# Chemokine Receptor Specific for IP10 and Mig: Structure, Function, and Expression in Activated T-Lymphocytes

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## Summary

A human receptor that is selective for the CXC chemokines IP10 and Mig was cloned and characterized. The receptor cDNA has an open reading frame of 1104-bp encoding a protein of 368 amino acids with a molecular mass of 40,659 dalton. The sequence includes seven putative transmembrane segments characteristic of G-protein coupled receptors. It shares 40.9 and 40.3% identical amino acids with the two IL-8 receptors, and 34.2–36.9% identity with the five known CC chemokine receptors. The IP10/Mig receptor is highly expressed in IL-2-activated T lymphocytes, but is not detectable in resting T lymphocytes, B lymphocytes, monocytes and granulocytes. It mediates $Ca^{2+}$ mobilization and chemotaxis in response to IP10 and Mig, but does not recognize the CXC-chemokines IL-8, GROα, NAP-2, GCP-2, ENA78, PF4, the CC-chemokines MCP-1, MCP-2, MCP-3, MCP-4, MIP-1α, MIP-1β, RANTES, I309, eotaxin, nor lymphotactin. The exclusive expression in activated T-lymphocytes is of high interest since the receptors for chemokines which have been shown so far to attract lymphocytes, e.g., MCP-1, MCP-2, MCP-3, MIP-1α, MIP-1β, and RANTES, are also found in monocytes and granulocytes. The present observations suggest that the IP10/Mig receptor is involved in the selective recruitment of effector T cells.

Chemokines constitute a family of small cytokines that are produced in inflammation and regulate leukocyte recruitment (1–3). Two subfamilies, CXC and CC chemokines, are distinguished by the arrangement of the first two of four conserved cysteines which are separated by one amino acid or are adjacent. Most CXC-chemokines attract neutrophil leukocytes whereas CC-chemokines are less selective and attract monocytes, eosinophil and basophil leukocytes, T-lymphocytes and natural killer cells. All chemokines act through G protein-coupled, seven transmembrane domain receptors (4, 5). Two of these, the interleukin-8 (IL-8)[1] receptors, IL-8R1 (6) and IL-8R2 (7), are largely restricted to neutrophil leukocytes and recognize the $NH_2$-terminal Glu-Leu-Arg (ELR) motif, an essential binding epitope in those CXC-chemokines that induce neutrophil chemotaxis (8–10). Five distinct CC-chemokine receptors have been described and designated CC-CKR1, 2, 3, 4, and 5 (11–17). They occur on several types of leukocytes, including monocytes, granulocytes and lymphocytes, and recognize CC but not CXC chemokines.

By contrast to monocytes and granulocytes, T-lymphocyte responses to chemokines are not well understood. Notably, none of the known receptors is expressed exclusively in lymphocytes and the chemokines that recognize these receptors cannot, therefore, account for the selective recruitment of T-lymphocytes that is observed in T cell–mediated inflammatory conditions. Here we describe a cDNA from human $CD4^+$ T cells, which was not present in monocyte or granulocyte derived cDNA libraries and which encodes a novel chemokine receptor that is selective for IP10 and Mig.

## Materials and Methods

*Human Chemokines.* The CXC-chemokines Mig, IL-8, GROα, NAP-2, GCP-2, ENA78, PF4, the CC-chemokines

[1] *Abbreviations used in this paper:* CC-CKR, CC-chemokine receptor; Mig, monokine induced by interferon-gamma; IP10, interferon-gamma inducible 10-kD protein; NAP-2, neutrophil-activating protein-2; ENA78, epithelial-derived neutrophil-activating peptide-78; GCP-2, granulocyte chemotactic protein-2; PF4, platelet factor-4; RANTES, regulated on activation, normal T cell expressed and secreted; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; $[Ca^{2+}]_i$, intracellular calcium ion concentration.

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MCP-1, MCP-2, MCP-3, MCP-4, MIP-1α, MIP-1β, RANTES, I309, eotaxin, and the chemokine-related lymphotactin were chemically synthesized according to established protocols (18). The CXC-chemokine IP10 was purchased from PeproTech (Rocky Hill, NJ).

