Dendritic Cells Express Multiple Chemokine Receptors Used as Coreceptors for HIV Entry[1]

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Cells of the dendritic lineage are thought to be among the first cells infected after mucosal exposure to HIV. In this study, we have identified the presence of multiple chemokine receptors on dendritic cells (DC) that may function as coreceptors for HIV entry. DC effectively used CCR5 for entry of macrophage (M)-tropic isolates. CCR3, the eotaxin receptor, initially identified on eosinophils, is expressed on DC and may be used as an entry coreceptor by certain dual-tropic strains. CXCR4 was not expressed on DC, although SDF-1 induced a calcium flux and DC could be infected by T cell line (T)-tropic HIV. Our findings provide evidence for the presence of a non-CXCR4 SDF-1 receptor on DC that is used mainly by T-tropic strains of HIV. DC from individuals homozygous for a 32-bp deletion of the *CCR5* gene are also infectable with M-tropic strains of HIV-1, and this infection is inhibited by stromal cell-derived factor (SDF)1, suggesting that this receptor can also be used by M-tropic HIV for entry. Delineation of the spectrum of coreceptor usage on DC may offer new approaches to interfere with the initiation and propagation of HIV infection. ***The Journal of Immunology*****, 1998, 160: 3933–3941.**

Dendritic cells (DC)[3] are potent APCs and are essential for the initiation of a primary immune response (1, 2). The role of DC in the initiation of SIV infection has been demonstrated in a macaque model (3). SIV was placed in the vaginal vault, and infected cells were identified and followed using in situ PCR technology. DC in the lamina propria of the cervicovaginal mucosa were among the first cells in which SIV DNA was detectable after inoculation. Subsequently, infected cells were observed in the subcapsular and paracortical regions of draining lymph nodes (3), suggesting that infected DC migrated from the mucosal tissue to regions of lymphoid organs, where viral replication occurs throughout the course of HIV-1 disease in humans (4–7). Since DC are also likely to be the first cell type infected after mucosal exposure in humans, study of DC infection should contribute to our understanding of the early events of HIV infection.

DC progenitors from the bone marrow enter the blood and migrate to nonlymphoid tissues, where they develop into immature DC, such as Langerhans cells (LC) or mucosal DC, with the capacity to take up and process foreign Ags. Upon encountering Ag, immature DC migrate to the regional lymphoid tissue and develop a more mature phenotype with a high capacity to stimulate T cells (8). Chemokines most likely play a role in the complex trafficking pattern of DC, and expression of various CC- and CXC-chemokine receptors on DC has been demonstrated (9).

Recently, the chemokine receptors CXCR4 and CCR5, which belong to a family of seven-transmembrane, G protein-coupled receptors, were identified as the predominant coreceptors for HIV-1 entry into target cells transfected with human CD4. The natural ligands for CCR5, the principal coreceptor for macrophage (M)-tropic isolates (10–14), are the β-chemokines MIP-1α, MIP-1β, and RANTES (15, 16); the natural ligand for CXCR4, a coreceptor for T cell line (T)-tropic strains (17), is the α-chemokine SDF-1 (18, 19). These chemokines block infection with M (20)- or T-tropic (18, 19) HIV isolates, respectively, by interfering with HIV-1 *env*-mediated membrane fusion or viral entry (10, 12, 14, 18, 19).

The chemokine receptors CCR2b and CCR3, the latter being the eotaxin receptor initially described on eosinophils (21), and the recently discovered orphan receptors STRL-33 (also named Bonzo), gpr15 (also known as BOB), and gpr1, have also been identified as entry coreceptors for certain strains of HIV and SIV (11, 13, 22–25). Microglial cells in the brain have been shown to express CCR3 and CCR5, and both serve as coreceptors for HIV entry, thus implicating CCR3 usage by certain viral isolates in the neurotropism of HIV (26).

Mature DC have been demonstrated to be infectable by both M- and T-tropic HIV-1 isolates (27). Although viral replication was inefficient, entry of T-tropic isolates was blocked by SDF-1, and entry of an M-tropic strain was inhibited by RANTES or MIP-1α, suggesting usage of CXCR4 and CCR5, respectively. However, DC in the tissue are immature and may express a different repertoire of chemokine receptors that could change as the cells mature during migration to lymphoid organs (28). Due to the difficulties in obtaining large numbers of immature tissue DC, methods have been developed to generate cells with dendritic morphology and

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[3] Abbreviations used in this paper: DC, dendritic cell; BA, butyric acid; GM-CSF, granulocyte-macrophage colony-stimulating factor; LC, Langerhans cell; LTR, long terminal repeat; M, macrophage; MCP, monocyte chemotactic protein; MDDC, monocyte-derived dendritic cell; MIP, macrophage-inflammatory protein; NIAID, National Institute of Allergy and Infectious Diseases; SDF, stromal cell-derived factor.

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function in vitro by culturing monocytes in the presence of GM-CSF and IL-4 (29). These monocyte-derived DC (MDDC) express high levels of CD1a, HLA-DR, and CD80 (29, 30); in addition, they chemotax to various β-chemokines, including MIP-1α, MCP-1, and RANTES (31), suggesting the expression of multiple chemokine receptors.

The present study was undertaken to characterize the chemokine receptor repertoire of immature MDDC, and to assess their usage as HIV-1 coreceptors.

