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MIP-3α, MIP-3β and fractalkine induce the locomotion and the mobilization of intracellular calcium, and activate the heterotrimeric G proteins in human natural killer cells

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SUMMARY

We demonstrate here that the CC chemokines macrophage inflammatory protein-3α (MIP-3α), macrophage inflammatory protein-3β (MIP-3β) and the CX_3C chemokine fractalkine induce the chemotaxis of interleukin-2 (IL-2)-activated natural killer (IANK) cells. In addition, these chemokines enhance the binding of [γ-^{35}S]guanine triphosphate ([γ-^{35}S]GTP) to IANK cell membranes, suggesting that receptors for these chemokines are G protein-coupled. Our results show that MIP-3α receptors are coupled to G_o, G_q and G_z, MIP-3β receptors are coupled to G_i, G_q and G_s, whereas fractalkine receptors are coupled to G_i, and G_z. All three chemokines induced a robust calcium response flux in IANK cells. Cross-desensitization experiments show that MIP-3α, MIP-3β or fractalkine use receptors not shared by each other or by the CC chemokine regulated on activation, normal, T-cell expressed, and secreted (RANTES), the CXC chemokines stromal-derived factor-1α (SDF-1α) and interferon-inducible protein-10 (IP-10), or the C chemokine lymphotactin.

INTRODUCTION

Depending on the presence and the arrangement of the cysteine residues in the amino terminal of the motif protein, chemokines have been divided into three subfamilies; these are C (γ), CC (β) and CXC (α), where X is any amino acid that separates the cysteine residue in the chemokine N-terminal region.[1] Recently, multiple new chemokines have been cloned. In fact, the pace at which new members of chemokines have been cloned has surpassed the rate at which their biological functions have been examined, and aside from their reported chemotactic effects on T lymphocytes, no other biological roles for these chemokines are known. Of these, macrophage inflammatory protein-3α (MIP-3α) and macrophage inflammatory protein-3β (MIP-3β),[2,3] which belong to the CC chemokine subfamily, whereas fractalkine or neurotactin,[4,5] which belongs to a fourth subfamily of chemokines known as CX_3C (δ), have been recently characterized.

The family of chemokines has gained much attention lately owing to the ability of members of the CC chemokines, and in particular MIP-1α, MIP-1β and regulated on activation, normal, T-cell expressed, and secreted (RANTES) to inhibit the replication of the non-syncytium-forming M-tropic human immunodeficiency virus-1 (HIV-1),[6] whereas a member of the CXC chemokine, stromal-derived factor-1 (SDF-1), inhibits the replication of the syncytium forming T-tropic HIV-1.[7,8] The anti-HIV-1 activity could be due to receptor occupancy by the chemokines: CCR5 is occupied by RANTES, MIP-1α and MIP-1β; CCR3 is occupied by Eotaxin, RANTES, MCP-3 and MCP-4; whereas CCR2b is occupied by members of the monocyte chemotactic peptide (MCP), hence inhibiting the entry and replication of the M-tropic HIV-1 strain into $CD4^+$ cells. Similarly, CXCR4 is occupied by SDF-1, inhibiting the entry and replication of the T-tropic HIV-1 strain into target cells. However, these chemokines also activate natural killer (NK) cells.[9,10] Recent findings showing that the CC chemokine macrophage-derived chemokine (MDC) inhibits both the M-tropic and T-tropic HIV-1 strains[11] further implicate NK cells as major players in the chemokine anti-HIV effect. MDC does not bind CCR5, CCR3, CCR2b or CXCR4, but it does activate NK cells.[12] NK cells are effector cells against viral infection, control tumour metastases, and play important roles in allogeneic transplantation.[13] We have previously reported that the CXC chemokines interleukin-8 (IL-8),[14] interferon-inducible protein-10 (IP-10),[15] or SDF-1α[16] influence the

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Abbreviations: ELC, EBI1-ligand chemokine; FK, fractalkine; G Proteins, guanine nucleotide-binding proteins; IANK cells, interleukin-2-activated natural killer cells; IP-10, interferon-inducible protein-10; Ltn, lymphotactin; MDC, macrophage-derived chemokine; MIP-3α, macrophage inflammatory protein-3α; MIP-3β, macrophage inflammatory protein 3β; NRS, normal rabbit serum; RANTES, regulated upon activation normal T-cell expressed and secreted; SDF-1, stromal derived factor-1.

