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Expression of CXCR4 in Eosinophils: Functional Analyses and Cytokine-Mediated Regulation

Hiroyuki Nagase,* Misato Miyamasu,† Masao Yamaguchi,‡ Takao Fujisawa,‡ Ken Ohta,§ Kazuhiko Yamamoto,† Yutaka Morita,* and Koichi Hirai2*‡

We examined the expression of transcripts of a panel of chemokine receptors in human eosinophils and found intense constitutive expression of CXCR4 mRNA. Although surface CXCR4 protein was hardly detectable in the peripheral blood or freshly isolated eosinophils, surface expression of CXCR4 became gradually apparent during incubation at 37°C. In contrast, the level of CCR3 expression was virtually unchanged during the incubation. Stromal cell-derived factor-1α (SDF-1α), the natural ligand of CXCR4, elicited an apparent Ca²⁺ influx in these cells and induced a strong migratory response comparable to that by eotaxin. The surface expression of CXCR4 in eosinophils was up-regulated by IFN-γ, TNF-α, and TGF-β while it was down-regulated by IL-4 and eosinophil-directed hemopoietins such as IL-5. The CXCR4 expression did not always parallel the apoptotic changes in cytokine-treated eosinophils. In contrast to IL-4 and IFN-γ, IL-5 potently reduced the level of CXCR4 mRNA. It seems unlikely that CXCR4 is fundamentally involved in the pathogenesis of allergic disorders by inducing the migration of eosinophils toward inflammatory sites, because a Th2-dominant state down-regulates eosinophil CXCR4 expression. However, CXCR4 may affect the size of the mobilizable pool by holding eosinophils at noninflamed tissues. Th2-dominant state may favor the liberation of eosinophils by down-regulating CXCR4 expression. The interplay between CXCR4 and SDF-1α in eosinophils potentially plays an important role in the accumulation of these cells at the allergic inflammatory sites. The Journal of Immunology, 2000, 164: 5935–5943.

M assive accumulation of eosinophils is a characteristic aspect of inflammation associated with allergic diseases. Historically, eosinophils had long been assumed to play an antiinflammatory role in allergic diseases by virtue of their antagonizing effects on mast cell-derived mediators such as histamine. However, it has become apparent that the eosinophils involved in these conditions are highly destructive: eosinophil-derived mediators, especially various cationic proteins, contribute to the tissue damage associated with allergic diseases. Along with the progression of allergic reactions, eosinophils migrate from the blood compartment to inflamed tissues and function as allergic inflammatory cells (1). The processes involved in tissue eosinophilia consist of a complex interplay of various pathways and are not fully understood. However, several chemoattractants generated at inflammatory sites potentially play a pivotal role in the recruitment of eosinophils in humans as well as in animals (2).

Chemotactic cytokines, termed chemokines, are now recognized as essential participants in the sequence of events by which circulating leukocytes migrate toward inflammatory sites. Chemokines are divided into two major subfamilies based on the sequence of cysteine groups: the CXC subfamily and the CC subfamily. Two minor subfamilies, i.e., the CX3C and the C chemokines, are also categorized. To date, ~40 chemokines have been identified, and 15 chemokine receptors, i.e., five CXC chemokine receptors (CXCRs), eight CCRs, one CX3CR, and one XCR, have been cloned. It has been reported that eosinophils express CXCR3, and ligands of CCR3 such as eotaxin induce strong migration of eosinophils (3, 4). Expression of CCCR1 (5), and under certain circumstances expression of CCR2 (6), have also been reported in eosinophils. The expression and function of these receptors have been extensively investigated. In contrast, there is little information regarding the expression and function of other chemokine receptors in eosinophils.

In this study, we have examined the expression of transcripts of a panel of chemokine receptors in human eosinophils and found intense constitutive expression of CXCR4 mRNA as well as CCR3 mRNA. Here, we demonstrate that surface expression of CXCR4 is inducible in eosinophils and that stromal cell-derived factor-1α (SDF-1α)3 elicits strong eosinophil migration comparable to that induced by eotaxin. The effects of cytokines on eosinophil CXCR4 expression have also been investigated.

