

# The LD78 $\beta$ isoform of MIP-1 $\alpha$ is the most potent CCR5 agonist and HIV-1-inhibiting chemokine

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PUBLICATION

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LD78 $\alpha$  and LD78 $\beta$  are 2 highly related nonallelic genes that code for different isoforms of the human CC chemokine macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ). Two molecular forms of natural LD78 $\beta$  (7.778 and 7.793 kDa) were identified from conditioned media of stimulated peripheral blood mononuclear cells. Although LD78 $\alpha$  and LD78 $\beta$  only differ in 3 amino acids, both LD78 $\beta$  variants were 100-fold more potent chemoattractants for mouse lymphocytes than was LD78 $\alpha$ . On the contrary, LD78 $\beta$  was only 2-fold more efficient than LD78 $\alpha$  in chemoattracting human lymphocytes and monocytes. Using CC chemokine receptor-transfected cells, both molecular forms of LD78 $\beta$  proved to be much more potent than LD78 $\alpha$  in inducing an intracellular calcium rise through CCR5. Compared with LD78 $\alpha$  and RANTES, this preferential binding of LD78 $\beta$  to CCR5 resulted in a 10- to 50-fold higher potency in inhibiting infection of peripheral blood mononuclear cells by CCR5-using (R5) HIV-1 strains. To date, LD78 $\beta$  is the most potent chemokine for inhibiting HIV-1 infection, and can be considered as a potentially important drug candidate for the treatment of infection with R5 HIV-1 strains.

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## Introduction

Chemokines are a family of chemotactic cytokines that interfere with normal (leukocyte homing, hematopoiesis, angiogenesis) and pathological (inflammation, HIV-1 infection, atherosclerosis) processes (1–5). Depending on the positioning of the cysteine residues, chemokines are classified as C, CC, CXC and CX<sub>3</sub>C chemokines. Each of the numerous chemokines stimulates migration of a unique set of leukocytic cell types. This selectivity is due to the restricted expression pattern of chemokine receptors on a particular cell type. Most chemokines recognize several receptors; e.g., human macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ) signals through the CC chemokine receptors CCR1 and CCR5. On the other hand, a single receptor can bind more than 1 chemokine; e.g., CCR5 is a receptor for RANTES, MIP-1 $\alpha$ , MIP-1 $\beta$ , and monocyte chemoattractant protein-2 (MCP-2) and is expressed on monocytes and

activated lymphocytes. Some of these 7 transmembrane-spanning, G-protein-coupled receptors are also necessary for HIV entry (1–5). Macrophage-tropic (M-tropic) (R5) HIV-1 strains predominantly use CCR5 as a coreceptor for infection, whereas T cell-tropic (T-tropic) (X4) HIV-1 strains enter via CXCR4, the unique but widely expressed receptor for stromal cell-derived factor-1 (SDF-1) (3, 5).

MIP-1 $\alpha$  has originally been isolated from murine macrophages as an inflammatory mediator and inhibitor of hematopoietic stem cell proliferation (6, 7). Human MIP-1 $\alpha$ , the first human CC chemokine for which the cDNA sequence has been reported, is encoded by 2 highly related nonallelic genes (8–11), designated LD78 $\alpha$  and LD78 $\beta$ , that are expressed in lymphocytes and monocytes. So far, only recombinant LD78 $\alpha$  has been investigated in detail; it was found to chemoattract monocytes, lympho-

cytes, natural killer cells, dendritic cells, eosinophils, and basophils (3–5). The 2 MIP-1 $\alpha$  isoforms differ only in 3 amino acids: the penultimate NH<sub>2</sub>-terminal residue and amino acids 39 and 47. Many studies have indicated the importance of the NH<sub>2</sub>-terminal regions of both CXC and CC chemokines for their biological activity (2, 12–15). Because modification or deletion of a single residue may result in altered chemokine receptor usage, and hence target cell specificity, we have investigated both isoforms of MIP-1 $\alpha$  in more detail. We believe that this study describes for the first time the isolation of natural LD78 $\beta$ . We show that this MIP-1 $\alpha$  isoform is a potent lymphocyte and monocyte chemoattractant that preferentially signals through CCR5 and is the most potent chemokine in inhibiting infection of peripheral blood mononuclear cells (PBMCs) by R5 HIV-1.