Materials and Methods

Reagents

RPMI 1640 (BioWhittaker, Walkersville, MD) was supplemented with 10% heat-inactivated FCS (HyClone Laboratories, Ogden, UT), 2 mM glutamine (Biofluids, Rockville, MD), 15 mM HEPES (Biofluids), and 100 U/ml penicillin/streptomycin (Biofluids). Recombinant human MIP-1α, MIP-1β, RANTES, MCP-1, MCP-3, TNF-α, IL-4, GM-CSF, and human recombinant SDF-1β (used for calcium flux) (R&D Systems, Minneapolis, MN); recombinant human eotaxin (Peprotech, Rocky Hill, NJ); an anti-human CD1a Ab (Biosource, Camarillo, CA); BioMag goat anti-mouse IgG beads (PerSeptive Diagnostics, Cambridge, MA); and an HIV-inhibiting anti-CD4 Ab and FITC HLA-DR (AMAC, Westbrook, ME) were used. Two sources of SDF-1 were used. Supernatants from a bone marrow stromal cell line (2N) (John Kehrl, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health, Bethesda, MD) known to produce SDF-1 were used. Results were confirmed using synthesized human SDF-1, which was a generous gift from Dr. Ian Clark-Lewis, Biomedical Research Centre, University of British Columbia (Vancouver, Canada). A mAb against CXCR4 (12G5) was generated by J. A. Hoxie (32), and CD83 (HB15a) mAb was obtained from T. F. Tedder (Duke University, Durham, NC) (30, 33). 12G5 Fab fragments were obtained using the ImmunoPure Fab preparation kit (Pierce, Rockford, IL), according to the manufacturer's instructions. The 12G5 Fab competed with 12G5 binding on PBMC, as assessed by flow cytometry, and partially inhibited entry of 89.6, but not HIV-IIIB, into PBMC (data not shown), which is in agreement with previously published data (34). Neutralizing Abs to CXCR4 (17) were kindly provided by E. A. Berger and G. Alkhatib (Laboratory of Viral Diseases, NIAID/National Institutes of Health). Abs to CCR5 (2D7) (35) and CCR3 (7B11) (36) were kindly donated by C. Mackay (LeukoSite, Cambridge, MA). A mouse mAb specific to CXCR1 was obtained from PharMingen (San Diego, CA). The mAbs 12G5 and 2D7 were directly conjugated to FITC (Fluorescein-Ex; Molecular Probes, Eugene, OR). Soluble CD4 was a kind gift from R. Sweet (SKB, King of Prussia, PA). Cells were obtained from subjects homozygous for a 32-bp deletion in *CCR5* who had been screened as previously described (37). Eosinophils derived from HL-60 clone 15 were generated in the absence (cl15) or presence of 0.5 μM butyric acid (BA) and 10 ng/ml IL-5 for 5 days (cl15 + BA + IL-5) (38).

Isolation of MDDC

MDDC were generated as described previously with minor modifications (29). After 7 days of culture, more than 80% of the cells showed typical DC morphology. Contamination of the MDDC preparations with T or B cells, as determined by flow cytometry, was less than 1%. In some experiments, MDDC were further purified by positive selection with CD1a Abs and goat anti-mouse IgG magnetic beads, according to the manufacturer's instructions. To allow further in vitro maturation of MDDC, TNF-α at 10 ng/ml was added on day 3 of culture, resulting in a cell population expressing high levels of CD83 (30) (data not shown). In some experiments, mature MDDC were positively selected using CD83 Abs and BioMag goat anti-mouse magnetic beads.

Epidermal cells containing LC were obtained by trypsin digestion (0.025% for 30 min) of epidermal sheets (39). The resulting suspension typically contained 2 to 3% HLA-DR$^+$, CD1a$^+$ LC.

Chemotaxis

Cell migration was evaluated using chemotaxis through 5-μm membranes, as described (40). Two hundred nanograms per milliliter of chemokines MIP-1α, MIP-1β, RANTES, IL-8, MCP-1, MCP-3, and eotaxin were placed in the lower wells of a 96-well microchemotaxis plate (Neuroprobe, Cabin John, MD) and covered with a polycarbonate filter. MDDC and LC were seeded at 10,000 to 20,000 cells in 25 μl in the upper chamber and incubated for 2 h. Migration was assessed by counting the cells in the lower well of the chemotaxis chamber. Samples were set up in triplicates and averaged. Epidermal cell suspensions containing LC were stained with fluorescein-labeled mAb specific for HLA-DR or CD1a (Becton Dickinson, San Jose, CA) before placement on the chemotaxis chamber, and the number of fluorescent cells in the lower well was counted.

Calcium flux assay

MDDC were loaded with 2 μM fura-2/AM (Molecular Probes), incubated for 30 min at 37°C in the dark, then washed twice in HBSS (BioWhittaker), and resuspended in HBSS at 1×10^6 cells/ml. Chemokines were added at indicated times to 1×10^6 cells in a 2 ml vol in a continuously stirred cuvette at 37°C in a Model MS-III fluorimeter (Photon Technology, South Brunswick, NJ). The relative ratio of fluorescence emitted at 510 nm following sequential excitation at 340 and 380 nm was recorded every 200 ms (41).

Flow cytometric analysis

Flow cytometric analysis was performed using the mouse mAbs CD83 (1/1000 dilution of ascites), 7B11 (anti-CCR3), and an anti-CXCR1 mAb, followed by staining with an FITC-labeled goat $F(ab')_2$ anti-mouse IgG (Caltag, Burlingame, CA) and FITC-labeled mAb to CXCR4 (12G5) and CCR5 (2D7). Cells were analyzed with a FACScan flow cytometer (Becton Dickinson).

Northern blot and RT-PCR analysis of mRNA for CXCR4, CCR5, and CCR3

For Northern blot analysis, total RNA was prepared using RNAzol following the manufacturer's instructions (Stratagene, La Jolla, CA). IsolatedRNA (15 μg/sample) was electrophoresed in a 1% agarose gel in a 10 mM 3-(*N*-morpholino)propanesulfonic acid buffer and blotted overnight to Nytran using a Turboblotter apparatus (Schleicher & Schuell, Keene, NH). Chemokine receptor probes were labeled using the Random Primer Labeling Kit (Boehringer Mannheim, Indianapolis, IN) and 6000 Ci/mmol [^{32}P]dCTP (Amersham, Arlington Heights, IL). Northern blots were prehybridized in 50% formamide, 20% dextran sulfate, 5× saline-sodium phosphate-EDTA, and 0.5% SDS at 37°C. Denatured ^{32}P-labeled probe at 2×10^6 cpm/ml was hybridized to the blot overnight at 37°C. Blots were then rinsed three times in 1× SSC, 0.1% SDS, washed at 60°C for 30 to 60 min in 1× SSC, 0.1% SDS, and then exposed to x-ray film. The probes all included the complete open reading frame and, in most cases, some untranslated sequence; they are p4 cDNA, CCR1 (41), and clone 3 cDNA, CCR3 (38). A 50-mer oligonucleotide probe specific for human β-actin was used as a control for loading.

