# Cutting Edge: Enhanced Anti-HIV-1 Activity and Altered Chemotactic Potency of $NH_2$-Terminally Processed Macrophage-Derived Chemokine (MDC) Imply an Additional MDC Receptor

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**Posttranslational processing of chemokines increases (IL-8) or decreases (monocyte chemotactic protein-1) their chemotactic potency. Macrophage-derived chemokine (MDC) attracts monocytes, dendritic cells, activated lymphocytes, and NK cells and has reportedly anti-HIV-1 activity. Here we report that truncation of MDC by deletion of two $NH_2$-terminal residues resulted in impaired binding to CC chemokine receptor (CCR)4, the only identified MDC receptor sofar. Truncated MDC(3-69) failed to desensitize calcium mobilization by MDC(1-69) or thymus- and activation-regulated chemokine (TARC), another CCR4 ligand. MDC(3-69) lacked HUT-78 T cell chemotactic activity but retained its capacity to attract monocytes and to desensitize chemotaxis. Compared with MDC(1-69), MDC(3-69) had weak but enhanced antiviral activity against M- and T-tropic HIV-1 strains. Furthermore, both MDC forms failed to signal through the orphan receptors Bonzo/STRL33 and BOB/GPR15 and to desensitize RANTES and stromal cell-derived factor (SDF)-1 responses in CCR5-transfected and CXC chemokine receptor (CXCR)4-transfected cells, respectively. These findings suggest that MDC recognizes another, yet unidentified, receptor. We conclude that minimal $NH_2$-terminal truncation of MDC differentially affects its various immunologic functions.** ***The Journal of Immunology,*** **1998, 161: 2672–2675.**

Chemokines are a family of chemotactic cytokines that attract and activate different leukocyte subtypes. Depending on the positioning of the first two cysteines, chemokines are divided into CC, CXC, $CX_3C$, and C subfamilies (1, 2). Within the CC chemokine subfamily, consisting of more than 20 different human molecules, redundant biologic activities have been observed. For most CC chemokines, the overlapping spectrum of target cells is explained by the use of shared G-protein-coupled receptors (3). At present, 10 CC chemokine receptors (CCR)[3] have been functionally characterized. CCR5, the receptor shared by RANTES, MIP-1$\alpha$, and MIP-1$\beta$ (4) has been identified as the coreceptor for M-tropic HIV-1 strains, whereas CXCR4 (5) is the main coreceptor for T-tropic virus strains (1, 2). Recently, we have shown that posttranslational modification of RANTES increases its anti-HIV-1 activity by enhancing its binding capacity to CCR5 but reduces its chemotactic potency due to loss of signaling through CCR1 and CCR3 (6, 7).

Macrophage-derived chemokine (MDC) has been described as a chemoattractant for monocytes, monocyte-derived dendritic cells, IL-2-activated NK cells, and chronically activated T cells (8, 9). So far, only CCR4 has been identified as a functional receptor for MDC (10). In addition, the closest related CC chemokine, TARC (thymus- and activation-regulated chemokine), is also a specific ligand for CCR4 (11, 12). Although no HIV-1 strains have been described that can enter $CD4^+$ cells using CCR4 as coreceptor (13), a truncated form of MDC missing two $NH_2$-terminal residues was found to inhibit HIV-1 infection (14). However, no data are available on the chemotactic potency and receptor usage of this truncated MDC (3-69) variant or on the antiviral potency of intact MDC (1-69). In this study, we report that MDC (3-69) has reduced chemotactic potency and increased HIV-1 inhibitory activity compared with intact MDC. Since MDC (3-69) showed reduced CCR4-binding capacity, its increased antiviral effect is probably mediated by interaction with another MDC receptor affecting HIV-1 infection.

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[3] Abbreviations used in this paper: CCR, CC chemokine receptor; MDC, macrophage-derived chemokine; TARC, thymus- and activation-regulated chemokine; SDF, stromal cell-derived factor; CXCR, CXC chemokine receptor; MIP, macrophage inflammatory protein; $[Ca^{2+}]_i$, intracellular calcium concentration; CI, chemotactic index.