Materials and Methods

Reagents and mAbs

The following reagents were purchased as indicated: recombinant human SDF-1α, eotaxin, IL-4, and TGF-β1 (PeproTech, London, U.K.); IFN-γ (Shionogi Pharmaceutical, Osaka, Japan); TNF-α (Dainippon Pharmaceutical, Osaka, Japan); C5a, cytochalasin B, and cycloheximide (Sigma, St. Louis, MO); ionomycin (Seikagaku, Tokyo, Japan); pertussis toxin (PTX) (Calbiochem-Behring, La Jolla, CA); anti-Fas mAb (CH-11, subclass IgM)

3 Abbreviations used in this paper: SDF-1α, stromal cell-derived factor-1α; PTX, pertussis toxin; MESF, molecules of equivalent soluble fluorochrome units; EPO, eosinophil peroxidase; OPEI, o-phenylenediamine dihydrochloride; EDN, eosinophil-derived neurotoxin.
and mouse IgMα1 with irrelevant specificity (Medical and Biological Laboratories, Nagoya, Japan and Organon Teknika, West Chester, PA, respectively). Anti-CCR3 mAb (7) was provided by Dr. H. Kawasaki (Institute of Medical Science, University of Tokyo, Tokyo, Japan). IL-3 and IL-5 were kindly donated by Kirin Brewery (Tokyo, Japan) and Suntory (Osaka, Japan), respectively. Anti-CCR3 mAb (7) was provided by Dr. H. Kawasaki (Institute of Medical Science, University of Tokyo, Tokyo, Japan). IL-3 and IL-5 were kindly donated by Kirin Brewery (Tokyo, Japan) and Suntory (Osaka, Japan), respectively.

**Eosinophil separation and culture conditions**

Eosinophils were separated from normal volunteers who had no history of allergy, as previously described (8). In brief, buffy coat cells were obtained from venous blood by dextran T500 sedimentation. Eosinophils were isolated by Percoll (1.088 g/ml; Pharmacia, Uppsala, Sweden) density centrifugation. Unless Percoll separation achieved purity of 90%, the eosinophils were further purified by negative selection using anti-CD16-bound micromagnetic beads (Miltenyi BioTech, Bergisch-Gladbach, Germany) and a magnetic-activated cell sorter column (Miltenyi BioTech) as the second step (9). After this negative selection, the mean eosinophil purity was 99%, and the viability was consistently >95%. Eosinophils (0.5–1.0×10⁸) were cultured in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% FCS (Life Technologies) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37°C in 5% CO₂ for 45 min. During flow cytometry procedures, contaminating neutrophils were discriminated on the basis of their different fluorescence properties.

**RT-PCR analysis of chemokine receptors**

RT-PCR analysis of eosinophil mRNA was performed as previously described (10). In brief, total RNA was extracted from 10⁶ eosinophils with a SNAP Total RNA Isolation kit (Invitrogen, Leek, The Netherlands). After precipitation with ethanol, the first-strand cDNA was reverse transcribed. Second-strand DNA synthesis and hot-start amplification were performed using a Takara Thermal Cycler MP (Takara, Ohtsu, Japan) under oil-free conditions. Amplification of cDNA was performed as previously described using AmpliTaq Gold DNA polymerase (Perkin-Elmer, Branchburg, NJ). Chemokine receptor cDNA was amplified by PCR with primers specific for each receptor. The direct and reverse oligo primers used and the expected product sizes are shown in Table I. The PCR conditions included 9 min of preheating at 95°C followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, elongation at 72°C for 30 s, and final elongation at 72°C for 10 min. PCR products were electrophoresed through a 2% agarose gel and visualized with ethidium bromide. To semiquantify CXCR4 mRNA, visualized bands were quantified using BIO-PROFIL densitometry (Vilber Lourmat, Marne La Vallée, France), and the density of CXCR4 mRNA was compared with that of β-actin mRNA.

**Flow cytometry of isolated eosinophils**

Isolated eosinophils were washed in PBS supplemented with 3% FCS and 0.1% NaCl, and then incubated with anti-CXCR4 mAb (12G5; Pharmingen, San Diego, CA) at 10 μg/ml for 60 min at 4°C. An isotype-matched mouse IgG2α with irrelevant specificity (UPC 10; Sigma) was used as a negative control. After washing, the cells were stained with FITC-labeled goat F(ab)₂ against mouse IgG (Jackson ImmunoResearch, West Grove, PA) at 7 μg/ml for 30 min at 4°C. During flow cytometry procedures, contaminating neutrophils were discriminated on the basis of their different fluorescence properties.