For RT-PCR, RNA was isolated from MDDC and unstimulated PBMC as control cells using TRIzol (Life Technologies, Grand Island, NY), according to the manufacturer's instructions. RNA was then treated with 25 U RNase-free DNase I for 60 min at room temperature. cDNA synthesis was performed in a 50 μl vol containing 2 μg total cellular RNA, 50 U avian myeloblastosis virus-reverse transcriptase (Boehringer Mannheim), 0.4 mM (each) dNTP, 40 U RNasin (Promega, Madison, MI), and 1 μg of oligo$(dT)_{12-18}$ primer (Life Technologies). One microgram of cDNA was added to a PCR mixture, containing 0.2 mM dNTP (each), 2.5 mM MgCl2, and 2.5 U *Taq* polymerase (Life Technologies)/reaction. Primers were used at 50 pmol each. The primers for CCR5 were: CCR5-up, 5′-TTC ATT ACA CCT GCA GCT CTC-3′; CCR5-down, 5′-CAG AGC CCT GTG CCT CTT CTT CTC ATT TCG-3′. The primers for CXCR4 were: CXCR4-up, 5′-ACT GAA GCT TGG AGA ACC AGC GGT TAC CAT G-3′; CXCR4-down, 5′-GTA CGG ATC CGT CTT TTA CAT CTG TGT TAG CTG G-3′. The conditions for amplification were: CCR5, 95°C, 3 min (1 min); 55°C, 2 min; and 72°C, 2 min for 1 (30) cycle. For CXCR4: 94°C, 30 s; 55°C, 45 s; and 72°C, 1 min 30 s for 5 cycles, then 94°C, 30 s; 62°C, 45 s; and 72°C, 1 min 30 s for 32 cycles. Eight microliters of the reaction mixture were run on a 1.2% agarose gel in the presence of ethidium bromide. To control for the presence of comparable amounts of cDNA, actin primers were included. Control cDNA reactions in which reverse transcriptase was omitted were run in parallel to exclude amplification of genomic DNA.

Viral strains

HIV-1 89.6 and HIV-1 ADA-M were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health. HIV-IIIB and Ba-L (Advanced Biotechnologies, Columbia, MD) were expanded by one-time passage on PHA-stimulated PBMC obtained from normal donors. Supernatants were cleared of cells by centrifugation, filtered through 0.2-μm pore-size filters, titered on PHA blasts by endpoint titration, and stored at −70°C until use. Before use, virus supernatants were treated with RNase-free DNase (50 U/ml; Boehringer

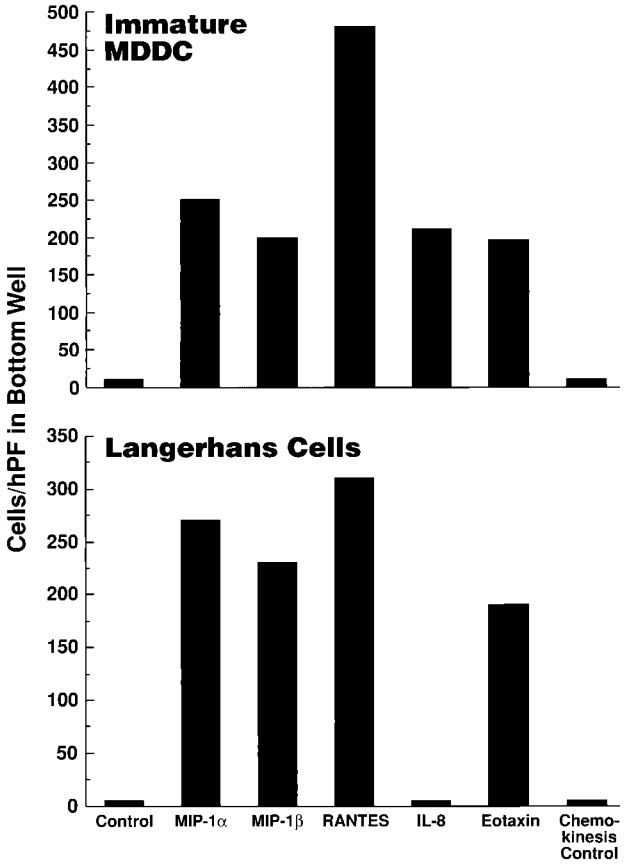


FIGURE 1. DC chemotax to multiple chemokines. Immature MDDC and LC were placed in the upper well of a 96-well chemotaxis chamber for 2 h, followed by counting the number of cells per high power field (hPF) that migrated to the lower well containing the specific ligand at 200 ng/ml. A single chemokinesis control, chemokine in top and bottom wells, for RANTES is shown; similar results were observed for the other chemokines tested. Data are representative of four separate experiments.

Mannheim) for 30 min at room temperature to remove contaminating viral DNA.

Infection systems

Cells were incubated at 100,000 cells/vial with the virus supernatants at a multiplicity of infection of 0.01 to 0.001 for 2 h (*gag*) or 7 h (R/U5 primers), followed by three washes to remove unbound virus. Cells for the R/U5-PCR were then pelleted and frozen at −70°C; cells for *gag*-PCR were incubated for an additional 46 h in RPMI/10% FCS, then washed, pelleted, and frozen at −70°C. To determine baseline contamination of DNA in the viral stocks, the *gag*- and R/U5-PCR were conducted after a 3-min or 2-h viral exposure, respectively; in addition, azidothymidine treatment of cells or pulsing with heat-inactivated virus (1 h at 56°C) was used. In selected experiments, cells were washed, trypsinized for 10 min, resuspended in RPMI/10% FCS to inactivate the trypsin, and used as an additional control to determine baseline contamination of DNA in the viral stocks. Preincubations were performed using anti-CD4 Abs at 40 μg/ml or chemokines alone or in combination for 1 h before the addition of virus.

Semiquantitative DNA-PCR

Semiquantitative DNA-PCR was performed as described (42). The oligonucleotide primer pair specific for the R/U5 region of the LTR (M667/AA55) was used to assess viral entry, as this primer pair flanks sequences within the first region of the viral DNA synthesized during reverse transcription, as described previously (43), and the SK38/39 (*gag* 1551–1578) primers were used to measure HIV *gag* DNA (42). The intensity of the signals was quantified by PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and compared with those from simultaneously run dilutions of ACH-2 cells, a chronically infected T cell line containing one proviral copy per cell.

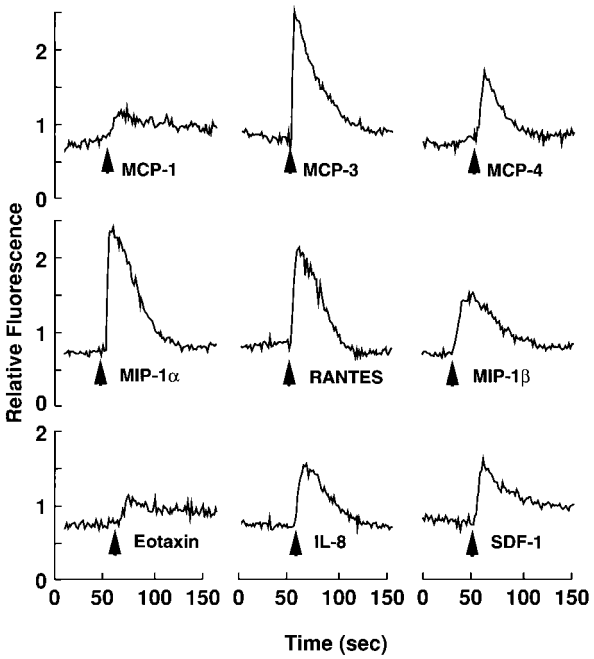


FIGURE 2. Immature MDDC show a calcium flux response to multiple chemokines. Intracellular Ca^{2+} was recorded in real time as the relative fluorescence in fura-2-loaded immature MDDC. Arrowheads mark the time points in which chemokines (as indicated) were added at 25 nM (MIP-1α, MIP-1β, MCP-4), 50 nM (IL-8, MCP-1, MCP-3, eotaxin), and 100 nM (RANTES, SDF-1). Results are representative of five separate experiments using cells from four different donors.

