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Sylvia Beaulieu

Davide E. Robbiani

Xixuan Du

Expression of a Functional Eotaxin (CC Chemokine Ligand 11) Receptor CCR3 by Human Dendritic Cells[1,2,3]

Sylvie Beaulieu,[4]* Davide F. Robbiani,* Xixuan Du,* Elaine Rodrigues,† Ralf Ignatius,[5]* Yang Wei,* Paul Ponath,[6]‡ James W. Young,† Melissa Pope,[7]* Ralph M. Steinman,* and Svetlana Mojsov[8]*

Critical to the function of Ag-presenting dendritic cells (DCs) is their capacity to migrate to lymphoid organs and to sites of inflammation. A final stage of development, termed maturation, yields DCs that are strong stimulators of T cell-mediated immunity and is associated with a remodeling of the cell surface that includes a change in the levels of expression of many molecules, including chemokine receptors. We show in this study that CCR3, a chemokine receptor initially discovered on eosinophils, is also expressed by human DCs that differentiate from blood monocytes, DCs that emigrate from skin (epidermal and dermal DCs), and DCs derived from $CD34^+$ hemopoietic precursors in bone marrow, umbilical cord blood, and cytokine-elicited peripheral blood leukapheresis. Unlike other chemokine receptors, such as CCR5 and CCR7, the expression of CCR3 is not dependent on the state of maturation. All DC subsets contain a large intracellular pool of CCR3. The surface expression of CCR3 is not modulated following uptake of particulate substances such as zymosan or latex beads. CCR3 mediates in vitro chemotactic responses to the known ligands, eotaxin and eotaxin-2, because the DC response to these chemokines is inhibited by CCR3-specific mAbs. We postulate that expression of CCR3 may underlie situations where both DCs and eosinophils accumulate in vivo, such as the lesions of patients with Langerhans cell granulomatosis. ***The Journal of Immunology*****, 2002, 169: 2925–2936.**

Dendritic cells (DCs)[9] are APCs that play several roles in controlling immunity (reviewed in Ref. 1). DCs are found in blood and in numerous anatomical locations throughout the body. A central feature is their ability to migrate from the periphery to lymphoid organs to initiate immunity and perhaps peripheral tolerance as well. DCs are especially abundant in the epidermis. Epidermal DCs, Langerhans cells (LC), are the prototype of the immature, Ag-capturing DC (2), and express distinct markers, such as E-cadherin (3), Birbeck granules (4, 5), and langerin (6, 7). Immature DCs are also found in blood, spleen, and lung. LCs and other immature DCs can undergo a set of phenotypic changes termed maturation, usually in association with inflammation or infection. Maturation is characterized by higher expression of MHC class II Ags; costimulatory molecules such as CD40, CD54, CD80, and CD86, and other Ags of unknown function at this time, like CD83 and DC-lysosome-associated membrane protein (LAMP). Mature DCs are the most potent stimulators of T cell immunity and can be found in the T cell-rich areas of lymphoid organs and also at sites of inflammation such as the lesions of psoriasis (8). Large numbers of DCs can be generated from precursors within human blood (9–11) or bone marrow progenitors (12, 13). These ex vivo expanded DC populations express the typical markers of DCs in vivo and their abundance makes them useful for examining certain aspects of DC physiology. One such area is the role of chemokines and chemokine receptors in DC migration and function.

Chemokines play a major role in leukocyte traffic and recruitment to sites of inflammation (14, 15). Their effects are mediated by cell surface receptors that belong to the 7-transmembrane class of G-protein-coupled receptors (16). At least 40 human chemokines have been identified (reviewed in Refs. 17 and 18), each

*Laboratory of Cellular Physiology and Immunology, The Rockefeller University and †Department of Medicine, Memorial Sloan-Kettering Cancer Center, Weill Medical College, Cornell University, New York, NY 10021; and ‡LeukoSite, Cambridge, MA 02139

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[3] According to the new proposed classification system (17), the nomenclature of the chemokines used in the experiments and described in this manuscript is as follows: CXCL12 for SDF-1, CCL11 for eotaxin, CCL19 for MIP-3β/ELC, CCL24 for eotaxin-2, CCL1 for I-309.

[4] Current address: Department of Microbiology and Immunology, Université de Montréal, Casier Postal 6128, Succursale Center-ville, Montréal, Québec, Canada, H3C 3J7.

[5] Current address: Department of Medical Microbiology and Infectious Diseases-Immunology, Institute of Infectious Diseases, University Hospital Benjamin Franklin, Free University of Berlin, 12203 Berlin, Germany.

[6] Current address: TolerRx, 675 Massachusetts Avenue, Cambridge, MA 02139.

[7] Current address: Center for Biomedical Research, Population Council, 1230 York Avenue, New York, NY, 10021.

[8] Address correspondence and reprint requests to Dr. Svetlana Mojsov, Laboratory of Cellular Physiology and Immunology, The Rockefeller University, 1230 York Avenue, New York, NY 10021. E-mail address: mojsov@mail.rockefeller.edu

[9] Abbreviations used in this paper: DC, dendritic cell; LC, Langerhans cell; LAMP, lysosome-associated membrane protein; SDF, stromal cell-derived factor; MIP, macrophage-inflammatory protein; ELC, EBV-induced molecule-1 ligand chemokine; HPC, hemopoietic progenitor cell; moDC, blood monocyte-derived DC; MCM, monocyte-condition medium; huCD40L, human CD40 ligand; HAT, hypoxanthine/aminopterin/thymidine; K/S, Kolmogorov-Smirnov.

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interacting with one or more specific receptor(s). Some chemokine receptors, like the stromal cell-derived factor (SDF)-1 receptor CXCR4 and the macrophage-inflammatory protein (MIP)-1α/MIP-1β/RANTES receptor CCR5 are expressed on a variety of cells including DCs (19). The expression of these and several other chemokine receptors is restricted to different stages of DC development. CCR5 and the MIP-3α receptor CCR6 are expressed at high levels on immature, but not mature, DCs (20–25), while the expression of the MIP-3β/EBV-induced molecule-1 ligand chemokine (ELC) receptor CCR7 is significantly up-regulated upon DC maturation (25, 26). Similarly, CXCR4 expression is increased on mature compared with immature DCs (20–22).

The eotaxin receptor, CCR3, is selectively expressed on eosinophils (14, 27–29), basophils (30, 31), and the Th2 subset (32). However, its expression on DCs has not been firmly established. Some reports indicate that CCR3 is expressed on moDCs (33, 34) while results reported in another study indicate that CCR3 is not found on DCs (19). In this study, we use the FACS, RT-PCR, and chemotaxis assays to study the expression and function of CCR3 on different populations of immature and mature DCs.

Materials and Methods

Preparation, culture, and enrichment of DCs

Emigrated skin DCs. Human split-thickness skin samples were obtained from the New York Firefighter's Skin Bank (New York Hospital-Cornell Medical Center, New York, NY) from cadavers within 36 h of death. Emigrated skin DCs were isolated as previously described by Pope et al. (35). Skin was washed twice in Dulbecco's PBS, cut (as 3 × 3-cm pieces), and cultured dermal side down in 100-mm dishes (four pieces per dish) in one of the following tissue-culture medium (15 ml final volume): 1) R10 (RPMI 1640; Life Technologies, Grand Island, NY; supplemented with 10% heat-inactivated FCS), 2) 1% human plasma (Sigma-Aldrich, St. Louis, MO), or 3) serum-free X-vivo15 medium (BioWhittaker, Walkersville, MD). After 2–3 days at 37°C, emigrated cells were treated with collagenase D (400 Mandl U/ml; Boehringer Mannheim, Indianapolis, IN) for 1 h at 37°C. Cells were pooled and washed in the appropriate medium. When cell enrichment was required, $CD3^+$ cells were depleted using magnetic beads (Dynabeads M-450; Dynal Biotech, Oslo, Norway) or by cell sorting as described by Pope et al. (35). Viability was assessed by trypan blue exclusion, and purity was verified routinely by staining for HLA-DR, CD83, p55, DC-LAMP, and CD3 as described below. Five independent experiments were performed for conditions described under 1) and 2), and two experiments for 3). For the RT-PCR experiments, single mature skin DCs, single skin T cells, and mature skin DC-T cell conjugates were obtained by cell sorting as previously described (35).

