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CUTTING EDGE

Cutting Edge: IL-16/CD4 Preferentially Induces Th1 Cell Migration: Requirement of CCR5[1]

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IL-16 binds to CD4 and induces a migratory response in CD4$^+$ T cells. Although it has been assumed that CD4 is the sole receptor and that IL-16 induces a comparable migratory response in all CD4$^+$ T cells, this has not been investigated. In this study, we determined that IL-16 preferentially induces a migratory response in Th1 cells. Because chemokine receptor CCR5 is expressed predominantly in Th1 cells and is physically associated with CD4, we investigated whether IL-16/CD4 stimulation was enhanced in the presence of CCR5. Using T cells from CCR5^{null} mice, we determined that IL-16-induced migration was significantly greater in the presence of CCR5. The presence of CCR5 significantly increased IL-16 binding vs CD4 alone; however, IL-16 could not bind to CCR5 alone. Because CD4$^+$ CCR5$^+$ cells are prevalent at sites of inflammation, this intimate functional relationship likely plays a pivotal role for the recruitment and activation of Th1 cells. The Journal of Immunology,* 2003, 171: 4965–4968.

Interleukin-16 was originally identified as a T cell chemoattractant with specificity for CD4$^+$ T cells (1, 2). Subsequent studies identified that IL-16 was a chemoattractant for a number of other immune cells such as monocytes, eosinophils, and dendritic cells (3–5). IL-16 has been characterized as a natural ligand for CD4 (6, 7), given that direct association with the D4 domain of CD4 results in activation of p56^{lck} and phosphatidylinositol 3-kinase (PI$_3$ kinase)[3] (8). Recent studies have suggested that IL-16 may signal through an alternative receptor in cells from the monocytoid lineage, as CD4^{null} epidermal dendritic cells and monocytes obtained from CD4^{null} mice are responsive to IL-16 (9, 10). In T cells, however, the data indicate a requirement of CD4 expression for induction of a cellular response to IL-16 stimulation (3, 11, 12). Although CD4 is required, it has not been established whether all CD4$^+$ T cells are responsive to IL-16 stimulation or whether there is a preferential subset effect. CD4 is expressed on primary T cells in a homogeneous fashion with no definable subsets; however, stimulation by IL-16 results in a migratory response in only 35–50% of the cells (our unpublished observations). Therefore, it is possible that a subset of responsive cells exists defined by a coreceptor, which facilitates maximal migratory effect to IL-16.

A membrane receptor with a high probability of functioning as a coreceptor for IL-16/CD4 signaling is chemokine receptor 5, CCR5. The association of CCR5 with CD4 was first identified in the context of HIV-1 binding and internalization (13, 14). Kornfeld et al. (15) reported that HIV-1 gp120-induced migration occurred as a result of CD4 signaling, whereas Weissman et al. (16) later identified that gp120-induced migration could also occur as a result of signaling through CCR5. It was later determined that CCR5 physically associates with CD4 (17). With this background and with our previous observation that IL-16 stimulation results in receptor cross-desensitization of CCR5 (18), we investigated whether the presence of CCR5 contributed to IL-16 stimulation.

Our studies demonstrate that IL-16 induces a greater migratory response in the Th1 subset as than in the Th2 subset. Although expression of CD4 is required, Th1 subset specificity is attributable to an increase in IL-16 binding and signaling facilitated by the presence of CCR5. Augmentation of IL-16 stimulation by CCR5 identifies an intimate functional relationship between CD4 and CCR5 that likely plays a role in regulation of Th1 cell recruitment and activation at sites of inflammation.

Materials and Methods

Animals and cells

Th1 and Th2 cells were generated from DO11.10 mice (The Jackson Laboratory, Bar Harbor, ME), which are transgenic for the TCR recognizing the OVA peptide p$_{323–339}$ (pOVA$_{323–339}$; Ref. 19). CCR5$^{-/-}$ null mice (B6;129P2-*Cmkbr5*^{tm1Kuz}), CD4$^{-/-}$ null mice (B6.129S2-*Cd4*^{tm1Mak}), and CCR5 and CD4 strain controls (B6129PF2/J and C57BL/6J, respectively) were also obtained from The Jackson Laboratory. All animals were used between 8 and 12 wk of age. The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health: HOS-CD4, HOS-CCR5, HOS-CCR3, HOS-CD4-CCR5, and HOS-CD4-CCR3 cell lines from Dr. N. Landau.

