Monocyte Chemotactic Protein-3 (MCP3) Interacts with Multiple Leukocyte Receptors

C-C CKR1, A RECEPTOR FOR MACROPHAGE INFLAMMATORY PROTEIN-1α/RANTES, IS ALSO A FUNCTIONAL RECEPTOR FOR MCP3*

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Adit Ben-Baruch, Luoling Xu, Peter R. Young, Kathleen Bengalj, J oost J. Oppenheim, and J i Ming Wang†

From the Laboratory of Molecular Immunoregulation, Biological Response Modifiers Program and the Biological Carcinogenesis and Development Program, SAIC Frederick, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, Maryland 21702, the Department of Molecular Immunology, Smith-Kline Beecham Pharmaceuticals, King of Prussia, Pennsylvania 19406

Monocyte chemotactic protein-3 (MCP3) is a recently identified and molecularly cloned C-C chemokine that is chemotactic for and activates a great variety of inflammatory cell types. MCP3 has been reported to interact with several C-C chemokine receptors, which can be simultaneously or selectively expressed on leukocyte subpopulations. In order to isolate receptor(s) for MCP3, a cDNA library was constructed using mRNA from a human NK-like cell line, YT. These cells showed high affinity binding sites for 125I-MCP3 and migrated in response to MCP3. A chemokine receptor cDNA clone, designated YT4, was sequenced and found to be identical to the known C-C CKR1 or macrophage inflammatory protein-1α (MIP1α)/Rantes receptor. YT4 cDNA was subcloned into a mammalian expression vector, and stable transfectants were prepared using the embryonic kidney cell line 293. The transfectants (YT4/293) showed high affinity binding for 125I-MCP3 in addition to specifically binding 125I-MIP1α and 125I-Rantes. All three C-C chemokines were able to cross-compete for binding sites on YT4/293 cells and induced directional migration of YT4/293 cells in vitro, with MCP3 being the most potent chemoattractant. MCP3, MIP1α, and Rantes were equally able to cross-attenuate the migratory response of YT4/293 cells to one another. In contrast, MCP1 and MIP1β had very limited capacity to compete for MCP3 binding on YT4/293 cells and had only a minor attenuating effect on MCP3-induced migration. Since MCP3 has been reported to use MCP1 receptor(s), our results with transfected 293 cells expressing only C-C CKR1 clearly establish that C-C CKR1 is also a functional receptor for MCP3.

The newly identified C-C chemokine, monocyte chemotactic protein-3 (MCP3) is chemotactic for and activates a variety of inflammatory cells (Refs. 1–8; reviewed in Ref. 9). MCP3 has 71% amino acid identity to MCP1, and both activate monocytes, T cells, and basophils (1–3, 4–6, 8, 10). However, MCP3, unlike MCP1, also activates eosinophils (6, 7). On the other hand, MIP1α and Rantes which are about 30% identical to MCP3, both also activate eosinophils, in addition to monocytes (6–8), T lymphocytes (10), and basophils (Refs. 6–8; reviewed in Refs. 9, 11, and 12). Based on calcium flux desensitization experiments and competition by MCP3 for binding of 125I-MCP1 and 125I-MIP1α on monocytes, MCP3 has been proposed to interact with the receptors for MCP1, MIP1α, and Rantes (4, 6, 7, 10).

Several chemokine receptors have been cloned and functionally expressed (reviewed in Refs. 13 and 14). These include two receptors for the C-X-C chemokine IL8 (15, 16) and receptors for the C-C chemokines MCP1 (MCP1R; Ref. 17) and MIP1α/Rantes (C-C CKR1; Refs. 18 and 19). All of these receptors belong to the seven-transmembrane, G-protein-coupled receptor superfamily (13, 14). Studies with cdls transfected with the MCP1 receptor showed that MCP3 is able to induce calcium flux in cells expressing only the MCP1 receptor (20). On the other hand, since MCP3 is able to interact with the receptors for MIP1α and Rantes on leukocytes (6, 7, 10), MCP3 has also been proposed to use the C-C CKR1, which is mainly activated by MIP1α and Rantes (18, 19). In addition, ongoing studies of the binding of 125I-MCP3 to human monocytes and neutrophils with 125I-MCP3 suggest that MCP3 may also share a receptor used by MIP1β as well as another unique receptor. Since human leukocytes usually express multiple C-C chemokine receptors, it is difficult to conclude from the interaction of MCP3 with normal leukocytes which of these receptors it may be using. To obtain more definitive data, we have therefore investigated the binding and signaling of MCP3 through a single receptor. In this report, by using human embryonic kidney cell line transfected with a C-C CKR1 cDNA, we show that C-C CKR1 is indeed a functional receptor shared by MCP3, MIP1α, and Rantes.