$CD34^+$-derived DCs from bone marrow, cord blood, and leukapheresis. Mononuclear leukocytes were isolated by Ficoll-Hypaque centrifugation from bone marrow or G-CSF-elicited leukapheresis specimens, obtained from healthy donors of allogeneic hemopoietic stem cell grafts. Cord blood was obtained from normal full-term deliveries. All samples were obtained under local Institutional Review Board-improved protocols. $CD34^+$ hemopoietic progenitor cells (HPCs) were isolated directly from the mononuclear cell populations by immunomagnetic cell separation using the MACS system according to the manufacturer's instructions (CD34 Progenitor Cell Isolation kit, no. 467-01; Miltenyi Biotec, Auburn, CA). Cells were passed over two sequential columns (LS column, no. 424-01; MS column, no. 422-01) to achieve $CD34^+$ purity in excess of 90%. $CD34^+$ HPCs were cultured initially at 2×10^5/3 ml of X-vivo15 medium (BioWhittaker) in six-well Costar plates (no. 3516; Corning, Corning, NY). Cultures were supplemented with the following final concentrations of recombinant human cytokines (minimum activity in IU given where available): GM-CSF (1000 IU/ml; Immunex, Seattle, WA); TNF-α 2.5 ng/ml (25 IU/ml; R&D Systems, Minneapolis, MN); TGF-β1 (5 ng/ml; R&D Systems) (36); c-*kit*-ligand/stem cell factor 20 ng/ml (R&D Systems); and FLT-3L 50 μg/ml (Immunex).

Half the medium was removed from the cultures on each of days 3, 7, 9, and 11. Fresh X-vivo15 (1.5 ml) was added together with 2× cytokines, to maintain the 1× cytokine concentrations given above in a final volume of 3 ml. FLT-3L and c-*kit*-ligand were included from day 0 through day 5–6. On day 5–6, cells were harvested, labeled with anti-CD34 (IgG clone 11.1.6; Memorial Sloan-Kettering Monoclonal Ab Core Facility, New York, NY) and anti-CD66b (IM 0166; Immunotech, Miami, FL), then panned at 4°C on goat-IgG-anti-mouse-IgG-coated plates (37, 38) to deplete any persistent $CD34^+$ HPCs or granulocytes and their immature precursors. Pan nonadherent cells were recultured at $\sim 2 \times 10^6$/3 ml fresh X-vivo15 and cytokines in the 1× concentrations given above. On day 12–13, the $CD34^+$ HPC-derived progeny were labeled with anti-CD1a-PE (PN IM 1942; Immunotech), sorted for $CD1a^+$ cells using a FACStar^{Plus} (BD Immunocytometry Systems, Mountain View, CA; with laser excitation of 200 mW at 480 nM (Innova 90-5 Argon laser; Coherent Radiation, Palo Alto, CA)) to enrich for LCs within the $CD1a^+HLA\text{-}DR^{++/+++}$ subset.

Blood monocyte-derived DCs (moDCs). moDCs were obtained according to Bender et al. (9). Briefly, PBMCs were isolated from Ficoll gradients (Pharmacia, Peapack, NJ). T cells were depleted either by rosetting with neuraminidase (Calbiochem, La Jolla, CA) treated-sheep RBC (Colorado Serum, Denver, CO) or by removing nonadherent cells after 1 h incubation at 37°C at 8×10^6 PBMCs per well of a six-well tray. The monocyte-enriched PBMC fraction was cultured in RPMI 1640 supplemented with either 1) 10% FCS, 2) 1% human plasma, or 3) X-vivo15 (BioWhittaker). At days 0, 2, 4, and 6, GM-CSF (Immunex) was added at a final concentration of 1000 U/ml and recombinant human IL-4 (R&D Systems) at 100 U/ml. These cells, after 6 or 7 days in culture, have many features of immature DCs. Immature DCs (day 6 or 7) were cultured for an additional 2 days in the presence of monocyte-conditioned medium (MCM) as described (9, 39–41). At day 8–9, mature DCs were washed with PBS to remove any chemokines that may have been present in the MCM, resuspended in chemotaxis medium, and tested. The DC phenotype was monitored by FACS and immunostaining of cytospins, as described below. In some experiments, immature and mature DCs were further enriched by depleting T and B cells using anti-CD2- and anti-CD19-coated magnetic beads (Dynabeads), respectively, or sorted with a FACStar (BD Immunocytometry Systems) based on large negative cells after a staining with anti-CD2 FITC and anti-CD19 PE. Purity and maturation states were verified by FACS analysis or alternatively by immunostaining of cytospins, as described below. For the RT-PCR experiments immature and mature moDCs were further purified by sorting on large cells (as determined by forward and side scatter) that were negative for CD3 and CD20 to exclude T cells and B cells.

RT-PCR for detection of CCR3 and other chemokine receptors transcripts in skin DCs and immature and mature moDCs

Total RNA from each cell population was extracted and prepared as described (23). Primers for detection of CCR3, CXCR4, CCR5, CCR7, CCR8, CXCR6, and the actin control were synthesized according to the published sequences. They were as follows: for CCR3 (sense, 5′-TAT CAC AGG GAG AAG TGA A-3′; antisense, 5′-ATC CAG TCT ACG TCT TTT TAA CGC-3′), for CXCR4 (sense, 5′-TGG TCT ATG TTG GCG TCT GGA-3′; antisense, 5′-CTT TTA CAT CTG TGT TAG CTG G-3′), for CCR5 (sense, 5′-CAG GAA TCA TCT TTA CCA GAT-3′; antisense, 5′-TCA CAA GCC CAC AGA TAT TTC C-3′), for CCR7 (sense, 5′-AGT GAG CAA GCG ATG CGA TGC T-3′; antisense, 5′-TCC AGG CAG AAG AGT CGC CTA T-3′), for CCR8 (sense, 5′-ACC TGG CTG TTG TCC ATG CCG T-3′; antisense, 5′-TCA CAA AAT GTA GTC TAC GCT G-3′), for CXCR6 (sense, 5′-TGC ATC ACT GTG GTA CGT TTC A-3′; antisense, 5′-GGC CTC TGT CAC CAT GAT GGT G-3′), for actin (sense, 5′-GTC GTC GAC AAC GCC TCC GGC ATG TG-3′; antisense, 5′-CAT TGT AGA AGG TGT GGT GCC ACA T-3′). The expected size of the amplified fragments was 1.1 kb for CCR3, 600 bp for CXCR4, 570 bp for CCR5, 550 bp for CCR7, 670 bp for CCR8, 460 bp for CXCR6, and 260 bp for actin. The conditions for the RT-PCR were the same as described (23). Actin primers were included in the reactions as an internal control. Amplified samples were resolved on ethidium bromide-stained agarose gels.

The effect of maturation stimuli on chemokine receptor expression on moDCs

The following stimuli were added to immature moDCs at day 6 for 48 h to look for effects on the cell surface expression of CCR5, CXCR4, and CCR3: 1) MCM (n = 17 to 26, depending on the particular chemokine receptor and cell surface or intracellular staining); 2) LPS (20 ng/ml, n = 3 to 4; Sigma-Aldrich); 3) PGE_2 (10 μg/ml, n = 3; Sigma-Aldrich); 4) TNF-α (1000 U/ml, n = 3; R&D Systems); and 5) combination of PGE_2 and TNF-α (n = 3). In a second set of experiments, moDCs were treated with TGF-β1 (5 ng/ml; R&D Systems) at day 0, 2, 4, and 6 (n = 3 to 5). In a third, immature moDCs were treated at day 6 with 1) soluble trimeric human CD40 ligand (huCD40L)/leucine-zipper fusion protein (huCD40L at 300 ng/ml; Immunex, Seattle, WA) for 48 h (n = 4 to 6), or with 2) a

combination of TGF-β1 and huCD40L for 48 h ($n = 4$), or with 3) irradiated huCD40L-transfected L cells (7500 rad) at the ratio of 1 CD40L L cell to 5 DCs, added for 48 h ($n = 3$).

The effect of uptake of particles on chemokine receptor expression on moDCs

To test whether particle uptake would influence CCR3 expression, immature DCs at day 6 were treated for 48 h with 1) zymosan (0.5 μl/ml, $n = 5$; ICN Pharmaceuticals, Costa Mesa, CA) and 2) latex particles (0.5 μl/ml, 0.65-μm diameter; Bangs Laboratories, Carmel, IN) alone ($n = 5$) or in the presence of MCM ($n = 5$) or LPS (20 ng/ml, $n = 3$). The zymosan and latex particles preparation were LPS-free as tested with the *Limulus* amebocyte lysate assay (Associates of Cape Cod, Woods Hole, MA).

Cell lines

The CXCR4-expressing A2.01 human T cell line, a hypoxanthine/aminopterin/thymidine (HAT)-sensitive derivative of A3.01cell line (42), stably transfected with CCR5 was a generous gift from Drs. Q. J. Sattentau (Imperial College of Science, London, U.K.) and A. Trkola (University Hospital, Zurich, Switzerland). It was propagated in RPMI 1640 containing 5% FCS and 1 mg/ml G418 (Life Technologies). G418 was added to the growth medium to maintain the selection for the CCR5 transgene.