## Methods

**Cell cultures and chemokines.** Human monocytic THP-1 cells were cultured in RPMI-1640 (BioWhittaker Europe, Verviers, Belgium) enriched with 10% FCS (FCS; GIBCO BRL, Paisley, Scotland, United Kingdom). Murine lymphocytic ESb/MP cells (16), kindly provided by J.M. Wang (National Cancer Institute/National Institutes of Health, Frederick, Maryland, USA), were grown in DMEM (BioWhittaker Europe) supplemented with  $\beta$ -mercaptoethanol and 10% FCS. Human osteosarcoma (HOS) cells transfected with CD4 and either CCR1 or CCR5 (17) were cultured in DMEM supplemented with 10% FCS and puromycin (1  $\mu$ g/mL; Sigma Chemical Co., St.

Louis, Missouri, USA). PBMCs were isolated by hydroxyethyl starch sedimentation and Ficoll-sodium metrizoate centrifugation (13). Lymphocytes and monocytes were further separated on magnetic beads, coated with anti-CD3 (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

RANTES and the human recombinant MIP-1 $\alpha$  isoform LD78 $\alpha$  were purchased from PeproTech Inc. (Rocky Hill, New Jersey, USA) and BioSource Europe (Fleurus, Belgium). The 7.793-kDa LD78 $\beta$  was synthesized by Fmoc solid-phase peptide synthesis (18). <sup>125</sup>I-RANTES was purchased from Amersham Pharmacia Biotech (Roosendaal, the Netherlands).

**Production, purification, and identification of natural MIP-1 $\alpha$  isoforms.** PBMCs ( $54 \times 10^9$  cells) from 132 blood donations were incubated for 48 hours in EMEM (GIBCO BRL) plus 2% FCS supplemented with 10  $\mu$ g/mL Con A (Calbiochem-Novabiochem Corp., San Diego, California, USA) and 2  $\mu$ g/mL LPS (from *Escherichia coli* 0111:B4; Difco Laboratories, Detroit, Michigan, USA). Natural MIP-1 $\alpha$  isoforms were isolated through a 4-step concentration and purification procedure (13) comprising adsorption to silicic acid, heparin-Sepharose chromatography, cation exchange chromatography, and reverse-phase HPLC (RP-HPLC). Fractions derived from RP-HPLC were checked for purity by SDS-PAGE under reducing conditions (13). MIP-1 $\alpha$  proteins were viewed by silver staining, and the following molecular weight mark-

ers were used: carbonic anhydrase ( $M_r = 31$  kDa), soybean trypsin inhibitor ( $M_r = 21$  kDa), lysozyme ( $M_r = 14$  kDa), and aprotinin ( $M_r = 6.5$  kDa). NH<sub>2</sub>-terminal amino acid sequences of pure MIP-1 $\alpha$  forms were obtained by Edman degradation, using a pulsed liquid-phase 477A/120A protein sequencer (Perkin-Elmer Applied Biosystems, Foster City, California, USA). To distinguish LD78 $\beta$  forms, purified proteins were subjected to electrospray mass spectrometry (LCQ-Iontrap; Finnigan, San Jose, California, USA).

**Chemokine assays.** MIP-1 $\alpha$  immunoreactivity was measured by a specific sandwich ELISA (BioSource Europe), kindly supplied by J.-M. Jaspar. The cellular expression of chemokine receptors was examined by flow cytometric analysis, using mAb's against CCR1 (R&D Systems Inc., Minneapolis, Minnesota, USA) and CCR5 (PharMingen, San Diego, California, USA). Competition for <sup>125</sup>I-RANTES binding was analyzed on CCR1- and CCR5-transfected HOS cells as previously described (19). MIP-1 $\alpha$  isoforms were tested for their chemotactic potency on freshly isolated monocytes ( $2 \times 10^6$  cells/mL), CD3<sup>+</sup> lymphocytes ( $10 \times 10^6$  cells/mL), monocytic THP-1 cells ( $0.5 \times 10^6$  cells/mL), and lymphocytic ESb/MP cells ( $2 \times 10^6$  cells/mL), in the Boyden microchamber (Neuro Probe Inc., Cabin John, Maryland, USA) (13, 16). The chemotactic index (number of chemoattracted cells divided by the number of randomly migrated cells) was used as a parameter for potency. Intracellular calcium con-

centrations ( $[Ca^{2+}]_i$ ) were measured by fluorescence spectrophotometry and calculated according to the Grynkiewicz equation (13).