MATERIALS AND METHODS

Chemokines and Cells—Recombinant human (rh) chemokines of the C-C or C-X-C subfamily were purchased from PeproTech Inc. (Rocky Hill, NJ). Radiodinated rhMCP3 was a kind gift from Dr. G. Brown (DuPont NEN). The other radiodinated human recombinant C-C chemokines were purchased from DuPont NEN. All radiodinated chemokines had a specific activity of 2200 Ci/mmol. Human natural killer-like cell line, YT (21), was maintained in RPMI 1640 containing 10% fetal calf serum (Hyclone Laboratories, Logan, UT) and antibiotics.

Isolation of C-C CKR1 cDNA from YT Cells—Total RNA was extracted from YT cells according to the usual procedure (22), and mRNA...
was isolated with a Poly(A) Quik™ mRNA purification kit (Stratagene, La Jolla, CA). cDNA library from YT cell mRNA was constructed by using a ZAP Express™ vector cloning kit (Stratagene) according to the manufacturer's instructions. The design of degenerate oligonucleotides and polymerase chain reaction were performed essentially as described elsewhere (23). One μg of template DNA prepared from YT cell cDNA library was used for each series of polymerase chain reactions using pairs of degenerate oligonucleotide primers. After polymerase chain reaction amplification, the reaction mixture was fractionated on agarose gel (Life Technologies, Inc.), and the DNA at size of 500–700 base pairs was purified and inserted into M13 mp19 phage vector for partial sequencing (Sequenase version 2.0 DNA sequencing kit, U.S. Biochemical Corp.) in order to be selected as probes for screening the YT cell cDNA library in Lambda Zap Express™ phage vector. Several positive clones were selected, and in vivo excision of the F-K-CMV plasmid from Zap Express™ vector was carried out. More detailed sequencing revealed some clones with consensus sequence of the G-protein-coupled receptor superfamily. One of the clones with the size of 2.2 kilobases whose coding region was found identical to the C-C CKR1 (18) was inserted and used for further studies (results not shown).

Establishment of Stable Transfectants—293 human embryonic kidney cell line (a gift from Dr. P. Gray, ICOS Corp., WA) was grown in monolayer in Dulbecco's modified Eagle's medium (BioWhittaker, Inc., Walkersville, MD) with 10% fetal calf serum (Hyclone) and antibiotics. Cells were grown to approximately 75% confluency at 37 °C with 5% CO₂. Transfections were performed with 40 μg of DNA by using DOTAP transfection reagent (Boehringer Mannheim, IN) according to the manufacturer's instructions. Stable transfectants were established by adding G418 (800 μg/ml; Life Technologies, Inc.) to cell culture from day 4 after the transfection to maintain selection pressure. The results presented stably transfected cells were designated YT4/293.

Binding Assays with Radiolabeled Chemokines—YT4/293 cells were trypsinized, washed twice in binding medium (BM; RPMI 1640 containing 1% bovine serum albumin, 25 mM Heps, and 0.05% Na₂EDTA) and detached from the surface. Duplicate samples of 200 μl of cells (1.5 × 10⁶) were incubated with a fixed concentration of radiolabeled chemokines in the presence of increasing concentrations of unlabeled ligands as required by a Macintosh computer-aided program, LIGAND (Dr. P. Munson, Division of Computer Research and Technology, NIH). After incubation and rotation at room temperature for 1 h, the cells were pelleted through a 10% sucrose, phosphate-buffered saline cushion for 1 min at 10,000 × g. The supernatant was removed, and the radioactivity associated with cell pellets was measured in a γ counter (CliniGamma, Pharmacia Biotech Inc.). This procedure did not cause substantial ligand-receptor complex internalization since most of the radioactivity (>85%) associated with the cell pellets could be eluted by a brief washing with acid solution (ice-cold 0.5% acetic acid in phosphate-buffered saline). Furthermore, the assays conducted at 4 °C yielded comparable results. The data generated were analyzed with the Macintosh computer program LIGAND.