Cytofluorometric analysis

The expression of CCR3, CCR5, and CXCR4 on the surface and in the cytoplasm of DCs was evaluated by two-color cytofluorometric analysis. Each staining was performed using $>2 \times 10^4$ cells per well of a "V" shaped bottom 96-well plate. Cells were washed in FACSWash (PBS supplemented with 5% FCS, 0.1% azide) and were either 1) not fixed or 2) prefixed by keeping them for 10 min on ice with 0.1% paraformaldehyde in PBS for cell surface analysis or 4% paraformaldehyde for intracellular staining.

For intracellular staining, cells were first permeabilized for 30 min on ice with 1% saponin. They were then washed three times with FACSWash containing 0.1% saponin to keep the pores open. All subsequent intracellular staining steps were performed in the presence of 0.1% saponin. For both surface and intracellular staining, cells were incubated at 4°C for 45 min with 100 μl of saturating amounts of unconjugated mAbs (listed below). After the incubation, cells were washed four times in FACSWash and incubated in the dark at 4°C for 30 min with FITC-conjugated goat anti-mouse IgG (Cappel Research Products; Organon Teknika, Durham, NC) or with FITC-conjugated mouse anti-rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Cells were washed in FACSWash and then incubated at 4°C with 5% normal mouse serum (Jackson ImmunoResearch Laboratories) for 10 min. PE conjugated to mouse-anti-human mAb were then added for 45 min in the dark at 4°C.

Samples stained for surface expression were washed in FACSWash and then fixed in the dark at 4°C for 10 min with 5% formalin in PBS. Cells were spun and half of the volume was replaced with FACSWash. Intracellularly stained samples were washed in PBS without additional fixation. Cells were analyzed using a FACScan with CellQuest software (BD Biosciences, San Jose, CA).

Phenotypic marker analysis of the DC populations was determined by direct or indirect immunofluorescence. Unconjugated mAbs included anti-HLA-DR clone L243, a mature DC marker anti-CD83 (clone HB15a; Immunotech), a mature DC marker anti-DC-LAMP (kindly provided by Dr. S. Lebecque, Schering-Plough, Dardilly, France), LC marker DC-GM4 (langerin) (kindly provided by Dr. S. Saeland, Schering-Plough), an integrin marker of LFA-1 complex anti-CD11c (clone BU15; Immunotech). PE-conjugated mAbs included anti-HLA-DR (BD Biosciences), a T cell costimulation molecule anti-CD86 (BD PharMingen, San Diego, CA), a monocyte/macrophage marker anti-CD14 (clone Leu-M3; BD Biosciences), an LC marker anti-CD1a (clone BL6; Immunotech), and a hemopoietic precursor cell marker anti-CD34 (BD Biosciences). T cell contamination was monitored with anti-CD3 (BD Biosciences).

Chemokine receptor expression was monitored using the following unconjugated mAbs: anti-CCR3 clone 7B11 provided by Drs. H. Heath and P. Ponath (LeukoSite, Cambridge, MA) (43) and clone 61828.11 (R&D Systems), anti-CCR5 (clone 2D7; BD PharMingen), and anti-CXCR4 (clone 12G5; kindly provided by Dr. J. Hoxie, University of Pennsylvania, Philadelphia, PA). In each experiment, parallel stainings with nonreactive Abs (isotype-matched controls) were performed as controls for nonspecific Ig binding (28, 43). Isotype-matched controls were 1) unconjugated isotypic mouse IgG2a (U7.27; Immunotech) for 7B11 (anti-CCR3), 12G5 (anti-CXCR4), and 2D7 (anti-CCR5); 2) rat Ab IgG2a (Immunotech) for mAb 61828.11 (anti-CCR3); and 3) simultest controls (BD Biosciences).

Statistics

The specificity of staining of the mAbs against CCR3 (7B11 and 61828.11), CXCR4 (12G5), and CCR5 (2D7) was further established through the application of the Kolmogorov-Smirnov (K/S) statistical test (44). For each experiment, the histogram representing the background staining with the isotype-matched Ab (negative control) was compared with the histogram representing the value obtained when DCs were stained with a mAb against a given chemokine receptor. The greatest difference between the two histograms is represented by a D value. They are between 0 and 1. When $D = 0$ it means that the two curves are identical and that the measured level of chemokine receptor expression is not different from the background staining with the isotype-matched Ab control. The average of the D values was calculated from several independent experiments performed. Their numbers are indicated in *Results* and in Figs. 2–4. The K/S statistical test was performed directly with the CellQuest software for FACS analysis.

Immunohistochemistry

Cytospins were prepared as previously described (35). Cells were routinely stained for several different markers including HLA-DR, CD86, and the intracellular sialoprotein marker CD68/macrosialin (DAKO, Carpinteria, CA). The mature DC markers p55 was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health. DC-LAMP was obtained from Dr. S. Lebecque (Schering-Plough). Slides were cover slipped with a PBS/glycerol mix (Sigma-Aldrich).

Chemotaxis assays

Chemotaxis assays were performed in 48-well chambers representing the modified version of the Boyden chamber (Neuro Probe, Cabin John, MD) based on method described by Falk et al. (45). Purified DCs were washed and resuspended in chemotaxis medium at pH 7.2 (RPMI supplemented with 2 mM L-glutamine, 20 mM HEPES and 0.5% BSA). Assays were performed in duplicates or triplicates using 5×10^4 DCs per well with a 5-μm polyvinyl-pyrrolidone-free polycarbonate filter. Each ligand was tested at 100 nM, 10 nM, and 1 nM. Eotaxin-2 was used at concentrations of 1000 nM, 500 nM, and 100 nM. SDF-1 and MIP-3β were used as positive controls in each assay with all the DC subsets. Maximum chemotactic response was observed with 10 nM SDF-1, 100 nM MIP-3β, and 100 nM eotaxin. These concentrations were used subsequently in all the experiments. Background migration (negative control) was measured in the absence of chemokines (medium alone). All incubations were for 2 h at 37°C in a final volume of 50 μl, because in preliminary experiments we found that these conditions supported a maximal chemotaxis response. After 2 h of migration, cells adhering to the bottom of the filter were fixed and stained with Diff-Quick and mounted on glass slides with entellan in xylol. The number of DCs that had migrated to the underside of the filters was counted at $\times$1000 magnification. The values for each assay represent the cell count of five fields as described by Sozzani et al. (46).

To test the specificity of DC migration in response to eotaxin, cells were preincubated with 20 μg/ml of anti-CCR3 (clone 61828.11 or 7B11) or isotype-matched control mAbs (anti-IgG2a rat anti-human or mouse anti-human, respectively) for 1 h at 37°C, and then assayed as above in the continued presence of mAb.

Results

We investigated the expression and function of CCR3 on different subsets of DCs, considering several environmental and maturation factors. In all experiments, we included an additional measure of DC phenotype by monitoring the cell surface and intracellular levels of CCR5 and CXCR4, whose expression have been shown to be regulated upon DC maturation (21, 22). In most experiments, cells were also permeabilized to monitor combined intracellular and surface expression. In control experiments, we determined that prefixation with 0.1% paraformaldehyde does not influence the level of CCR3, CCR5, or CXCR4 expression on the cell surface, as compared with the nonfixed cells. For detection of CCR3 expression on DCs, we used primarily the mAb 7B11 that has been extensively characterized and shown to be highly selective for CCR3 (43). We confirmed the specificity of surface and intracellular mAb7B11 staining in the A2.01 T cell line that does not express CCR3, but expresses CXCR4 (endogenously) and CCR5 (as a transgene) (Fig. 1). We found that mAb 7B11 did not detect

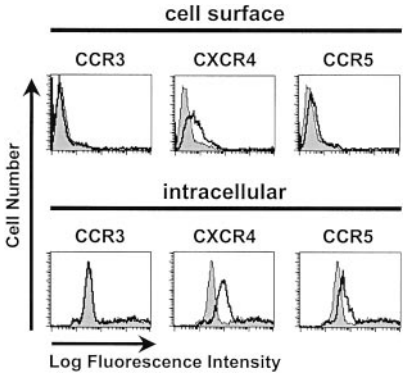

FIGURE 1. mAb 7B11 is specific for detection of the cell surface and intracellular CCR3. The A2.01 human T cell line derived from the A2.03 cell line (42) that does not express CCR3, but expresses CXCR4 endogenously and CCR5 as a transgene, was maintained in culture as described in *Materials and Methods*. Expression of cell surface and intracellular CCR3, CXCR4, and CCR5 were analyzed by FACS. Histograms were obtained using the same conditions as described for DC subsets (see *Materials and Methods* and Figs. 2–4). Each histogram shows the background staining with the nonspecific Ig Ab (negative isotype-matched control; shaded profile) and the staining with the mAb specific for the indicated chemokine receptor (solid line). For CCR3, the two histograms are superimposible, indicating the absence of any specific cell surface and intracellular staining with the mAb 7B11.