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motility of NK cells. Furthermore, we and others have shown that members of the CC chemokines,[17–20] or the C chemokine lymphotactin[15,21,22] induce the chemotaxis of these cells.

Receptors for chemokines are G protein-coupled.[1,23] Similarly, the ability of the CXC, C or CC chemokines to induce the motility of NK cells is linked to the ability of these chemokines to activate the heterotrimeric G proteins in the membranes of NK cells.[14–17] These G proteins are composed of three subunits α, β, and γ. About 20 α, 6 β and 12 γ subunits have been reported.[24] The α subunits belong to four subfamilies: A. α_s (α_s and α_{olf}), B. α_i (α_{i1}, α_{i2}, α_{i3}, α_{o1}, α_{o2}, α_z, α_t, α_{gus}, α_{con}, and α_{rod}), C. α_q (α_q, α_{11}, α_{14}, α_{15}, and α_{16}), and D. α_{12} (α_{12} and α_{13}). We were the first to report that the CC chemokine receptors expressed in NK cells are promiscuously coupled to multiple G proteins.[25] Similar coupling of chemokine receptors in various other cell types has been recently reported by other investigators.[26,27] Here, we have investigated whether MIP-3α, MIP-3β or fractalkine influence the motility of NK cells, and whether they induce the mobilization of intracellular calcium. Also, we examined the coupling of these chemokine receptors to G proteins.

MATERIALS AND METHODS

Culture medium

Culture medium (CM) consisted of RPMI-1640 supplemented with 10 U/ml penicillin, 100 μg/ml streptomycin, 1 mM L-glutamine, 1% non-essential amino acids (GIBCO BRL, Paisley, UK), 10% human AB serum and 5×10^{-5} M 2-mercaptoethanol (Sigma Chemicals, St Louis, MO).

Reagents

Human recombinant fractalkine (FK), MIP-3α, MIP-3β, IP-10 and RANTES were purchased from R & D Systems Europe Ltd (Oxford, UK). SDF-1α was from Peprotech EC Ltd (London, UK), and lymphotactin (Ltn) was from Genzyme (Cambridge, MA). Leupeptin, aprotinin, pepstatin A, PMSF, DTT, Tris HCl, HEPES, EDTA, EGTA, and $MgCl_2$ were purchased from Sigma Chemical Co. [γ-^{35}S]GTP (1000 Ci/mM) was from NEN-Dupont (Brussels, Belgium). Rabbit polyclonal antibodies to G_q, G_s, G_z G_i, and G_o were purchased from either NEN Life Science Products (Brussels, Belgium), or from Gramsch Laboratories (Schwabhausen, Germany).

Preparation of IANK cell membranes

IANK cells were prepared as described.[14–17] Their membranes were prepared in a lysis buffer containing 10 mM HEPES buffer, pH 7·5, 3 mM $MgCl_2$, 2 mM EDTA, 40 μg/ml PMSF, 10 μg/ml leupeptin, 2 μg/ml pepstatin A and 2 μg/ml aprotinin. After homogenization and sonication, they were centrifuged at 1000 *g* for 10 min and the supernatant was transferred into Beckman tubes and ultracentrifuged at 150 000 *g* for 30 min at 4°. The membranes were transferred into a buffer containing 10 mM HEPES, 3 mM $MgCl_2$, and 2 mM EDTA.

Chemotaxis assay

This was carried out as previously described.[14–17] Nuclepore blind-well chemotaxis chambers with a lower well volume of 200 μl were used. A maximum volume of 200 μl medium containing RPMI plus 1% bovine serum albumin was placed in the lower wells in the presence or absence of various agents. Cells (4×10^5) were placed in the upper compartments of Boyden chambers above the filters. The chambers were incubated for 2 hr at 37° in a 5% CO_2 incubator. The filters were removed, dehydrated, and stained with 15% modified Wright stain for 7 min and then mounted on glass slides using a drop of immersion oil between the filters and the slides. Cells in 10 high-power fields from two filters were counted and averaged for each sample. The migration index was calculated as the number of cells migrating toward the concentration gradients of chemokines, divided by the number of cells migrating toward medium only.