**Flow cytometry of eosinophils in whole blood**

FACS analysis of eosinophils in whole venous blood was performed as previously described (11). In brief, blood was anti-coagulated with EDTA, and an equal volume of FACS buffer (PBS with 3% FCS and 0.1% NaN₃) was added. Cells were stained with anti-CXCR4-FITC (12G5; R&D Systems, Minneapolis, MN) and anti-CD16-PE (Coulter) at 10 μg/ml for 45 min at 4°C. An isotype-matched mouse IgG2α-FITC (R&D Systems) and

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Table I. Primers for PCR amplification

| Receptor | Sense/Antisense Sequence (5’ to 3’ | bp | Reference |
|----------|-----------------------------------|----|-----------|
| CCR1     | Sense TGG AAA CTC CAA ACA CCA CAG  | 297| Originally constructed |
|          | Antisense CCC AGT CAT CCT TCA ACT TG |    |           |
| CCR2     | Sense CTG TCC ACA TCT CGT CGG TTT A  | 324| Originally constructed |
|          | Antisense CCC AAA GAC CCA CTC ATT TGC AGC |    |           |
| CCR3     | Sense CTG CTA CAC AGG AAT CAT CAA AAC  | 366| Originally constructed |
|          | Antisense GTT CTT TCG AGT TCA GGA AGG |    |           |
| CCR4     | Sense TAC TGG GTG GCC TTT TAC AG  | 382| Originally constructed |
|          | Antisense TCA TCT TCA CCG CCT TGT TC |    |           |
| CCR5     | Sense AAT AAT TGC AGT AGC TCT AAG AGG  | 272| Originally constructed |
|          | Antisense TGG ATG CCG TAT CAC CCC |    |           |
| CCR6     | Sense GAG CCC ATC AGG TGG AAG CTT CTG  | 316| Originally constructed |
|          | Antisense GGC AGC AGT GCA GGA AAG CCA GGA C |    |           |
| CCR7     | Sense TCC TTC TCA TCA AGC GTC TGT C  | 530| Originally constructed |
|          | Antisense GAG GCC GCC CAG GTC GTT GAG G |    |           |
| CCR8     | Sense GTG GTG TCT GTC TTT TAT TAC ATT GG  | 495| Originally constructed |
|          | Antisense ACA TCC ATC CAA GAT GTG CA |    |           |
| CXCR1    | Sense CAG ATC CAC AGA TGT GGG T  | 468| Originally constructed |
|          | Antisense AGC AGCCAA GAA CAC ACT T |    |           |
| CXCR2    | Sense CTT TTC TAC TAG ATG CCG C  | 417| Originally constructed |
|          | Antisense AGA TGC TGA GAC ATA GAG AT T |    |           |
| CXCR3    | Sense TTC TGG AGG TGG GTG ACC ACA AA  | 584| Originally constructed |
|          | Antisense CTC GTT GTG GTG GGC CGA CAG |    |           |
| CXCR4    | Sense AGT TGT CGG AAA AGG TGG TCT ATG  | 260| Originally constructed |
|          | Antisense GGC TTT CTG GTG GCC CTT GGA GTG TG |    |           |
| CXCR5    | Sense AAG TTC ATC TCG ACA TCT CTG CT  | 347| Originally constructed |
|          | Antisense TCT TAG TGG AAA TAT CAG CAT CAG |    |           |
| CXCR1    | Sense TCC AGG TGG AGT CCT CAG GCA AAC  | 531| Originally constructed |
|          | Antisense CAC AGC CCG AAG AAA GCA CCT T |    |           |
| CX,CR    | Sense GTA GTG TTT GCC CTC ACC AAC A  | 502| Originally constructed |
|          | Antisense ACA GCG TCT GGA TGA TTC TGA A |    |           |

* The direct and reverse oligo primers for chemokine receptor sequences and their expected bp size are shown.
mouse IgG1-PE (Immunotech, Marseille, France) with irrelevant specific-
ity were used as negative controls. Contaminating erythrocytes were eli-
mated with a lysis buffer (Ortho Diagnostic Systems, Tokyo, Japan). Gran-
ulocytes were discriminated on the basis of different forward/side scatter
properties, and electronic gates were set on CD16-negative cells to identify
eosinophils.