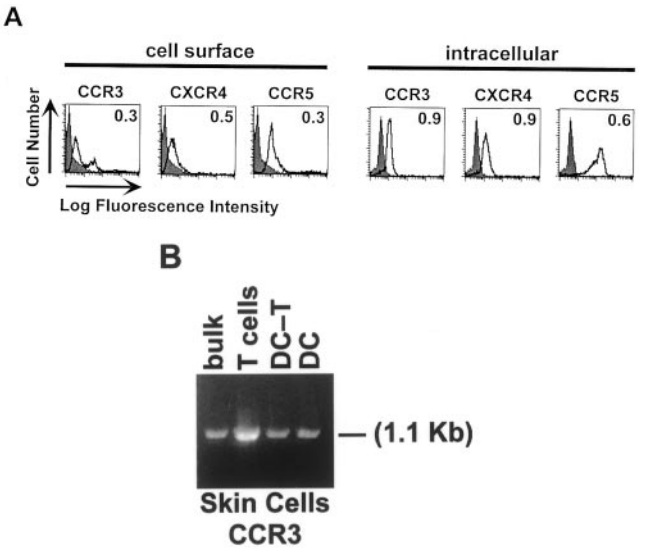

FIGURE 2. *A*, CCR3 is expressed inside and at the surface of DCs emigrating from skin. Expression of cell surface and intracellular CCR3, CXCR4, and CCR5 were analyzed by FACS. The emigrating leukocytes were double-stained with anti-HLA-DR-PE to identify the MHC class II-rich DCs, and with control Ig or mAb to CCR3, CXCR4, or CCR5, detected with goat anti-mouse FITC. One of five similar experiments is shown in which cells were maintained in complete R10. Each histogram shows the background staining with the nonspecific Ig Ab (negative isotype matched control; shaded profile) and the staining with the mAb specific for the indicated chemokine receptor (solid line) by the gated HLA-DR-positive population. The number in each panel indicates the average of the *D* value, which is the greatest difference between the maximum fluorescence intensity (MFI) of the background staining (negative isotype-matched control) and the staining with the mAb specific for the given chemokine receptor, according to the K/S statistical test for all five independent experiments performed. *B*, Detection by RT-PCR of CCR3 mRNA in skin DCs. Purified skin DCs, T cells, and DC-T cell conjugates were isolated from the bulk emigrated DC-T cell mixtures (bulk) as described (35). The 1.1-kb marker indicates the expected size of the full-length CCR3 mRNA. Amplified samples were resolved on ethidium bromide-stained agarose gels.

specific staining on A2.01 cells above the isotype-matched control background. However, 12G5 (anti-CXCR4) and 2D7 (anti-CCR5) detected the expression of CXCR4 and CCR5, both on the cell surface and in the cytoplasm of A2.01 cells. In the later part of our studies, we also used the mAb 61828.11, when it became commercially available, and obtained similar results for the cell surface and intracellular staining of CCR3 as the ones obtained with mAb 7B11.

Results from each experiment were evaluated according to the K/S statistical tests to provide an additional assessment for the specificity of the mAb stainings and to account for the variation of receptor expression that we observed between donors for CCR3, as well as for CCR5 and CXCR4. The histograms in Figs. 2–4 show representative examples of the entire phenotypic and chemokine receptor expression analysis of the given subset of DCs derived from single donors. Isotype-matched negative controls (shaded areas) are shown in each case. At the same time, *D* values for each chemokine receptor shown in these figures were calculated as an average of multiple experiments, and therefore do not represent a single histogram.

Skin-derived DCs express CCR3

DCs emigrate from skin explants via afferent lymphatic vessels (47). This population is a mixture of mature LCs and dermal DCs (35, 47–49). Their morphology and phenotype are typical of mature DCs with high levels of expression of HLA-DR and CD86, as well as CD83 (50), p55 (51), and DC-LAMP (52, 53). As shown in Fig. 2, CCR3, as well as CXCR4 and CCR5 expression were clearly detected by FACS both intracellularly and on the cell surface of HLA-DR$^+$ gated cells. CD1a$^+$ gated cells displayed identical results (data not shown). There was variation in the level of surface CCR3 expression between samples from different donors. However, in all five experiments, we found that CCR3 expression was independent on the tissue culture medium (10% heat-inactivated FCS vs 1% human plasma vs X-vivo15) used for DC culture (data not shown). In the example shown in Fig. 2*A*, CCR3 is also detected in a subset of cells, possibly corresponding to the DC-T cell conjugates.

Expression of CCR3 mRNA by skin DCs was also confirmed by RT-PCR analysis (Fig. 2*B*). For comparison, in a single experiment we used bulk skin populations (containing DCs, T cells, and DC-T cell conjugates), single T cells, and DC-T cell conjugates obtained from the same donor. We found in all experiments, including the one shown in Fig. 2*B*, that skin DCs from multiple donors express CCR3 mRNA, but these levels were lower than those seen in T cells.

CD34$^+$-derived DCs express CCR3

Expression of CCR3, CCR5, and CXCR4 was examined during different stages of the development of DCs originating from CD34$^+$ HPCs. CD34$^+$ HPCs from umbilical cord blood, adult bone marrow, or G-CSF elicited peripheral blood stem cells give rise to DCs under the aegis of cytokines, like stem cell factor (c-*kit*-ligand), FLT-3L, GM-CSF, and TNF-α (13, 54). One population, LCs (CD1a$^+$CD14$^-$HLA-DR^{bright}) develops directly from CD34$^+$ HPCs (11, 37, 55). The other population develops from CD34$^+$ HPC into CD14$^-$HLA-DR^{bright} interstitial or dermal DCs via a CD14$^+$HLA-DR$^+$ intermediate, which can alternatively develop into macrophages (11, 37). The addition of TGF-β1 in serum-free medium supports the specific generation of LCs (36, 56)

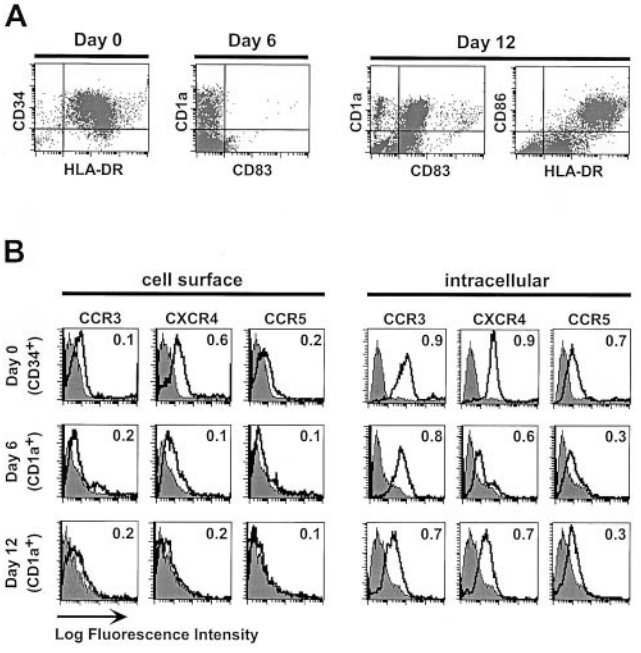

FIGURE 3. Phenotype and chemokine receptor expression of bone marrow-derived DCs. Bone marrow cells were studied at different time points (days 0, 6, and 12). *A*, Cell phenotype was monitored using double-staining for CD34 and HLA-DR (day 0), CD1a and CD83 (days 6 and 12), and CD86 and HLA-DR (day 12). Representative examples for each time point are shown. *B*, Corresponding histograms of cell surface and intracellular CCR3, CXCR4, and CCR5 expression from 1 representative experiment. Cells were double-stained with anti-CD34 (day 0) or anti-CD1a-PE (days 6 and 12) and combined with the appropriate negative isotype-matched controls vs anti-CCR3, -CXCR4 or -CCR5 mAbs (FITC). Histograms represent results on $CD34^+$ gated cells (day 0), $CD1a^+$ gated cells (day 6), and $CD1a^+$ sorted cells (day 12). Each histogram shows the background nonspecific staining with the isotype-matched control (shaded profile) and the staining with the mAb specific for the indicated chemokine receptor (solid line). The number in each panel indicates the average of the *D* value, which is the greatest difference between the background staining with the negative isotype-matched control and the staining with the mAb specific for the given chemokine receptor, according to the K/S statistical test for all three to six independent experiments performed (see *Materials and Methods* and *Results*).

