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*J Immunol* 2000; 164:4048-4054; doi: 10.4049/jimmunol.164.8.4048

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Human NK Cells Express CC Chemokine Receptors 4 and 8 and Respond to Thymus and Activation-Regulated Chemokine, Macrophage-Derived Chemokine, and I-309

Marit Inngjerdingen,* Bassam Damaj,† and Azzam A. Maghazachi‡

NK cells respond to various chemokines, suggesting that they express receptors for these chemokines. In this paper, we show that IL-2-activated NK (IANK) cells express CC chemokine receptor 4 (CCR4) and CCR8, as determined by flow cytometry, immunoblot, and RNase protection assays. Macrophage-derived chemokine (MDC), the ligand for CCR4, induces the phosphorylation of CCR4 within 0.5 min of activating IANK cells with this ligand. This is corroborated with the recruitment of G protein-coupled receptor kinases 2 and 3 and their association with CCR4 in IANK cell membranes. Also, CCR4 is internalized between 5 and 45 min but reappears in the membranes after 60 min of stimulation with MDC. MDC, thymus and activation-regulated chemokine (TARC), and I-309 induce the chemotaxis of IANK cells, an activity that is inhibited upon pretreatment of these cells with pertussis toxin, suggesting that receptors for these chemokines are coupled to pertussis toxin-sensitive G proteins. In the calcium release assay, cross-desensitization experiments showed that TARC completely desensitizes the calcium flux response induced by MDC or I-309, whereas both MDC and I-309 partially desensitize the calcium flux response induced by TARC. These results suggest that TARC utilizes CCR4 and CCR8. Our results are the first to show that IL-2-activated NK cells express CCR4 and CCR8, suggesting that these receptors are not exclusive for Th2 cells.

Chemokines play major roles in combating HIV-1 infection. The CC chemokines macrophage inflammatory protein (MIP)-1α, MIP-1β, and RANTES inhibit the replication of the M-tropic HIV-1 strains (13), whereas the CXC chemokine stromal-derived factor (SDF)-1α inhibits the replication of the T-tropic HIV-1 strains (14, 15). Recently, I-309 was reported to inhibit the replication of several HIV-1 strains (16). Also, the truncated form of MDC inhibits the replication of both HIV-1 strains (17, 18). One possible mechanism for this inhibition is the ability of chemokines to activate NK cells (reviewed in Ref. 9). The present work attempts to examine the expression of chemokine receptors (in particular CCR4 and CCR8 in NK cells) and to investigate the biological significance of these receptors.

Materials and Methods

Culture medium and other reagents

Culture medium consisted of RPMI 1640 supplemented with 10% human AB serum (Ullevål Hospital, Oslo, Norway), 10 U/ml penicillin, 100 µg/ml streptomycin, 1 mM d-glutamine, 1% nonessential amino acids (all from Life Technologies, Paisley, U.K.), and 5 × 10^-3 M 2-ME (Sigma, St. Louis, MO). Ionomycin was from Biomol (SMS Norway, Oslo, Norway).

Preparation of NK cells and their membranes

IL-2-activated NK (IANK) cells were prepared by adherence to plastic flasks for 10 days (in the presence of IL-2) of nylon-wool column nonadherent cells generated from buffy coats of human volunteers (Ullevål Hospital), as previously described (19). Depending on the donor, the plastic adherent cells contained between 17 and 35% CD3^+ T cells. Hence, it was necessary to deplete contaminating T cells. This was accomplished by binding these cells twice to M-450 CD3-coupled beads (Dynal, Oslo, Norway). This procedure resulted in more than 95% of the cells expressing the CD56 cell surface marker and less than 5% of the cells expressing the CD3 cell surface marker. IANK cell membranes were prepared in a buffer containing 25 mM Tris, 50 mM NaCl (pH 7.5), 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 supernatants were transferred into Beckman tubes and ultracentrifuged at 150,000 × g for 45 min at 4°C. The membranes were transferred into a buffer containing 1% Brij, 25 mM Tris, and 150 mM NaCl.

Received for publication June 24, 1999. Accepted for publication February 4, 2000.

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1 This work was supported by grants from the Norwegian Research Council, the Norwegian Cancer Society, Anders Jahres Fond, and Hydro, Norway. A.A.M. is a Senior Scientist of the Norwegian Cancer Society.