Measurement of intracellular calcium concentration
Purified eosinophils (purity, >99%) were resuspended in HBSS with Ca2+
and Mg2+ (Life Technologies) and 2% BSA at a cell density of 2.0 ×
106/ml. Fura-2 AM (Dojindo, Tokyo, Japan) was added at a final con-
centration of 2 μM. After incubation for 20 min, excess dye was removed by
centrifugation, and the cells were resuspended in a buffer containing 119
mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 0.03% human serum albumin,
and 25 mM PIPES, pH 7.4, at a concentration of 1.6 × 106
cell/ml. Ca2+ influx was measured using excitation at 340 and 380 nm on a
Hitachi F-2500 fluorescence spectrometer (Hitachi, Tokyo, Japan). Cal-
ibration was performed using 0.1% Triton X-100 for total Ca2+ release and
10 mM EGTA to chelate free Ca2+.

Chemotaxis assay of eosinophils
Eosinophil migration was measured using a 96-well multiwell Boyden
chamber (Neuroprobe, Bethesda, MD) and a 10-μm-thick polycarbonate
lidone-free polycarbonate membrane filter with pores of 5 μm in diameter
(Neuroprobe), as previously described (8). Aliquots of 362 μl of triplicate
samples were transferred into the lower wells, while 200 μl of a cell sus-
pension that contained 1.5 × 104 eosinophils was introduced into each well
of the top compartment. After incubation for 90 min, the eosinophil per-
oxidase (EPO) activity of cells at the bottom of wells was determined with
200 μl of 0.05 M Tris-HCl, pH 8.0, containing 0.1% (v/v) Triton X-100,
0.1 mM o-phenylenediamine dihydrochloride (OPD; Sigma) and 50 mM
hydrogen peroxide. The OD was read at 490/570 nm in an ELISA reader
(Model 550; Bio-Rad, Hercules, CA). Data were analyzed with the Micro-
plate Manager III program (Bio-Rad), and the numbers of migrated eosin-
ophils were calculated based on a standard curve established with varying
known numbers of eosinophils.

Eosinophil degranulation
Freshly isolated eosinophils (purity, >99%) were cultured for 24 h with or
without IFN-γ (10 ng/ml). These cells were pretreated with 5 μg/ml of
cytoclasin B for 5 min and then stimulated with SDF-1α (333 ng/ml) or
C5a (5 × 10−9 M) for 5 h. The level of eosinophil-derived neurotoxin
(EDN) was measured with an EDN ELISA kit (Medical and Biological
Laboratories).

Analysis of apoptotic cells
Differential analysis of apoptotic and necrotic cells was performed using a
MEBCYTO apoptosis kit (Medical and Biological Laboratories). Apop-
totic cells were quantitatively determined by their ability to bind annexin V
and exclude propidium iodide. Cells stained with propidium iodide were
determined by their ability to bind annexin V.

Results
Expression of CXCR4 in eosinophils
Eosinophils were highly purified by means of Percoll density gradient
centrifugation followed by negative selection using anti-CD16 microbeads
(purity, >99%), and RT-PCR was performed as described in Materials and
Methods. The PCR products were electrophoresed and then visualized with
ethidium bromide. A representative example of four separate experiments
is shown, and the other experiments all showed similar results. BA; β-actin.