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[3] Abbreviations used in this paper: PI$_3$ kinase, phosphatidylinositol 3-kinase; pOVA, OVA peptide; MIP-1β, macrophage-inflammatory protein-1β; SDF-1α, stromal cell-derived factor 1α; IP-10, IFN-γ-inducible protein-10; wt, wild type; PT, pertussis toxin.

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Generation of Th1 and Th2 cell lines

DO11.10 $CD4^+$ T cells were isolated from lymph nodes by a density gradient centrifugation-positive cell selection technique (Stem Cell Technologies, Vancouver, Canada). $CD4^+$ T cell purity was assessed by flow cytometric analysis. APCs were generated from syngeneic T cell-depleted splenocytes prepared by positive selection using a magnetic bead pan-T cell Ab to Thy-1.2 (Dynal, Lake Success, NY). Ag-presenting splenocytes were treated with mitomycin C (50 μg/ml) (Sigma-Aldrich, St. Louis, MO) for 20 min on ice in the dark and washed twice with PBS. Th1 and Th2 cell cultures were generated, as previously described (20), by adding 2×10^6 $CD4^+$ T cells/ml with 2×10^6 APCs/ml and $pOVA_{323–339}$, 1 μg/ml (Invitrogen, Grand Island, NY). Th1 or Th2 cell skewing mixture was then added to the cell cultures. Th1 cell mixture included IL-12 (5 ng/ml) and anti-IL-4 (2.5 μg/ml) (R&D Systems, Minneapolis, MN). Th2 cell mixture included IL-4 (5 ng/ml), anti-IL-12 (10 μg/ml) (R&D Systems, Minneapolis, MN), and anti-IFN-γ (2.5 μg/ml) (Sigma-Aldrich).

Cell culture

Th1 and Th2 murine lymphocytes were maintained in complete medium (RPMI 1640 from Sigma-Aldrich, containing 200 U/ml penicillin, 200 μg/ml streptomycin, 2 mM glutamine, 20 mM HEPES, and 10% FBS). Th1 and Th2 cells cultures were propagated after initial skewing in 24-well plates with complete medium and recombinant murine IL-2 at 50 ng/ml (Sigma-Aldrich) every 24 h. Primary Th1 and Th2 cultures were harvested after 5-7 days, washed with PBS and stored at −80°C in 5% DMSO. These T cell subsets were then thawed and secondarily propagated in complete medium containing mitomycin C-treated splenocytes (2×10^6 cells/ml) pulsed with pOVA (1 ng/ml). Th1 and Th2 cells were propagated with complete medium and recombinant murine IL-2 (50 ng/ml) for 5–7 days until the resting phase. Resting secondary Th1 and Th2 cells (2×10^6 cells/ml) were stimulated for 24 h at 37°C with plate-bound anti-TCR Ab (10 μg/ml) (BD PharMingen, San Diego, CA), before use for migration. Functional Th1 and Th2 cell purity was confirmed by cytokine production and by the migration studies. The HOS cell lines were grown in DMEM with 10% FBS with 1.0 μg/ml puromycin in all cultures except for the HOS-CD4 cultures.

Lymphocyte chemotaxis assays

In vitro chemotaxis assays were performed as previously described (7, 21). Cell migration was assessed by using a 48-well microchemotaxis chamber separated by a nitrocellulose membrane with an 8-μm pore size (Neuroprobe, Cabin John, MD). Isolated Th1, Th2, $CCR5^{-/-}$, $CD4^{-/-}$, or wild-type (wt) control T lymphocytes (5×10^6/ml) were stimulated by various concentrations of IL-16, macrophage-inflammatory protein-1β (MIP-1β), stromal cell-derived factor 1α (SDF-1α), IFN-γ-inducible protein-10 (IP-10), and RANTES (R&D Systems) or control buffer. The chamber was incubated at 37°C for 4 h, after which the filters were fixed in ethanol and stained with hematoxylin. Light microscopy was used to quantify the number of cells that migrated beyond 60 μm. Under basal conditions, 15–20 cells/high power field were routinely counted. The Student *t* test was used for statistical analysis.