GTP binding assay

IANK cell membranes were incubated with various concentrations of MIP-3α, MIP-3β or fractalkine for 3 min, and then added to the binding buffer that contained 20 mM HEPES/NaOH, pH 7·4, 0·1 mM EDTA, 0·125 mM $MgCl_2$ and 10 nM (γ-^{35}S)GTP (1000 Ci/mM). The mixtures were incubated at 37° for 15 min, and the reactions were terminated by the addition of 900 μl of ice-cold buffer containing 100 mM Tris-HCl, pH 8·0, 25 mM $MgCl_2$, 100 mM NaCl, and 20 μM cold GTP. The mixtures were incubated on ice for 1 hr, washed several times with ice-cold phosphate-buffered saline (PBS) plus 0·05% Tween 20, and centrifuged at 14 000 r.p.m. at 4° using an Eppendorf centrifuge. The pellets were then suspended in a scintillation cocktail and counted in a beta counter. Non-specific binding was determined by the addition of cold GTP.

In addition, another method[28] was utilized to determine the exact nature of G protein activated with the chemokines. In this, immunomagnetic beads (Dynabeads, Dynal, Oslo, Norway) coated with sheep anti-rabbit IgG were incubated for 2 hr at 4° with rabbit anti-G proteins, rabbit immunoglobulin G (IgG) or normal rabbit serum (NRS) in a PBS buffer containing 1% bovine serum albumin (BSA). IANK cell membranes incubated first with 100 pg/ml MIP-3α, MIP-3β or fractalkine, were added to the GTP binding buffer plus (γ-^{35}S)GTP and then mixed with anti-G protein-, IgG-or NRS-coated beads, washed with PBS buffer plus 0·05% Tween 20, suspended in the scintillation mixture and transferred to scintillation vials. All assays were performed in triplicate.

Measurement of intracellular Ca^{++}

IL-2-activated NK cells were incubated in a buffer containing 145 mM NaCl, 5 mM KCl, 10 mM Na/MOPS, 1 mM $MgCl_2$, 10 mM glucose, 0·25% BSA, 10 mM HEPES, pH 7·4, and 2·5 μM Fura-2 AM (Sigma Chemicals) for 30 min at 37°. The cells were washed and resuspended at a concentration of 1×10^5 cells/ml and then incubated with the appropriate concentration of chemokines. Accumulation of intracellular calcium was measured according to Grynkiewicz *et al.*[29] A dual-excitation luminescence spectrometer system (Perkins Elmer LS 50B; Perkins Elmer, Buckinghamshire, UK) containing 340 nm and 380 nm band-pass filters was used. The emission was determined at 510 nm and the intensity was measured by using the photomultiplier tube system. Cell-free conditions were used to determine the background fluorescence. Correction of the data was performed before the calculation of relevant fluorescence intensity (340:380 ratio).

Statistics
Significant values were determined using the two-tailed Student's *t*-test.

RESULTS

MIP-3α, MIP-3β and fractalkine induce the motility of NK cells

To determine whether MIP-3α, MIP-3β or fractalkine may induce the motility of IANK cells, various concentrations (ranging between 10 fg/ml–10 ng/ml) of these chemokines were placed in the upper and lower wells of Boyden chambers. Figure 1, upper panel (a), shows that concentrations of 0·1–10 ng/ml of MIP-3α are chemoattractants for these cells ($P<0·003$, compared with cells migrating in the presence of medium only). Similarly, MIP-3β induced the chemotaxis of IANK cells as shown in Fig. 1, middle panel. The minimum concentration required was 10 pg/ml ($P<0·001$). However, as low as 100 fg/ml of fractalkine induced the chemotaxis of NK cells ($P<0·0001$, compared with cells migrating in the medium only). Fractalkine induced a typical bell-shaped chemotactic response in IANK cells, where 1 pg/ml, 10 pg/ml and 100 pg/ml doses are chemoattractants ($P<0·0002$, $P<0·001$ and $P<0·0002$, respectively), whereas 1 ng/ml and 10 ng/ml doses are not. In addition, fractalkine induced the motility of IANK cells in the no-gradient as shown in Fig. 1, lower panel (b) ($P<0·01$, as compared with cells migrating in the absence of the chemokine). Higher concentrations of fractalkine (0·1 ng/ml and 1 ng/ml) also induced the motility of these cells in the negative gradients of Boyden chambers ($P<0·01$) as shown in Fig. 1, lower panel (c).