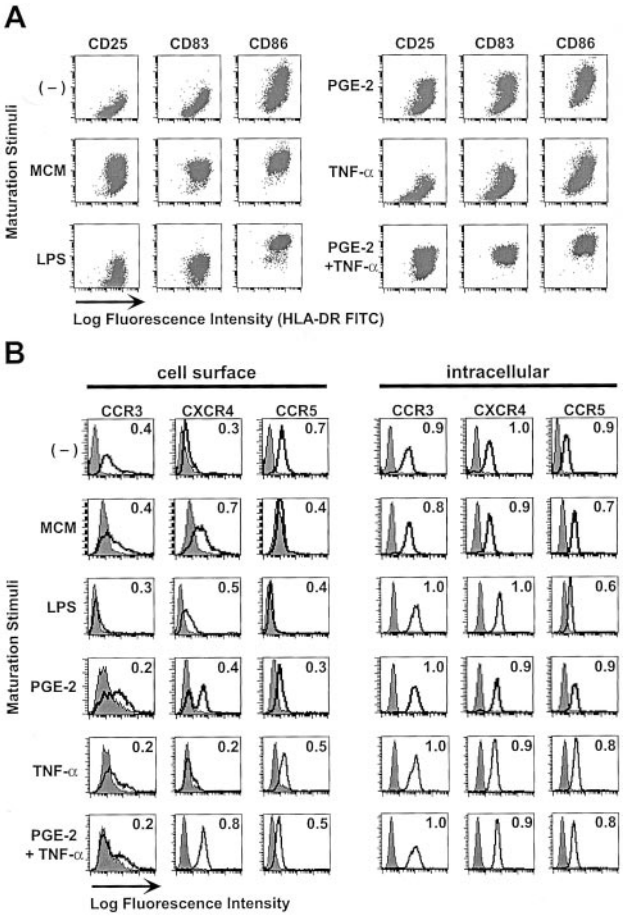

FIGURE 4. Different maturation stimuli have little impact on CCR3 expression by moDCs. Blood-derived monocytes were cultured for 6 days in the presence of GM-CSF and IL-4 to generate immature DCs. At day 6, immature DCs were cultured for 48 h with different maturation stimuli as indicated and flow cytometric analysis was performed. *A*, For each maturation stimulus, DC phenotype was monitored using double-staining with anti-HLA-DR-FITC and PE-conjugated anti-CD25, -CD83, and -CD86. Representative data from 1 of 3 to 40 experiments are shown (see *Materials and Methods*). *B*, Corresponding histograms illustrating cell surface and intracellular CCR3, CXCR4, and CCR5 expression from DCs of the same individual as in *A*. Cells were double-stained with anti-HLA-DR-PE and isotype-matched control vs anti-CCR3, -CXCR4, or -CCR5 mAbs (detected with goat anti-mouse-FITC). One representative experiment is shown for immature moDCs (−) of 1) 32 performed for cell surface and 23 for intracellular CCR3 expression, 2) 40 for cell surface and 28 for intracellular CXCR4 expression, and 3) 38 experiments for cell surface and 26 for intracellular CCR5 expression. For mature moDC, 1 representative experiment of 26 for MCM, and of 3 for LPS, PGE-2, TNF-α, and PGE-2 + TNF-α is shown. Each histogram shows the background staining with the nonspecific Ig Ab (negative isotype-matched control; shaded profile) and the staining with a mAb specific for the indicated chemokine receptor (solid line) by the $HLA\text{-}DR^+$ gated cells. The number in each panel indicates the average of the *D* value, which is the greatest difference between the background staining with the negative isotype-matched control and the staining with the mAb specific for the given chemokine receptor, according to the K/S statistical test for all 3 to 40 independent experiments performed (see *Materials and Methods*). Values of *D* were calculated as an average of all the experiments performed for a given condition and do not correspond to the level of chemokine receptor expression illustrated by the histograms. Results in *A* and *B* were obtained with moDCs derived from one individual.

that express E-cadherin (3), Birbeck granules (4), and the associated Lag Ag (57), or langerin (6).

FACS analyses at days 0, 5–6, and 12–13 of culture demonstrated the DC phenotype (Fig. 3*A*) and CCR3, CXCR4, and CCR5 expression (Fig. 3*B*) of $CD34^+$-derived bulk progeny from bone marrow. Results shown in Fig. 3 represent one of three independent experiments for day 0 DCs, three (intracellular staining) to six (cell surface staining) for day 6 DCs, and three (intracellular staining) to four (cell surface staining) for day 12 DCs. At day 0, the majority of cells were $CD34^+$, $HLA\text{-}DR^+$ cells (Fig. 3*A*, *left panel*). At days 6 and 12, cells were analyzed for the presence of $CD1a^+$ DC precursors. $CD1a^+$ intermediates were evident at day 6 (Fig. 3*A*, *middle panel*). By day 12, even before exogenous maturation stimuli, a significant population of $CD1a^+CD83^+$ and $CD86^+HLA\text{-}DR^{+++}$ cells were present (Fig. 3*A*, *right panels*). The expression of cell surface CCR3 was detected at all stages of DC development with similar levels detected at days 0, 6, and 12. The expression of CCR5 was similar to that of CCR3, while cell surface expression of CXCR4 decreased after day 0 and remained low thereafter. Similar results were obtained with DC populations generated from umbilical cord blood and leukapheresis-derived DCs. For cord-blood-derived DCs, one (intracellular staining) to three (cell surface staining) independent experiments were performed for day 0 DCs, two for day 6 DCs, and two for day 12 DCs

(data not shown). For peripheral blood stem cell-derived DCs, one (intracellular staining) to two (cell surface staining) independent experiments were performed for day 0 DCs, two (intracellular staining) to four experiments (cell surface staining) for day 6 DCs, and three (intracellular staining) to four experiments (cell surface staining) for day 12 DCs (data not shown). As was the case with the skin-derived DCs (Fig. 2), all the populations of $CD34^+$-derived DCs contained a significant level of intracellular CCR3, CXCR4, and CCR5.

TGF-β1 plays a critical role in the differentiation of LCs in vitro and in vivo (36, 58, 59). However, we found that TGF-β1 did not influence the observed (above) expression of CCR3, CCR5, and CXCR4 on DCs originating from $CD34^+$-precursors (data not shown).

Mature and immature moDCs express CCR3

DCs can be obtained in large numbers from blood monocytes, and these DCs were used to study the effects of different stimuli on the surface expression of CCR3, CCR5, and CXCR4. After an initial differentiation of monocytes to immature DCs with GM-CSF and IL-4, maturation was induced by culturing for an additional two days with 1) MCM, 2) LPS, 3) PGE_2, 4) TNF-α, and 5) a combination of PGE_2 and TNF-α (Fig. 4*A*). As was the case with skin- and $CD34^+$-derived DCs, CCR3, CXCR4, and CCR5 levels on the cell surface varied between donors. Fig. 4, *A* and *B*, illustrates the phenotypic characteristics and CCR3, CXCR4, and CCR5 expression by moDCs derived from one individual. In this example, there might be a slight down-regulation of the cell surface CCR3 levels on mature DCs as compared with immature. However, the average *D* values, shown in the right corners of the histograms (Fig. 4*B*), calculated from the analysis of cell surface CCR3 expression (vs the isotype-matched controls) on immature and mature DCs (obtained upon treatment with MCM) derived from 32 individuals highlight that mature and immature DCs expressed similar levels of CCR3 on their cell surface. The level of expression of CCR3 on the cell surface of mature DCs obtained upon treatment with LPS ($n = 3$), PGE_2 ($n = 3$), TNF-α ($n = 3$), and combination of PGE_2 and TNF-α ($n = 3$) was again not significantly different from the level detected on immature DCs. All cells contained significant amounts of intracellular CCR3 (*right panels*).

As expected, the surface expression of CXCR4 was significantly increased on mature moDCs compared with immature blood DCs (Fig. 4*B*, *middle panels*) (20–22). This pattern of CXCR4 expression is different from the one that we observed with different populations of $CD34^+$-derived DCs (Fig. 3*B*, *middle panels*). The surface expression of CCR5 was down-regulated upon maturation (Fig. 4*B*, *right panels*) in agreement with previous findings (20, 23). Incomplete DC maturation induced with PGE_2 or TNF-α alone (Fig. 4*A*) correlated with "intermediate" expression of CXCR4 and to a lesser degree CCR5 (Fig. 4*B*, *middle* and *right panels*). Again, all three receptors were detected intracellularly.

Several reports have indicated that other stimuli, such as TGF-β1 and huCD40L, can influence the differentiation and phenotype of moDCs (22, 36, 58, 59). In the next set of experiments, TGF-β1, huCD40L, or a combination of the two were added to immature DC culture. TGF-β1 increased CD1a expression but did not fully mature the DC. The presence of huCD40L resulted in DC maturation as monitored by FACS analysis, and the combination of TGF-β1 and huCD40L resulted in an intermediate pattern of maturation (data not shown). However, both populations of mature DCs expressed similar levels of CCR3 on their surface, while the levels of CXCR4 and CCR5 correlated with the DC maturation state in agreement with previous findings with moDCs (data not shown).