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Abbreviations used in this paper: CCR, CC chemokine receptor; TARC, thymus and activation-regulated chemokine; MDC, macrophage-derived chemokine; MIP, macrophage inflammatory protein; SDF, stromal-derived factor; IANK, IL-2-activated NK; PT, pertussis toxin; fura-2-AM, fura-2-acetoxymethyl ester; GRK, G protein-coupled receptor kinases; RPA, RNase protection assay; hCR5, human cytokine receptor set 5.
Pretreatment with pertussis toxin (PT)

PT (Calbiochem-Novabiochem, La Jolla, CA) was activated with 20 mM DTT and 20 mM HEPEs for 10 min at 37°C. IANK cells (1 × 10⁶) were either left intact or treated with 2, 20, or 200 ng/ml of activated PT in a final concentration of 10 ml per flask and were incubated for 18 h at 37°C. The cells were collected and washed three times with complete medium. Only preparations that contained more than 95% viable cells were used in the assays described here.

Chemotaxis assay

This procedure has been described in detail (19, 20). In brief, blind-well chemotaxis chambers with a lower-well volume of 200 μl were used. A maximum volume of 200 μl of RPMI medium containing 1% BSA was placed in the lower wells in the presence or absence of various chemokines (MDC, TARC, or I-309; R&D Systems, Abingdon, Oxon, U.K.). Cells (4 × 10⁷) were washed, and upper compartments of Boyden chambers were exten

Calcium measurement

For the association of G protein-coupled receptor kinases (GRKs) with CCR4 in IANK cell membranes, these cells were either left intact or were stimulated with CCR4 (as above). Membranes were prepared from these cells and were immunoprecipitated with anti-CCR4. These membranes were subjected to SDS-PAGE and then immunoblotted with a 1:100 dilution of rabbit anti-human GRK2 or rabbit anti-human GRK3 (Santa Cruz Biotechnology, Santa Cruz, CA). Goat anti-rabbit (Bio-Rad, Hercules, CA) diluted at 1:5000 was used as a secondary Ab.

Analysis of CCR4 and CCR8 expression by multiprobe RNase protection assay (RPA)

Total RNA was prepared by guanidium thiocyanate/cesium chloride gradients following standard protocols. The specific mRNA for CCR4 and CCR8 was detected by the human cytokine receptor set 5 (hCR5) multiprobe template set (RiboQuant; PharMingen, San Diego, CA), which contains templates for CCR1, CCR2, CCR3, CCR4, CCR5, and CCR8. In brief, antisense RNA probes were generated from DNA templates using T7 DNA-dependent RNA polymerase in the presence of [α-32P]UTP (specific activity, 10 μCi/μl; Amersham Pharmacia Biotech, Uppsala, Sweden). Labeled probes were hybridized with total RNA (10 μg) overnight at 56°C. Unhybridized RNA was digested with RNase according to PharMingen’s supplied procedures. RNase-protected probes were resolved on denaturing 5% polyacrylamide gels. The gels were dried and exposed to film (BIOMAX MS; Eastman Kodak, Rochester, NY) at −70°C for 3 h.

Statistical analysis

Significant values were determined by using the two-tailed Student t test.

Results

Detection of mRNA for CCR8 and CCR4 in IANK cells

The expression of CCR4 and CCR8 at the mRNA level was examined utilizing the RPA. hCR5, which has templates for CCR4 and CCR8 among other chemokine receptors including CCR1, CCR2, CCR3, and CCR5, was utilized. Results in Fig. 1 show that IANK cells contain mRNA for CCR4, CCR8, and CCR2 (lane 3). The expression of CCR1 and CCR3 was variable in different donors and is not shown in this figure. Surprisingly, CCR5 was absent in IANK cells. Receptors other than CCR4 and CCR8 have not been fully examined in this report. Yeast tRNA was included as a negative control and is shown in lane 2 of Fig. 1.