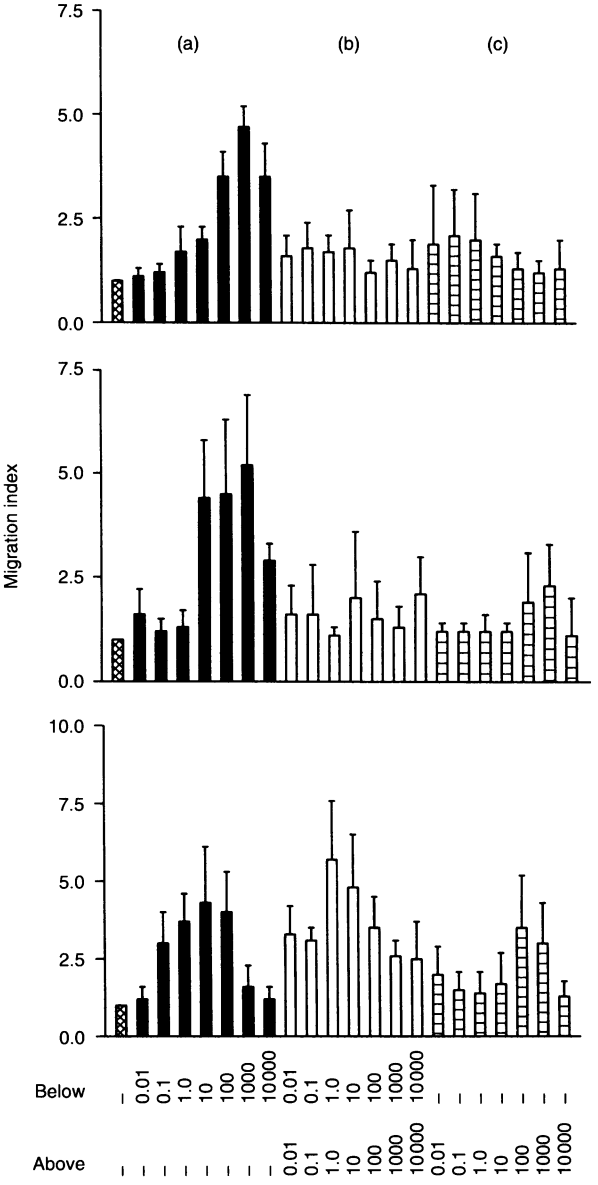

Figure 1. Effect of MIP-3α (upper panel), MIP-3β (middle panel) and fractalkine (lower panel) on the *in vitro* motility of IANK cells. Various concentrations (0·01–10 000 pg/ml) of these chemokines were placed in the upper (above) or the lower (below) wells of Boyden chambers. (a) (solid columns) shows the chemotactic response in the positive gradients of the chambers. (b) (blank columns) shows the chemokinetic response in the no-gradients of the chambers. (c) (shaded columns) shows the response in the negative gradients of Boyden chambers. The migration index was calculated as the number of cells migrating in the presence of the chemokines divided by the number of cells migrating in the presence of medium only. Mean ± SD of six separate experiments.

MIP-3α, MIP-3 β and fractalkine activate the heterotrimeric G proteins in NK cell membranes

Receptors for chemokines are coupled to G proteins. In order to demonstrate whether MIP-3α, MIP-3β or fractalkine receptors are coupled to G proteins, we activated IANK cell membranes with various concentrations of these chemokines in the presence of (γ-^{35}S) GTP. Figure 2 shows that concentrations between 0·01 ng/ml and 10 ng/ml of either MIP-3α (a), MIP-3β (b) or fractalkine (c) enhance the GTP binding in IANK cell membranes ($P<0·04$, as compared with basal GTP binding). To determine the nature of the G protein subtypes coupled to these chemokine receptors, we activated IANK cell membranes with these chemokines, incubated the membranes with (γ-^{35}S) GTP, and added them to Dynabeads coated with either anti-G proteins, IgG or normal rabbit serum (NRS). The results in Fig. 3(a), shows that MIP-3α enhanced the GTP binding to G_o, G_q and Gz ($P<0·01$, compared with NRS- or rabbit IgG-activated membranes), whereas MIP-3β enhanced the GTP binding to G_i, G_s and G_q ($P<0·01$; Fig. 3b), and fractalkine to G_i and G_z ($P<0·01$; Fig. 3c).