*Cloning of Receptor cDNA.* Novel DNA fragments coding for putative T-lymphocyte-restricted chemokine receptors were generated using the following polymerase chain reaction (PCR) protocol. Two degenerate oligonucleotide primers to conserved motifs of chemokine receptors (5′-GGGCTGCAGCIIT(T/G) (T/G)C (C/A)GAC(A/C)TICTI(C/T)T and 5′-GGGTCTAGAIGG-GTTIAI(G/A)CA(G/A)C(T/A)(G/A)(T/C)G, I = inosine) were used to PCR amplify DNA fragments from human genomic DNA. The designed primer sequences were based on the highly conserved nucleotide sequences within transmembrane domain-2 (TM2) and TM7 of the chemokine receptors IL-8R1, IL-8R2, CC-CKR1, CC-CKR2 and the orphan receptors EBI I (19), LESTR (20) and BLR1/MDR15 (21, 22). 100 μl reaction mixture containing 2 μg human genomic DNA, 1×-DynaZyme buffer (Finnzymes OY, Espoo, Finland), 1.5 mM $MgCl_2$, 500 μM of each deoxynucleotide, 1 μM of both primers and 2.5-U of DynaZyme DNA polymerase was subjected to 30-cycles (94°C for 1 min, 55°C for 1 min, and 72°C for 2 min) on a DNA thermal cycler (Techne PHC-2, Brouwer, Switzerland). PCR products of the predicted size (~700-bp) were cloned into the Gene Scribe-Z vectors pTZ18/19-U/R (USB, Cleveland, OH), partially sequenced (23), and evaluated for their similarity to known chemokine receptors and expression of their corresponding mRNA in leukocytes. The DNA fragment 2MLC22 revealed 64% nucleotide sequence identity with IL-8R2 and specifically hybridized to RNA from T cells but not monocytes or neutrophils. Using 2MLC22 as screening probe, 23 positive clones were isolated from a human tetanus toxoid-specific $CD4^+$ T cell (KT30) cDNA library, prepared in lambda-ZAP Express (Stratagene, Zurich, Switzerland). The clone with the largest insert (1,670 bp) was sequenced to completion.

*Northern Blot Analysis.* 10-μg samples of total RNA were examined from freshly isolated human blood monocytes, neutrophils, lymphocytes (PBL), nylon wool-purified T cells, and from cultured cells, including cloned human $CD4^+$ and $CD8^+$ T cells (KT30 and ERCD8, respectively), cloned NK-cells (ERNK57) and PBL cultured for 10 d (1–2.5 × $10^6$ cells/ml in RPMI-1640 medium containing 2 mM glutamine, 1× non-essential amino acids, 1 mM sodium pyruvate, 100 μg/ml kanamycin, 5 × $10^{-5}$ M 2-mercaptoethanol, and 5% human serum) in the presence of 400 U/ml hrIL-2. RNA samples were analyzed with $^{32}P$-labeled 5′-fragment of the IP10/MigR DNA ($10^9$ cpm/μg DNA) at 5 × $10^6$ cpm/ml hybridization solution as described (20).

*Receptor Transfectants.* To generate stable transfectants, 4 × $10^6$ of either mouse pre-B cells (300-19) (24), human promyelocytic cells (GM-1) (25) or human acute T cell leukemia cells (Jurkat) (26) were transfected by electroporation with 20 μg of receptor cDNA in pcDNA3, linearized with BglII as described (27). IP10/MigR transfected cells were cloned by limited dilution under selection of G-418 (Life Technologies, Inc., Gaithersburg, MD) at 1.0 mg/ml for 300-19 and 0.8 mg/ml for Jurkat and GM-1 cells. G 418-resistant clones were screened for receptor expression by RNA Dot blot analysis.

*$Ca^{2+}$ Changes.* Changes in the cytosolic free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) were measured in cells loaded with fura-2 by incubation for 30 min at 37°C with 0.1 nmol fura-2 acetoxymethylester per $10^6$ cells in a buffer containing 136 mM NaCl, 4.8 mM KCl, 1 mM $CaCl_2$, 5 mM glucose, and 20 mM Hepes, pH 7.4. After centrifugation, loaded cells were resuspended in the same buffer ($10^6$ cells/ml), stimulated with the indicated chemokine at 37°C, and the $[Ca^{2+}]_i$-related fluorescence changes were recorded (28).