Chemotaxis Assays—The migration of 293 cells transfected with YT4 cDNA was assessed by a 48-well microchamber technique (24). Different concentrations of chemokines were placed in the lower wells of the chamber. The cells (50 μl, 0.5–1 × 10⁵/ml) were loaded in the upper wells. Chemokines and cells were diluted in RPMI 1640 containing 1% bovine serum albumin and 25 mM Heps. The lower and upper wells were separated by a polycarbonate filter (pore size, 0.4 μm; Porous Pore Corp., Livermore, CA) precoated with 20 μg/ml mouse collagen type IV (Collaborative Biomedical Products, Bedford, MA) for 2 h at 37 °C. The chamber was incubated at 37 °C for 5–6 h at 37 °C in humidified air with 5% CO₂. At the end of the incubation period, the chamber was disassembled, and the cells remaining on the upper surface of the filter were removed by scraping the filter over a rubber scraper. The filter was then fixed and stained with Diff-Quik (Baxter, Miami, FL). After coding the samples, the migrates across the filter onto the lower surface were counted in three high power fields under light microscopy in triplicate. Data are presented as the chemotaxis index calculated by the following ratio: number of cells migrating on the upper surface of the filter (chemotaxis index). This was probably due to the formation of aggregates of YT4 cells at high concentrations (25).

C-C chemokines do not bind or induce migration of parental 293 cells

| Chemokine | Total Binding (cpm) | Non-specific Binding (cpm) | Specific Binding (cpm) | Migration (chemotaxis index) |
|-----------|-------------------|----------------------------|-----------------------|-----------------------------|
| MCP3      | 120               | 130                        | 0                     | 0.9                          |
| MIP1α     | 98                | 105                        | 0                     | 1.1                          |
| MIP1β     | 133               | 128                        | 5                     | 0.9                          |
| MIP1γ     | 144               | 154                        | 0                     | 0.99                         |
| Rantes    | 255               | 433                        | 1                     | 1.1                          |

The binding assays were performed as described under "Materials and Methods." Total binding was obtained with the cells incubated with radiolabeled chemokines in the absence of unlabeled ligands. Non-specific binding represents the radioactivity on cells incubated in the presence of a 100-fold excess of labeled ligands.

The chemotaxis indices were calculated as described under "Materials and Methods." The spontaneous migration (in response to medium alone) was taken as 1. No significant migration of parental 293 cells to chemokines was detected.

was calculated as 1 – (migration of cells incubated with chemokines/migration of cells incubated with medium) × 100.

The significance of the difference between test and control groups was analyzed using Student's t-test.

RESULTS

Cloning of C-C CKR1—YT, a human NK-like cell line was established from a patient with thymic lymphoma (21). These cells have the phenotype of NK cells and were reported to possess NK-like killing activity (21). YT cells expressed approximately 40,000 specific binding sites/cell for MCP3, with estimated Kd ranging from 0.6 to 2 nM. YT cells also migrated in response to MCP3 in a dose-dependent manner with the optimal migratory response to 10–50 ng/ml (1.2–6 nM) MCP3. In addition, other C-C chemokines including MIP1α, MIP1β, and Rantes, but not MCP1, specifically bound to and induced migration of YT4 cells. Thus the binding and signaling of MCP3 on YT cells is clearly dissociated from MCP1. A cDNA library was therefore established from YT cell mRNA, and several cDNA clones were isolated using degenerate polymerase chain reaction with primers from conserved regions of seven-transmembrane domain chemoattractive receptors. One of these clones, designated YT4, was identical in nucleotide sequence to the known C-C CKR1 (18), otherwise known as MIP1α/Rantes receptor (19) or LD78 receptor (23).