IL-16 binding

Binding of ^{125}I-IL-16 was conducted on HOS cells transfected to express CD4, CCR5, CCR3, or a combination of CD4 with either CCR5 or CCR3. Binding assays were conducted by stimulating 3×10^6 cells in 100 μl of culture medium with varying concentrations of ^{125}I-IL-16 (1–600 pM) for 120 min at 4°C. The samples were then aspirated through GF/C microfiber filters (Whatman, Maidstone, U.K.) using a vacuum harvester. The filters were air dried and counted in a gamma counter. To determine specific binding, 100-fold excess of unlabeled IL-16 was added for each condition. Residual counts were subtracted from total bound counts to yield specific binding. An approximate K_D was calculated based on half-maximal binding, where maximal specific binding was achieved after addition of 455 pM IL-16.

Flow cytometric analysis and ELISA

CD4, Thy-1.2, and CCR5 expression were analyzed using PE or FITC fluorescently conjugated Abs (Sigma-Aldrich). Cells were analyzed with a FACScan (BD Biosciences, San Jose, CA). Th1 and Th2 cell culture supernatants were analyzed for IFN-γ and IL-4 with ELISA kits purchased from BioSource International (Camarillo, CA). The Student *t* test was used for statistical analysis.

Results and Discussion

Effect of IL-16 on Th1 and Th2 subsets

To address whether IL-16 has a preferential effect on a subset of T cells, we conducted migration assays on murine $CD4^+$ Th1 and Th2 cell lines. Th1 and Th2 cells at 5×10^6/ml were stimulated with MIP-1β (50 ng/ml), SDF-1α (50 ng/ml) or IL-16 (10^{-10} M) for 4 h. There was a small but consistent difference in baseline migration as Th1 cells averaged 15 cells/high power field, vs 20 cells for the Th2 population, at a depth of 60 μm. As shown in Fig. 1*A,* IL-16-stimulated Th2 cells demonstrated a migratory response that was significantly different from control cell migration; however, the response in Th1 cells was ~80% greater than migration of Th2 cells (221 ± 25% vs 145 ± 19%). The functional purity of the subsets was demonstrated by a lack of a response to MIP-1β and SDF-1α by Th2 and Th1 cells, respectively. There was no subset specificity for RANTES stimulation. These data identify for the first time a selective effect of IL-16 on a subset of $CD4^+$ T cells.

A dose response to IL-16 indicated that peak migratory responses were achieved at 10^{-11} M for Th1 cells, whereas Th2 cells required a log higher concentration (Fig. 1*B*). Increasing the concentration of IL-16 could not compensate for the decreased migratory response of the Th2 cells. To determine whether the preferential response by Th1 cells could be explained by differences in CD4 expression, the cells were assessed by FACS. FACS revealed comparable CD4 receptor expression for both subsets (~97% for each) (Fig. 1*C*). This finding suggested that CD4 alone could not account for the difference in responsiveness and raised the possibility of either an alternative receptor or the existence of a functional coreceptor for CD4 present on Th1 cells.