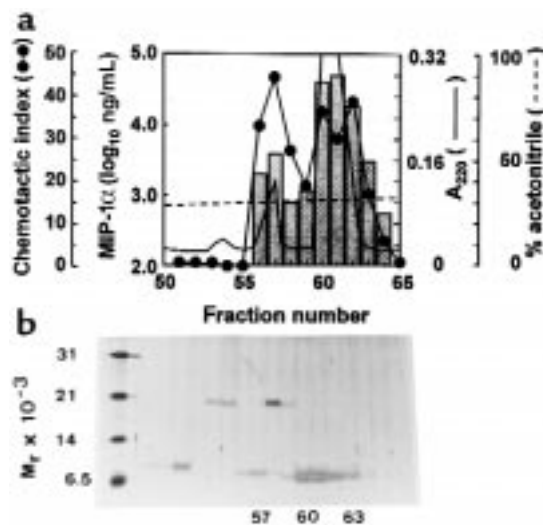
**Inhibition of HIV-1 infection.** The R5 HIV-1 strain BaL was obtained through the Medical Research Council AIDS Reagent Project (National Institute for Biological Standards and Control, Herts, United Kingdom). Purified PBMCs, stimulated with PHA (1  $\mu$ g/mL for 3 days), were infected with virus in the presence of 25 U/mL of IL-2 and various concentrations of LD78 $\alpha$ , LD78 $\beta$ , or RANTES (12). Cell supernatant was collected at day 10, and HIV-1 core antigen in the supernatant was analyzed by a p24 antigen ELISA kit (NEN Life Science Products Inc., Boston, Massachusetts, USA).

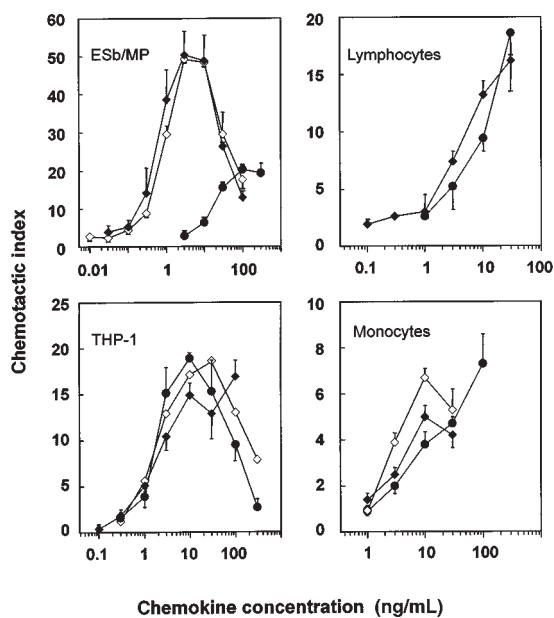
## Results

**Identification of natural LD78 $\beta$  isoforms from stimulated mononuclear cells.** Conditioned medium from PBMCs stimulated with mitogen and endotoxin was analyzed for its MIP-1 $\alpha$  content using a specific ELISA and the sensitive lymphocyte (ESb/MP cells) chemotactic assay. With an established procedure for chemokine isolation (13), MIP-1 $\alpha$  isoforms were fractionated and finally purified to homogeneity by RP-HPLC (Figure 1a). MIP-1 $\alpha$  immunoreactivity and chemotactic activity corresponded to a minor peak containing a single protein of 8 kDa and to a major peak of 7–8 kDa MIP-1 $\alpha$  (Figure 1b). NH<sub>2</sub>-terminal amino acid sequence analysis revealed that the minor peak (fraction no. 56–57) corresponded to a pure, intact LD78 $\beta$  isoform of MIP-1 $\alpha$ , whereas the major peak (fraction no. 60) contained a mixture of intact LD78 $\beta$  and truncated LD78 $\alpha$  or  $\beta$ , each missing 4 NH<sub>2</sub>-terminal residues. Because the sequences of LD78 $\alpha$  and LD78 $\beta$  differ only in 3 amino acids, including the penultimate and 2 internal but interchanged residues, NH<sub>2</sub>-terminal sequence analysis and mass spectrometry allowed us to discriminate between the intact  $\alpha$  and  $\beta$  forms but not between the truncated LD78 isoforms. Nevertheless, extended Edman degradation (up to 45 amino acids identified) showed the presence of

