL-16 pretreatment and CXCL10-induced migration. Cells pretreated with wortmannin in addition to IL-16 demonstrated no inhibitory activity after IL-16 stimulation (Fig. 5B). These findings strongly suggest that p56\textsuperscript{Lck} enzymatic activity, not PI3K activity, is involved in the transmission of a CD4-induced signal that results in the blockade of CXCR3 signaling. However, the possibility exists that these kinase inhibitors may have some other, as yet unidentified, effect on cellular responses.
For this reason confirmation studies were conducted using murine T cell hybridomas that express mutated constructs of p56\(^{\text{Lck}}\). These cells were previously used to determine the requirement for p56\(^{\text{Lck}}\) enzymatic activity for desensitizing CCR5 and CXCR4 (9, 10). These cells were infected to express either the full-length chimeric CD4-p56\(^{\text{Lck}}\) protein or a chimeric protein containing CD4 and p56\(^{\text{Lck}}\) that lacks the SH1 (enzymatic) domain. When stimulated with murine CXCL10, both of these cell lines demonstrated a migratory dose response (Fig. 5C). Pretreating the cells that express full-length CD4-p56\(^{\text{Lck}}\) with IL-16 (10\(^{-9}\) M) for 20 min before CXCL10 stimulation resulted in significant inhibition of the migratory response. IL-16 stimulation had no inhibitory effect, however, on hybridoma cells expressing the SH1-truncated chimeric protein. These data indicate that CD4-mediated signaling requires the enzymatic activity of p56\(^{\text{Lck}}\) to induce receptor cross-desensitization of CXCR3.

**Dependence on CCR5 for CD4-mediated CXCR3 desensitization**

Because the mechanism involved in CD4-induced desensitization of CXCR3 was identical with that required for desensitizing CCR5, and because CCR5 and CXCR3 are coexpressed on Th1 cells, we next determined whether CCR5 is involved in the desensitization of CXCR3. For these studies lymph node T cells were obtained from CCR5\(^{-/-}\) mice. The cells were pretreated with IL-16 for 20 min before stimulation with CXCL10. As shown in Fig. 6A, cells from a strain control mouse exhibited the expected desensitization response when IL-16-treated cells were stimulated with CXCL10. However, cells obtained from the CCR5\(^{-/-}\) mouse did not demonstrate any desensitization response after IL-16 pretreatment, because the migratory response to CXCL10 was statistically identical with that of untreated cells. The presence...
of CCR5 was also required for IL-16-induced desensitization of CXCL9, because IL-16-treated CCR5<sup>−/−</sup> cells responded to CXCL9-induced migration (data not shown). CCR5 had no effect on CXCL11 stimulation, because wild-type and CCR5<sup>−/−</sup> cells responded similarly whether pretreated, or not, with IL-16 (data not shown).

CCR5<sup>−/−</sup> murine T cells were next pretreated with CXCL10 for 30 min before stimulation by IL-16. The absence of CCR5 had no effect on CXCL10-induced desensitization of CD4/IL-16-stimulated chemotaxis (Fig. 6B). This indicates that the mechanism for the CD4 effect on CXCR3 is different from the mechanism used by CXCR3 to desensitize CD4.

The data suggest that activation of CCR5 by IL-16/CD4 results in transmission of a regulatory signal that inhibits CXCR3 signaling. We next determined whether direct stimulation of CCR5 by CCL4 (monokine induced by IFN-1β) could also induce desensitization of CXCR3. Human T cells were pretreated with 50 ng/ml CCL4 for 30 min, then placed in a chemotaxis chamber and allowed to migrate toward various concentrations of CXCL10. As shown in Fig. 7, pretreatment with CCL4 inhibited CXCR3/CXCL10-induced chemotaxis. The time required for desensitization to occur was between 10 and 15 min, and the duration of desensitization was ∼8–9 h (data not shown). Taken together, these findings suggest that desensitization of CXCR3 can occur through stimulation of either CD4 or CCR5, and that CCR5 can act as an adaptor molecule for this process with CD4. It is likely that signaling through CCR5 is involved with induction of the desensitization effect, because ligation of CCR5 by Ab (incapable of inducing a migratory signal) had no effect on CXCL10 stimulation (213 ± 5% for CXCL10 alone and 206 ± 7% with anti-CCR5 pretreatment). Similar numbers were observed for CXCL9 stimulation (data not shown). Similarly, the addition of anti-CCR5 Abs, added simultaneously with or before IL-16 stimulation of primary T cells, did not alter the desensitization effect of IL-16 on CXCL10-induced migration (data not shown). These data again suggest that signaling by, and not just the presence of, CCR5 is required. Expression studies using truncated CCR5 protein lacking the capacity to signal should be conducted to confirm these findings and to identify regions on CCR5 responsible for transmission of the inhibitory signal.