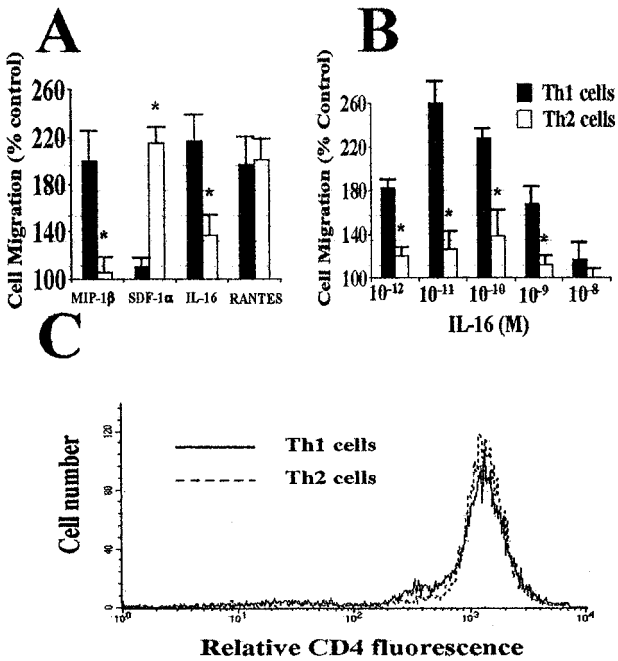

FIGURE 1. Subset specificity for IL-16-induced migration. *A,* Murine $CD4^+$ Th1 and Th2 cell migration in response to specific chemokine stimulation. Th1 (■) or Th2 (□) cells were stimulated with MIP-1β (50 ng/ml), SDF-1α (50 ng/ml), IL-16 (10^{-10} M), or RANTES (10 ng/ml) for 4 h. Results are expressed as a percent of control cell migration, designated as 100%. *B,* A dose-dependent effect of IL-16-induced migration of murine $CD4^+$ Th1 (■) vs Th2 (□) cells. Data for both *A* and *B* represent the averages ± SD from four separate experiments. *, Significant difference in cell migration of Th1 compared with Th2 cells, $p < 0.05$. *C,* Flow cytometric analysis of Th1 and Th2 cells for $CD4^+$ cell expression.

Effect of IL-16 on CD4^{null} cell migration

Several reports have identified cellular responsiveness to IL-16 in CD4^{null} cells (9, 10, 22). It is possible therefore that in Th1 cells IL-16 binds to an alternative receptor that augments the migratory response. To determine whether CD4 expression is required for IL-16 signaling and whether an alternative receptor can function in the absence of CD4, we studied IL-16 dose-dependent migration in CD4^{null} vs strain control T cells. A dose response for IL-16 stimulation resulted in a migratory response peaking at 10^{-11}M in wt cells (Fig. 2*A*). There was no detectable migration in this dose range of IL-16 from the CD4^{null} cells. In fact, IL-16 concentrations up to 10^{-6} M did not induce a migratory response in the CD4$^{-/-}$ mouse (data not shown). These findings indicate that for T cells, CD4 expression is required for an IL-16-induced migratory signal. These studies do not eliminate the possibility of a coreceptor that can augment IL-16/CD4 signaling but alone is insufficient to transmit a migratory signal. Such a mechanism has been reported for HIV-1 gp120 binding (16). We therefore investigated whether CCR5 was contributing to IL-16-induced migration for the following reasons: 1) CCR5 has been identified as a major coreceptor for HIV-1 binding (13, 14); 2) CCR5 has been shown to physically associate with CD4 (23); 3) CCR5 is present predominantly on Th1 cells (24, 25); and 4) there is reciprocal cross-desensitization between CD4 and CCR5 (18).

IL-16-induced migration of, and binding to, CCR5$^{-/-}$ cells

To address this, cells were isolated from CCR5^{null} or strain control (wt) mice, and chemotaxis was conducted using IL-16, MIP-1β, or IP-10. The absence of CCR5 resulted in a decrease in the migratory response from 202 ± 12% in cells from wt mice to 160 ± 6% in cells from CCR5 null mice (Fig. 2*B*). As expected, cells from the CCR5^{null} mice failed to migrate to MIP-1β. There was no significant difference, compared with wt cells, in migration to IP-10, indicating that CCR5^{null} cells could respond equally when stimulated through another receptor. An IL-16 dose response in CCR5^{null} cells indicated peak migration at 10^{-10} M compared with 10^{-11}M for the wt strain (Fig. 2*C*). This is similar to the differential responses observed in Th1 vs Th2 cells, shown in Fig. 1*B*. The addition of neutralizing Abs to CCR5 ligands MIP-1β, MIP-1α, and RANTES in the chemotaxis assay did not alter the responses (data not shown), indicating that IL-16 does not induce these factors, which could secondarily influence CCR5^{+} cell migration.