*Chemotaxis.* Cell migration was assessed in 48-well chambers (Neuro Probe, Cabin John, MD) using polyvinylpyrrolidone-free polycarbonate membranes (Nucleopore) with 5-μm pores for IP10/MigR transfected cells (26) or 3-μm pores for human PBL (29). RPMI 1640 supplemented with 20 mM Hepes, pH 7.4, and 1% pasteurized plasma protein solution (Swiss Red Cross Laboratory, Bern, Switzerland) was used to dissolve the chemokines (lower wells), and to dilute the cells (100,000 receptor transfectants or PBL in the upper well). After 60 min at 37°C, the membrane was removed, washed on the upper side with PBS, fixed and stained. All assays were done in triplicate, and the migrated cells were counted in five randomly selected fields at 1,000-fold magnification. Spontaneous migration was determined in the absence of chemoattractant.

## Results and Discussion

*Receptor cDNA.* During a search for T-lymphocyte-specific chemokine receptors we have isolated a cDNA from a human $CD4^+$ T cell library, which was not present in commonly used monocyte or granulocyte derived cDNA libraries. This cDNA, which is shown below to encode the IP10/Mig receptor, has an open reading frame of 1,104 bp corresponding to a protein of 368 amino acids with a molecular mass of 40,659 dalton. The sequence includes seven putative transmembrane segments, which are characteristic for G protein-coupled receptors, three potential *N*-glycosylation sites ($Asn^{22}$, $Asn^{32}$, and $Asn^{199}$), and one threonine and nine serine residues in the intracellular COOH-terminal region as potential phosphorylation sites for receptor kinases (30–32) (Fig. 1). A truncated version of this clone, with an incomplete coding sequence, was previously isolated from a human genomic DNA library (33). Alignment with the other chemokine receptors reveals several conserved motifs particularly in the transmembrane domains and the second intracellular loop. Considerable identity with IL-8R1 and IL-8R2, but not with CC-chemokine receptors is observed in the third and the sixth transmembrane domains (Fig. 1). The novel sequence shares 40.9 and 40.3% identical amino acids with the two IL-8 receptors, and 34.2 to 36.9% identity with the five known CC chemokine receptors (Table 1). A lower degree of similarity was found with seven-transmembrane-domain receptors that are expressed in T cells but do not bind chemokines, e.g., 27.2% identity with the thrombin receptor (34).

*Receptor Function in Transfected Cells.* To determine whether the receptor was functional, clones of murine pre-B cells (300-19), human promyelocytic cells (GM-1), and human T cell leukemia (Jurkat) were stably transfected with the new receptor cDNA. Activation of chemokine receptors leads to a transient rise of the cytosolic free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$), and this assay was used to monitor signaling in the transfected cells. A rapid $[Ca^{2+}]_i$ rise was observed in response to IP10 and Mig. IP10 was described more than ten years ago, and shown to be expressed in cu-

