Macrophage Inflammatory Protein 3α Is Involved in the Constitutive Trafficking of Epidermal Langerhans Cells

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Summary

Certain types of dendritic cells (DCs) appear in inflammatory lesions of various etiologies, whereas other DCs, e.g., Langerhans cells (LCs), populate peripheral organs constitutively. Until now, the molecular mechanism behind such differential behavior has not been elucidated. Here, we show that CD1a+ LC precursors respond selectively and specifically to the CC chemokine macrophage inflammatory protein (MIP)-3α. In contrast, CD14+ precursors of DC and monocytes are not attracted by MIP-3α. LCs lose the migratory responsiveness to MIP-3α during their maturation, and non-LC DCs do not acquire MIP-3α sensitivity. The notion that MIP-3α may be responsible for selective LC recruitment into the epidermis is further supported by the following observations: (a) MIP-3α is expressed by keratinocytes and venular endothelial cells in clinically normal appearing human skin; (b) LCs express CC chemokine receptor (CCR)6, the sole MIP-3α receptor both in situ and in vitro; and (c) non-LC DCs that are not found in normal epidermis lack CCR6. The mature forms of LCs and non-LC DCs display comparable sensitivity for MIP-3ß, a CCR7 ligand, suggesting that DC subtype-specific chemokine responses are restricted to the committed precursor stage. Although LC precursors express primarily CCR6, non-LC DC precursors display a broad chemokine receptor repertoire. These findings reflect a scenario where the differential expression of chemokine receptors by two different subpopulations of DCs determines their functional behavior. One type, the LC, responds to MIP-3α and enters skin to screen the epidermis constitutively, whereas the other type, the “inflammatory” DC, migrates in response to a wide array of different chemokines and is involved in the amplification and modulation of the inflammatory tissue response.

Key words: Langerhans cell • dendritic cell • chemokine • migration • epidermis

Dendritic cells (DCs) are bone marrow–derived leukocytes with specialized antigen uptake, processing, and presentation functions (for reviews, see references 1 and 2). Under normal, noninflammatory conditions, immature DCs are found in trace quantities in parenchymatous organs and contact interfaces between the body and the environment. The main DC of the epidermis is the Langerhans cell (LC), a cell type which is anchored to neighboring epithelial cells via homotypic E-cadherin (E-cad) binding (3, 4). LCs display CD1a and exhibit typical ultrastructural features (i.e., Birbeck granules) that allow their discrimination from other members of the DC family. Apparently, the still-enigmatic LC precursor migrates to the skin in the virtual absence of inflammation-related stimuli. On the other hand, other types of DCs (e.g., peripheral blood [PB]-DCs) selectively cross activated, but not resting, endothelia (5), or emerge from transmigrated blood-derived precursors in the presence of inflammatory stimuli (6). In certain disease states, inflammation-related DC types may reach the body’s outermost tissues, as evidenced by the appearance of an epidermal non-LC DC population in atopic dermatitis skin (7).

Recent attention has focused on the impact of the chemokine–chemokine receptor system on DC migration. Currently, chemokines are subdivided into four subfamilies according to the position of the first cysteine pair (CXC,
CCs), the lack of the second and the fourth cysteine (C), or the presence of three spacing amino acids in the first cysteine tandem (CX3Cs; for reviews, see references 8 and 9). Until now, five and nine receptors have been identified for CXC and CC chemokines, respectively. Monocyte-derived (md)DCs display migratory responsiveness to a broad array of inflammatory CC chemokines, including monocyte chemoattractant protein (MCP)-1, MCP-2, and MCP-4; regulated upon activation, normal T cell expressed and secreted (RANTES), macrophage inflammatory protein (MIP)-1α, MIP-1β, and MIP-5/human CC chemokine 2 (HCC2); macrophage-derived chemokine; and the CXC chemokines IL-8 and stromal cell–derived factor (SDF)-1α (10-16). In correlation to this response profile, mdDCs express CC chemokine receptor (CCR)1, CCR2, CCR3, and CCR5, and CXC chemokine receptor (CXCR)1 and CXCR4. Interestingly, DCs generated in vitro from CD34+ stem cells, but not mdDCs, express CCR6 and migrate in response to MIP-3β/liver and activation-regulated chemokine (LARC) (17, 18). During terminal maturation, mdDCs and CD34+ hematopoietic precursor cell (HPC)-derived DCs acquire responsiveness to MIP-3β/EBV-induced molecule 1 ligand chemokine (ELC) because of the de novo expression of CCR7, a specific receptor for MIP-3β and secondary lymphoid tissue chemokine (SLC) (10, 13, 18-21). In keeping with an important role of CCR7 signaling for the proper function of mature DCs in vivo, the observation that plt mice, which lack expression of SLC, have reduced numbers of mature DCs in T cell areas of lymph nodes but, importantly, display an epidermal LC population that is normal in terms of cell numbers and distribution (22).