RT-PCR analysis was also performed to assess the presence of CCR3 mRNA in immature and mature moDCs. Fig. 5*A* shows the results obtained in mature moDCs from one individual. The expression of CCR3 mRNA was compared with the expression of CXCR4, CCR5, CXCR6, and CCR8 mRNAs. As seen from Fig. 5*A*, mature moDCs express significantly lower levels of CCR3 transcripts compared with CXCR4 and CCR5, but similar to the levels of CXCR6. The relative levels of CCR3, CXCR4, and CCR5 mRNA in mature moDCs detected in this experiment correlated with the levels measured by FACS analysis (Fig. 4*B*). The expression of CCR3 in mature moDCs was further confirmed when full-length CCR3 cDNA was obtained from total RNA prepared from purified mature mo DCs. However, immature and mature moDCs from another individual did not express CCR3 transcripts, although the pattern of expression of CXCR4, CCR5, and CCR7 mRNAs in immature vs mature DCs was in agreement with the published observations (20, 21, 23, 25). In addition, the relative levels of CXCR4 and CCR5 mRNA in immature and mature DCs of this individual correlated with the levels detected by FACS analysis and represented by the *D* values calculated as an average of multiple experiments(Fig. 4*B*).

As expected, in both individuals immature and mature moDCs did not express transcripts for CCR8, the I-309 receptor constitutively expressed in monocytes and the thymus (60, 61).

Uptake of particles by DCs does not alter CCR3 expression

Our experiments demonstrated that DCs derived from different progenitors and at various stages of maturation expressed low levels of CCR3 on their cell surface. However, there was always a

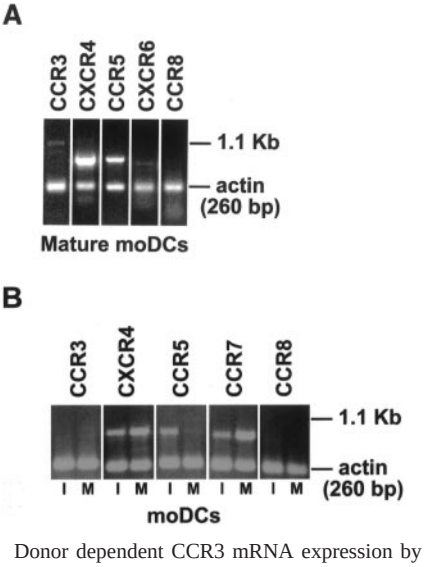

FIGURE 5. Donor dependent CCR3 mRNA expression by mature (*A*) and immature and mature (*B*) moDCs detected by RT-PCR. Expression of CCR3 mRNA in purified mature (*A*) and immature and mature moDCs (*B*) was compared with the expression of CXCR4, CCR5, CXCR6, and CCR8 mRNAs (*A*) and to CXCR4, CCR5, CCR7, and CCR8 mRNA (*B*). DCs were purified by sorting on large cells (as determined by forward and side scatter) that were negative for CD3 and CD20 to exclude T and B cells, respectively (41). Representative examples are from two individuals. The 1.1-kb marker indicates the expected size of full-length CCR3 mRNA. Immature and mature moDCs in *B* do not express CCR3 mRNA. Actin primers were added in each sample as an internal control. Amplified samples were resolved on ethidium bromide-stained agarose gels.

significant intracellular pool of CCR3. Therefore, we next investigated whether the uptake of particles would stimulate the release of CCR3 from this intracellular pool and increase its levels on the cell surface. Immature moDCs (day 6) were cultured without or with 1) zymosan or 2) latex beads in the presence or absence of maturation stimuli (MCM or LPS). DC phenotype and CCR3, CXCR4, and CCR5 expression were monitored at different time points (1, 5, 24, and 48 h). Within 1 h, many latex and zymosan particles were taken up and were retained for the 48-h observation period (data not shown). DC phenotype and CCR3 expression were unchanged at the 1 h time point. Within 5 h, evidence of phenotypic maturation (up-regulation of CD25, CD83, CD86) was observed with zymosan alone, and also with zymosan in combination with MCM (data not shown). At 48 h, a typical mature DC phenotype was seen (Table I). In contrast, uptake of latex beads did not affect the phenotype even after 48 h (Table I). However, none of these conditions had a significant impact on the levels of CCR3 on the surface of mature moDCs (Table I). Uptake of latex beads during maturation in the presence of LPS may have somewhat increased the surface expression of CCR3 on mature DCs, as measured by the changes of *D* values from 0.4 to 0.6. CCR5 expression was in accordance with expected levels of these receptors in immature and mature moDCs in the absence of a particle meal (Ref. 21; Figs. 4*B* and 5*B*). The particle uptake prevented the up-regulation of CXCR4 on mature moDCs upon stimulation of immature DCs with LPS but not MCM. These observations indicate that different pathways regulate the levels of cell surface expression of CCR5 and CXCR4 on immature and mature moDCs, but have little impact on CCR3.

CCR3 expression by DCs enables migration to eotaxin and eotaxin-2

Chemotaxis assays were performed on skin-, $CD34^+$-derived (bone-marrow, umbilical cord-blood, and leukapheresis), and immature and mature moDCs. The tested chemokines interacted with CCR3 (eotaxin and eotaxin-2), CXCR4 (SDF-1), and CCR7 (MIP-3β/ELC). For the chemotaxis experiments, DCs were selected on the basis of CCR3 expression on their cell surface, because the main aim was to determine whether DCs have a chemotactic response to eotaxin. The average of the *D* values, calculated according to the K/S statistical test (44) (see Figs. 2–4 and *Statistics*), was taken as an index representing the level of CCR3 expression on the cell surface. The range of *D* values on all different DC subsets used in the chemotaxis experiments was between 0.1 and 0.8. In all experiments, DC chemotaxis in response to SDF-1 (10 nM) and MIP-3β (100 nM) was the positive control, while DC chemotaxis in the absence of any chemokines (culture medium alone) was the negative control.

Chemotactic responses to eotaxin (100 nM) were observed with all DC populations (Figs. 6 and 7). However, there was a large variation in the migratory responses observed between different experiments, regardless of the DC origin (Fig. 6*A*). The number of migratory cells correlated roughly with the *D* values of CCR3 expression. For example, the high migratory response was consistently observed with DCs where *D* values were above 0.5.

We pursued this observation further with immature and mature (obtained upon MCM treatment) moDCs, because we could perform larger number of experiments. In this set of experiments ($n = 12$), we compared the chemotactic response of cells with *D* values above 0.4 (high) and below 0.4 (low). This *D* value was chosen as a cut-off number because we observed that, in the majority of experiments, much higher chemotactic responses to eotaxin were observed when *D* values were 0.4 and higher, and also because it corresponded to the average *D* values (Fig. 4 and Table I). As seen in Fig. 6*B*, both immature and mature moDCs with *D* values above 0.4 ($n = 4$ for immature and $n = 7$ for mature DCs) demonstrated larger chemotactic responses to 100 nM eotaxin than DCs with *D* values below 0.4. These differences were statistically significant (Student's *t* test, $p < 0.026$ for immature DCs, and $p < 0.00015$ for mature DCs).

The immature moDCs consistently demonstrated lower chemotactic responses than the mature moDCs, even when the *D* values were identical. The extent of chemotactic migration of immature and mature DCs was dependent on the eotaxin concentrations (Fig. 6*C*) and continued to be segregated at concentrations of 1 and 10 nM according to the *D* values, as was the case with 100 nM eotaxin (Fig. 6*B*). The responses of immature and mature DCs to 100 nM eotaxin shown in Fig. 6*B* were measured in the same experiments as the responses to 1 and 10 nM eotaxin ($n = 12$) shown in Fig. 6*C*. The values for 100 nM eotaxin from Fig. 6*B* are plotted again in Fig. 6*C* to highlight the dose-dependent chemotaxis. In a few instances, mature moDCs did not show chemotactic response upon treatment with eotaxin, although *D* values were in the range of 0.4, and chemotaxis was observed in response to SDF-1 and MIP-3β.

In all the control experiments, immature moDCs showed only a low response to SDF-1 (10 nM) and MIP-3β (100 nM) (data not shown). In contrast, mature moDCs showed strong chemotactic activity to SDF-1 (10 nM) and MIP-3β (100 nM) (Fig. 7*A*). The chemotactic responses of immature and mature DCs to SDF-1 correlated well with the increased expression of CXCR4 on mature DCs (Figs.