Detection of surface expression of CCR4 and CCR8

Fig. 2a shows that IANK cells express CCR4 and CCR8 as detected by intracellular staining by Abs that bind the carboxy terminal of these receptors. Because the amount of anti-CCR8 in our possession was very scarce, we examined the expression of only CCR4 by other assays such as immunoblotting. Results in Fig. 2b show that CCR4 is detected by this method (lane 1). Pretreatment of 4 × 10⁷/ml IANK cells with 50 ng/ml MDC or TARC for 25 min at 37°C significantly reduced the expression of this receptor (Fig. 2b, lanes 2 and 3, respectively), suggesting that CCR4 is...
internalized after pretreatment with these ligands. SDF-1α, which does not bind CCR4, did not induce the endocytosis of CCR4 (Fig. 2b, lane 4). To examine in detail the kinetics of CCR4 endocytosis, we pretreated IANK cells with MDC for various periods of time and examined the presence of this receptor. Fig. 2c shows that 5 min after stimulation, this receptor expression was low, with maximal internalization occurring between 15 and 45 min. Interestingly, this receptor started to reappear in the cell membranes 60 min after stimulation. This result suggests that the mechanism of disappearance of this receptor is due to its internalization and not to other mechanisms such as impediment of its recognition by the Ab due to ligand binding.

Phosphorylation of CCR4
Phosphorylation of chemokine receptors is the first step in the process of endocytosis (reviewed in Ref. 3). Hence, we examined whether CCR4 is phosphorylated as a result of stimulation with MDC. Fig. 3 shows that pretreatment of IANK cells with MDC for 0.5 min resulted in the phosphorylation of CCR4 (upper panel, lane 2) compared with unstimulated cells (upper panel, lane 1), as detected by Ab to phosphorylated serine/threonine residues. This phosphorylation was maximal by 1 min (upper panel, lane 3). It was also observed after 2 min but disappeared after 5 min of stimulation with MDC (upper panel, lanes 4 and 5, respectively). Immunoprecipitating NK cell membranes with goat IgG did not result in detecting the phosphorylated CCR4. Stripping the membranes and reimunoblotting them with anti-CCR4 showed that the same amount of CCR4 is present in all samples (Fig. 3, lower panel).

The serine/threonine kinases GRK2 and GRK3 have been implicated in phosphorylating chemokine receptors such as CCR5 (22). To address the possibility that CCR4 phosphorylation may be related to the activity of these enzymes, we examined whether CCR4 is associated with GRK2 or GRK3. Fig. 4 shows that these enzymes are not present in IANK cell membranes. However, 0.5 min after stimulation with MDC, an association between CCR4 and GRK2 (Fig. 4a) but not GRK3 (Fig. 4b) occurred in these membranes. In contrast, after 1 min of stimulation, both GRK2 and GRK3 were associated with CCR4 at this time point. A similar amount of CCR4 was observed in these membranes upon stripping and reimunoblotting with anti-CCR4 (Fig. 4c).

MDC, TARC, and I-309 induce the chemotaxis of IANK cells
To investigate the biological significance of the presence of CCR4 and CCR8, we examined the ability of IANK cells to respond to the ligands recognizing CCR4 and CCR8. Fig. 5 shows that MDC, TARC, and I-309 induce the chemotaxis of IANK cells util-

FIGURE 1. Detection of CCR4 and CCR8 mRNA in IANK cells utilizing RPA. hCR5, which detects mRNA for CCR4 and CCR8 among other chemokine receptors, has been utilized. Detection of mRNA was as described in the procedures supplied by PharMingen. Lane 1, mRNA of RNase- unprotected probes. Lane 2, Yeast tRNA as a control. Lane 3, mRNA of protected probes in IANK cells. Arrows indicate the presence of mRNA for CCR4 and CCR8. Other chemokine receptors marked in the figure have not been examined in detail.