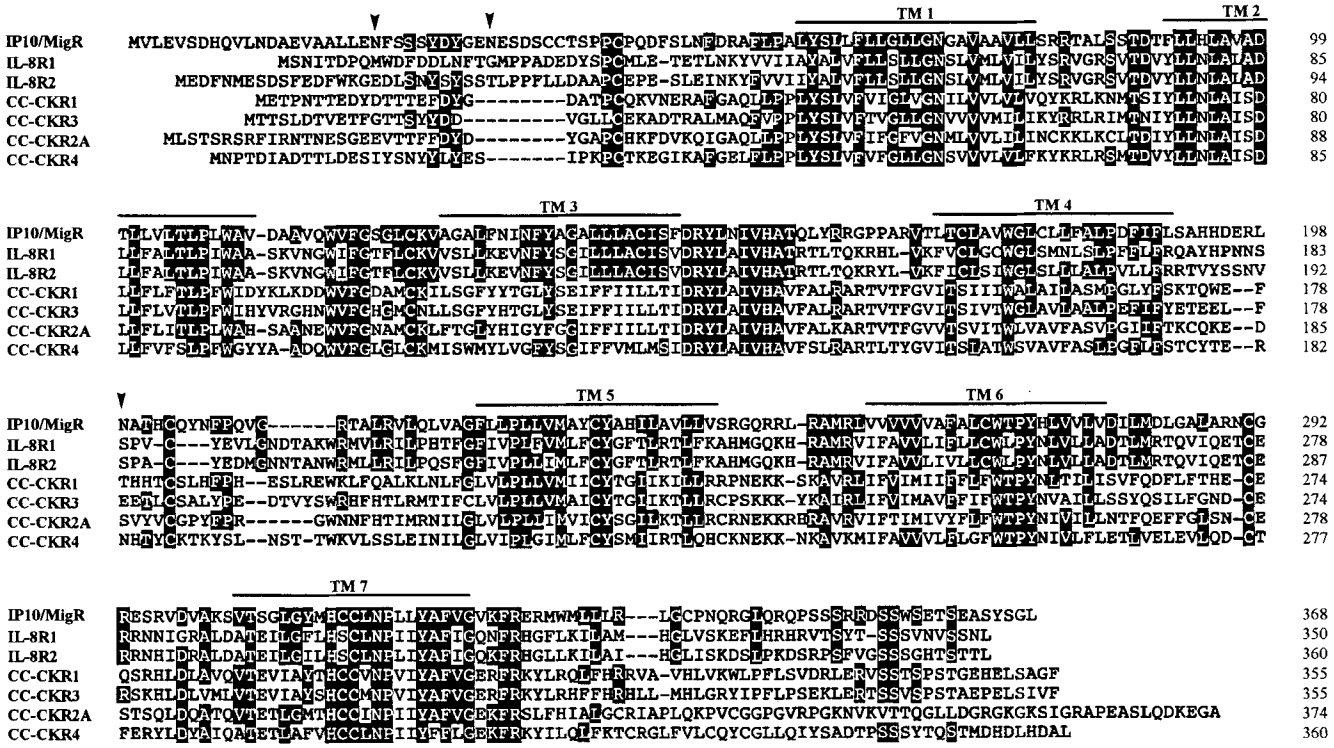


**Figure 1.** Amino acid sequence alignment of the novel receptor (IP10/MigR) with other human chemokine receptors. Multiple protein alignment was performed according to Higgins and Sharp (51). The black areas show regions of identity between IP10/MigR and at least two other chemokine receptors. Arrowheads indicate potential N-linked glycosylation sites and horizontal lines the putative transmembrane domains (TM1-TM7). Amino acids are abbreviated: *A*, Ala; *C*, Cys; *D*, Asp; *E*, Glu; *F*, Phe; *G*, Gly; *H*, His; *I*, Ile; *K*, Lys; *L*, Leu; *M*, Met; *N*, Asn; *P*, Pro; *Q*, Gln; *R*, Arg; *S*, Ser; *T*, Thr; *V*, Val; *W*, Trp; and *Y*, Tyr. These sequence data are available from EMBL/Genbank/DDBJ under accession number X95876.

taneous delayed-type hypersensitivity reactions (35, 36) while Mig was identified more recently (37, 38). Both chemokines have the CXC arrangement of the first two cysteines like IL-8, but are not chemotactic for neutrophil leukocytes. It was recently shown that IP10 attracts T-lymphocytes (39, 40) and that Mig is chemotactic for tumor-associated lymphocytes (41).