**Effect of cholesterol depletion and reloading on receptor desensitization**

Receptor sequestration and signaling are mediated by cholesterol-rich lipid microdomains, lipid rafts. These domains can be disrupted by cholesterol chelation, using MCD. To determine whether disruption of the rafts was associated with altered CXCR3 desensitization, human T cells were pretreated with MCD (1 μM) for 30 min before IL-16 exposure. The cells were subjected to CXCL10-induced chemotaxis. As shown in Fig. 8A, MCD alone at this concentration did not significantly affect CXCL10-induced migration. T cells exposed to MCD before IL-16, however, did not demonstrate desensitization upon stimulation with CXCL10 (Fig. 8A). Because CD4-mediated inhibition of CXCR3 appears to be a two-step process, we next investigated the effects of MCD treatment on CXCR3 desensitization. As shown in Fig. 8B, MCD treatment disrupted CXCL4 desensitization of CXCR3/CXCL10-induced chemotaxis. To confirm this concept, cells that had been treated with MCD were reloaded with cholesterol or an oxidized form of cholesterol as previously described (27), and reconstitution of the inhibitory signal was investigated. As shown in Fig. 8C, the ability of CCL4 to desensitize CXCR3 was restored when cells were reloaded with cholesterol, but not with 4-choleren-3-one, the oxidized and inert form of cholesterol. MCD treatment did not disrupt IL-16/CXCL4-induced inhibition of CCR5 (data not shown).

Lipid rafts are capable of regulating receptor signaling by selective recruitment or exclusion from the raft of cell surface or intracellular molecules (30). To initially investigate the mechanism by which lipid rafts facilitate desensitization of CXCR3 by CCR5,
human T cells were again exposed to MCD (1 μM) for 30 min, then the presence of CCR5 and CXCR3 within the lipid raft component was assessed. As shown in Fig. 8D, before MCD exposure both receptors were detected within the lipid raft fractions after Triton X-100 solubilization, SDS-PAGE chromatography, and Western blotting. However, after exposure to MCD, CXCR3 was also detected in the nonraft fractions, suggesting that MCD treatment resulted in movement of CXCR3 out of the lipid raft. This effect was not detected for CCR5 (Fig. 8D); this suggests that MCD treatment results in spatial rearrangement of CXCR3 with CCR5. Taken together, these data indicate that the inhibitory effect of IL-16/CD4 on CXCR3 is dependent on the presence of CCR5, and that CCR5 requires intact cholesterol in the plasma membrane to facilitate the inhibitory signal.

Discussion
Regulation of T cell migration is a complex series of events that requires the proper interpretation of many simultaneous and sequential stimuli. The ability of chemokine receptors to either augment or desensitize each other is well established, although a complete understanding of which chemokine receptors have this ability and the mechanisms involved has not been clearly elucidated. We have previously reported that stimulation through CD4, a receptor outside the chemokine receptor family, results in desensitization of both CCR5- and CXCR4-induced migration. The mechanism for this effect is different for each receptor, because desensitization of CCR5 requires p56lck enzymatic activity, whereas inhibition of CXCR4 requires activation of PI3K through the SH3 domain of p56lck (9, 10). We have expanded those observations to include a novel dependence on the presence of CCR5 for CD4-mediated heterologous chemotactic receptor desensitization of CXCR3.

In this study we show that stimulation of CD4 by IL-16 results in receptor cross-desensitization of CXCR3. This effect not only appears to be selective for CXCR3, because we have previously demonstrated that other chemokine receptors, such as CXCR3 and CCR2, are not affected, but is also selective for certain ligands of CXCR3. IL-16/CD4 stimulation of human T cells resulted in desensitization of only CXCL9- and CXCL10-induced, not CXCL11-induced, migration. Signaling by chemokine receptors is complex and involves a number of domains related to ligand binding and intracellular signaling. This is particularly true for induction of chemotaxis. Colvin et al. (31) have identified at least two distinct intracellular domains that relate to transmission of the migratory signal. Interestingly, although the domains were consistent for all three ligands for induction of chemotaxis, CXCL11 induced receptor internalization via a different domain than CXCL9 and CXCL10. This suggests that CXCL11 interacts with and signals through CXCR3 differently than either CXCL9 or CXCL10. Our data support this finding and indicate that signaling after CXCL11 binding is not altered by prestimulation by IL-16/CD4. A distinction between CXCL11 and the other two ligands for induction of a calcium flux was also reported in myofibroblasts (32).