Based on the above considerations, we asked whether signals governing constitutive DC trafficking are at least partly different from those regulating the influx of DCs into inflammatory sites. To this end, we analyzed the chemokine responsiveness of in vitro–generated LCs and non-LC DCs, as well as of their CD1a+ and CD14+ precursors. Furthermore, comparative studies on the chemokine receptor profile of in vitro–generated and ex vivo–isolated LCs, as well as of inflammation-related DCs (i.e., PB-DCs), and the analysis of chemokine expression in normal human skin provided insight into the molecular interactions involved in LC homing.

Materials and Methods

mAbs and Recombinant Growth Factors and Chemokines. FITC–conjugated mAbs used were anti-CD1a, anti-CCR5, and anti-B7-2 (PharMingen); anti-CD2 and anti-CD11b (Becton Dickinson); and anti-CD3 (Immunotech). PE-conjugated mAbs included anti-CD14 (Becton Dickinson), anti-CD1a, and anti–CXCR4 (PharMingen), and anti-CXCR2 and anti–CCR6 (R&D Systems). The peridinin chlorophyll protein (PerCP)-conjugated anti-CD34 and anti-CD3 mAbs were from Becton Dickinson, and anti-CD1a CyChrome was from PharMingen. Biotin-conjugated anti–CCR1 was from R&D Systems. The anti–e-cad mAb (Immunotech) was biotinylated according to standard protocols. The binding of biotinylated mAbs was revealed either by PE- (Becton Dickinson) or Cy5–phycoerythrin (R PE)-conjugated streptavidin (SA-Cy5; Dako). Isotype control mAbs included biotinylated, FITC-, PE-, or PerCP-labeled mouse IgG1 or IgG2a (Becton Dickinson). Recombinant human (rh)GM-CSF and IL-4 were from Novartis Forschungsinstitut. rhTNF-α and rhFlt3-ligand (Flt3-L) were obtained from Genzyme and Serum, respectively. rhMIP-1α, MIP-3α, MIP-3β, SDF-1α, RANTES, and MCP-1 were obtained from R&D Systems.

Isolation of CD34+ HPCs. Cord blood (CB) was obtained according to institutional guidelines. CD34+ cells were separated from CB-MNCs by a positive immunoselection procedure (CD34 MultiSort Kit; Miltenyi Biotec). In brief, CB mononuclear cells (MNCs) (1×106/ml) were incubated with anti-CD34 mAb–coated paramagnetic microbeads for 30 min at 4°C. After several washings, bead-bound CD34+ HPCs were isolated on Miltenyi separation columns using a magnet (Miltenyi Biotec). CD34+ cells (0.5–1.5×107) were recovered at a purity of >95%, as determined by immunostaining with a PerCP-labeled anti-CD34 mAb (clone HPCA-2) recognizing a CD34 epitope distinct from that bound by the mAb used for immunoselection.