**Figure 1**

Purification of PBMC-derived MIP-1 $\alpha$  to homogeneity by RP-HPLC. (a) Purified MIP-1 $\alpha$  immunoreactivity from conditioned medium of PBMCs was loaded on a C<sub>8</sub> RP-HPLC column. Proteins were eluted with an acetonitrile gradient (dashed line) and detected by measuring UV absorption at 220 nm (solid line). Fractions were tested for the presence of MIP-1 $\alpha$  immunoreactivity (histogram) and ESb/MP cell chemotactic activity (solid line broken by circles). (b) Proteins eluted from the HPLC column were analyzed for purity by SDS-PAGE under reducing conditions and were stained with silver. Markers used are indicated in Methods.





**Figure 2**

Comparison of the chemotactic potencies of LD78α and the LD78β isoforms. The chemotactic activities of LD78α (filled circles), 7.778-kDa LD78β (filled diamonds), and 7.793-kDa LD78β (open diamonds) were determined in the Boyden microchamber assay, using ESb/MP lymphocytic cells, THP-1 monocytic cells, and PBMC CD3<sup>+</sup> lymphocytes and monocytes. Results represent the mean chemotactic index ± SEM of 3 or more independent experiments (each performed in triplicate).

both truncated LD78α and LD78β in the major MIP-1α peak. Intact LD78α could not be recovered from any of the natural MIP-1α preparations analyzed. When both MIP-1α peaks were subjected to mass spectrometry, a different molecular mass was obtained for NH<sub>2</sub>-terminally intact LD78β in RP-HPLC fraction no. 56 (7.7776 ± 0.0012 kDa) and fraction no. 60 (7.7939 ± 0.6 kDa). Because the theoretical monoisotopic molecular mass of LD78β is 7.7927 kDa, it was concluded that only fraction no. 60 contains authentic LD78β. The loss of 16 kDa, observed in the LD78β form present in fraction no. 56, may correspond to an amino acid substitution (polymorphism) or the reduction (loss of oxygen) or deamination of a single residue. To biologically compare these 2 forms of LD78β, we have synthesized the authentic 7.793-kDa molecule, because the latter was not obtained in its pure natural form.

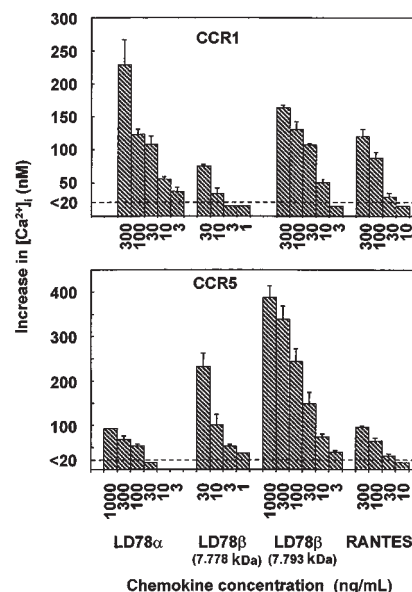
*Diverging chemotactic potencies of LD78α and LD78β isoforms on monocytic and lymphocytic cells.* Because natural LD78β protein has not yet been biologically evaluated, pure NH<sub>2</sub>-terminally intact LD78β forms (natural = 7.778 kDa; synthetic = 7.793 kDa) were compared with intact LD78α for their capacity to stimulate monocytic (freshly isolated blood monocytes and THP-1 cells) and lymphocytic (freshly isolated CD3<sup>+</sup> blood lymphocytes and ESb/MP cells) cell migration in vitro. In the microchamber assay, both LD78β forms induced lym-