Results

DC chemotax and flux calcium in response to multiple chemokines

Immature MDDC were obtained by culturing peripheral blood-derived monocytes from healthy donors in the presence of GM-CSF and IL-4 for 6 to 7 days, as described previously (29). The resulting cell population expressed CD1a, CD4, HLA-DR, and CD80. Expression of CD14 and CD83 was low or absent (Fig. 3 and data not shown).

To determine the expression of chemokine receptors on MDDC, we first measured chemokine-induced chemotaxis and calcium fluxes. MDDC and LC, obtained after trypsinization of epidermal sheets, chemotaxed in response to RANTES, MIP-1α, MIP-1β, and eotaxin (Fig. 1). MDDC also migrated in response to IL-8, whereas LC did not (Fig. 1); in addition, MDDC chemotaxed to MCP-1 and MCP-3 (data not shown). Immature MDDC exhibited strong calcium flux responses when stimulated with the same panel of chemokines; in addition, they responded to SDF-1 and MCP-4 (Fig. 2).

Chemokine receptor expression on MDDC as determined by flow cytometry

Treatment of monocytes with GM-CSF and IL-4 results in DC with an immature phenotype similar to tissue DC and LC. Culturing of immature MDDC in the presence of TNF-α induces maturation, including an increase in T cell-stimulatory activity and CD83 (a mature DC marker) expression (30) (data not shown). Immature MDDC expressed CCR5, CCR3, and CXCR1 on the cell surface, as determined by staining with the mAb 2D7, 7B11, and an anti-CXCR1 mAb (Fig. 3). A similar pattern was obtained for mature MDDC (data not shown). Neither immature MDDC nor mature MDDC expressed CXCR4 on their surface (Fig. 3 and data not shown). In contrast, the same Ab stained freshly isolated $CD4^+$

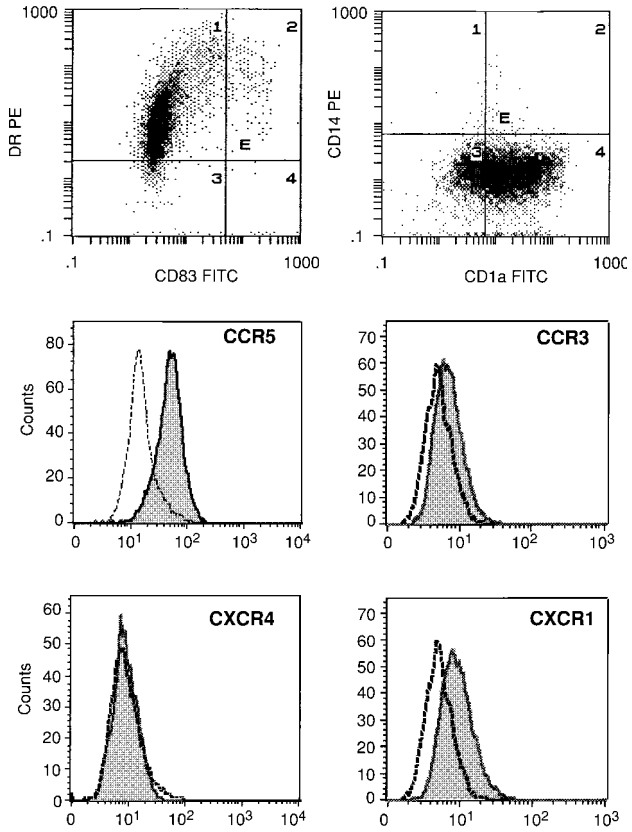


FIGURE 3. Expression of cell surface markers on immature MDDC. Immature MDDC were stained either with a mAb against the indicated marker (——) or an isotype control mAb (– – – –). CD1a and DR are highly expressed on immature DC, whereas expression of CD14 or CD83 is low or absent (*top*). Both CCR5 and CCR3 are expressed on immature MDDC (*middle*). CXCR1 is expressed on immature MDDC; however, CXCR4 (using the mAb 12G5) was not detected (*bottom*). Data are representative of four separate MDDC preparations. Staining for CXCR4 was performed on nine different MDDC preparations.

T cells, monocytes, macrophages (data not shown), and CXCR4-transfected cells (32).

MDDC express mRNA for CCR1, CCR3, CCR5, and CXCR4

Using Northern blot analysis, it has been demonstrated that mature MDDC express CCR5 and CXCR4 transcripts (27). However, the CCR5 cDNA can cross-hybridize to transcripts for the closely related receptor CCR2 (16). RT-PCR was therefore performed on immature and mature MDDC as a more sensitive and specific analysis. CCR5 transcripts were detected in both mature (*lane 1*) and immature MDDC (*lane 2*), as well as in unstimulated PBMC (*lane 3*) (Fig. 4*A*). RT-PCR was also used to detect CXCR4 mRNA transcripts that were present in PBMC (*lane 3*) and immature MDDC (*lane 2*), but expression in mature MDDC (*lane 1*) was variable (Fig. 4*A* and data not shown). Northern blot analysis dem-