Fig. 2 summarizes the effects of IP10 and Mig on cells transfected with the cDNA and expressing the functional IP10/MigR. As shown by the $[Ca^{2+}]_i$ changes the action of IP10 and Mig was concentration dependent and already detectable at 1 nM, indicating that both chemokines have high affinity for the novel receptor. The IP10/MigR transfectants, by contrast, did not respond to any of 16 other

**Table 1.** *Amino Acid Sequence Comparison of IP10/MigR with Human Chemokine Receptors*

| | IL-8R1 | IL-8R2 | CC-CKR1 | CC-CKR2A | CC-CKR3 | CC-CKR4 | CC-CKR5 | ThromR |
|---|---|---|---|---|---|---|---|---|
| IP10/MigR | 40.9* | 40.3 | 34.9 | 34.2 | 34.4 | 35.8 | 36.9 | 27.2 |
| IL8R1 | | 77.1 | 33.7 | 32.9 | 34.3 | 39.7 | 34.3 | 29.1 |
| IL8R2 | | | 34.9 | 33.6 | 34.1 | 40.8 | 34.4 | 29.7 |
| CC-CKR1 | | | | 54.1 | 63.1 | 49.3 | 56.3 | 26.8 |
| CC-CKR2A | | | | | 50.7 | 46.1 | 68.8 | 24.6 |
| CC-CKR3 | | | | | | 46.5 | 52.3 | 27.3 |
| CC-CKR4 | | | | | | | 50.0 | 29.2 |
| CC-CKR5 | | | | | | | | 23.6 |

*Numbers refer to percentage amino acid identity. Pairwise protein sequence alignments were carried out using the program PALIGN with an open gap cost and unit gap cost of 3 and 2, respectively.

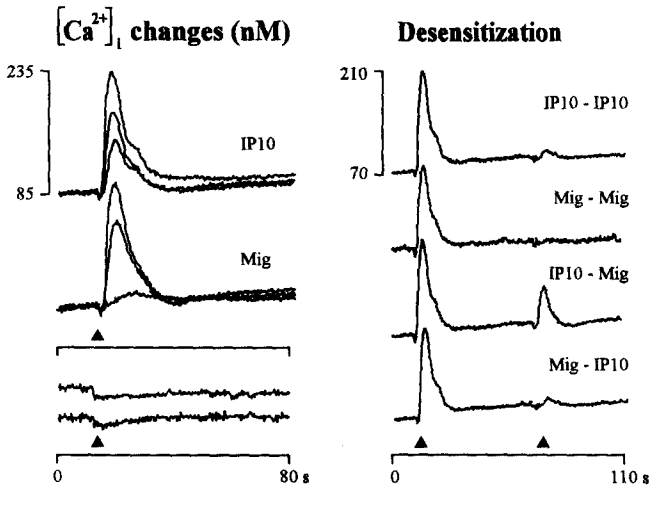


**Figure 2.** Responses induced by IP10 and Mig in stably transfected cells expressing IP10/MigR. Concentration-dependent $[Ca^{2+}]_i$ changes in IP10/MigR transfected 300-19 cells. IP10 and Mig were added at 1, 10, and 100-nM to Fura-2/AM loaded cells (*arrowhead*), and $[Ca^{2+}]_i$-dependent fluorescence changes were recorded. Non-transfected cells (*lower tracings*) were stimulated with IP10 or Mig at 100 nM under identical conditions. To test for receptor desensitization and cross-desensitization IP10/MigR expressing 300-19 cells were sequentially stimulated with 100 nM IP10 or Mig, and with IP10 followed by Mig or vice versa, and fluorescence changes were recorded. Chemotaxis of IP10/MigR expressing Jurkat cells stimulated with IP10 (*closed circles*) and Mig (*closed squares*) was determined as described in Materials and Methods. The lower panel shows responses of non-transfected Jurkat cells (*open symbols*). Mean numbers (± SD) of migrating cells per five high-power fields are presented.

potential agonists at concentrations up to 100 nM, including the CXC-chemokines IL-8, GROα, NAP-2, GCP-2, ENA78, PF4, the CC-chemokines MCP-1, MCP-2, MCP-3, MCP-4, MIP-1α, MIP-1β, RANTES, I309, eotaxin, and the chemokine-related lymphotactin. Identical results were obtained with the murine and the human transfected cells. These observations demonstrate that the novel receptor is highly selective for IP10 and Mig, and we propose to name it IP10/Mig receptor (IP10/MigR). As shown in Fig. 2, repeated stimulation with IP10 or Mig resulted in desensitization as commonly observed for chemokine receptors. Furthermore, cross-desensitization occurred when the cells were stimulated with IP10 followed by Mig or vice versa, confirming that the receptor has high affinity for both chemokines. At 100 nM concentration, it became evident that Mig was more potent in cross-desensitization than IP10, suggesting higher affinity or binding stability of IP10/MigR for Mig.