In Vitro Generation of DCs from CD34+ HPCs and Isolation of DC Precursors. CD34+ HPCs were cultured in RPMI 1640 medium containing 10% FS cells (both from Gibco BRL) supplemented with 200 U/ml GM-CSF, 50 U/ml TNF-α, and 50 ng/ml Flt3-L. CD34+ HPCs were cultured in 75-cm² tissue culture flasks (Costar) at a density of 1×10⁶/ml. At day 3 or 4, cell suspensions were split and diluted in fresh RPMI/10% FCS supplemented with GM-CSF and TNF-α. At day 10, cells were collected and resuspended in fresh cytokine-conditioned medium at a density of 1×10⁶/ml, and further cultured until days 12 and/or 14. Where indicated, cells were harvested at day 6 and labeled with anti-CD1a-FITC and anti-CD14–PE. CD1a+CD14+ cells (26.2±2.0% of the total population, mean ± SEM, n = 20) and CD1a–CD14+ cells (36.1±2.2%) were isolated using a FACStarSP Plus™ flow cytometer (Becton Dickinson). The purity of the sorted cell populations was always >98%. Sorted cells were either used in chemotaxis assays, subjected to lysis and RNA extraction, or further propagated in the presence of GM-CSF and TNF-α until days 12 and/or 14.

Isolation and Culture of Epidermal Celis and PB-DCs. Normal human skin was obtained from patients undergoing plastic surgery upon informed consent. Keratome split-thickness skin was incubated in dispase (50 U/ml; Collaborative Biomedical Products) for 1 h at 37°C, or overnight at 4°C. Thereafter, epidermal sheets were peeled off and exposed to trypsin/0.05% EDTA (Gibco BRL) containing 0.025% DNase 1 (Sigma Chemical Co.) for 15 min at 37°C, and cell suspensions were prepared by mechanical agitation. Residual aggregates and cellular debris were removed by filtering and density gradient centrifugation on Lymphoprep (Nycomed Amersham plc). For cell sorting experiments, epidermal cell suspensions were exposed to anti-CD11b/CD2-FITC, anti-CD1a–PE, and anti-CD3–PerCP. After several washings, CD1a+CD2+CD3+CD11b+ LCs and keratinocytes (CD1a+CD2–CD3+CD11b+ cells) were isolated using a FACStarSP Plus™ flow cytometer. Resulting cell populations were resorted to yield purities of >99%. In some experiments, sorted epidermal LCs or LC-enriched epidermal cell suspensions were cultured for the indicated time periods in GM-CSF (200 U/ml)–conditioned RPMI 1640/10% FCS. PB-DCs were prepared as described previously (23, 24). In brief, PB-MNCs from healthy donors were prepared by density gradient centrifugation on Ficoll-Hypaque (Amersham Pharmacia Biotech) and subjected to counter current elutriation centrifugation (JE-6B elutri-
ation system; Beckman Instruments [24]). From the resulting cell population, T, B, and NK cells, HPCs, monocytes, and basophils were removed by anti-CD3/CD11b/CD16/CD19/CD34/CD56 immunolabeling and anti-mouse IgG immunomagnetic depletion (MACS; Miltenyi Biotec). PB-DC–enriched cell populations were further reacted with anti-CD11c-FITC, anti-CD13–PE, and anti-CD4–Cy5 mAbs, and CD11c<sup>+</sup>CD13<sup>+</sup>CD4<sup>+</sup> cells were isolated on a FACStar<sup>®</sup> PLUS™ flow cytometer. Purified CD11c<sup>+</sup>PB-DCs were seeded at a density of 0.5–1 <sup>3</sup>10<sup>6</sup>/ml in 96-well flat-bottomed microtiter plates (Costar) in RPMI 1640/10% AB serum supplemented with GM-CSF (1,000 U/ml) and IL-4 (800 U/ml).

**Isolation and Culture of Dermal Microvascular ECs and Human Umbilical Vein ECs.** Dermal microvascular ECs (DMECs) were isolated from human foreskins according to a protocol approved by the institutional ethics committee. Foreskins were cut into 1-cm<sup>2</sup> pieces and exposed to dispase (25 U/ml) for 20 min at 37°C. Cells recovered after gentle teasing of the tissue were resuspended in low-serum endothelial cell medium (PromoCell) and plated on dishes coated with fibronectin (1 μg/ml; Endogen, Inc.). For passaging, cells were recovered from the dishes by trypsin/0.05% EDTA treatment. Cells were used for further experiments at passage four. Human umbilical vein ECs (HUVECs) were isolated as described previously (25) and passaged in IMDM/20% FCS, streptomycin (100 μg/ml), penicillin (100 U/ml), β-glutamine (2 mM; all from GIBCO BRL), EC growth factor (50 μg/ml; Collaborative Biomedical Products), and heparin (5 U/ml; Sigma Chemical Co.). Activated DMECs and HUVECs were prepared by exposing the cells for 6 h to 50 ng/ml IFN-γ (Endogen, Inc.).