Chemokine Binding by 293 Embryonic Kidney Cells Transfected with C-C CKR1—The parental 293 cells or cells transfected with pCDNA3 vector alone did not bind or migrate in response to any of the C-C chemokines (Table I). The 293 cells stably transfected with the C-C CKR1 cDNA (designated YT4/293 cells) showed a high level of specific binding for 125I-MIP1α (40,000 ± 5000 sites/cell, Kd = 5–9 nM, Figs. 1A and 2B) as previously reported for C-C CKR1 (18). Competition analyses showed that unlabeled MIP1α competed completely for its own binding (Fig. 1A), while the competition of 125I-Rantes binding by unlabeled Rantes was incomplete and biphasic as described (Ref. 18 and Fig. 1B). This was perhaps due to the formation of aggregates of Rantes at high concentrations (25). However, unlabeled MIP1α was able to effectively displace 125I-Rantes binding. The total binding of 125I-MIP1β to YT4/293 cells was low, and less than 40% of the total binding was competed for by unlabeled MIP1α at concentrations greater than 10 nM, reflecting a low binding affinity (data not shown). No binding of 125I-MCP1 was detected on YT4/293 cells.

YT4/293 cells also specifically bound 125I-MCP3 with 21,000 (± 2400) sites/cell with high affinity (Kd = 0.7 nM) (Fig. 2A). The binding affinity of 125I-MCP3 was consistently higher (0.5–1.8 nM) than that obtained with 125I-MIP1α (5–9 nM, Fig. 2B) on

3 L. L. Xu, unpublished observation.
these cells, suggesting that MCP3 may have a better interaction with C-C CKR1 than MIP1α. These results suggest that C-C CKR1 is also a receptor for MCP3.

Cross-competition of Chemokine Binding to C-C CKR1—In order to further characterize the interactions between MCP3 and other C-C chemokines on C-C CKR1, we performed cross-competition experiments. Fig. 3A shows that MCP3 was able to compete for 125I-MIP1α binding on YT4/293 cells with potency comparable with that of unlabeled MIP1α. MCP3 also competed for 125I-Rantes binding on YT4/293 cells more effectively than unlabeled Rantes in a dose-dependent manner (Fig. 3B). The low level binding of 125I-MIP1β on YT4/293 cells was more effectively inhibited by unlabeled MCP3 than by MIP1β itself (Fig. 3C). Moreover, Fig. 4 shows that MIP1α and Rantes competed for 125I-MCP3 binding with less potency than that of unlabeled MCP3. Unlabeled MCP1 and MIP1β partially displaced 125I-MCP3 binding with IC50 greater than 100 nM (Fig. 4). None of the C-X-C chemokines including IL8, neutrophil-activating protein 2, and interferon-γ-inducible protein 10 showed any competition for 125I-MCP3 binding on YT4/293 cells (data not shown).

Chemotactic Response of C-C CKR1-transfected 293 Cells—In an effort to address the functional role of MCP3 on C-C CKR1, we first tested the ability of MCP3 as well as other chemokines to induce calcium flux in YT4/293 cells. Although at times observed calcium flux in YT4/293 cells in response to MCP3, MIP1α, and Rantes, the effect was generally weak and variable. Therefore, we investigated the ability of MCP3 to induce directional migration of YT4/293 cells in vitro. Previous studies established that 293 cells stably transfected with IL8 receptor cDNA migrated consistently in response to low doses of IL8 (26), proving that this method was sensitive and reliable in the evaluation of the function of IL8 receptor transfected into 293 cells. Fig. 5 shows that MCP3 and MIP1α as well as Rantes all induced significant migration of YT4/293 cells in vitro. The optimal concentrations for these C-C ligands to induce YT4/293 cell migration was about 6–12 nM (50–100 ng/ml). However, MCP3 consistently was the most potent chemoattractant (Fig. 5A) compared with MIP1α (Fig. 5B) and Rantes (Fig. 5C). MCP1β was able to induce a significant but relatively low level of migration of YT4/293 cells only at a concentration of 30 nM (250 ng/ml) (Fig. 5D), which correlated with a low binding level of the radiolabeled ligand to these cells. On the other hand, MCP1 was unable to attract YT4/293 cells over a wide range of concentrations tested (data not shown). Thus, the efficacy of C-C ligand binding to YT4/293 cells appears directly correlated to the potency of chemotactic response of these cells to C-C chemokines.