To augment IL-16-induced migration, it is conceivable that CCR5 influences IL-16 binding and/or IL-16 signaling, similar to CD4's influence on MIP-1β binding and signaling through

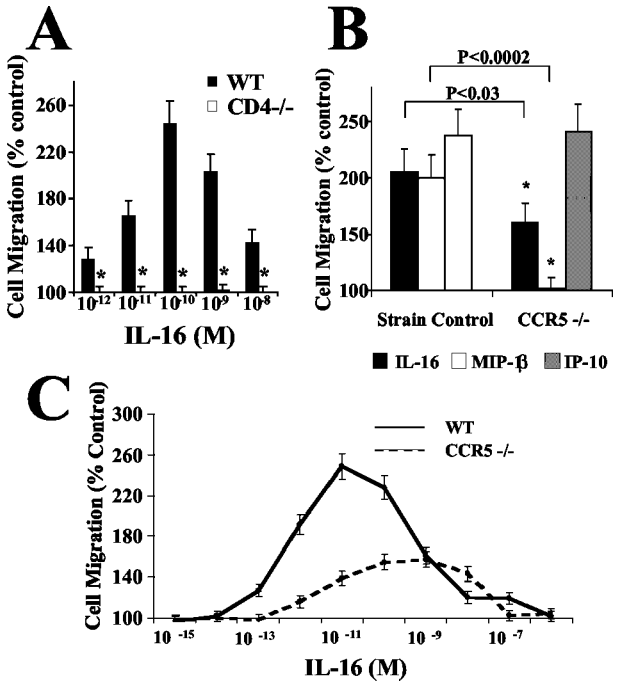

FIGURE 2. Contribution of CCR5 on IL-16-induced migration. *A,* Dose-dependent effect of IL-16-induced migration on CD4^{null} T cells vs strain control. T lymphocytes from either CD4^{null} (□) or strain control (■) mice were stimulated with IL-16. Data are the averages ± SD from three separate experiments. *, Significant difference in cell migration between the two groups, $p < 0.05$. *B,* Migratory response of CCR5^{null} or strain control T cells to chemokine stimulation. T cells from either CCR5^{null} or strain control mice were stimulated with IL-16 (10^{-9} M) (■), MIP-1β (50 ng/ml; □), or IP-10 (50 ng/ml) (▨). Data represent the averages ± SD from three separate experiments. *, Significant difference in cell migration as designated. *C,* Dose-dependent effect of IL-16-induced migration of CCR5^{null} T cells vs strain control. T cells from CCR5^{null} (– – – –) or strain control (——) mice were stimulated with IL-16 (10^{-15}–10^{-6} M). Data are the averages ± SD from three separate experiments. *, Significant difference in cell migration of CD4^{null} T cells compared with strain control T cells.