**mRNA Extraction and Reverse Transcription PCR Amplification.** Primary human cells isolated and cultured as described above were lyzed at a cell density of 4 × 10<sup>6</sup>100 μl in Tris-lithium-dodecylsulfate (LiDS)-containing buffer (100 mM Tris-HCl, 500 mM LiCl, 10 mM EDTA, 1% LiDS, 5 mM dithiothreitol, pH 8.0) for 5 min at 4°C. Poty(A) RNA was isolated using oligo(dT)<sub>25</sub>–conjugated Dynabeads (Dynal) according to the manufacturer’s recommendations. In brief, 30 μl of the Dynabead oligo(dT)<sub>25</sub> suspension was added to 100 μl cell lysate, and beads were allowed to hybridize with mRNA poly(A) tails for 5 min at room temperature. bead-bound poly(A) RNA was retrieved using a magnetic particle concentrator (Dynal), and mRNA was eluted for 3 min at 65°C in H<sub>2</sub>O. Reverse transcription (RT) of purified mRNA was performed using the avian myeloblastosis virus (AMV) first strand cDNA synthesis kit (Boehringer Mannheim). In brief, eluted mRNA was incubated with 10 mM Tris-HCl, 5 mM MgCl<sub>2</sub>, 1 mM dNTP, 0.04 A<sub>260</sub> U Oligo-p(dT)15 primer, 50 U RNase inhibitor, and 20 U AMV reverse transcriptase for 1 h at 42°C. Thereafter, the reverse transcriptase was inactivated by heating the reaction mix to 99°C for 5 min. PCR amplifications were performed in 50 μl reaction buffer (20 mM [NH<sub>4</sub>]<sub>2</sub>SO<sub>4</sub>, 75 mM Tris-HCl, pH 8.8, 0.01% Tween 20, 1.5 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP, and 2.5 U thermostable DNA polymerase; all from Advanced Biotechnologies Ltd.), and

| Transcript | Primer sequences (5’-3’) | Product size (bp) |
|------------|-------------------------|-----------------|
| CCR1       | AAG CTT CAG AGA GAA GCC GGG ATG GAA ACT CC (ref. 46) | 1,094 |
|            | CTC GAG CTA GAT CAG AAC CCA GGA GAG AGT TC (ref. 46) | |
| CCR2       | TGG CTT TGT TGT CTT CTT CTC TC | 229 |
| CCR3       | AAG CTT CAG GGA GAG ATG AAA TAA AAC ACA ATA GAG ATG TGC (ref. 46) | 1,084 |
|            | CTC GAG CAG ACC TAA AAC ATA GAG ATG TCC (ref. 46) | |
| CCR5       | TTC GGC TAC AAG AAA TGC TC | 328 |
| CCR7       | CAT GTA CCT GGT GAA ACC AA | 1,174 |
|            | GIG GAG GTC CCT CTA GT | |
| CXCR4      | AAG CTT GGA GAA CCA GGC GTG GGT ACC ATG GAG GGG ATC (ref. 46) | 1,084 |
| MIP-3α     | CTC GAG CAT CTT TGT CTT TAG CAG TGA AAA CTT GAA GAG TC (ref. 46) | |
| MIP-3β     | GCA CAA ATT TCA CCA CCA CAG | 254 |
| β-actin    | ATC TGG CAC CAC ACC TCT TAC ATT GAG CTG CG | 286 |
|            | CGT CAT ACT CCT CTT TGC TGA TCG TGC AAC TCT GC | 838 |

ref., reference.  
* Sequences from Clontech.
forming the upper compartments. 50 μl of cell suspension (10^6 cells/ml) was placed into the upper compartment of each well. Chambers were incubated for 2 h at 37°C, filters were harvested, nonmigrated cells were removed, and the migrated filter-bound cells were stained with hemacolor. Chemokine-induced cell migration was analyzed by computer-assisted counting (Optomax V; Bestobell M obrey) of the cells adherent to the bottom side of the membrane.