The effect on CXCR3 is reciprocal, because stimulation of CXCR3 by CXCL10 results in desensitization to IL-16-induced 4-cholesten-3-one, as previously described (27). The cells were then subjected to CXCL10-induced chemotaxis. The data for all experiments are expressed as a percentage of control migration. * Statistically different migration between IL-16-treated and untreated cells (p < 0.05). D, Lipid raft association of CCR5 and CXCR3 with and without MCD treatment. After sucrose density centrifugation, the fractions were run on SDS-PAGE, and Western blotting was performed using anti-CCR5 and anti-CXCR3 Abs. These data are representative of three separate experiments.
migration. Similar to the effects on CCR5 and CXCR4, IL-16 stimulation does not induce modulation of CXCR3, nor does it displace CXCL10 binding, indicating that the desensitizing effect on CXCR3 was mediated via intracellular signaling pathways. Indeed, the presence and enzymatic activity of the Src tyrosine kinase family member (p56\(^{lck}\)) is required, because desensitization was not detected after treatment with herbimycin A or using cells expressing p56\(^{lck}\) lacking the SH1 domain.

Both CCR5 and CXCR3 are members of the G protein-coupled receptors (GPCRs) family. Many studies have reported that prolonged activation through one GPCR results in heterologous desensitization of one or several other GPCRs (4–7). In general, this effect is thought to be mediated by the activation of either protein kinase A or protein kinase C (PKC). Activation of PKC occurs after the formation of diacylglycerol with the increase in intracellular calcium. Along those lines, we have reported that IL-16/CD4 stimulation in T cells results in the formation of diacylglycerol, an increase in intracellular calcium, as well as activation and translocation of PKC (33, 34). It is conceivable, therefore, that IL-16 stimulation through CD4 induces activation and translocation of PKC, resulting in heterologous desensitization of CCR5 and CXCR3. Studies to determine the relationship between CD4-induced activation and translocation of PKC with chemokine receptor desensitization have been initiated.

The CD4 signaling pathway required to desensitize CXCR3 is identical with that described for desensitizing CCR5 (9). In fact, desensitization of CXCR3 is dependent on the expression of CCR5, because there was no inhibitory effect of IL-16 on CCR5 in cells lacking CCR5. In addition, direct ligation of CCR5 by CCL4 desensitizes CXCR3. This represents a previously unidentified adaptor function for CCR5. The mechanism by which CCR5 transmits the IL-16-induced desensitizing signal has not as yet been identified, nor have we determined the underlying functional reason for the apparent interaction between CCR5 and CXCR3. However, the MCD and cholesterol repletion studies indicate that intact cholesterol is required for CCR5 to relay the regulatory signal and suggest the possibility that all three receptors are contained within the same lipid raft. The presence of CD4, CCR5, and CXCR3 in a common lipid raft microdomain could facilitate receptor cross-talk. Lipid rafts play an essential role in cell motility by asymmetrically partitioning molecules involved in migration at the uropod and leading edge (35). In addition, the cytoplasmic side of lipid rafts is associated with numerous signaling molecules that play an important role in the immunological synapse and as a direct link to cytoskeletal proteins involved in chemotaxis (35–37). Initial studies of MCD-treated cells indicated that CCR5 and CXCR3 are located primarily within lipid raft fractions after sucrose density centrifugation. MCD treatment results in the loss of CXCR3 from the lipid raft fraction, whereas CCR5 remains predominantly within the raft. This dissociation may account for the loss of receptor desensitization as the functional receptor complex is disrupted. Alternatively, Nygèn et al. (27) have demonstrated that MCD treatment of human T cells alters the structural integrity of CCR5 such that CCL4 can no longer bind. The concentration of MCD used for these studies was lower than those used to demonstrate loss of receptor binding. We did observe CCL4-induced migration after MCD treatment, indicating sufficient receptor integrity for that function; however, it is possible that other structural components of CCR5 are altered, thus preventing transmission of the desensitizing signal.

The transregulatory effects of CD4, CCR5, and CXCR3 have significant implications in disease states. The ligands for each of these receptors are produced in diseases predominated by Th1 cells, such as rheumatoid arthritis, inflammatory bowel disease, and type 1 diabetes (38–45). Proper recruitment and subsequent activation of these cells require a tightly regulated process by which the appropriate cells respond, and unwanted cells can be selectively excluded. Heterologous desensitization, in particular inhibition of multiple receptors induced by a single cytokine, would represent an effective and efficient mechanism to facilitate selective cell recruitment. It is likely that other members of the chemokine receptor family are also affected by the CD4-CCR5 receptor complex, and additional investigation is required to fully understand the capacity of this complex to regulate T cell motility and activation.

Disclosures
The authors have no financial conflict of interest.

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