phocytic ESb/MP cell chemotaxis from 0.3 ng/mL onward, whereas for LD78α, 30 ng/mL was necessary (Figure 2). Moreover, at optimal concentrations, 2.5 times more ESb/MP cells migrated in response to LD78β compared with LD78α. In contrast, LD78α and LD78β were equally potent as chemoattractants for monocytic THP-1 cells, with a minimal effective concentration of 1 ng/mL. Similarly, LD78α was equipotent to LD78β in inducing calcium mobilization in THP-1 cells (data not shown). Additional experiments on freshly isolated monocytes and CD3<sup>+</sup> lymphocytes indicated that LD78β was only 2-fold more potent than LD78α. Taken together, these findings indicated that, despite minor structural differences between the MIP-1α isoforms, LD78α and LD78β show diverging chemotactic potencies. Because monocyte and lymphocyte populations were found to express both CCR1 and CCR5, as determined by flow cytometric analysis (data not shown), the effect of the ligands LD78α and LD78β on these chemokine receptors was further investigated.

*Inverse chemokine receptor recognition by LD78α and LD78β.* First, we studied whether the 7.793-kDa LD78β differed from LD78α in order to compete with <sup>125</sup>I-labeled RANTES for receptor binding on CCR1- and CCR5-transfected HOS cells. LD78α and LD78β displaced equally well, whereas on CCR5 transfectants, LD78β was superior to LD78α (data not shown).

LD78α strongly induced calcium mobilization in CCR1 transfectants (minimal effective con-

centration: 3 ng/mL), whereas LD78β (7.778 and 7.793 kDa) yielded a significant effect only from 10 ng/mL onward (Figure 3). In contrast, in CCR5 transfectants, both LD78β forms induced calcium mobilization more efficiently than RANTES and LD78α (minimal effective concentration: 3 vs. 30 and 100 ng/mL, respectively), indicating an inverse receptor preference of these MIP-1α isoforms. A small difference between both LD78β forms, in favor of the 7.778-kDa variant, was observed on CCR5 transfectants. The maximal calcium response of LD78β on CCR5 was 3-fold higher than that of LD78α and RANTES. The 30-fold higher potency of LD78β, compared with LD78α, to signal through CCR5 suggested that LD78α and LD78β might differ in their ability to block CCR5 binding by M-



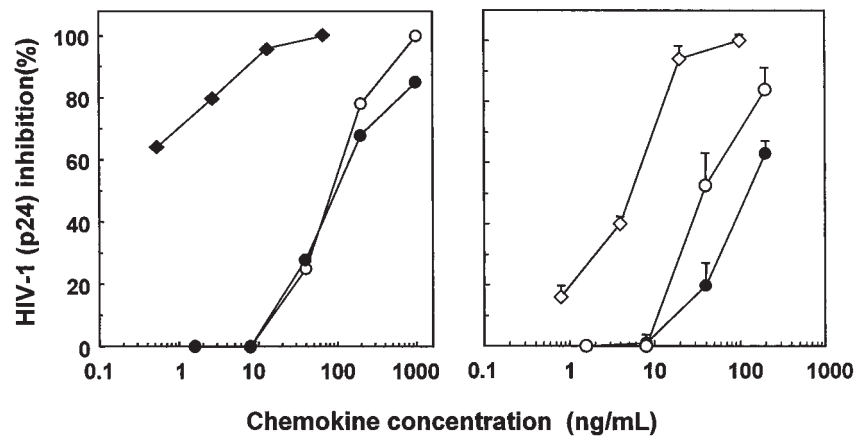
**Figure 3**

Diverging signaling capacities of LD78α, LD78β, and RANTES through CCR1 and CCR5. Intracellular calcium mobilization experiments were performed on CCR1- and CCR5-transfected HOS cells. Results represent the mean [Ca<sup>2+</sup>]<sub>i</sub> rise ± SEM, obtained in 2 or more independent experiments. The limit for significant increases in [Ca<sup>2+</sup>]<sub>i</sub> (20 nM) is indicated by the dashed line.