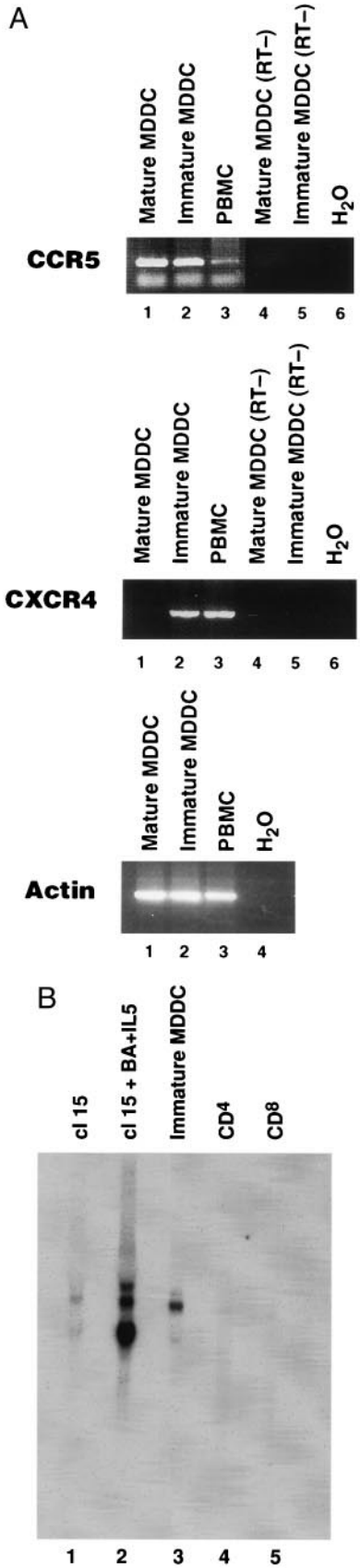
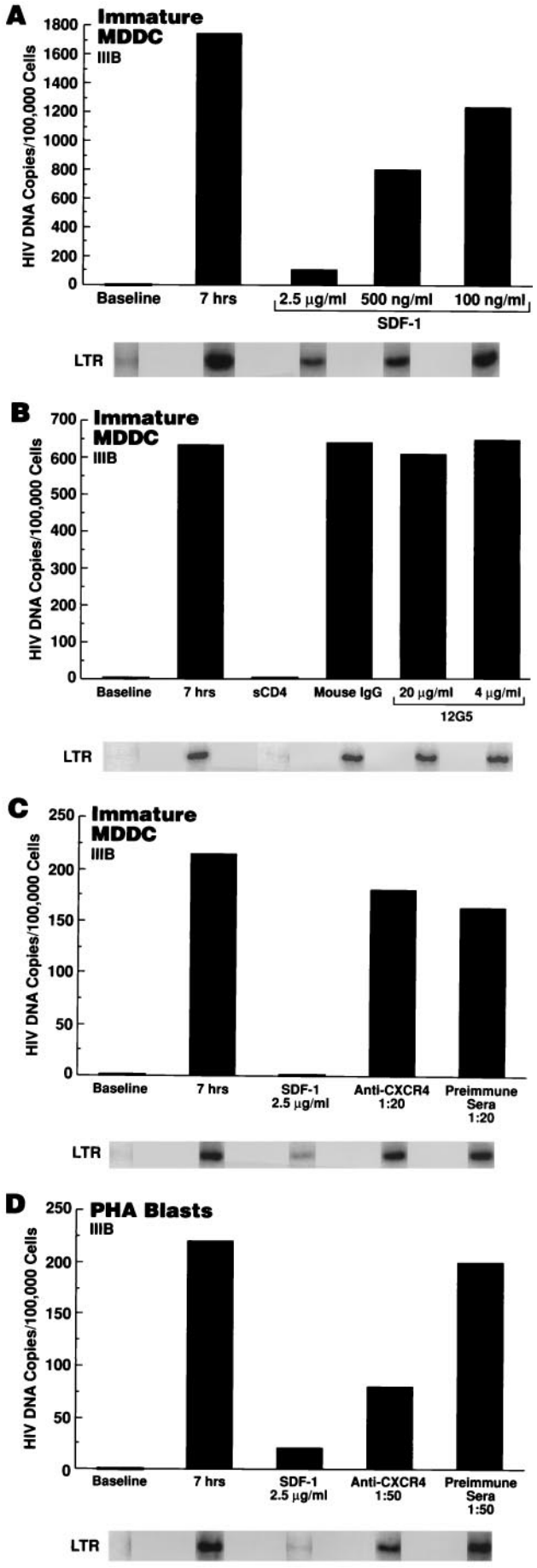


FIGURE 4. Expression of chemokine receptor mRNA in MDDC: RT-PCR (*A*). RNA from the cell types indicated above each lane were reverse transcribed and then amplified using primers specific for CCR5, CXCR4, or actin. Products were resolved by agarose gel electrophoresis and visualized by staining with ethidium bromide. RT refers to samples without reverse transcription to exclude amplification of genomic DNA. Data are representative of three (CCR5) and five (CXCR4) separate experiments. *B*, Northern blot analysis. A Northern blot containing total cellular RNA, 10 μg/lane, from HL-60 clone 15 grown in the absence (cl15) or presence of BA and IL-5 (cl15 + BA + IL-5), immature MDDC, $CD4^+$ T cells, and $CD8^+$ T cells was hybridized with a full-length cDNA probe for CCR3. After washing at high stringency, the blot was exposed to x-ray film in a Quanta III cassette at −70°C with an intensifying screen for 7 days. Data are representative of two experiments.



onstrated the presence of mRNA for CCR1 (data not shown) and CCR3 in immature MDDC. In primary eosinophils and HL-60 cell-derived eosinophils (cl15), CCR3 mRNA exists as a major 1.6-kb transcript and a minor 3.3-kb transcript (38). However, we observed that in immature MDDC, the larger transcript is the predominant species with relatively low amounts of the 1.6-kb transcript (Fig. 4*B*).

Both T- and M-tropic strains of HIV enter MDDC

Semiquantitative DNA-PCR, using primers for the R/U5 region of the LTR (M667/AA55) and *gag* (SK38/39), was used to assess viral entry and infection of MDDC, respectively. Products of both the R/U5-PCR and the *gag*-PCR were observed after exposure of immature MDDC to M-tropic (Ba-L, ADA) and T-tropic (IIIB, SF-2) isolates, indicating that both M-tropic and T-tropic viruses enter and initiate replication in these cells (Figs. 5 and 6 and data not shown). Viral entry was dependent on CD4 since preincubation with an inhibitory anti-CD4 Ab blocked infection with both T-tropic and M-tropic HIV strains (data not shown).

Entry of T-tropic strains of HIV into immature MDDC is inhibited by SDF-1, but not by the anti-CXCR4 mAb 12G5 or a polyclonal Ab against CXCR4

T-tropic strains of HIV use CXCR4 for entry (17), and its ligand SDF-1 specifically inhibits infection (18, 19). SDF-1 inhibited viral entry in immature (Fig. 5*A*) and mature (data not shown) MDDC infected with the T-tropic strain HIV-IIIB. However, no inhibition of HIV-IIIB entry into both immature and mature MDDC was observed using 12G5 Fab (Fig. 5*B* and data not shown). Preincubation of the virus with soluble CD4 completely prevented viral entry (Fig. 5*B*). A neutralizing polyclonal Ab against CXCR4 (kindly provided by G. Alkhatib and E. A. Berger, NIAID, National Institutes of Health) did not inhibit entry of HIV-IIIB into immature MDDC (Fig. 5*C*). In contrast, the Ab inhibited viral entry of HIV-IIIB into PHA blasts (Fig. 5*D*) (17). Given the absence of CXCR4 on the cell surface of MDDC (Fig. 3), these data strongly suggest that T-tropic strains of HIV may use a non-CXCR4, SDF-1 receptor for entry into MDDC.