FIGURE 2. Expression of CCR4 and CCR8 in IANK cells. a, Expression of CCR4 and CCR8 was determined by flow cytometric analysis. Goat anti-human CCR4 or goat anti-human CCR8 was introduced inside permeabilized IANK cells, respectively. The cells were washed and then incubated with F(ab)2 FITC-conjugated rabbit anti-goat as a secondary Ab. Filled columns represent the background controls in the presence of goat IgG plus the secondary Ab. b, Expression of CCR4 by immunoblot assay. IANK cell membranes were immunoprecipitated with anti-CCR4 and then immunoblotted with anti-CCR4 (lane 1). In lanes 2-4, the cells (4 × 10^7/ml) were incubated first with 50 ng/ml of TARC, MDC, or SDF-1α, respectively, for 25 min at 37°C before the membranes were prepared from these cells. These membranes were immunoprecipitated and then immunoblotted with anti-CCR4 as above. Lane 5 shows membranes immunoprecipitated with goat IgG as a control. c, Time kinetic study of CCR4 internalization. IANK cells (4 × 10^7/ml) were stimulated with 50 ng/ml MDC for 0, 5, 15, 30, 45, and 60 min. These cells were examined for the presence of CCR4 as determined in a.
TARC, and I-309 induced a typical bell-shaped chemotactic response. As low as 1 pg/ml of TARC induced the chemotaxis of these cells \((p < 0.0001\) compared with cells migrating in the absence of chemokines), whereas 100 pg/ml is the minimum concentration of MDC and I-309 required to induce the chemotaxis of IANK cells \((p < 0.01\) and \(p < 0.001,\) respectively, compared with cells migrating in the presence of medium only). No significant migration was observed when chemokines were placed in the upper wells’ “negative gradients” or in the upper and lower wells’ “no gradients” (data not shown). Hence, the effect of these chemokines is a chemotactic rather than a chemokinetic response.

Almost all receptors for chemokines are coupled to heterotrimeric G proteins \((1–3, 9).\) To investigate whether this is true for TARC, MDC, or I-309, we pretreated IANK cells with various concentrations of PT for 18 h at 37°C. Results in Fig. 5 show that 2 ng/ml PT did not inhibit the chemotactic response induced by these chemokines. However, as low as 20 ng/ml inhibited TARC- or I-309-induced IANK cell chemotaxis \((p < 0.004\) and \(p < 0.005,\) respectively). A dose of 200 ng/ml PT inhibited MDC, TARC, and I-309-induced chemotaxis \((p < 0.02,\) \(p < 0.001,\) and \(p < 0.001,\) respectively), suggesting that the chemotactic response induced by these three chemokines is mediated by PT-sensitive G proteins.

MDC, TARC, and I-309 induce the mobilization of \([Ca^{2+}]_i\) in IANK cells

Because the pattern of calcium signaling in activated cells closely correlated to the pattern of receptor expression \((23),\) we examined the ability of these chemokines to induce the mobilization of intracellular calcium. Fig. 6 shows that MDC induced a potent calcium flux in IANK cells, an activity that was desensitized by the addition of MDC. Similarly, TARC induced a robust calcium flux in these cells, an activity that was desensitized by the addition of TARC. Surprisingly, MDC only partially inhibited the calcium flux response induced by TARC. The concentration of MDC utilized \((20 \text{ ng/ml})\) was enough to desensitize CCR4, as shown in Fig. 6. In contrast, TARC desensitized the calcium flux response induced by MDC. These results suggest that TARC in addition to CCR4 may utilize other receptors. Recent work has shown that in addition to...
CCR4, TARC utilizes CCR8 (24). Results in Fig. 6 show that I-309, the ligand for CCR8, induced a robust calcium flux response in IANK cells. I-309 only partially inhibited the calcium flux induced by TARC, whereas TARC completely desensitized the calcium response induced by I-309. There was no cross-desensitization of the calcium flux response among MDC and I-309. These results suggest that in IANK cells, TARC shares the receptors utilized by MDC (CCR4) and I-309 (CCR8). However, MDC and I-309 do not share the same receptor. Because all three chemokines induced only a transient calcium flux in IANK cells, we included ionomycin (ION) as a control. This ionophore induced a sustained calcium flux in these cells in the presence of extracellular calcium, suggesting that it facilitates the influx of calcium across the plasma membranes.

To investigate whether the calcium flux response induced by these chemokines is PT-sensitive, we pretreated IANK cells with 200 ng/ml PT for 18 h before examining them in the calcium assay. Ionomycin was used as a control. In these assays, extracellular calcium was not added to the buffer. Ionomycin induced a transient calcium flux, which returned to a baseline level (Fig. 7A), a normal response to this ionophore when incubated with cells in nominally Ca$^{2+}$-free medium (25). Our results indicate that this ionophore can also induce the release of calcium from intracellular stores in NK cells. More importantly, PT did not inhibit the calcium response induced by this ionophore (Fig. 7B). The calcium mobilization effect of MDC and TARC was completely inhibited upon pretreatment of IANK cells with PT (Fig. 7, C–F). Surprisingly, the calcium flux induced by I-309 was not affected by prior treatment of these cells with PT (Fig. 7, G and H).