**Figure 4**

Inhibition of R5 HIV-1 infection by LD78 $\beta$ . PBMCs were protected from infection with the R5 BaL strain by the CC chemokines LD78 $\alpha$  (filled circles), 7.778-Da LD78 $\beta$  (filled diamonds), 7.793-kDa LD78 $\beta$  (open diamonds), or RANTES (open circles). HIV-1 inhibition was evaluated by measuring viral p24 core antigen. Results at left show 1 representative experiment out of 2, whereas the results at right represent the mean  $\pm$  SEM of 4 independent experiments.



tropic HIV-1 strains.

*Higher potency of LD78 $\beta$ , compared with LD78 $\alpha$  and RANTES, in inhibiting HIV-1 infection.* LD78 $\alpha$  and LD78 $\beta$  were compared for their capacity to prevent HIV-1 infection of PBMCs by the M-tropic BaL strain, which uses CCR5 as a coreceptor to enter the cells. As could be predicted by the receptor recognition patterns, LD78 $\beta$  (7.778 and 7.793 kDa) was found to be a more potent inhibitor of HIV-1 infection than LD78 $\alpha$  and RANTES, both known to block M-tropic strains via CCR5 (Figure 4). The 7.778-kDa LD78 $\beta$  form seemed to be more effective compared with the 7.793-kDa LD78 $\beta$  form. On average, the IC<sub>50</sub> values were about 1, 5, 60 and 100 ng/mL for 7.778-kDa LD78 $\beta$ , 7.793-kDa LD78 $\beta$ , RANTES, and LD78 $\alpha$ , respectively. This more than 20-fold difference in antiviral potency between LD78 $\alpha$  and LD78 $\beta$  reflects their differences in CCR5 signaling capacity.

## Discussion

The human macrophage inflammatory proteins MIP-1 $\alpha$  and MIP-1 $\beta$ , mainly produced by mononuclear leukocytes (2), display different chemotactic activities despite their high structural relationship (70% identical amino acids). The diverging effects of MIP-1 $\alpha$  and MIP-1 $\beta$  on leukocytes can be explained by the fact that MIP-1 $\alpha$  signals through CCR1 and CCR5, whereas MIP-1 $\beta$  does not bind CCR1 (1–5). The anti-HIV-1 activities of MIP-1 $\alpha$  and MIP-1 $\beta$  are both mediated through CCR5 binding (17, 20–23). For MIP-1 $\alpha$ , 2 isoforms (LD78 $\alpha$  and LD78 $\beta$ ) have been reported, but so far only the biological activities and receptor binding properties of

LD78 $\alpha$  have been investigated in detail (8–11, 20–23). The primary structure of LD78 $\alpha$  and LD78 $\beta$  differs in 3 amino acids, including 1 residue in the NH<sub>2</sub>-terminal region, which may be important for receptor binding, as shown for other chemokines (2, 12–15).

This study describes the isolation and biological characterization of natural LD78 $\beta$  from stimulated leukocytes. Two variants of natural NH<sub>2</sub>-terminally intact LD78 $\beta$ , characterized by different molecular masses, were recovered. The 7.794-kDa LD78 $\beta$  variant, with a molecular mass corresponding to the theoretical mass (7.793 kDa), was impure and was synthesized to be compared with the 7.778-kDa variant, lacking 0.016 kDa. Compared with LD78 $\alpha$ , both LD78 $\beta$  variants were found to be 100-fold more potent in chemoattracting ESb/MP lymphocytic cells, whereas no difference was observed on THP-1 monocytic cells. On freshly isolated blood monocytes and CD3<sup>+</sup> lymphocytes, both LD78 $\beta$  variants seemed to be only 2-fold better chemoattractants than LD78 $\alpha$ . By means of the calcium mobilization assay, using CCR transfectants, it was further shown that LD78 $\alpha$  signals predominantly via CCR1. In contrast, LD78 $\beta$  activates CCR5 more efficiently than LD78 $\alpha$  or RANTES. This probably explains the differences in chemotactic potencies observed between LD78 $\alpha$  and LD78 $\beta$ . Finally, the preference of both LD78 $\beta$  isoforms for CCR5 was biologically confirmed on PBMCs by their 10- to 50-fold higher antiviral activity against M-tropic HIV-1 strains compared with RANTES and LD78 $\alpha$ . The reported rank order amongst CC chemokines for antiviral potency is