Attenuation of YT4/293 Cell Migration in Response to C-C Chemokines—We further confirmed by showing attenuation of chemotaxis that the C-C CKR1 is a common receptor for MCP3, MIP1α, and Rantes. As shown in Table II, when incubated in upper wells of the chemotaxis chamber in the presence of MCP3, MIP1α, or Rantes, YT4/293 cells failed to migrate in response to any one of these C-C chemokines in the lower wells. In contrast, MCP1 inhibited MCP3-induced migration only to a
the cell migration to MCP3 possibly by competing with MCP3 for binding to the C-C CRK1, despite the fact that the binding affinity of MCP1 to C-C CRK1 is too low to detect. This indicates that both the chemotaxis attenuation and binding competition assays are sensitive in detecting the weak interaction of MCP1 with the C-C CRK1.

**DISCUSSION**

MCP3 activates a variety of inflammatory cells through a number of different binding sites for C-C chemokines expressed on these cells. Previous studies based on desensitization of calcium flux predicted that MCP3 would be able to interact with at least three types of receptors: 1) MCP1 receptor on monocytes and basophils (4, 6, 7), 2) selective Rantes receptor on basophils and eosinophils (6, 7), and 3) selective MIP1α receptor on basophils, eosinophils, and neutrophils (6). Results obtained from binding studies using 125I-MCP1 and 125I-MIP1α on monocytes suggested that MCP3 may also interact with C-C CRK1, the MIP1α/Rantes receptor. By using 293 cells transduced with the MCP1 receptor, it has been shown that MCP3 was able to signal through MCP1 receptor (20). However, all of these observations were based on cross-desensitization of calcium mobilization between MCP3 and other C-C chemokines or on the ability of MCP3 to compete for 125I-MCP1 and 125I-MIP1α binding. No data concerning direct binding of radiolabeled MCP3 to native cells or to cells transfected with cloned receptors has been reported. Recently, by using 125I-MCP3, we have characterized the binding behavior of MCP3 on human monocytes and neutrophils and provided evidence that in addition to interacting with binding sites for MCP1, MIP1α, and Rantes, MCP3 also interacts with a binding site for MIP1β as well as with as yet undefined unique site(s) not shared by other C-C chemokines.

Since human leukocytes usually express multiple C-C chemokine receptors, they exhibit a complex pattern of shared as well as selective utilization of the receptors by different C-C chemokines (4–8, 10, 25, 27). In order to more precisely evaluate the C-C receptor promiscuity, it is crucial to examine the binding and signaling of each C-C chemokine through a single isolated receptor. Using this approach, cells transfected with the MCP1 receptor clearly were shown to bind and signal in response to MCP1 but not to MIP1α, MIP1β, or Rantes (17). It was subsequently shown that MCP3 was also able to induce calcium flux in MCP1 receptor transfectants confirming the use of the MCP1 receptor (20).

In an effort to characterize the binding sites for MCP3 on human cell lines, we observed that YT cells exhibited a high level of specific binding for 125I-MCP3. The 125I-MCP3 binding on YT cells was progressively less well competed for by unlabeled MCP3, Rantes, MIP1α, MIP1β, and MCP1, with MCP1 being the least potent competitor for MCP3 binding. No 125I-MCP1 binding was detected on YT cells. The YT cells also migrated in response to various C-C chemokines but not to MCP1. Thus the binding and biological activity of MCP3 on YT cells was separable from that of MCP1. A cDNA library was therefore constructed with the mRNA extracted from YT cells, and several cDNA clones with seven-transmembrane domain, G-protein-coupled receptor features were obtained. One of these clones was identical to the C-C CRK1 (18), known as MIP1α/Rantes receptor (19), which has been shown to be mainly activated by MIP1α and Rantes, although MCP1 and MIP1β could also weakly signal through C-C CRK1. Since it was proposed that MCP3 might interact with receptors for MIP1α and Rantes (6, 7, 10), we decided to examine this possibility by using cells transfected to express only C-C CRK1 binding sites. Our binding and functional studies with YT4/293 cells confirmed that C-C CRK1 was very well activated by limited extent (Table II), while MIP1β was totally ineffective at the concentrations of 6–30 nM (50–250 ng/ml; data not shown). Consequently, MCP1 has low but significant capacity to reduce

**FIG. 3.** Competition for binding of C-C chemokines on YT4/293 cells by unlabeled MCP3. YT4/293 cells were incubated with radiolabeled chemokines MIP1α (A), Rantes (B), or MIP1β (C) in the presence of unlabeled chemokines at increasing concentrations. Five experiments were performed with similar results. C, MCP3; △, Rantes; ●, MIP1α and MIP1β in panels A and C, respectively. LOG(T), concentration of unlabeled chemokines.