Discussion

Among other functions, chemokines inhibit the replication of HIV-1 (13–17). One possible scenario for this inhibition is the ability of NK cells to secrete and to respond to these chemokines. Activated NK cells collected from HIV-1-infected individuals have been shown to secrete MIP-1α, MIP-1β, and RANTES and to inhibit the in vitro replication of the M-tropic HIV-1 (26). In addition, NK cells that are recruited and activated by the CXC chemokines Mig and Crg-2 contributed significantly to the eradication of murine vaccinia virus (27). Also, NK cells respond by migrating toward the concentration gradients of the anti-HIV-1 chemokines (reviewed in Ref. 9). In this paper, we have added to the list of chemokines the CC chemokines TARC, MDC, and I-309. These chemotactic activities were inhibited upon pretreatment of IANK cells with PT. This toxin ADP-ribosylates the α subunit of members of Gi and Go proteins and hence uncouples them from the membranes. Our results suggest that TARC, MDC, and I-309 receptors are coupled to members of the Gi, or Go proteins (3, 9).

Also, we showed that these chemokines induce the accumulation of intracellular calcium in IANK cells. Extravasation of NK cells into various tissues and their accumulation at the sites of HIV-1 replication must be accompanied by the expression of chemokine receptors in these cells. Also, expression of chemokine receptors corresponds to the chemotaxis and the calcium signaling induced by chemokines in T cells (23). In this paper we have clearly shown by several criteria including flow cytometric, biochemical, and molecular analyses that NK cells express CCR4. Messenger RNA for CCR4 or CCR8 was also detected in IANK cells. The expression of CCR4 and CCR8 explains how TARC, MDC, and I-309 induced the chemotaxis and the mobilization of intracellular calcium in these cells. When TER-1 (CCR8) was first cloned, its mRNA was detected in certain NK cell lines, such as NK 3.3, but not in others, such as NKL and YT-5, or in activated cells (6). In our study, it is clear that CCR8 is expressed in NK cells cultured with IL-2 both at the mRNA and on the surface and in terms of the response of these cells to the CCR8 ligands TARC and I-309. The difference between our study and that reported by Napolitano et al. (6) is that we used adherent
IANK cells. These cells may express different chemokine receptors than nonadherent cells and may resemble the NK 3.3 chemokine receptor expression. In fact, both adherent IANK cells and the NK 3.3 cells respond similarly to various CC chemokines (19). Although not examined in any detail, we noticed that IANK cells lack mRNA for CCR5, the receptor for MIP-1β, MIP-1α, and RANTES.

Pretreatment of IANK cells with MDC or with TARC induced the internalization of CCR4. This occurred after 5 min of stimulation and was persistent until 45 min. Several steps must take place before G protein-coupled receptors such as CCR4 are internalized. These include the recruitment of GRKs, which phosphorylate the receptors and consequently bind β-arrestin (reviewed in Ref. 28). Chemokine receptors form appropriate targets for GRKs because these are serine/threonine kinases, and the carboxy terminal of the chemokine receptors are rich with these residues (reviewed in Refs. 1 and 3). In this paper, we have shown that CCR4 is phosphorylated 0.5 min after incubation of NK cells with MDC. GRKs are most probably responsible for phosphorylating CCR4 because we observed that there is a sequential association of GRK2 and GRK3 with CCR4 shortly after stimulation with MDC.