CXCR4-mediated eosinophil activation
In the next series of experiments, we investigated whether eosin-
ophil CXCR4 is functionally activated by a specific ligand for
CXCR4, SDF-1α. As shown in Fig. 2, freshly isolated eosinophils
did not express significant amounts of CXCR4 on their surface, but
SDF-1α elicited a small but apparent Ca2+ influx in these cells
(Fig. 4A). In cells incubated for 24 h at 37°C, much stronger influx
of Ca2+ was observed (Fig. 4C). The magnitude of Ca2+ influx
elicited by SDF-1α was comparable to that induced by eotaxin
(Fig. 4D). Furthermore, SDF-1α-induced Ca2+ influx was com-
pletely attenuated by treatment with PTX (Fig. 4E), indicating the
involvement of G proteins of the Gi class in the signal transduction
pathways. To determine whether CXCR4 expressed in eosinophils
has functional relevance, we tested the migration-inducing ability of
SDF-1α. As shown in Fig. 5, SDF-1α was capable of inducing a
migratory response in eosinophils: although SDF-1α did not in-
duce significant migration in freshly isolated cells, eosinophils in-
cubated for 24 h at 37°C exhibited a migratory response toward
SDF-1α, indicating the involvement of CXCR4 and the other eosinophil activa-
tion receptors in the signal transduction pathways.
granulation was noted in cells that had migrated in response to SDF-1α compared with freshly isolated or 24-h-cultured eosinophils (Fig. 6). In fact, in vitro experiments showed that CXCR4 was not involved in the process leading to degranulation: SDF-1α failed to induce significant release of EDN from eosinophils cultured for 24 h with or without IFN-γ or from freshly isolated cells (Fig. 7).

Modulation of surface CXCR4 expression in eosinophils by cytokines

In the next series of experiments, we examined the effects of cytokines on CXCR4 expression by eosinophils during 24 h of incubation. As shown in Table II, TGF-β1, IFN-γ, and TNF-α up-regulated the expression of CXCR4. In contrast, eosinophil-directed hemopoietins, i.e., IL-3, IL-5, and GM-CSF, almost completely attenuated the surface expression of CXCR4. In addition, IL-4 also drastically down-regulated the CXCR4 expression (Table II and Fig. 8A). As shown in Fig. 8B, as small as a femtomedlar level of IL-5 was sufficient to inhibit the CXCR4 expression. Half-maximal inhibition was observed at a concentration of ~100 fM of IL-5 with dose-dependent inhibition seen between 0.1 fM and 10 pM. For IL-4, half-maximal inhibition was observed at a concentration of ~1 pM with dose-dependent inhibition seen between 10 fM and 10 pM. On a molar basis, IL-5 was 10-fold more potent than IL-4. Furthermore, delayed addition of IL-5 or IL-4 effectively down-regulated CXCR4 expression. When eosinophils were cultured for 24 h without addition of any factors and then treated with IL-5 or IL-4, the level of CXCR4 expression in the

FIGURE 2. Time course of surface CXCR4 expression in purified eosinophils. Eosinophils were purified by means of Percoll density gradient centrifugation followed by negative selection using anti-CD16 microbeads. Purified eosinophils (purity, >99%) were cultured at 4°C (A) or 37°C (B) for the indicated times in culture medium. The cells were then treated with anti-CXCR4 mAb or isotype control mouse IgG (shaded peak) for 60 min at 4°C, stained with FITC goat F(ab')2 against mouse IgG for 30 min at 4°C and analyzed by flow cytometry. A and B are representatives of three separate experiments, and the others showed similar results. C. The time course of surface CXCR4 expression is shown. All data are expressed as the mean ± SEM (n = 3) of MESF values calculated as described in Materials and Methods. **, p < 0.01 vs MESF values of eosinophils cultured at 37°C. D, Expression of CCR3 on freshly isolated (0 h) and 24-h-cultured eosinophils (a representative of three separate experiments) is shown.

FIGURE 3. Expression of CXCR4 on eosinophils in whole blood. Eosinophils in whole blood were double-stained with anti-CXCR4-FITC and anti-CD16-PE and analyzed by flow cytometry. Granulocytes were discriminated on the basis of different forward/side scatter properties (A) and electronic gates on CD16-negative cells were set to eliminate neutrophils (B). C. Surface CXCR4 expression on eosinophils in fresh whole blood and eosinophils in whole blood cultured for 24 h at 37°C. The shaded area shows the fluorescence of cells stained with isotype-matched mouse IgG2a-FITC. The data are representative of three independent experiments, all showing similar results.

FIGURE 8. Expression of CXCR4-FITC on freshly isolated eosinophils (0 h) and 24-h-cultured eosinophils (A and B) are representatives of three separate experiments, and the others showed similar results. C, The time course of surface CXCR4 expression is shown. All data are expressed as the mean ± SEM (n = 3) of MESF values calculated as described in Materials and Methods. ***, p < 0.001 vs MESF values of eosinophils cultured at 37°C. D, Expression of CCR3 on freshly isolated (0 h) and 24-h-cultured eosinophils (a representative of three separate experiments) is shown.
eosinophils was decreased at 6 and 24 h after the addition of each cytokine (data not shown).