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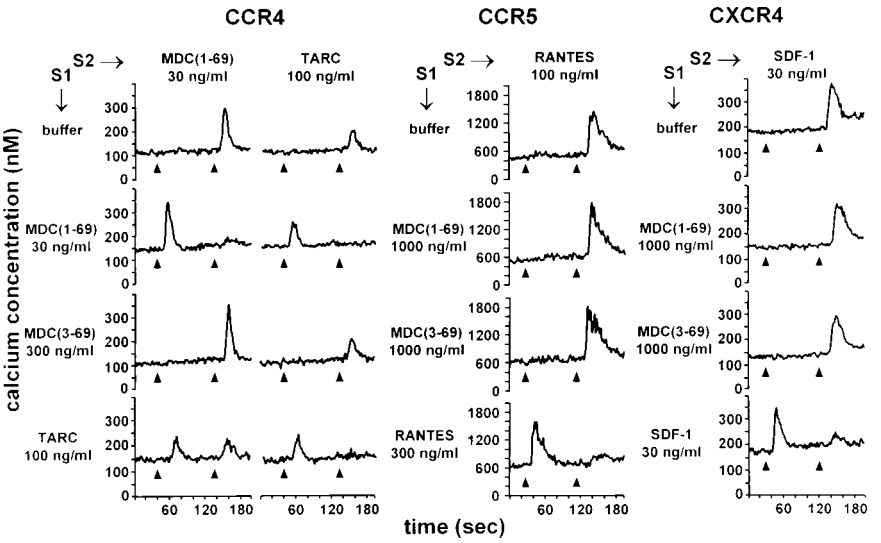


**FIGURE 1.** Desensitization of calcium mobilization by MDC and TARC in CCR4, CCR5, and CXCR4 transfectants. HOS cells transfected with CD4 and CCR4, or CXCR4 and 3T3 cells transfected with CD4 and CCR5, were loaded with the fluorescent probe fura 2. The $[Ca^{2+}]_i$ was monitored by a spectrophotometer. The time of addition of the first (S1) and second (S2) stimulus is indicated by an arrowhead. The spectra from one representative experiment of two (CCR5, CXCR4) or three (CCR4) are shown.

## Materials and Methods

### *Cell cultures and chemokines*

Human osteosarcoma (HOS) cells (15) and murine 3T3 cells (16) transfected with CD4 and one of the chemokine receptors were cultured in DMEM, containing 1 $\mu$g/ml puromycin as selection agent. The T cell line HUT-78 (10, 12) was grown in RPMI 1640. All growth media were enriched with 10% FCS. PBMC from healthy donors, purified by centrifugation on a Ficoll gradient (Biochrom, Berlin, Germany), were used for monocyte chemotaxis. Recombinant human chemokines (RANTES, SDF-1, MDC, TARC) were purchased from PeproTech (Rocky Hill, NJ) or R&D Systems (Abingdon, U.K.). Synthetic MDC was obtained from Tecnogen (Piana di Monte Verna, Italy).

### *Calcium assay*

The increase in the intracellular calcium concentration ($[Ca^{2+}]_i$) induced by chemokines was monitored by fluorescence spectrophotometry as reported (7). Briefly, cells were loaded with fura 2-AM and used at $10^6$ cells/ml. Fura 2 fluorescence was measured in an LS50B luminescence spectrophotometer (Perkin-Elmer, Norwalk, CT). The $[Ca^{2+}]_i$ was calculated from the Grynkiewicz equation (17). For desensitization experiments, cells were first stimulated with buffer or chemokine; as a second stimulus, chemokines were added at a concentration inducing a significant increase in the $[Ca^{2+}]_i$. The percentage inhibition of the second stimulus was calculated using the increase in $[Ca^{2+}]_i$ after prestimulation with buffer as 100% value.

### *Chemotaxis assay*

The chemotactic potency of chemokines was determined with the Boyden microchamber assay (Neuroprobe, Cabin John, MD) (6, 8). Monocyte migration ($1 \times 10^6$/ml) through 5-$\mu$m pore size polyvinyl pyrrolidone (PVP)-treated polycarbonate filters (Nuclepore, Pleasanton, CA) was evaluated after 1.5 h incubation. For HUT-78 cell migration ($8 \times 10^6$/ml, 4-h incubation time), collagen-coated membranes (5 $\mu$m, polyvinyl pyrrolidone-free polycarbonate membranes) were used. Chemotactic indexes (CI) were calculated by dividing the number of (microscopically counted) migrated cells toward the chemokine by the number of cells migrated toward the dilution buffer.