Hence, GRK2 is recruited first, because it is associated with CCR4 0.5 min after incubation with MDC and seems to be responsible for the initial phosphorylation of CCR4 in IANK cells. Thereafter, GRK3 is recruited and remains associated with the phosphorylated receptors for the duration of the phosphorylation process, i.e., up to 2 min. It appears that GRK3 may maintain the receptor in its phosphorylated forms. Both GRK2 and GRK3 are dissociated from the receptor 5 min after stimulation. This is coincided with the loss of phosphorylation. Also, we observed that there is a lapse of time between the phosphorylation of the receptor (within seconds) and its endocytosis (between 5 and 45 min). This is most probably due to the fact that phosphorylated receptors are not internalized right after stimulation. We have evidence showing that after activation with MDC or TARC, phosphorylated CCR4 recruits β-arrestin and nonreceptor tyrosine kinases. Such recruitment starts 5 min after stimulation (data not shown). These molecules are important for directing phosphorylated receptors toward endocytic pits to start the process of internalization. Interestingly, CCR4 reappeared in NK cell membranes 60 min after stimulation, suggesting that this receptor is not degraded by endogenous proteolytic enzymes but instead is recycled into the membranes. This is important when one considers CCR4 as a target for therapy. Our results support those of others who observed that GRK3 phosphorylates CCR2B (29). In addition, GRK2 and GRK3 were reported to phosphorylate CCR5 and to induce its desensitization and endocytosis upon activation of various cell types with RANTES (22, 30).

It was suggested that in Th2 cells (31) or in L1.2 transfected cells (18), receptors other than CCR4 must bind MDC. Such receptors, if found, are most probably not expressed on NK cells, because in these cells TARC inhibited the calcium mobilization induced by MDC, suggesting that CCR4 is the only receptor utilized by MDC. Alternatively, a novel receptor that binds both MDC and TARC may be identified, which could explain the present results. However, our results suggest that in addition to CCR4, TARC utilizes some other receptors because MDC, the ligand for CCR4, partially inhibited the calcium mobilization response induced by TARC. This receptor is probably CCR8 because I-309, the ligand for CCR8, also partially inhibited the calcium mobilization.

FIGURE 7. Effect of PT on the mobilization of intracellular calcium induced by MDC, TARC, and I-309. IANK cells (1 × 10^6/ml) were either left untreated (UNT) or were pretreated with 200 ng/ml of activated PT for 18 h at 37°C. The cells were extensively washed, labeled with fura 2-AM, and then examined in the calcium release assay. Ionomycin (ION) was used at 20 μM. The concentrations of chemokines are the same as in Fig. 6. Arrows indicate the times at which these reagents were added. One of three representative experiments.
response induced by TARC. This result supports the findings of Ber- 
ardini et al. (24), who showed that TARC utilizes CCR5 in the Jurkat cell line.

In the calcium release assays, we observed that the response induced by MDC, TARC, or I-309 is sharply declined 10–15 s after stimulation with these chemokines, suggesting a transient re-

lease of calcium from intracellular sources such as the endoplasmic reticulum. The calcium channels on these sources are there-

after closed until further stimulation. This stimulation is facilitated by G proteins, which recruit and activate phospholipase Cβ and hence the inositol 1,4,5 trisphosphate/inositol 1,4,5 trisphosphate receptor pathway or the ADP-ribosyl cyclase and hence the cyclic ADP-ribose/ryanodine receptor pathway (32). Both of these path-

ways are activated by chemokines in IANK cells (33). The calcium flux response induced by MDC and TARC is dependent on PT-
sensitive G proteins because PT inhibited this response. Because the heterotrimeric G proteins are dissociated from IANK cell membranes shortly after stimulation with chemokines (21), it is not surprising that there is an initial calcium flux response induced by the three chemokines, which is desensitized until further association of the various subunits of G proteins with the receptors. Hence, this response will only be restored upon the reappearance of the receptor and its reassociation with the α, β, and γ subunits of G proteins, waiting for another round of stimulation. On the other hand, the calcium flux response induced by I-309 is not in-
hhibited by PT, suggesting that this response is mediated through PT-insensitive G proteins. The nature of these G proteins is not known at the present time. This result marked a distinction be-
 tween the chemotactic response, which is PT-sensitive, and the calcium response induced by I-309, supporting the findings of oth-
ers who reported such a distinction between the two activities (chemotaxis and calcium flux) induced by chemokines in T cells (23).

In conclusion, our results are the first to show that NK cells express CCR4 and CCR8, among other chemokine receptors. This, combined with the ability of NK cells to secrete various chemokines, may explain how these cells extravasate into various tissues and may shed some light on the possible involvement of NK cells in eradicating HIV-1-infected cells. Also, our results indicate that CCR4 and CCR8 are not exclusive for Th2 cells.

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