β-Chemokine-mediated inhibition of entry of M-tropic HIV isolates is dependent on the maturation of the DC

The effect of the CCR5 ligands MIP-1α, MIP-1β, and RANTES on entry of the M-tropic strain HIV-BaL into MDDC was dependent on the maturation stage of the cells. Significant and sometimes even complete inhibition of viral entry into mature MDDC was achieved using MIP-1α, MIP-1β, and RANTES in concentrations ranging from 200 to 500 ng/ml of each β-chemokine, suggesting usage of CCR5 (Fig. 6*A*), as has been recently described (27). In

FIGURE 5. Entry of T-tropic HIV isolates into immature MDDC is inhibited by SDF-1, but not by 12G5 Fab fragments or by a polyclonal Ab to CXCR4. Entry of HIV-IIIB in MDDC (*A*, *B*, and *C*) and PHA blasts (*D*) is inhibited by SDF-1. 12G5 did not inhibit entry of HIV-IIIB into immature MDDC (*B*); however, inhibition was observed when IIIB was preincubated with sCD4 (*B*). Mouse IgG at 10 μg/ml was used as a control. A rabbit anti-CXCR4 antiserum, but not rabbit preimmune serum, blocks entry into PBMC (*D*), but not into MDDC (*C*). HIV PCR products are shown below the corresponding densitometry values. Baseline refers to cells pulsed with HIV and washed immediately as a control for DNA in the viral stock. In each condition, HIV PCR was conducted on cells incubated with HIV-1 for 7 h using the primer pair M667/AA55 for the R/U5 region of the LTR. Data are representative of four, three, two, and two experiments for *A*, *B*, *C*, and *D*, respectively.

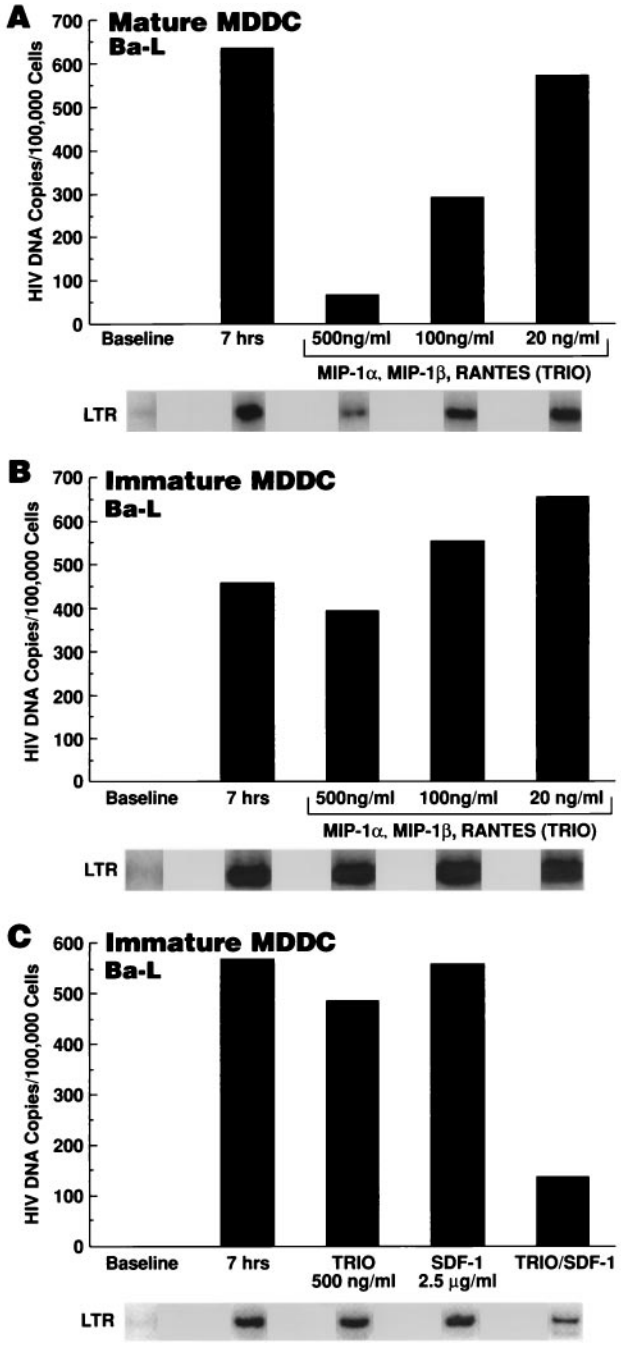


FIGURE 6. Entry of M-tropic isolates into mature MDDC is inhibited by MIP-1a, MIP-1β, and RANTES, while the addition of SDF-1 is required for efficient blockade of entry into immature MDDC. *A*, Entry of HIV Ba-L into mature $CD83^+$ MDDC is inhibited by MIP-1α, MIP-1β, and RANTES. *B*, Entry of HIV Ba-L into immature MDDC is much less sensitive to this combination. *C*, Entry of HIV Ba-L into immature MDDC is inhibited by MIP-1α, MIP-1β, and RANTES together with SDF-1. The concentrations indicated are for each chemokine tested. Trio refers to combined addition of MIP-1α, MIP-1β, and RANTES. In each condition, HIV PCR was conducted on cells incubated with HIV-1 for 7 h. HIV PCR products are shown below the corresponding quantitation, which was calculated from densitometry values from a standard curve of serial dilutions of ACH-2 cells. Data are representative of three, six, five, and three experiments for *A*, *B*, *C*, and *D*, respectively.

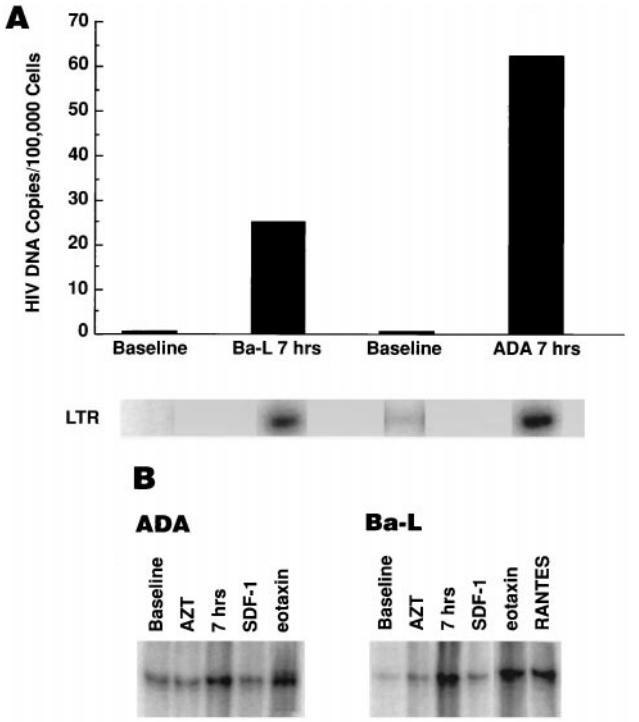


FIGURE 7. M-tropic HIV enters immature MDDC from individuals homozygous for the 32-bp deletion in *CCR5*, which is inhibitable by SDF-1. *A*, The M-tropic HIV-1 isolates Ba-L and ADA enter immature MDDC that do not express functional CCR5. *B*, Entry of both ADA and Ba-L into Δ32 immature MDDC is inhibited by SDF-1, but not by eotaxin or RANTES. Cells infected with virus in the presence of azidothymidine (30 μM) were used as a control. Data are representative of three (*A*) and two (*B*) experiments.