RANTES > MIP-1 $\beta$  > MIP-1 $\alpha$ /LD78 $\alpha$  (24). Our findings that RANTES and LD78 $\alpha$  have an IC<sub>50</sub> of about 60 and 100 ng/mL, respectively, are in agreement with these published data (24). The fact that the IC<sub>50</sub> for SDF-1, when evaluated against different T-tropic HIV isolates, was as high as 200–3,500 ng/mL demonstrates that LD78 $\beta$  (IC<sub>50</sub>: 1–5 ng/mL) is the most potent chemokine reported to date for inhibiting HIV-1 infection. This superior anti-HIV-1 activity of LD78 $\beta$  needs to be reconsidered in the light of the isolation of MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES from cultured T cells as suppressors of HIV-1 infection (20). Although not investigated in the latter study (20), it can be deduced from our data that the antiviral effect corresponding to MIP-1 $\alpha$  was likely due to LD78 $\beta$ . A reported overproduction of the CC chemokines MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES correlated with the protection against HIV-1 infection (25–26). Moreover, MIP-1 $\alpha$  appeared faster and reached higher concentrations than MIP-1 $\beta$  or RANTES (25–26). These clinical data demonstrating natural resistance to HIV-1 infection, which was not linked to a mutation deletion in the CCR5 gene, are in agreement with the higher antiviral potency of the LD78 $\beta$  isoforms of MIP-1 $\alpha$  observed in our study.

The fact that we could only recover intact LD78 $\beta$  from stimulated mononuclear cells points to a higher resistance of LD78 $\beta$  to posttranslational processing by proteolytic cleavage. Nevertheless, both truncated LD78 $\alpha$  and LD78 $\beta$ , missing 4 NH<sub>2</sub>-terminal residues, were purified from leukocyte-conditioned media. In a previous study,

using the HTLV-1-infected MT4 cells as a cellular source, only NH<sub>2</sub>-terminally processed MIP-1 $\alpha$  was recovered (27). NH<sub>2</sub>-terminal truncation of 2 amino acids, including a penultimate proline, by dipeptidyl peptidase IV/CD26 differentially affected the chemotactic and anti-HIV-1 activities of both CXC (SDF-1) and CC (RANTES, macrophage-derived chemokine [MDC]) chemokines (12, 14–15). Although posttranslational processing of MDC and RANTES seemed to improve their antiviral capacity, both truncated MDC and RANTES remained far inferior for blocking HIV-1 replication in PBMCs when compared with LD78 $\beta$  (12, 15). In view of the importance of the NH<sub>2</sub>-terminal region of chemokines for receptor recognition, the substitution of the penultimate serine residue in LD78 $\alpha$  by a proline in LD78 $\beta$  is probably responsible for the receptor switch from CCR1 to CCR5.

In addition to HIV-1 infection, the role of MIP-1 $\alpha$  has been studied in other human pathologies, including airway inflammation and autoimmune disease (1). Using immunotests recognizing both LD78 $\alpha$  and LD78 $\beta$ , elevated MIP-1 $\alpha$  levels have been detected in bronchoalveolar lavage fluids of asthma patients. Similarly, MIP-1 $\alpha$  immunoreactivity is significantly present in the synovial fluid during rheumatoid arthritis. The involvement of MIP-1 $\alpha$  in eosinophilic and mononuclear leukocyte infiltration has been confirmed in animal models by blocking antibodies (1). Because no MIP-1 $\alpha$  isoforms have been described in the mouse, and human LD78 $\alpha$  and LD78 $\beta$  are immunologically indistinguishable, the individual roles of LD78 $\alpha$  and LD78 $\beta$  in these human diseases remain, at present, unknown.

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