### *Inhibition of HIV-1 infection*

The HIV-1 M-tropic strain BaL and the T-tropic strain NL4.3 were obtained through the Medical Research Council (MRC) AIDS reagent project (Herts, U.K.) and from the National Institute of Allergy and Infectious Diseases (NIAID) AIDS reagent program (Bethesda, MD), respectively. PBMC from healthy donors were stimulated with PHA for 3 days before infection. At the time of infection, chemokines (RANTES, SDF-1, MDC) were added (6, 18). Cell supernatants were collected after 8 days of culture in the presence of IL-2, and HIV-1 core Ag was analyzed by a p-24 Ag ELISA kit (NEN, Boston, MA).

## Results

### *$NH_2$-terminal processing of MDC impairs its signaling capacity through CCR4*

Since CCR4 is the only known receptor for MDC, recombinant preparations of truncated MDC (3-69) and intact MDC (1-69) were compared for calcium mobilization in CCR4-transfected cells. Intact MDC (1-69) concentration-dependently increased the $[Ca^{2+}]_i$ in CCR4 transfectants, 10 ng/ml being the minimal effective concentration. In contrast to this strong effect of MDC (1-69), truncated MDC (3-69) failed to induce calcium mobilization in CCR4 transfectants at a concentration as high as 1000 ng/ml (not shown). Intact MDC (1-69) concentration-dependently inhibited (87% at 30 ng/ml) the calcium response in CCR4 transfectants toward a second stimulation with 30 ng/ml of intact MDC (Fig. 1). In contrast, pretreatment of the cells with 300 ng/ml of MDC (3-69) failed to inhibit the calcium increase induced by 30 ng/ml of MDC (1-69) or by 100 ng/ml of TARC. Nevertheless, intact MDC at 30 ng/ml completely inhibited the calcium response to 100 ng/ml of TARC ($>$ 79% inhibition), in accordance with the shared CCR4 usage of MDC and TARC described previously (10). From these data it can be concluded that the truncated MDC (3-69) has reduced binding to, and signaling through, CCR4.

### *Truncated MDC (3-69) has reduced chemotactic activity for lymphocytes, but not for monocytes*

In accordance with the literature (10), intact MDC concentration-dependently induced HUT-78 cell migration, 3 ng/ml being the minimal effective concentration (Fig. 2, *upper panel*). However, truncated MDC (3-69) was 100-fold less potent than MDC (1-69), indicating that the $NH_2$-terminal dipeptide of MDC is important for biologic activity. In contrast, the chemotactic potency of MDC (3-69) for monocytes remained unaffected (Fig. 2, *lower panel*), and MDC (3-69) cross-desensitized the chemotaxis toward both MDC variants (Table I). In addition, MDC (3-69) reduced MDC (1-69)- and TARC-induced migration of HUT-78 cells (not shown). The differential behavior of intact and truncated MDC in chemotaxis assays was confirmed with synthetic chemokines (not shown). These findings suggest that CCR4 and maybe another receptor are important for lymphocyte chemotaxis. MDC-induced monocyte chemotaxis, however, seems to depend on such an additional receptor, which recognizes truncated MDC (3-69).

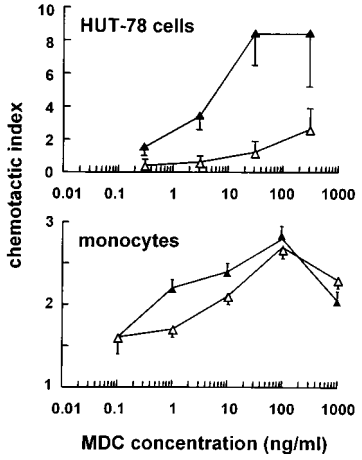


**FIGURE 2.** Comparison of the chemotactic potency of intact (▲) MDC (1-69) with truncated (Δ) MDC (3-69). The lymphocyte (HUT-78 cells) and monocyte chemotactic activity was determined in the microchamber assay. Results represent the mean CI ± SEM of four (HUT-78) and three (monocytes) independent experiments. In each experiment, samples were tested in triplicate. The number of randomly migrated cells in ten microscopic fields was 1.2 ± 0.7 and 28 ± 2 for HUT-78 cells and monocytes, respectively.