contrast, variable and incomplete inhibition of viral entry was observed when immature MDDC were preincubated with MIP-1α, MIP-1β, and RANTES even in concentrations up to 500 ng/ml each before infection with HIV Ba-L (Fig. 6*B*) or ADA (data not shown). The addition of MCP-1, MCP-3, or IL-8 to the triple combination of MIP-1α, MIP-1β, and RANTES did not increase inhibition of viral entry. We next tested the possibility that M-tropic strains of HIV use the non-CXCR4, SDF-1 receptor referred to above for entry when CCR5 is blocked by MIP-1α, MIP-1β, or RANTES. Preincubation of immature MDDC with the triple combination of β-chemokines (MIP-1α, MIP-1β, and RANTES) only minimally inhibited M-tropic viral entry, while preincubation with SDF-1 alone did not inhibit entry of Ba-L. However, addition of SDF-1 to the triple combination of β-chemokines (MIP-1α, MIP-1β, and RANTES) resulted in marked inhibition of entry (Fig. 6*C*).

Individuals with the 32-bp deletion of *CCR5* do not express CCR5 on their cell surface, and their PBMC have been shown to be resistant to infection with M-tropic HIV isolates (37, 44–48). MDDC were generated from three individuals homozygous for the 32-bp deletion of *CCR5* (CCR5Δ32). Of note, both Ba-L and ADA were able to infect MDDC derived from these individuals (Fig. 7*A*), although copy numbers were low, suggesting inefficient entry. Preincubation with SDF-1, but not RANTES or eotaxin, inhibited entry of Ba-L or ADA, suggesting usage of the novel SDF-1 receptor (Fig. 7*B*). Taken together, these data suggest that a non-CXCR4, SDF-1 receptor on immature MDDC is used for entry by both T-tropic and certain M-tropic viral isolates. In addition, this receptor may be used for viral entry of M-tropic strains into MDDC derived from individuals with the 32-bp deletion of *CCR5*.

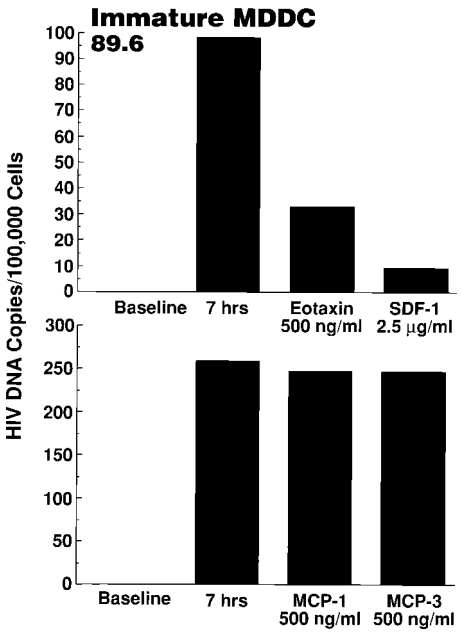


FIGURE 8. The dual-tropic HIV-1 isolate 89.6 enters immature MDDC from individuals with the 32-bp deletion of the *CCR5*; entry is inhibited by eotaxin. Data are representative of three separate experiments.

CCR3 may be used by certain dual-tropic viruses as a coreceptor for entry

CCR3 is expressed on MDDC, as determined by chemotaxis (Fig. 1), calcium flux (Fig. 2), flow-cytometric analysis (Fig. 3), and mRNA analysis (Fig. 4*B*). Analysis of the role of CCR3 as an entry cofactor on MDDC is complicated by the expression of CCR5, which is also used by M- and dual-tropic HIV-1 isolates, and by the observation that RANTES binds to both CCR3 and CCR5 (49). To analyze directly the role of CCR3 as a coreceptor for HIV entry, immature MDDC derived from three individuals homozygous for the 32-bp deletion of *CCR5* (CCR5Δ32) were used. The HIV-1 isolate 89.6 has been shown previously to use CCR2b, CCR3, CCR5, and CXCR4 as coreceptors in nonhuman cell lines transfected with CD4 and human chemokine receptors (11, 13). Entry of 89.6 into CCR5Δ32 MDDC was blocked by eotaxin or SDF-1 (Fig. 8*A*), but not by MCP-1 or MCP-3 (Fig. 8*B*), suggesting that CCR3 present on MDDC was functioning as an HIV entry coreceptor for certain dual-tropic isolates.

Discussion

The present study provides evidence that cells of the DC lineage express multiple chemokine receptors, and that a subset of these receptors may function as coreceptors for HIV entry. Our data indicate the presence of a novel SDF-1 receptor on immature MDDC that is distinct from CXCR4, and that may be used by T-tropic, and under certain circumstances, M-tropic viral isolates. MDDC obtained from individuals with the 32-bp deletion of the *CCR5* gene were infectable with two M-tropic HIV-1 isolates, and entry was most likely mediated by this novel SDF-1 receptor. In addition, CCR3, the eotaxin receptor previously described on eosinophils and microglial cells (21, 26), was detected on LC by chemotaxis and on MDDC additionally by flow cytometry, calcium flux, and mRNA, and was used for viral entry by the dual-tropic HIV-1 isolate 89.6.