**FIG. 4.** Competition of 125I-MCP3 binding on YT4/293 cells by unlabeled chemokines. YT4/293 cells were incubated with 125I-MCP3 in the presence of increasing concentrations of unlabeled chemokines. Five experiments were performed with similar results. ●, MCP3; ○, MIP1α; ●, MIP1β; △, Rantes; △, MCP1. LOG(T), concentration of unlabeled chemokines.
MIP1α and Rantes. Furthermore, our study demonstrated that C-C CKR1 exhibited even higher affinity binding for 125I-MCP3. MCP3 was also the most potent C-C chemokine in inducing the migration of C-C CKR1-transfected cells. MCP3 not only exhibited better binding and activation of cells transfected with C-C CKR1, it also competed more effectively for 125I-Rantes and 125I-MIP1β binding on C-C CKR1 than unlabeled Rantes and MIP1β. This may be attributable to the possibility that MCP3 may have more binding domains for C-C CKR1 than either Rantes or MIP1β, or that MCP3 may have a better interaction with the same number of domains.

Studies with IL8 and its receptors have shown that more than one site either on the ligand or on the receptor are responsible for the binding and signaling (28, 29). Accordingly, studies with mutants derived from MCP3 ligand and cloned C-C chemokine receptors may provide more information about the greater capacity of MCP3 to interact with multiple C-C chemokine receptors. It will also be interesting to investigate whether MCP3 can interact with two recently identified promiscuous chemokine receptors, Duffy Antigen (30, 31) and the cytomegalovirus DNA open reading frame, US28 (19, 32).

Overall, we have shown that 293 embryonic kidney cells

### TABLE II

Attenuation of YT4/293 cell migration to C-C chemokines

YT4 cDNA-transfected cells were placed in the upper wells of the chemotaxis chamber in the presence or absence of various chemokines at a concentration of 6 nM. The lower wells contained chemokines or control medium alone. After incubation, the filters were stained, and the migrated cells were counted.

| Upper wells chemotaxis index (% inhibition)a | Medium | MCP3 | MIP1α | Rantes | MCP1 |
|---------------------------------------------|--------|------|-------|--------|------|
| Lower wells                                 | Medium |      |       |        |      |
| Medium                                      | 1      |      |       |        |      |
| MCP3                                        | 7.0 ± 0.2 | 1.2 ± 0.1 (97)b | 1.4 ± 0.3 (93)b | 1.9 ± 0.2 (85)b | 5.6 ± 0.4 (24)b |
| MIP1α                                       | 5.1 ± 0.4 | 1.5 ± 0.2 (88)b | 1.1 ± 0.1 (96)b | 2.2 ± 0.3 (71)b | 4.4 ± 0.2 (17) |
| Rantes                                      | 4.5 ± 0.7 | 0.7 ± 0.1 (100)b | 0.8 ± 0.1 (100)b | 1.3 ± 0.2 (91)b | 4.4 ± 0.2 (3) |

a The results are expressed as chemotaxis indices (± S.E.) calculated as described under “Materials and Methods.” The values in the parentheses are percentage of inhibition of chemotaxis.

b p < 0.05 compared with migration of cells incubated with medium in the upper wells.

![Graphs and images](image-url)
stably transfected to express C-C CKR1, known as MIP1α/ C-C chemokine receptor type 1. MIP1α/ Rantes receptor, bind 125I-MCP3 with higher affinity (0.5–1.8 nM) than the affinity for 125I-MIP1δ (5–9 nM). MCP3, of all the C-C chemokines tested, was the most potent chemoattractant for C-C CKR1-transfected 293 cells (YT4/293 cells). Since MCP3 acts on a variety of inflammatory cells and utilizes multiple receptors for its function, characterization and isolation of the shared as well as unique receptors for MCP3 will provide further insights into the pathophysiological roles of MCP3. C-C chemokines are mediators of a number of pathological conditions such as chronic inflammation, tumor, allergy, and atherosclerosis (reviewed in Refs. 9, 11, and 12). Since the binding and signaling of MCP3 is most promiscuous, the development of compounds or peptides that can block MCP3 binding to the receptor may prove to be useful in the treatment of diseases mediated by a number of C-C chemokines.

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