Flow Cytometry Analysis. For two- or three-color immunolabeling, cells were washed twice in ice-cold PBS and incubated in 50 μl PBS containing the appropriate biotin- and/or fluorochrome-labeled mAbs (2.5 μg/ml each) for 30 min on ice. For the detection of cell-bound biotinylated mAbs, cells were further exposed to 1 μg/ml SA-PE or SA-Cy5. At least 10,000 cells were analyzed on a FACScan™ (Becton Dickinson). For the detection of intracellular antigens, cells were subjected to fixation and permeabilization after surface immunostaining using a kit according to the manufacturer’s recommendations (Fix & Perm; An der Grub Bioresearch).

Actin Polymerization. HPC-derived cells were harvested on day 6, washed, and resuspended in migration buffer (1 x 10^6 cells/ml). After allowing the cells to equilibrate for 15 min at 37°C, buffer only or MIP-1α at the indicated final concentration was added. After the indicated incubation periods, the reaction was stopped by adding PFA (final concentration 0.1%), and individual samples were incubated for 30 min at 4°C. Thereafter, cells were washed and sequentially exposed to anti-CD1a-CyChrome/anti-CD14-PE and saponin (final concentration 0.1%)/phalloidin-FITC (final dilution 1:5,000; both from Sigma Chemical Co.), and subjected to FACS® analysis.

Immunohistochemistry. Normal human skin was removed during elective plastic surgery upon informed consent. The skin samples were snap-frozen in liquid nitrogen-chilled isopentane. Frozen sections were mounted onto glass slides and fixed for 10 min in acetone at room temperature. Thereafter, the slides were kept at –20°C for up to 8 wk, until the staining procedure. For immunostaining, the slides were rehydrated and incubated with the following primary Abs: rabbit anti-MIP-3α, anti-MIP-3β (Peprotech), and anti-CCR 6 (gift of Joshua Farber, National Institutes of Health, Bethesda, MD [28]); monoclonal mouse anti-HLA-DR (Bioresearch), mouse anti-CCR 6, anti-MIP-1α, anti-MIP-1β, and anti-RANTES; and goat anti-SDF-1α and anti-MCP-1 (all from R&D Systems). The bound primary Abs were detected by sequential incubations with alkaline phosphatase-conjugated goat anti-rabbit, rabbit anti-mouse, or rabbit anti-goat secondary Abs and an alkaline phosphatase anti-alkaline phosphatase staining kit (Dako), according to the manufacturer’s instructions. To control the specificity of Ab binding, rabbit, goat, and mouse IgG control Abs (Dako) were used at equimolar concentrations. Each immunostaining protocol was performed on skin from at least three different donors.

Results and Discussion

LCs and N on-LC DC Precursors Respond to Different Sets of Chemokines. In a first series of experiments, we investigated whether chemokines can induce the migration of well-defined DC types and their precursors in a selective manner. As a model system, we used the cellular progeny of GM-CSF/TNF-α-stimulated CD34+ HPCs. Under these culture conditions, virtually nonoverlapping CD1a+ and CD14+ cell subsets appear around day 6 until day 12, they
develop into E-cad^CD11b^-CD1a^+ LCs and E-cad^CD11b^CD1a^- non-LC DCs, respectively (29; data not shown). Chemokines that have been found to induce the migration of bulk DC populations of various maturation stages and lineages (i.e., MIP-1α, RANTES, MIP-3α, MIP-3β, MCP-1, and SDF-1α) were tested. Fig. 1 shows that, on the basis of their chemokine response pattern, CD1a^-LCs and CD14^-non-LC DC progenitors are functionally diverse cell populations. Most importantly, MIP-3α, a cytokine with chemotactic activity for CD34^-HPC-derived DCs but not mDCs (18), attracted almost exclusively CD1a^-LC precursors (Fig. 1A). The maximal MIP-3α-induced response measured for CD14^-cells and for the residual, presumably poorly differentiated, CD14^-CD1a^- cell population was at least threefold lower and only occurred at an MIP-3α concentration 100-fold higher than that needed to achieve a similar migratory response with CD1a^-LC precursors (Fig. 1A). We also confirmed, by a checkerboard assay, that the CD1a^-LC precursors, as well as the few CD14^-cells that were attracted by MIP-3α, migrated in a directional rather than a random fashion (data not shown).