Transfected cells expressing the IP10/MigR readily migrated toward IP10 and Mig while the non-transfected, parental cells did not respond (Fig. 2). Both agonists showed a typically biphasic concentration dependence. IP10 induced migration at concentrations above 1 nM whereas the response of Mig became detectable above 10 nM. The efficacy, which is measured by the maximum number of migrating cells, was about twice as high for Mig than for IP10. These results demonstrate that the IP10/MigR, like all known chemokine receptors in leukocytes, signals for locomotion.

Despite the expression of functional IP10/MigR, we were unable to perform satisfactory binding experiments with radioactive ligands. Nonspecific binding was always between 60 and 80% of the total, preventing the determination of the binding parameters. Since IP10 and Mig are highly cationic (pI values of 10.8 and 11.1), unspecific interaction with cell surface proteoglycans may explain the anomalous behavior. Indeed, chemokine receptor-unrelated, heparinase-sensitive binding sites for IP10 (and PF4) were detected on a variety of blood and tissue cells (42), and we have observed that heparan sulfate binds IP10 and

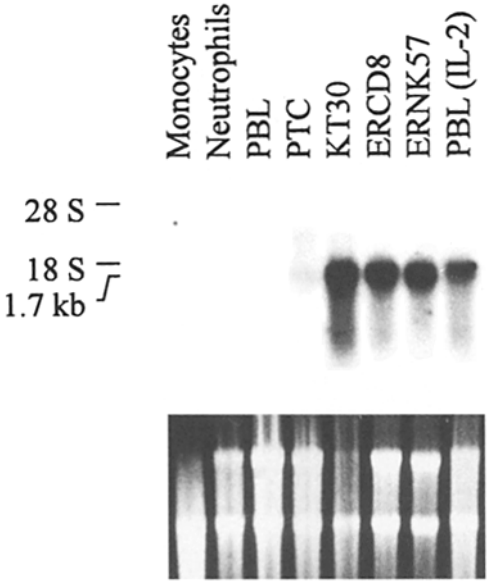


**Figure 3.** Expression of IP10/MigR RNA in human blood leukocytes. Northern blot analysis was performed with 10 μg of total RNA from freshly isolated human blood monocytes, neutrophils, lymphocytes (*PBL*), nylon wool-purified T cells (*PTC*), and from cultured cells, including cloned human $CD4^+$ and $CD8^+$ T cells (*KT30* and *ERCD8*, respectively), cloned NK cells (*ERNK57*) and PBL cultured for 10 d in the presence of IL-2 (400 U/ml). RNA samples were analyzed with a IP10/MigR DNA probe as described in Materials and Methods. Lower panel shows ethidium bromide-stained RNA in the agarose gel before blotting.

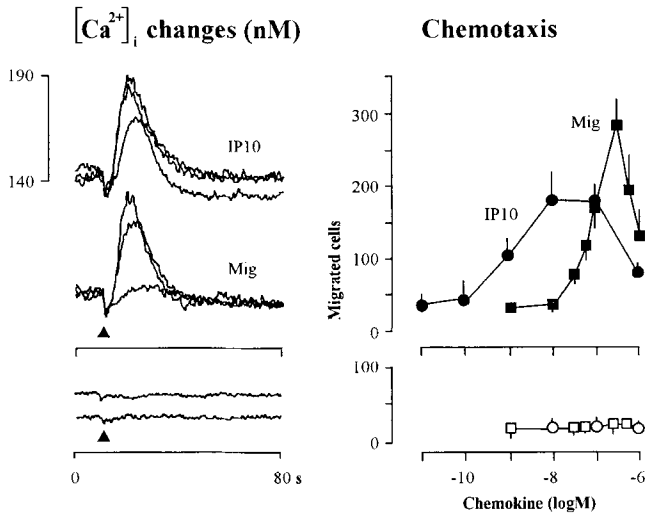


**Figure 4.** IP10 and Mig responses of peripheral blood lymphocytes (*PBL*). Freshly isolated PBL from donor blood buffy coats were used as such (*lower tracings* and *open symbols*) or after culturing for 10 d in the presence of IL-2 (400 U/ml) (*upper tracings* and *closed symbols*). $[Ca^{2+}]_i$ changes and chemotaxis were determined as in Fig. 2.