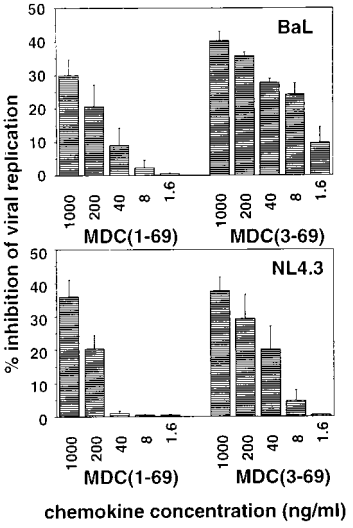


**FIGURE 3.** Inhibitory effect of MDC (1-69) and MDC (3-69) on infection of mononuclear cells by HIV-1. PHA-activated PBMC were infected with the M-tropic BaL strain or the T-tropic NL4.3 strain in the presence of various concentrations (1.6–1000 ng/ml) of recombinant MDC. After 8 days of culture, cell supernatants were evaluated in a p-24 Ag ELISA. The mean percentage inhibition ± SEM from three independent experiments (three different donors) is shown.

*Truncated MDC (3-69) has enhanced anti-HIV-1 activity*

It was confirmed that, in agreement with intact MDC, recombinant MDC (3-69) failed to signal (calcium mobilization) through CXCR4 and CCR5 and to desensitize the response induced by SDF-1 and RANTES, respectively (Fig. 1). Furthermore, on monocytic cells, both MDC forms were unable to desensitize the calcium flux to MIP-1$\beta$, a selective CCR5 agonist (not shown). Since MDC (3-69) has been reported to inhibit certain HIV-1 strains (14), the activity against the M-tropic BaL and T-tropic NL4.3 HIV-1 strains of both intact and truncated MDC was compared in PBMC. RANTES and SDF-1 were evaluated in parallel and were able to block the viral replication completely at 1 $\mu$g/ml. The $IC_{50}$ of RANTES was 14 ng/ml against the BaL strain, and the $IC_{50}$ of SDF-1 was 250 ng/ml against the NL4.3 strain. Both intact MDC (1-69) and MDC (3-69) at 1 $\mu$g/ml caused only partial inhibition of both virus strains (30–40% inhibition). However, the antiviral potency of MDC (3-69) was significantly higher compared with that of intact MDC. The minimal concentration that still inhibited viral replication (20% inhibition) was 25-fold (BaL strain) and 5-fold (NL4.3 strain) lower for MDC (3-69) than for intact MDC (Fig. 3). This finding suggests that, for its antiviral effect, MDC (3-69) probably blocks at least one additional chemokine receptor, e.g., Bonzo/STRL33, which can function as a fusion cofactor for both M- and T-tropic HIV-1 strains (16, 19). However, intact and truncated MDC failed to induce calcium mobilization in 3T3 cells transfected with Bonzo/STRL33. Similar results were obtained using cells transfected with BOB/GPR15, reported to be a coreceptor used by SIV strains, some M-tropic and dual-tropic HIV-1 strains, and by some HIV-2 strains tested (16).

Table I. *Desensitization of peripheral blood monocyte migration by MDC*

| Desensitization by[a] | Chemotactic Response (CI) to[b,c] | |
|---|---|---|
| | MDC (1-69) | MDC (3-69) |
| Buffer | 2.5 ± 0.2 | 2.2 ± 0.1 |
| MDC (1-69) | 1.0 ± 0.1 | 1.0 ± 0.0 |
| MDC (3-69) | 0.9 ± 0.1 | 0.9 ± 0.0 |

[a] For MDC cross-desensitization, monocytes were preincubated (30 min, 37°C) with either buffer, 100 ng/ml MDC (1-69), or 100 ng/ml MDC (3-69) and washed before transfer to the upper wells of the microchamber.

[b] Intact or truncated MDC (100 ng/ml) was added as chemoattractant to the lower wells of the microchamber.