MIP-3α, MIP-3β and fractalkine induce the accumulation of $(Ca^{++})i$ in NK cells

The ability of MIP-3α, MIP-3β or fractalkine to induce the accumulation of $(Ca^{++})_i$ in IANK cells generated from six donors, was investigated. Figure 4 shows that MIP-3α (4a), MIP-3β (4g) or fractalkine (4m) induced a robust calcium flux response in these cells. Desensitization experiments show that MIP-3α desensitized the calcium mobilization effect of MIP-3α (4a), but not of fractalkine (4b), RANTES (4c), IP-10 (4d), lymphotactin (4e) or SDF-1α (4f). Similarly, MIP-3α did not desensitize the effect of MIP-3β (not shown). MIP-3β desensitized the calcium mobilization activity of MIP-3β (4g), but there was no desensitization among MIP-3β

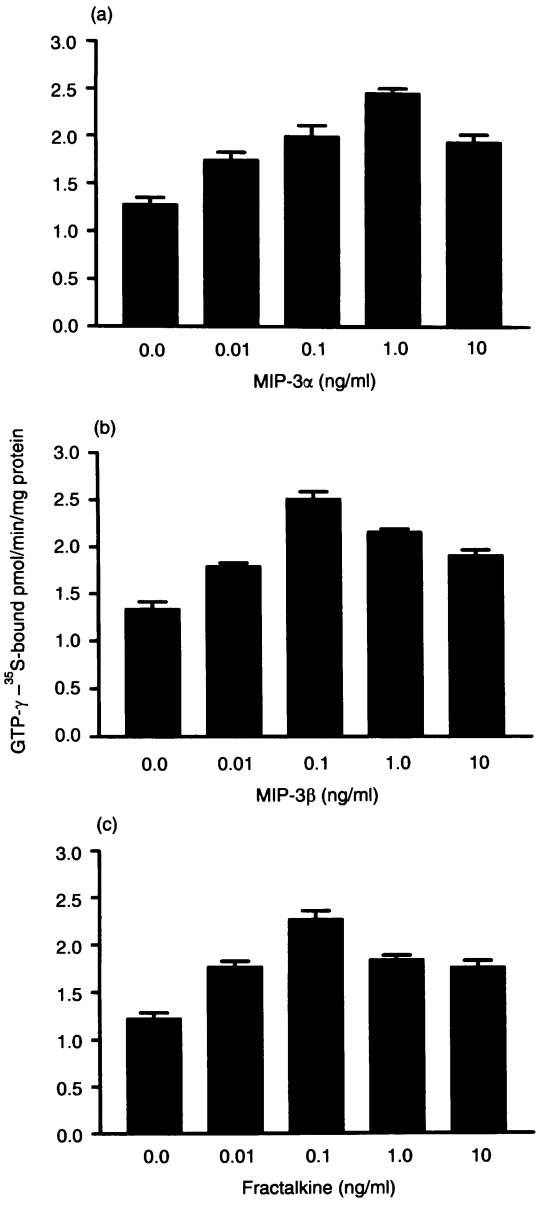

Figure 2. MIP-3α, MIP-3β and fractalkine enhance the GTP binding in IANK cell membranes. IANK cell membranes were incubated with buffer (basal binding) or with various concentrations of MIP-3α (upper panel), MIP-3β (middle panel) and fractalkine (lower panel) in the presence of (γ-^{35}S)GTP. Values are the mean ± SD of triplicate determinations. One of three experiments.