Mig and prevents lymphocyte chemotaxis (data not shown).

*Expression in Activated T-Lymphocytes.* In view of the observed chemokine selectivity it was of interest to examine the occurrence of the IP10/MigR in leukocytes and related cell lines. As shown in Fig. 3, abundant expression of mRNA of the expected size was found in the cloned $CD4^+$ T cells, KT30, that were used for isolation of the receptor cDNA. Similar levels of expression were observed in the $CD8^+$ T cell clone, ERCD8, and the NK cell clone, ERNK57. In freshly isolated blood lymphocytes and nylon wool-purified T cells, by contrast, IP10/MigR transcripts were barely detectable. However, when these cells were cultured in the presence of IL-2, a strong upregulation was obtained, and the level of receptor mRNA approached that of T and NK cell clones. No IP10/MigR transcripts were found in freshly isolated blood monocytes, neutrophil leukocytes, eosinophil leukocytes. Additional leukocyte-related cells that did not express IP10/MigR mRNA include the mast cell line, HMC-1, the promyelocytic leukemia line, HL60, the histiocytic lymphoma, U937, the chronic myelogeneous leukemia line, K562, the acute T cell leukemia line, Jurkat, the acute lymphoblastic leukemia line, Molt, the B-lymphoblastic cell lines Daudi and Raji, lymphocytes from patients with chronic and acute B-lymphoid leukemia (B-CLL and B-ALL), mature basophils from a patient with basophilic leukemia, and the erythroleukemia cell line, HEL. By contrast, the receptors for chemokines which have been shown previously to attract lymphocytes, i.e. MCP-1, MCP-2, MCP-3, MIP-1α, MIP-1β and RANTES (29, 43–46), are also found in monocytes and granulocytes. Therefore, the exclusive expression of IP10/MigR in activated T-lymphocytes is an exciting finding and suggests that this novel receptor may mediate selective lymphocyte recruitment.

*Responses of Human T-Lymphocytes.* In agreement with the cellular distribution of the IP10/MigR, we found that activated human T-lymphocytes are highly responsive to IP10 and Mig (Fig. 4). The activity of IP10 and Mig as inducers of $[Ca^{2+}]_i$ changes and in vitro chemotaxis was consistent with the effects observed in the transfected cells expressing the IP10/MigR as IP10 was more potent but less efficacious than Mig. Activation of the T-lymphocytes by culturing in the presence of IL-2 was required, and no response was observed with freshly isolated blood lymphocytes.

Two aspects of the present study are noteworthy: The ligand selectivity of the novel receptor and its restricted expression in activated T-lymphocytes. The receptor recognizes two unusual chemokines, IP10 and Mig. They both belong to the CXC-subfamily, but their target cells are lymphocytes and not neutrophil leukocytes which respond to IL-8 and its numerous CXC-chemokine analogs. The expression is also unusual. IP10 and Mig are induced by interferon-gamma which down-regulates the expression of IL-8 (47, 48). In recent years, chemokines were recognized as the long-sought mediators for the recruitment of lymphocytes. Several CC-chemokines were found to elicit lymphocyte chemotaxis (29), but they are also active on monocytes and granulocytes (49, 50). The situation is different for IP10 and Mig which do not share receptors with other chemokines, and are selective for activated T-lymphocytes. From the present observations it may be inferred that the formation of the characteristic infiltrate in delayed-type hypersensitivity lesions, sites of viral infection and certain tumors may be regulated via IP10/MigR expression. T-lymphocytes that bear this receptor as a result of activation are recruited into the lesion by IP10 and Mig which are induced locally by interferon-gamma. So far this is the only mechanism that can be proposed for the selective recruitment of T cells.

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