Table I. *Uptake of particles does not modulate cell surface CCR3 expression by moDCs*[a]

	Chemokine Receptor Expression (*D* values[b])			DC Phenotype[c] (48 h)		
Stimulus	CCR3	CXCR4	CCR5	CD25	CD83	CD86
None	0.4	0.3	0.7	−	−/weak	++
MCM	0.4	0.7	0.4	++	++	++++
LPS	0.3	0.5	0.4	++	++	++++
Latex	0.4	0.1	0.5	−	−/weak	++
Latex + MCM	0.3	0.6	0.4	++	++	++++
Latex + LPS	0.6	0.3	0.3	++	++	++++
Zymosan	0.4	0.7	0.5	++	++	++++
Zymosan + MCM	0.2	0.5	0.2	++	++	++++
Zymosan + LPS	0.3	0.2	0.3	++	++	++++

[a] The average *D* values from three to five independent experiments are shown for CCR3, CXCR4, and CCR5 surface expression on HLA-DR^+ moDCs activated by the indicated stimuli.

[b] Values of *D* represent the greatest difference between the mean fluorescence of the isotype negative control and the specific Ab stain according to the K/S statistical test.

[c] Each + reflects 1 log of fluorescence staining as determined by FACS.

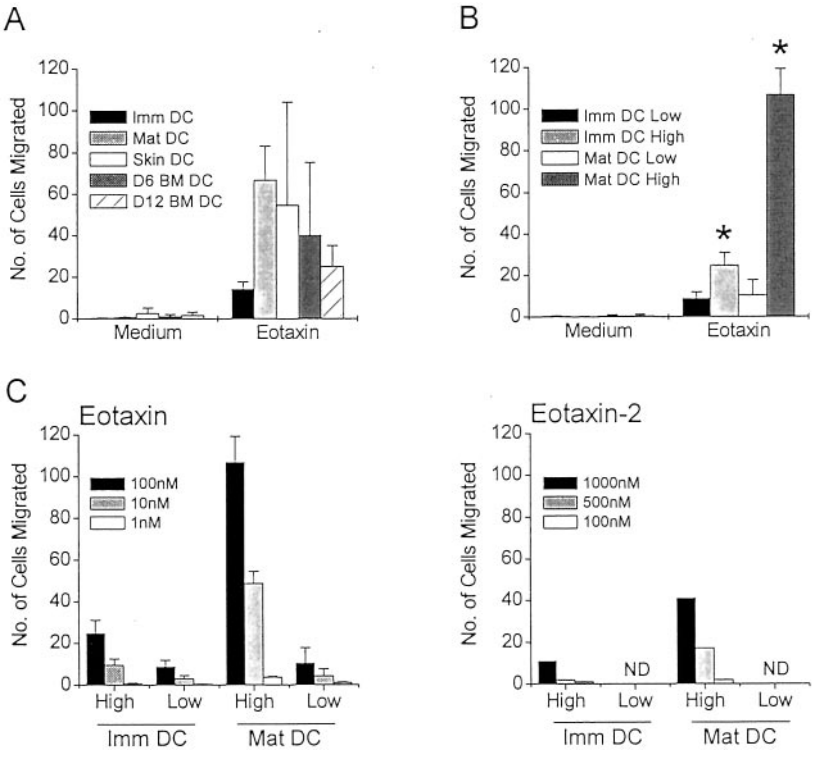

FIGURE 6. Chemotactic responses of different subsets of DCs. Immature moDCs, mature moDCs, emigrated skin DCs, and $CD34^+$ bone marrow (BM)-derived DCs at days 6 and 12 were isolated and prepared as described in *Materials and Methods*. Cells (5×10^4 cells/well) were incubated in a final volume of 50 μl for 2 h without (medium alone) or in the presence of a gradient of eotaxin (1 nM, 10 nM, 100 nM) (in triplicate wells) and tested for their ability to migrate across a 5-μm pore-size polycarbonate filter. For each well, the number of DCs that had migrated through the filter and were still attached to it was determined. Migrated DCs were counted twice in five fields at $\times$1000 magnification and averaged. Chemotaxis in response to SDF-1 (10 nM) and MIP-3β (100 nM) were the positive controls (not shown) and in the absence of any chemokines (medium) was the negative control. *A*, Results (mean $\pm$ SEM) represent an average of 12 independent experiments measuring the chemotactic response to 100 nM eotaxin of immature and mature moDCs and 2 for skin- and $CD34^+$- bone marrow days 6 and 12 DCs. Chemotaxis experiments were also performed with days 6 DCs ($n = 2$) and 12 DCs ($n = 2$) derived from $CD34^+$ progenitors from cord-blood and by leukapheresis. Results from both experiments were similar to the ones shown for $CD34^+$ bone marrow-derived days 6 and 12 DCs (data not shown). *B*, Chemotactic responses to 100 nM eotaxin of immature and mature moDCs expressing different levels of cell surface CCR3 as determined by *D* values (see *Statistics*, Figs. 2–4 and Table I). Results (mean $\pm$ SEM) are an average of 8 independent experiments for immature, and 5 for mature, moDCs with *D* values below 0.4 (low) and 4 independent experiments with immature, and 7 for mature, moDCs with *D* values above 0.4 (high). $*$, Statistically significant differences in *B*. *C*, Dose-dependent chemotactic response to eotaxin (1, 10, and 100 nM) and eotaxin-2 (100, 500, and 1000 nM) of immature and mature moDCs expressing low and high levels of cell surface CCR3 as determined by *D* values. Responses to 1 and 10 nM eotaxin were measured in the same experiments as the ones shown in *B*. Results for 100 nM eotaxin from *B* are plotted again in *C* to highlight the dose-dependent migratory responses. Results for eotaxin-2 represent an average of triplicate wells.

4*B* and 5*B*), while the strong migratory response of mature DCs to MIP-3β correlated with increased mRNA expression of CCR7 by mature DCs (Ref. 25 and Fig. 5*B*).

We also tested eotaxin-2, another CCR3 ligand, but with lower affinity than eotaxin. As seen in Fig. 6*C*, eotaxin-2 did induce a dose-dependent chemotactic response, albeit lower than that induced by eotaxin. The maximum chemotactic response to eotaxin-2 was observed at 1000 nM and was lower than the migratory response to 100 nM eotaxin, consistent with the published observations (62).

Checkerboard analysis conducted in preliminary experiments confirmed that the actions of eotaxin and eotaxin-2 on mature moDCs were chemotactic and not chemokinetic (data not shown).

To confirm that CCR3 was mediating the response to eotaxin, mature moDCs were preincubated with anti-CCR3 mAbs (clone 61828.11 and mAb 7B11) at concentrations of 20 μg/ml before measuring chemotaxis to eotaxin, eotaxin-2, SDF-1, and MIP-3β. Both Abs inhibited the chemotactic response to eotaxin and eotaxin-2, but not as completely as reported for eosinophils (43, 62). The migration in response to SDF-1 and MIP-3β were unaffected. The results shown in Fig. 7 represent the chemotactic responses obtained in the presence of the mAb clone 61828.11 and are similar to the ones obtained with the mAb 7B11 Ab.

Discussion

Our results demonstrate that the eotaxin receptor CCR3 is expressed and is functional on immature and mature moDCs, mature skin-DCs, as well as on DCs derived from $CD34^+$ hemopoietic progenitors. However, we found that the level of CCR3 expression was donor-dependent. A few individuals, from the pool of $\sim$50 examined in our studies, did not express CCR3 on their DCs, an observation that may explain the conflicting reports in the literature regarding the expression of CCR3 on DCs (19, 33, 34). The expression of CCR3 on the cell surface was lower, as determined by FACS analysis, than CCR5 and CXCR4, the other two chemokine receptors that we used as controls in our studies. This observation was confirmed in the RT-PCR experiments that also detected in mature moDCs lower levels of CCR3 mRNA as compared with CXCR4 and CCR5 mRNAs (Fig. 5*A*).