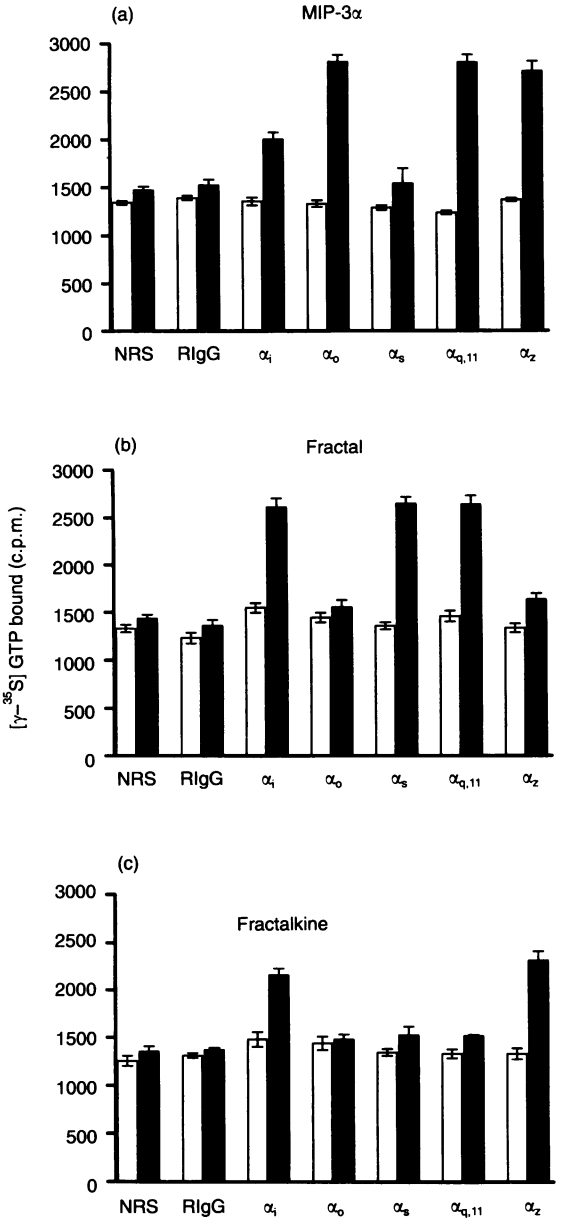

Figure 3. Nature of G proteins activated by MIP-3α, MIP-3β or fractalkine in IANK cell membranes. Dynabeads were coated with sheep anti-rabbit IgG, and were incubated with rabbit antibody to various G protein α-subtypes, rabbit IgG or normal rabbit serum (NRS). IANK cell membranes were stimulated with 100 pg/ml MIP-3α, MIP-3β or fractalkine, incubated with (γ-^{35}S) GTP for 15 min, and added to the beads. White columns represent unstimulated membranes, and solid black columns represent MIP-3α- (upper panel), MIP-3β-(middle panel) or fractalkine- (lower panel) stimulated membranes. Values are the mean ± SD of triplicate determinations. One of four experiments.

and fractalkine (4h), RANTES (4i), IP-10 (4j), lymphotactin (4k) or SDF-1α (4l). On the other hand, fractalkine desensitized the calcium accumulation effect of fractalkine (4m), but there was no desensitization with MIP-3β (4n), RANTES (4o), IP-10 (4p), lymphotactin (4q) or SDF-1α (4r). However, in IANK cells generated from one donor, we observed that addition of MIP-3β first, desensitized the calcium mobilization flux induced by fractalkine, but addition of fractalkine first did not desensitize the activity of MIP-3β (data not shown). This result suggests that MIP-3β receptors may distribute at NK clonal level. However, this activity was not examined further, as blood samples from this donor are no longer available.

DISCUSSION

Chemokines are important mediators of inflammatory reactions, and are required to induce the extravasation of various