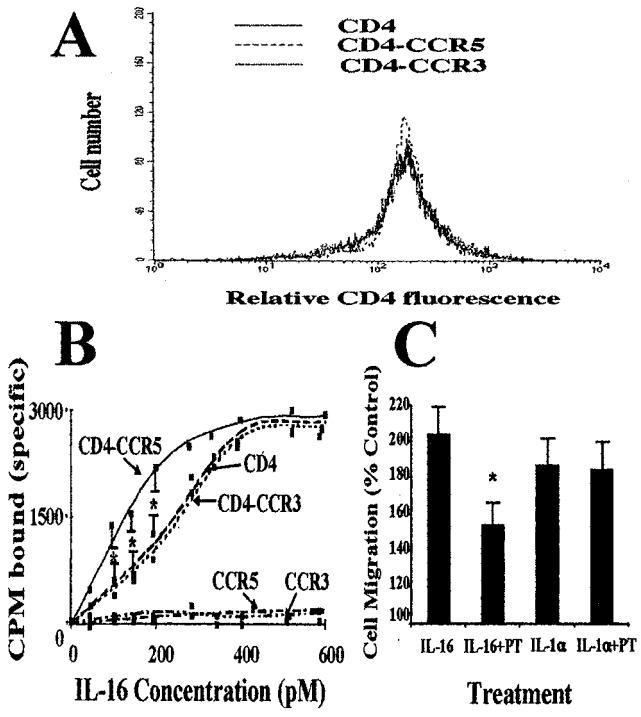

FIGURE 3. Contribution of CCR5 to IL-16 binding and signaling. *A,* HOS cells expressing CD4, CD4-CCR5, or CD4-CCR3 were labeled with fluorescently conjugated anti-CD4 Ab and assessed for CD4 surface expression. *B,* HOS cells (3×10^6) expressing CD4, CCR5, or CCR3 alone or coexpressing CD4-CCR5 or CD4-CCR3 were exposed to varying concentrations of radiolabeled IL-16 (1–600 pM) for 120 min at 4°C. For each condition, bound counts were obtained in the absence and presence of a 100-fold excess of unlabeled IL-16. Data represent average counts from three separate experiments; *, Significantly different counts between cells expressing CD4 with cells expressing CD4-CCR5, at $p < 0.05$. *C,* Effects of PT (100 ng/ml for 10 h) on IL-16-induced migration. T cells were stimulated with IL-16 (10^{-10} M) or IL-1α (1 U/ml). Data are the averages ± SD from three separate experiments. *, Significant difference in T cell migration, $p < 0.05$.

CCR5 (26). To determine whether CCR5 could affect IL-16 binding, HOS cells transfected to express human CD4, CCR5 or CCR3 alone, or cotransfected to express CD4 and CCR5 (CD4-CCR5) or CCR3 (CD4-CCR3) were used. All these cell lines were determined to have comparable levels of CD4 (Fig. 3*A*). Expression of CCR3 was chosen as a control, given that previous reports had not identified any functional association between CD4 and CCR3 (27). Radiolabeled IL-16 was bound to the cells for 2 h before harvesting and counting. IL-16 did not demonstrate any binding to the parental cells (data not shown) or to cells expressing either CCR5 or CCR3 alone (Fig. 3*B*). As expected, IL-16 did bind to cells expressing CD4 alone (Fig. 3*B*), with an apparent K_D of 291 pM. IL-16 binding was increased in cells expressing both CD4 and CCR5, with an apparent K_D of 93 pM. CD4 expression was comparable for both cell lines, which suggests that the presence of CCR5 alters CD4 binding affinity for IL-16. The affinity was not altered in cells expressing CD4-CCR3 (Fig. 3*B*). Although the mechanism by which CCR5 increases IL-16 binding has yet to be determined, IL-16 stimulation for 2 h did not alter CCR5 expression in the cell line (data not shown). It is possible that IL-16 binds in a manner similar to that of gp120 whereby association with CD4 structurally alters CCR5, allowing for direct binding to CCR5. Alternatively, the presence of CCR5 associated with CD4 may structurally alter CD4, thus allowing for stronger interaction of IL-16 with the D4 domain of CD4 (6). The ability of CCR5 to increase IL-16 binding may also result in signal transduction mediated by CCR5. To initially investigate this possibility, mixed T cells were incubated in the presence of a Giα-signaling antagonist, pertussis toxin (PT), before IL-16 induced migration. As shown in Fig. 3*C*, cells incubated with PT demonstrated ~40% decrease in responsiveness to IL-16. There was no loss of migration to IL-1α stimulation, indicating a specific inhibitory effect. These data do not definitively identify CCR5 as the signaling G-protein receptor but, combined with the binding data, strongly suggests its involvement.

It is now becoming clear that the functions of CD4 and CCR5 are intimately connected and reciprocally regulated. The presence of CD4 influences MIP-1β binding, and costimulation with IL-16 results in increased MIP-1β-induced phosphorylation of CCR5 (26). We now report that the presence of CCR5 enhances IL-16 binding and induced migration. Preliminary data indicate that IL-16 can induce signaling through CCR5; however, the lack of CCR5 phosphorylation following IL-16 stimulation (26) suggests activation of an alternative pathway. This functional relationship may also explain in part the preferential efficiency of infection by HIV-1 for $CD4^+CCR5^+$ cells, whereby binding, internalization, and signaling are readily accomplished by this receptor complex. We are currently conducting studies to address the direct effect of IL-16 signaling through CCR5. This functional relationship appears to be based on the physical interaction between the two receptors and suggests that they function much like a heterodimeric complex. Because CCR5 has multiple chemokine ligands and CD4 functions as a coreceptor for TCR signaling, this functional association between the two receptors likely plays a key role in augmenting selective recruitment and activation of Th1 cell at sites of inflammation.

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