The presence of receptors for SDF-1, MIP-1α, MIP-1β, RANTES, MCP-1, MCP-3, IL-8, and eotaxin on MDDC was demonstrated by measuring calcium flux responses; chemotaxis; the detection of mRNA for CCR1, CCR3, CCR5, and CXCR4; and cell surface expression of CCR5, CCR3, and CXCR1. A recent publication describes the expression of mRNA for CCR1, CCR2, CCR5, CXCR1, CXCR2, and CXCR4; however, these authors detected only a faint signal for CCR3 in MDDC (9). We could demonstrate cell surface expression of CXCR1 and CCR3 by flow cytometry. Our results differ with regard to the fact that we detected a chemotactic and calcium flux response to IL-8 and eotaxin. RT-PCR analysis of peripheral blood-derived DC revealed the expression of mRNA for CCR1, CCR2, CCR3, CCR4, CCR5, and CXCR4 in this subset of DC (50). The discrepancy in results may reflect the heterogeneity of cells of the dendritic lineage or may be related to different preparation techniques used when generating MDDC or blood-derived DC. The expression of CXCR4 on the cell surface of DC is still controversial. The expression of CXCR4 mRNA does not necessarily establish expression of CXCR4 protein on the cell surface. We were unable to detect CXCR4 expression on either immature or mature MDDC, as determined by flow cytometry, yet the cells responded to SDF-1. However, monocytes (as the precursor cell population from which the MDDC were derived) expressed cell surface CXCR4. Positive staining was also obtained for PBMC and macrophages. Interestingly, LC were found to express CCR5, but not CXCR4 on their surface, yet mRNA for both CCR5 and CXCR4 was detectable (51) and CXCR4 was detected by intracellular staining techniques. In contrast, peripheral blood-derived DC were shown to express CXCR4 on their surface (50). These results suggest that expression of chemokine receptors on cells of the DC lineage may depend on their stage of differentiation and maturation or on the source of DC. Although the absence of CXCR4 on LC or MDDC was interpreted as a potential explanation for preferential sexual transmission of M-tropic HIV isolates, we have shown entry of T-tropic strains of HIV-1 into immature MDDC. Entry of HIV-IIIB was blocked by SDF-1, but not by polyclonal Abs to CXCR4 or by 12G5. Taken together, these findings suggest the presence of an SDF-1 receptor on MDDC that functions as a coreceptor for entry of T-tropic HIV-1 isolates, but that is distinct from CXCR4. Of note, our data suggest that this receptor may also be used by M-tropic strains of HIV-1 when cell surface CCR5 is absent or blocked. Entry of the M-tropic strains Ba-L and ADA into MDDC from CCR5Δ32 individuals was detected, although in low copy numbers, and was inhibited by preincubation with SDF-1. Interestingly, in the absence of a functional CCR5 coreceptor, usage of other coreceptors, at least with regard to entry of M-tropic isolates, seems to be inefficient. Envelope glycoproteins from M-tropic, but not T-tropic HIV isolates were shown to signal through CCR5 (52), thus raising the question as to whether signal transduction via CCR5 might influence viral entry and replication. Individuals with the 32-bp deletion of *CCR5* have been shown to be resistant to HIV infection, although a few individuals have been described who are HIV-infected despite the presence of this mutation (53–56). According to our data, infection of cells of the dendritic lineage with M-tropic HIV isolates may be possible in these individuals, although initiation of productive infection in autologous $CD4^+$ T cells is most likely restricted to dual-tropic and T-tropic isolates (Dybul M et al., unpublished observations).

DC have been demonstrated to be infected by both T- and M-tropic strains of HIV (27). However, there is disagreement with regard to the productivity of infection (27, 50, 57) and the infectability of certain types of DC by T-tropic viruses (58), which is most likely due in part to the diversity of the DC family as well as to the fact that methods of isolation vary considerably. Entry of

M-tropic HIV isolates into immature MDDC is not blocked or is only incompletely blocked by MIP-1α, MIP-1β, and RANTES, which may be explained by usage of coreceptors other than CCR5 for entry such as a non-CXCR4, SDF-1 receptor. Recently, it was shown that infection of mature, CD83$^+$ MDDC by M-tropic viruses could be inhibited by MIP-1α and RANTES (27), which is in agreement with our results and supports the role of CCR5 being the predominant coreceptor for entry of M-tropic HIV isolates into mature MDDC. However, these data suggest that maturation has an impact on the expression of coreceptors on immature and mature DC, as it was observed previously for Fc receptors and probably mannose receptors (8). In contrast, expression of CCR3 and CXCR1 was detected on both immature and mature DC. It is also possible that as a result of the dynamic membrane fluidity of immature MDDC, which are specialized in the uptake of external Ags (59), CD4, HIV, and CCR5 may be present in regions such as clefts or vesicles or previously described trypsin-resistant regions (60, 61) that are less accessible to a variety of factors including chemokines. With maturation, there is a down-regulation of Ag processing (59) that may be associated with diminished membrane activity, increased accessibility of receptors, and more opportunity for chemokines to inhibit viral entry via their respective coreceptor.

CCR3 has been demonstrated to function as an HIV coreceptor when stably transfected into cell lines (11, 13). Binding of the CCR3 ligand, eotaxin, inhibited infection by a restricted subset of viruses in this system (11). The expression of CCR3 was demonstrated on eosinophils (21, 62, 63) and basophils (64), which are not considered to be targets of HIV infection in vivo, and most recently on Th2 cells (65). The presence of CCR3 in addition to CCR5 was reported on microglia cells of the brain (26), and the detection of CCR3 mRNA was described in peripheral blood-derived DC (50). In this study, CCR3 has been identified on MDDC by multiple approaches, including cell surface staining, and on LC by chemotaxis. To determine whether CCR3 on MDDC was used as a cofactor for viral entry, we generated MDDC from individuals homozygous for the 32-bp deletion of the *CCR5* gene to exclude the usage of CCR5 since CCR3 and CCR5 share certain ligands (49). Viral entry of the dual-tropic isolate 89.6 into immature MDDC derived from such individuals was inhibited by eotaxin, demonstrating that CCR3 may serve as a coreceptor on MDDC for viral entry. These data are in agreement with a recently published study (50) in which eotaxin inhibited replication of HIV YU-2 in a coculture system of blood-derived DC with activated autologous T cells, thus giving indirect evidence for CCR3 being involved as a cofactor for HIV entry.

LC within the lamina propria of the mucosa are among the first cells to encounter SIV after vaginal exposure (3), and a similar scenario most likely exists for HIV-1. Thus, the observation that CCR3 and a non-CXCR4, SDF-1 receptor on MDDC may serve as coreceptors for entry of certain HIV isolates, including M-tropic isolates, may be important with regard to the initial events of HIV infection. The expression of CCR3 on cells of the DC lineage and the observation that both MDDC and LC can chemotax in response to its agonist, eotaxin, may have important implications not only with regard to the potential function of CCR3 as a coreceptor for HIV entry, but also for allergic and autoimmune diseases. The results presented in this work contribute to our understanding of the initial events of HIV infection and have potential implications with regard to the development of therapeutic reagents to block the interactions between HIV and its coreceptors on cells of the DC lineage.

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