[c] Mean CI ± SEM ($n$ = 4) using different donors.

## Discussion

Posttranslational modifications of chemokines have been observed since the initial isolation of natural IL-8 forms. The importance of $NH_2$-terminal processing to activate CXC chemokines was previously demonstrated by the observation that the precursor platelet basic protein (PBP) needs to be cleaved to the neutrophil activating protein-2 (NAP-2) to become a chemoattractant (20). Multiple proteases have been reported to be involved in this chemokine processing. In contrast, $NH_2$-terminal truncation of CC chemokines such as the monocyte chemotactic proteins (MCPs) results in reduction or complete loss of chemotactic activity (21). In previous studies, it has been demonstrated that truncated chemokine forms, both natural (21) and synthetic (22), can act as receptor antagonists. In particular, RANTES has been shown to be $NH_2$-terminally processed by the aminopeptidase CD26 into RANTES (3–68), resulting in impaired chemotactic potency due to loss of binding to CCR1 and CCR3 (7). However, as a consequence of its enhanced binding capacity to CCR5, RANTES (3–68) has increased anti-HIV-1 activity on PBMC infected with M-tropic virus (6). Although a truncated form of the novel chemokine MDC, i.e., MDC (3-69), has been identified as an inhibitor of HIV-1 (14), no data were available on the antiviral activity of intact MDC. Therefore, it was investigated whether $NH_2$-terminal processing alters the receptor recognition of MDC and affects its chemotactic or antiviral potency.

In this study it is evidenced that deletion of two $NH_2$-terminal residues from MDC resulted in impaired signaling through CCR4 (23). Furthermore, the chemotactic potency of MDC (3-69) on the T cell line HUT-78, expressing CCR4 (12), was 100-fold reduced. However, intact MDC (1-69) and truncated MDC (3-69) remained

equally chemotactic for monocytes, suggesting that these cells are probably activated through yet another receptor. Indeed, no CCR4 mRNA could be detected in monocytes (12). Additional evidence for the existence of another MDC receptor was provided by the finding that MDC (3-69) retained its capacity to reduce infection of PBMC by M- and T-tropic HIV-1 strains. In fact, MDC (3-69) seems to have a higher affinity for this receptor(s), since it was found to reduce viral replication at lower concentrations than intact MDC. Since intact MDC and MDC (3-69), up to 1 $\mu$g/ml, neither induced calcium mobilization nor desensitized RANTES or SDF-1 responses in CCR5 or CXCR4 transfectants, these receptors are probably not involved. CCR2 and CCR3 usage by MDC (3-69) can be excluded since these CCR are no coreceptors on activated PBMC for the BaL strain (13). MDC (1-69) and MDC (3-69) also failed to induce calcium mobilization in cells transfected with the orphan chemokine receptors Bonzo/STRL33 and BOB/GPR15, which can function as fusion cofactors for M- and/or T-tropic HIV-1 strains. It must be concluded that yet another (orphan or unidentified) receptor is recognized by MDC and HIV-1, unless a mechanism distinct from inhibition of viral entry is involved.

The human MDC gene is located in the same region on chromosome 16 (16q13) as the gene coding for TARC, the chemokine most homologous to MDC (8, 10). Unlike TARC, MDC mRNA is expressed in LPS-stimulated monocytes, resting and activated macrophages, and monocyte-derived dendritic cells (8, 9). However, both genes are constitutively expressed in the thymus (8, 11). In addition to a partly shared expression pattern, MDC and TARC share CCR4 on T lymphocytes (10, 12). This suggests that, similarly to TARC and other recently identified lymphocyte-specific chemokines, MDC is involved in trafficking and homing of particular subsets of lymphocytes (24). Indeed, CCR4 expression is restricted to Th2 lymphocytes (25). In addition, intact MDC is chemotactic for dendritic cells and NK cells. But no data are available yet concerning CCR4 expression in these cell types, and MDC (3-69) has not yet been investigated on these cell types. This broad spectrum of target cells indicates that MDC is an important mediator of Ag presentation, lymphocyte recirculation, and inflammation. In conclusion, posttranslational modification of MDC has differential effects on its biologic activities, involving the use of at least two receptors, and may have far reaching consequences in terms of host resistance to foreign pathogens, including HIV.

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