Substantial evidence has shown that isolated eosinophils rapidly undergo apoptosis. In fact, when apoptotic cells were quantita-

![Figure 4](image1.png)

**FIGURE 4.** SDF-1α caused calcium influx to eosinophils. Calcium influx in highly purified eosinophils (purity, >99%) just after purification (A and B) and eosinophils cultured for 24 h at 37°C without (C and D) or with (E) further treatment with PTX. E. Cultured cells were treated with PTX at 100 nM for 2 h at 37°C, and then stimulated sequentially with SDF-1α and ionomycin. The data shown are representative of two independent analyses from different donors, each showing similar results. The concentration of chemokines and ionomycin were 400 ng/ml and 1 μM, respectively.

![Figure 5](image2.png)

**FIGURE 5.** SDF-1α induced migration of eosinophils. The migration-inducing activity of SDF-1α and eotaxin was analyzed using eosinophils just after purification (purity, 96.3 ± 1.3%) and eosinophils cultured for 24 h at 37°C. The data are expressed as the percentage of total cells introduced into each of the upper wells (mean ± SEM, n = 4). The effects of the concentration of chemokines were analyzed by the two-way ANOVA test. When this test indicated a significant differences between concentrations (indicated as + +, p < 0.05), Fisher’s protected least significance (PLSD) test was used to compare individual groups. ***, p < 0.01 vs values of eosinophil migration in control buffer at the same time point.

![Figure 6](image3.png)

**FIGURE 6.** Photomicrographs of eosinophils that were freshly isolated (A), 24-h-cultured (B), and migrated in response to SDF-1α (C). The photos are representatives of two different experiments and another showed similar results. Magnification, ×600.
iodide-negative cells are defined as apoptotic, and propidium iodide-positive cells are defined as necrotic. Data are expressed as the mean ± SEM of the percentage of EDN release in four duplicate experiments. 

cells, while it failed to suppress and actually up-regulated the eosinophil CXCR4 expression (Table II). Finally, treatment of eosinophils with anti-Fas mAb slightly but significantly decreased the number of living cells, whereas it failed to up-regulate CXCR4 expression (Table II).

Modulation of CXCR4 mRNA expression in eosinophils by cytokines

The surface expression of CXCR4 by eosinophils was significantly but partially inhibited by treatment with a protein synthesis inhibitor, cycloheximide (MESF: 86.9 ± 3.0% and 59.5 ± 7.7% of inhibition for 4-h and 24-h cultured cells, respectively, n = 3), indicating that de novo protein synthesis is involved in the process leading to the surface expression of CXCR4. In the last series of experiments, we studied cytokine-mediated regulation of CXCR4 expression at the mRNA level. When we examined the expression of CXCR4 mRNA by RT-PCR amplification, we observed that treatment with IL-5, but not IL-4, apparently decreased the level of CXCR4 mRNA, although both cytokines suppressed surface protein expression of CXCR4. In addition, CXCR4 mRNA remained at the baseline level in eosinophils cultured with IFN-γ. In contrast, none of these cytokines had any effect on the level of CCR3 mRNA (Fig. 10).

Discussion

In this study, we have demonstrated the functional expression of CXCR4 in human eosinophils. Among the 15 chemokine receptors, intense expression of CXCR4 as well as CCR3 transcripts was always detected in freshly isolated eosinophils (Fig. 1). While we were preparing this manuscript, Chelucci et al. reported the surface expression of CXCR4 in the eosinophil lineage during the culture of purified hemopoietic progenitors (12), although they did not examine the functional relevance of CXCR4 in these cells. In contrast to those cultured eosinophils, we observed that surface CXCR4 protein was hardly detected in freshly isolated eosinophils (Fig. 2). However, surface CXCR4 expression gradually became apparent during incubation at 37°C. Because surface CXCR4 in cultured eosinophils was widely variable, it is of importance to clarify the control mechanisms of eosinophil CXCR4 expression. In this study, we observed that several cytokines exerted reciprocal and divergent effects on eosinophil CXCR4 expression (Table II). The patterns of cytokine-mediated regulation of CXCR4 expression in eosinophils seem to be distinct from those in other types of leukocytes such as T lymphocytes. In T lymphocytes, IL-4 up-regulates (13–15) and IFN-γ down-regulates (16) CXCR4 expression, while both cytokines exerted completely opposite effects on eosinophils.