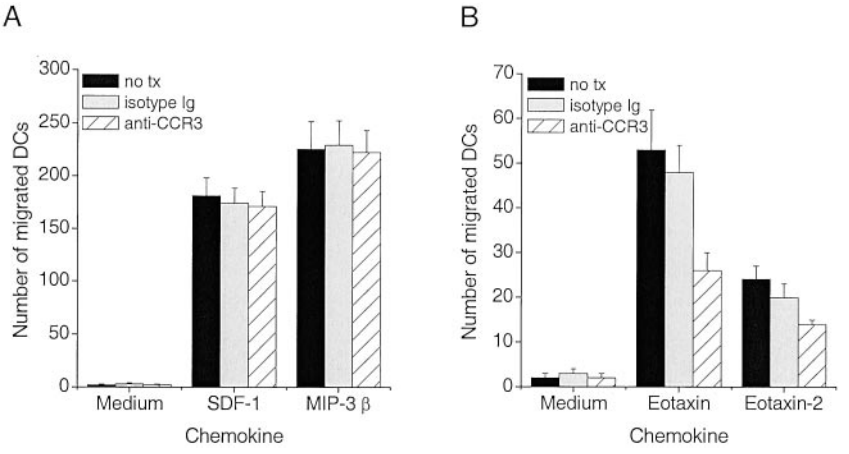

FIGURE 7. Inhibition of eotaxin-induced chemotaxis by anti-CCR3 mAb. Mature moDCs were generated as described, using MCM as the maturation stimulus. DCs were preincubated for 1 h at 37°C with 20 μg of either nonspecific Ig Ab (isotype-matched control) or anti-CCR3 mAb (clone 61828.111; R&D Systems) or left untreated (no tx), before addition of (*A*) SDF-1 (10 nM), MIP-3β (100 nM), and (*B*) eotaxin (100 nM) or eotaxin-2 (1000 nM) in the chemotaxis chamber for 2 h. Control wells contained only the CCR3-specific mAbs or isotype-matched controls. For each well, the migrated DCs were counted in five fields at a ×1000 magnification and averaged as for Fig. 6. Results represent an average of triplicate wells.

In contrast to CCR5 and CXCR4, the level of CCR3 expression on the cell surface of DCs was similar on all DC subsets and was not dependent on their developmental stage. In our experiments, immature DCs expressed higher levels of CCR5 than the mature DCs (Figs. 4*B* and 5*B*), in agreement with previously published observations (20, 22, 23). The expression of CXCR4 on different DCs subsets showed a more complex pattern. CXCR4 levels on mature moDCs were up-regulated as compared with immature moDCs after incubation of immature moDCs with the typical maturation stimuli (MCM or LPS) (Figs. 4*B* and 5*B*), as expected. (20–22). However, in $CD34^+$-derived DCs the level of expression of CXCR4 decreased on the mature subset (days 6 and 12) compared with the immature (day 0; Fig. 2). Finally, the addition of latex beads or zymosan to MCM and LPS had different impacts on CXCR4 levels (Table I). In the presence of LPS, uptake of particles by immature moDCs inhibited the up-regulation of CXCR4, typically observed during maturation in the presence of MCM (Table I).

The uniform expression of CCR3 on different DC subsets seems to be so far a unique characteristic of CCR3 because the expression of all the other chemokine receptors that have been tested, like CCR6 and CCR7, is also dependent on the origin of DCs and their developmental stage (25, 26, 63). Our results indicate that the expression of some chemokine receptors, like CCR3 and CXCR4, on different DC subsets is regulated by pathways that are not entirely connected to the phenotypic changes leading to mature DCs.

To investigate whether the expression of CCR3 on DCs was functional, we measured the chemotaxis of DCs in responses to its natural ligands. We found that all different subsets of DCs demonstrated consistent and dose-dependent chemotactic responses to eotaxin and eotaxin-2. (Figs. 6 and 7). Maximum migratory responses were observed at 100 nM eotaxin (Fig. 6*C*) and 1000 nM eotaxin-2 (Fig. 6*D*), identical to the ones described for eosinophils and basophils (31, 62, 64, 65). As was the case with eosinophils (43), we found a considerable variation between donors in the migratory responses to eotaxin (Fig. 6, *A–C*). However, there was a good correlation between the cell surface levels of CCR3, represented by the *D* values (see *Materials and Methods* and *Results*) and the extent of the chemotactic response of DCs to eotaxin at all three tested concentrations (Fig. 6, *B* and *C*). Immature moDCs demonstrated consistently lower migratory responses than the mature moDCs even when similar levels of CCR3 were expressed on immature and mature moDCs. In few instances, we also observed that mature moDCs did not migrate in response to eotaxin, although CCR3 was expressed on their cell surface and chemotaxis was observed in response to SDF-1 and MIP-3β. These limited observations suggest that some other factor(s), beside CCR3 expression on the cell surface, might influence DC chemotaxis in response to eotaxin. In our experiments, the migratory responses of DCs to eotaxin were consistently lower than to SDF-1 and MIP-3β at all concentrations tested (1, 10, and 100 nM), but nonetheless they were in the similar range as reported for eosinophils (31, 64, 65). Migratory responses of DCs to SDF-1 and MIP-3β also varied significantly between donors, but we did not investigate this variation in depth as we did for eotaxin.

Dose-dependent chemotaxis of immature and mature moDCs obtained from multiple donors was also observed in response to eotaxin-2 (Fig. 6*C*, *right panel*; Fig. 7*B*), which is a functional homologue of eotaxin. However, this required a 10-fold higher concentration (Figs. 6*C* and Fig. 7*B*), consistent with the published results describing the chemotactic response of eosinophils to eotaxin and eotaxin-2 (62). Pretreatment of DCs with two different CCR3-specific mAbs inhibited the chemotaxis in response to eotaxin and eotaxin-2, but not to SDF-1 or MIP-3β (Fig. 7), thus confirming that CCR3 is the principal chemokine receptor mediating chemotaxis of DCs to eotaxin and eotaxin-2.

Preincubation of eosinophils with the murine mAb 7B11 led to a complete inhibition of chemotaxis (43, 62). In contrast to these findings in eosinophils, preincubation with two different CCR3-specific Abs, one of them being mAb 7B11, did not completely inhibit in our experiments the chemotaxis of DCs in response to eotaxin and eotaxin-2 (Fig. 7). Analogous to our findings in DCs, partial inhibition of SDF-1 induced chemotactic responses of lymphocytes and monocytes has been reported for the CXCR4-specific mAb 12G5 (66). Possibly, DCs undergo a rapid exchange of CCR3 between the cell surface and the intracellular stores, making complete Ab blocking more difficult. Alternatively, DCs may have another, yet to be identified chemokine receptor that recognizes eotaxin and eotaxin-2. It is unlikely, though, that the chemotactic responses of DCs to eotaxin are mediated through the other eotaxin receptor CCR5 (67), due to the low affinity of CCR5 toward eotaxin (68). However, it is clear from our experiments that DCs continue to respond to eotaxin and eotaxin-2 during their transition from an immature to a mature state.

We also observed that all DC subsets contained significant intracellular pools of CCR5, CXCR4, and CCR3. Several known agents that promote DC maturation and activation, like MCM, LPS, PGE_2, TNF-α, CD40L, TGF-β1, or uptake of zymosan or latex beads, did not significantly up-regulate the cell surface expression of CCR3 (Table I). These observations suggest that surface recruitment of CCR3 from the intracellular stores, if it occurs,

will be triggered by factors not related to these forms of DC maturation. They reinforce our earlier conclusions that the expression of CCR3 on DCs, unlike CCR5 and CCR7, may not be regulated by the known pathways leading to the phenotypic changes that characterize the immature and mature DCs.

The existence of intracellular pools of chemokine receptors is not restricted to DCs. For example, CXCR4 is found in the cytoplasm of a variety of lymphocytes (69). The significance of trafficking of the chemokine receptors between the cell surface and the cytoplasm of different cell types has been addressed for some chemokine receptors. Most studies in this area have been focused on ligand-induced receptor internalization. Earlier work has demonstrated that in neutrophils, the binding of IL-8 triggers a rapid internalization and recycling to the cell surface of the IL-8R, suggesting that this event is important in regulating chemotaxis to IL-8 (70). More recent work has demonstrated rapid and prolonged internalization of CXCR4 and CCR5 induced by their ligands in different subsets of lymphocytes (69, 71, 72), as well as internalization of CCR3 in eosinophils induced upon binding of eotaxin and its other ligand RANTES (73).

Our results raise the possibility that CCR3 mediates a coordinated influx of DCs and other cells during certain types of inflammation. Eotaxin expression is typically associated with the recruitment of eosinophils to inflamed tissues (64, 74) and in their accumulation during some inflammatory processes, like allergy and asthma (75–77). Expression of CCR3 on basophils (30, 31), human T lymphocytes (78), and Th2 subsets (32) has also been implicated in the recruitment of these cell types to sites of allergic inflammation where they colocalize with eosinophils. A combination of eosinophils and LCs is observed in the tissue infiltrates of the disease LC granulomatosis or histiocytosis (79–82). This disease of unknown origin is characterized by granulomatous lesions mainly in the bone and the lung, and occasionally in the skin, lymph node, spleen, posterior pituitary, and liver (83, 84). The diagnostic LCs have Birbeck granules and the $CD1a^+$ marker (79, 81). Several inflammatory cytokines are detected in the lesions, including GM-CSF, IL-4, and TNF-α (80). These cytokines mediate developmental changes in DCs, especially viability and maturation, with signs of the latter reported in histiocytosis (82). CCR3 ligands, like eotaxin and RANTES, could also be involved and could mediate the combined accumulation of eosinophils and LCs that are observed in these rare but clinically significant granulomas.

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