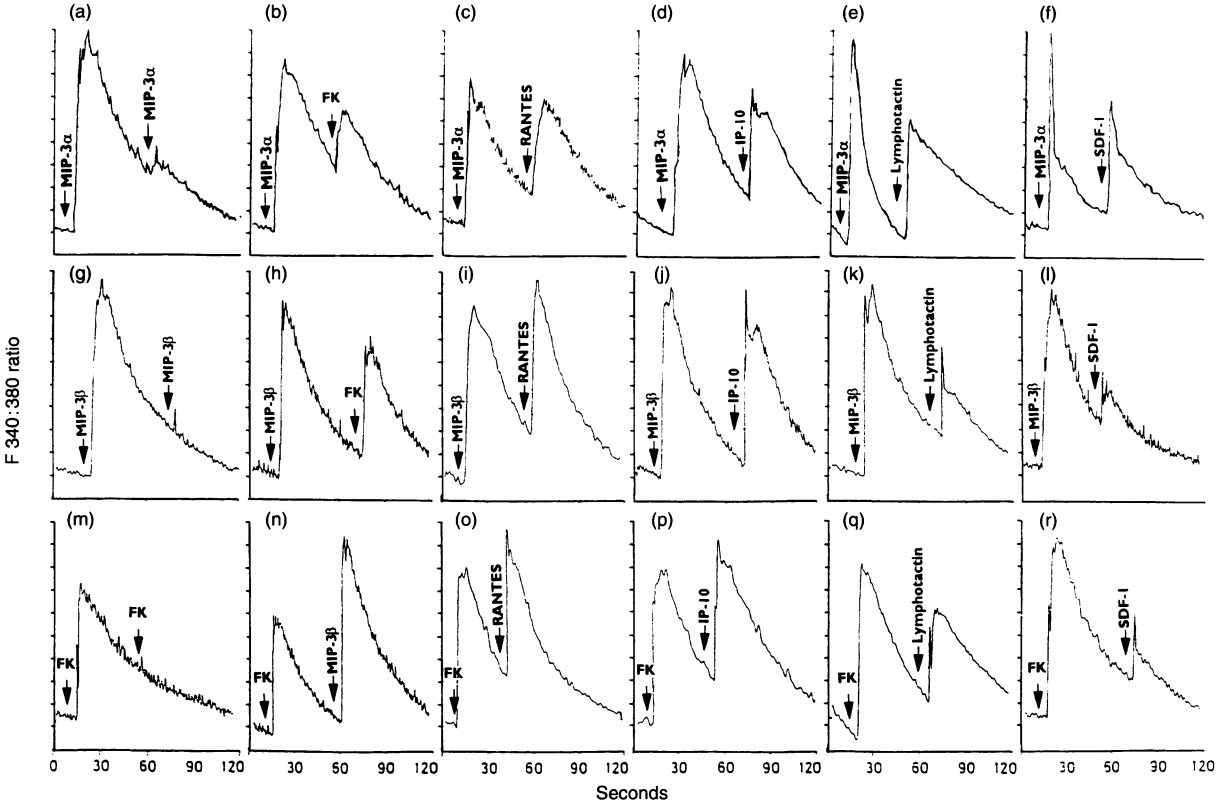

Figure 4. MIP-3α, MIP-3β and fractalkine induce the mobilization of $(Ca^{++})_i$ in IANK cells. In panels (a)–(f), 100 pg/ml of MIP-3α was added prior to the addition of 100 pg/ml fractalkine, 100 pg/ml RANTES, 200 pg/ml IP-10, 100 pg/ml lymphotactin, or 1 ng/ml SDF-1α. In panels (g)–(l), 100 pg/ml of MIP-3β was added prior to the addition of other chemokines, whereas in panels (m)–(r) 100 pg/ml of fractalkine was added prior to the other chemokines. Arrows indicate the time of addition of chemokines. Representative experiment of six donors.

cell types into inflammatory tissues.[1] Their importance is highlighted by the ability of certain chemokines to inhibit the replication of HIV-1.[6–8] Although this activity has been suggested to be due to competition with the virus for the chemokine receptors that act as the virus co-receptors, chemokines also activate the anti-viral effectors NK cells,[9,10,18] providing an alternative and/or an additional mechanism to the anti-HIV effect of chemokines.

Chemokines are divided into four subfamilies: 1. C chemokine (lymphotactin); 2. CC chemokines (MIP-1α, MIP-1β, MCP1-5, Eotaxin, RANTES, I-309, HCC, MIP-3α, MIP- 3β, and 6Ckine, among others); 3. CXC chemokines (IL-8, NAP-2, GRO-α, GRO-β, GRO- γ, IP-10, SDF-1, GCP-2, PF4, MIG, and ENA, among others); and 4. CX_3C (fractalkine or neurotactin). MIP-3α, MIP-3 β, and fractalkine are newly cloned chemokines. Their biological significance is not yet known. In addition, five CXC chemokine receptors (CXCR 1–5), and nine CC chemokine receptors (CCR 1–9) have been cloned. Of these, CCR6 binds MIP-3α,[30,31] and CCR7 binds MIP-3β.[3] Recently, fractalkine receptors have been identified,[32] and are shown to be highly expressed in NK cells. Imai *et al.*[32] also reported that fractalkine induces the chemotaxis of freshly isolated NK cells. However, the effect of this chemokine on IL-2-activated NK cells was not examined, and neither was an indication that this chemokine induces the mobilization of intracellular calcium. More important, it is not known whether fractalkine activates G proteins in NK cell membranes. These issues are examined in the present study.

Our results show that fractalkine induces both the chemotaxis and the chemokinesis activities in NK cells. This finding suggests that this chemokine is not only important during inflammation where chemokines form concentration gradients, but also during normal physiological conditions where the cells migrate from one place to another, and extravasate into various organs such as the liver, the lungs, and possibly the lymph nodes. This activity of fractalkine may fulfil its unusual structure; in contrast to other chemokines, fractalkine is present not only in a soluble form, but also as a part of an adhesion vehicle, being bound to the surface of the endothelial cells.[4,5] On the other hand, MIP-3α and MIP-3β induce only the chemotaxis of NK cells, resembling the effect of other CC chemokines such as MIP-1α, MCP-1, and RANTES.[17–20]