Studies of time course and various conditions for CXCR4 expression raised a possibility that the expression of CXCR4 is related to apoptotic changes. IL-5, IL-3, and GM-CSF, all of which are known to rescue eosinophils from apoptosis (17–19), completely attenuated CXCR4 expression in eosinophils. Furthermore, the concentration of IL-5 necessary for the inhibition of CXCR4 induction matched the reported range required for inhibition of eosinophil apoptosis (20) (Fig. 8B). Consequently, it would be reasonable and even attractive to speculate that CXCR4 is preferentially induced in eosinophils undergoing apoptosis, and that these cells are then eliminated from the circulation by the interplay between CXCR4 and ubiquitously expressed SDF-1α. However, the level of eosinophil CXCR4 expression did not always parallel

![FIGURE 7. Effect of SDF-1α on EDN release from eosinophils. Freshly isolated eosinophils (purity, >99%) were cultured for 24 h with or without IFN-γ (10 ng/ml). Degranulation experiments were performed as described in Materials and Methods. Percent release of EDN was calculated as the ratio of EDN content in the supernatant to total EDN in the lysate. Results are expressed as the mean ± SEM of the percentage of EDN release in four duplicate experiments. * p < 0.05 vs values of EDN release in control buffer at the same time point.](http://www.jimmunol.org/Downloaded from by guest on July 24, 2018)

Table II. Modulation of CXCR4 expression and eosinophil survival by cytokines or anti-Fas mAb

| CXCR4 Expression (% of control) | Cell Status       | Alive | Apoptotic | Necrotic |
|---------------------------------|-------------------|-------|-----------|----------|
| Nil                             |                   | 53.3 ± 4.2 | 23.2 ± 2.7 | 23.1 ± 3.3 |
| IL-3                            |                   | 64.0 ± 2.6** | 43.0 ± 0.2* | 10.0 ± 0.2 |
| IL-4                            |                   | 19.9 ± 1.2** | 21.8 ± 2.2 | 25.5 ± 9.4 |
| IL-5                            |                   | 20.3 ± 4.0** | 2.7 ± 0.3* | 11.1 ± 3.8 |
| GM-CSF                          |                   | 7.1 ± 0.9** | 3.1 ± 0.3* | 12.7 ± 1.8 |
| TGF-β1                          |                   | 133.3 ± 6.0** | 22.3 ± 5.1 | 23.0 ± 5.0 |
| IFN-γ                           |                   | 139.0 ± 11.0* | 3.6 ± 0.6** | 18.9 ± 4.4 |
| TNF-α                           |                   | 196.7 ± 31.0* | 12.0 ± 2.8** | 24.2 ± 1.5 |
| Control IgM                     |                   | 100    | 45.5 ± 7.5 | 26.9 ± 6.6 | 26.9 ± 5.1 |
| anti-Fas mAb                    |                   | 76.1 ± 8.8 | 33.1 ± 6.0* | 29.0 ± 6.8 | 37.5 ± 4.8 |

* Eosinophils (purity, >99%) were incubated at 37°C for 24 h with the indicated cytokines, and CXCR4 expression was analyzed by flow cytometry. The concentration of each cytokine was 10 ng/ml, except for TNF-α, which was used at 100 ng/ml. In separate experiments, eosinophils were incubated with 1 μg/ml of anti-Fas mAb or the same concentration of control IgM for 24 h and then analyzed by flow cytometry. The data are expressed as the percentage of the calculated MESF of control cells, which were incubated in culture medium alone or incubated with control IgM (mean ± SEM, n = 4). 