Receptors for chemokines are G protein-coupled.[1,23,33] Members of the heterotrimeric G proteins perform a unique biological function. For example, G_s induces the accumulation of cAMP, whereas G_i inhibits such accumulation. G_o activates ion channels, G_q activates phospolipase β, and G_z induces cellular proliferation, and NK cell cytotoxicity (reviewed in 33). We have previously shown that chemokine receptors are promiscously coupled to various heterotrimeric G proteins.[25] Similar coupling has been observed by others who examined the coupling of CC chemokine receptors to G proteins in

co-transfected COS-7 cells,[26] or the coupling of the CXC chemokine SDF-1 to the heterotrimeric G proteins in astrocytes and microglial cells.[27] Similarly, we observed that the CXC chemokine IP-10[15] or SDF-1α[16] receptors are coupled to various heterotrimeric G proteins. Also, we suggested that there must be a cross-talk between these G proteins in order for the signal to be ultimately transduced inside NK cells.[15,33] Recent work[34] supported this conclusion. Those investigators reported that the β_2-adrenergic receptor (a heptahelical receptor) is coupled to G_s and G_i, and that the signal can switch between these G proteins, depending on the activation of second messengers such as protein kinase A. An interplay among cholera toxin-sensitive and pertussis toxin-sensitive G proteins through cyclic nucleotides has been previously suggested by us.[35] Therefore, coupling of receptors such as the chemokine receptors to multiple heterotrimeric G proteins should be viewed as a normal physiological phenomenon provided to fulfil the need of the cells to amplify the intracellular signals, and to appropriately transduce these signals in order to perform the function desired (e.g. chemotaxis).

Although it was suggested that CCR6, which binds MIP-3α, is not present on NK cell clones,[31] the presence of this receptor on freshly isolated or IL-2-activated NK cells has not been examined. Our results showing that IL-2-activated NK cells respond to MIP-3α, suggest that receptors for this chemokine are present on these cells. Work is in progress to resolve this issue. MIP-3β binds to the CCR7[3] which has been previously cloned and was termed as Burkitt's lymphoma receptor 2 'BLR2'[36] or Epstein–Barr virus-induced gene 1 'EBI'.[37] This receptor has been shown to be G protein-coupled.[36,37] In order to determine whether MIP-3α, MIP-3β or fractalkine receptors are coupled to G proteins in NK cells, we examined the ability of these chemokines to enhance the GTP binding in NK cell membranes. Our results clearly show that all three chemokines increase the binding of GTP to these membranes. It is worth noting that 1 ng/ml and 10 ng/ml of fractalkine enhance the GTP binding, but not the chemotaxis in NK cells. It is possible that these high concentrations of fractalkine may induce receptor desensitization by activating the G protein-coupled receptor kinases (GRKs). These GRKs are recruited to the membranes by the βγ subunits of the activated G proteins as a result of ligand–receptor interaction. GRK phosphorylate G protein-coupled receptors, and inhibit the coupling of these receptors to G proteins leading to receptor desensitization.[38] This may explain why high concentrations of fractalkine activated G proteins, but did not induce chemotaxis.

To examine the nature of G proteins coupled to fractalkine, MIP-3α or MIP-3β receptors, we stimulated IANK cell membranes with these chemokines, and then bound them to anti-G proteins immobilized on immunobeads. This method specifically picks those G proteins that are activated in the membranes. It appears that MIP-3α activates G_o, G_q and G_z but not G_i or G_s, MIP-3β activates G_i, G_q and G_s but not G_o or G_z, and fractalkine activates G_i and G_z but not G_o or G_s. Hence, receptors for these chemokines discriminate at their coupling to the hetetrotrimeric G proteins, providing some possibility of signalling specificity.

MIP-3α, MIP-3β and fractalkine induce robust calcium fluxes in IANK cells seconds after their addition to Fura-2AM-loaded cells. In cross-desensitization experiments performed on IANK cells generated from six donors, homologous but not heterologous desensitization was observed, i.e. MIP-3α desensitized the calcium response to MIP-3α but not to the C chemokine lymphotactin, the CC chemokines RANTES and MIP-3β, the CXC chemokines IP-10 and SDF-1α, or the CX_3C chemokine fractalkine. Similarly, MIP-3β desensitized the calcium mobilization to MIP-3β but not to lymphotactin, RANTES, IP-10, SDF-1α or fractalkine. Similar homologous, but not heterologous, desensitization was observed when fractalkine was used as a stimulus. These results indicate that MIP-3α, MIP-3β and fractalkine utilize receptors not shared among these three chemokines or among other chemokines. In summary, our results are the first to show that the newly described CC chemokines MIP-3α, MIP-3β and the CX_3C chemokine fractalkine induce IL-2-activated NK cell chemotaxis, and enhance the accumulation of intracellular calcium in these cells.

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