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* p < 0.05; ** p < 0.01 vs each control (NS = 100%). Eosinophils were also double-stained with FITC-conjugated annexin V and propidium iodide. Annexin V-negative and propidium iodide-negative cells are defined as alive. Annexin V-positive and propidium iodide-negative cells are defined as apoptotic, and propidium iodide-positive cells are defined as necrotic. Data are expressed as the mean ± SEM of the percentage of total cell number (n = 4).
the apoptotic changes (Table II). IL-4, which is devoid of anti-apoptotic effects, almost completely attenuated CXCR4 expression (Table II). On the contrary, IFN-γ, which is known to block eosinophil apoptosis (Ref. 20 and Table II), efficiently up-regulated CXCR4 expression. It might be possible that each cytokine may regulate eosinophil CXCR4 expression via different pathways or mechanisms. However, these discrepancies observed between apoptotic changes and CXCR4 expression in cytokine-treated eosinophils, together with the direct determination of CXCR4 expression in nonapoptotic populations (Fig. 9), strongly suggest that eosinophil CXCR4 expression cannot be explained merely as a consequence of apoptotic changes in these cells.

Spontaneous sustained increase in CXCR4 expression similar to our present findings has also been reported for monocytes and lymphocytes (21, 22). In these cells, surface expression of CXCR4 seems not to be regulated at the mRNA level but mainly at the protein level, such as receptor trafficking between the intracellular space and cell surface (23). In the present study, despite the constitutive expression of CXCR4 mRNA (Fig. 1), freshly isolated eosinophils did not express significant amounts of CXCR4 protein on their surface (Fig. 2). Furthermore, spontaneous sustained increase in CXCR4 expression was reversibly down-regulated by delayed addition of IL-4 and IL-5. These findings strongly suggest that surface CXCR4 expression in eosinophils is regulated, at least in part, at the level of receptor trafficking. However, the expression of surface CXCR4 dose not seem to be regulated merely at the level of posttranscription. The process leading to the expression of CXCR4 in eosinophils was partly inhibited by cycloheximide, indicating that the sustained induction of surface CXCR4 expression involves a cycloheximide-sensitive component. Although a direct relationship between the level of mRNA and the amount of surface protein was not always observed in cytokine-mediated alteration of CXCR4 expression, IL-5 apparently down-regulated the level of both mRNA and surface protein of CXCR4 (Table II and Fig. 10). Taken together, it seems plausible that surface CXCR4 expression in eosinophils is modulated not only at the level of posttranscription but also at least in part at the level of transcription.

The most striking findings of our study are that surface expression of CXCR4 in eosinophils has functional relevance, and that SDF-1α induced strong eosinophil migration comparable to that
induced by eotaxin (Fig. 5). Eotaxin, a specific ligand for CCR3, has been known as the most potent chemokine for eosinophil chemotaxis (5), and an essential role of CCR3/eotaxin in selective accumulation of eosinophils has been established in vivo as well as in vitro. In contrast, the in vivo role of CXCR4/SDF-1α-mediated chemotaxis is yet to be established and remains totally speculative, but it seems unlikely that CXCR4/SDF-1α is fundamentally involved in the pathogenesis of allergic diseases by inducing the migration of eosinophils toward inflammatory sites. Firstly, in contrast to CCR3, the expression of which is restricted only to eosinophils (4) and basophils (24, 25), CXCR4 is expressed in various white blood cells (reviewed in Ref. 26), indicating that CXCR4 is inducible in eosinophils, and that SDF-1α might be crucial for retaining eosinophils in noninflamed tissues. Th2-dominant state may favor the liberation of eosinophils by down-regulating CXCR4 expression, which in turn would permit enhanced accumulation of eosinophils at allergic inflammatory sites by eosinophil-active chemokines such as eotaxin. The presence of IL-5 in the serum, which is often observed in patients with allergic and helminthic disorders, may increase the distribution of eosinophils to inflamed tissues.

In summary, we have demonstrated that functional expression of CXCR4 is inducible in eosinophils, and that SDF-1α elicits strong migration comparable to that induced by eotaxin. Th2 cytokines such as IL-4 and IL-5 drastically inhibited the expression of CXCR4. The interplay between CXCR4 and SDF-1α may affect the size of the mobilizable pool, and anchorage of mature eosinophils by SDF-1α may be crucial for retaining eosinophils in noninflamed tissues. Th2-dominant state may favor the liberation of eosinophils by down-regulating CXCR4 expression, which in turn would permit enhanced accumulation of eosinophils at allergic inflammatory sites by eosinophil-active chemokines such as eotaxin. The presence of IL-5 in the serum, which is often observed in patients with allergic and helminthic disorders, may increase the distribution of eosinophils to inflamed tissues.

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