In addition to being a selective chemoattractant for LC precursors, MIP-3α was the only chemokine in the panel tested that induced potent and efficacious migratory responses of these cells. LC precursors responded only weakly to SDF-1α and MIP-3β (Fig. 1, C and D), and did not migrate at all in response to MCP-1, which, in contrast, attracted some CD14^-non-LC DC precursors (Fig. 1B). Another chemokinesis profile was observed for the CD14^-CD1a^- cell population. As shown previously with HSCs, day 6 DC precursors were attracted by SDF-1α (Fig. 1 C [30]) and responded to MCP-1 (Fig. 1 B), but not to MIP-3α or MIP-3β (Fig. 1, A and D). As shown in Fig. 2 A, MIP-1α failed to induce migration of day 6 CD34^-HPC-derived DC precursors. The apperent lack of migratory response to this chemokine was surprising, since day 6 DC precursors expressed CCR1 and CCR5, both of which are receptors for MIP-1α (see below). The transwell migration assay used allows only the analysis of cells that have passed the membrane and detached into the lower chamber, whereas the cells that remain membrane bound escape detection. To control for the cells adherent to the lower side of the membrane, we also tested MIP-1α in the Boyden-type chamber assay, which detects the migrated, membrane-bound cells only. The direct comparison of the results obtained in these two migration assays, plus the analysis of the transwell mem-

![Figure 1](image1.png)

**Figure 1.** Chemotactic response of LC and non-LC DC precursors generated in vitro from CD34^-HPCs. CB-derived HPCs were stimulated for 5 days with GM-CSF/TNF-α and then studied in the transwell chemotaxis assay. MIP-3α (A), MCP-1 (B), SDF-1α (C), or MIP-3β (D) was added to the lower compartments of 24-well plates, transwell devices were inserted, and cell suspensions were layered into the transwell inserts. After 2 h at 37°C, the cells that transmigrated into the lower compartment were harvested, stained with anti-CD1a-FITC and anti-CD14-PE, and subjected to FACS® analysis. Results are given as the number of transmigrated cells in percentage of the input cell number of individual subpopulations (CD1a^-CD14^-, filled circles; CD14^-CD1a^-, open squares; CD14^-CD1a^-). Mean ± SEM values obtained in three different experiments are shown.

![Figure 2](image2.png)

**Figure 2.** MIP-1α and MIP-3α elicit different migratory and/or adhesive responses in CD34^-HPC-derived LC and DC precursors. Results shown in A and B were obtained using the transwell chemotaxis assay and the 48-well Boyden-type chamber chemotaxis assay, respectively. For both assays, day 5 LC and/or DC precursors were harvested and tested for their migratory responses to MIP-1α (filled circles), MIP-3α (open squares), and MIP-3β (open triangles). Mean percentages (± SEM) of migrated and detached (A; n = 3) and migrated, membrane-bound cells (B; n = 2) are shown. (C) Representative vertical sections through 5-μm pore size membranes used in the transwell chemotaxis assay. The assays were performed using the indicated stimuli (MIP-1α at 100 ng/ml, MIP-3α at 1 μg/ml, and the combination of both), or buffer alone. Membrane-bound cells were fixed and labeled, and membranes were subjected to confocal laser scanning microscopy. Broken lines denote the position of the membrane.
branes by confocal microscopy, showed that MIP-1α was eliciting migratory and/or adhesive responses in CD34+ HPC-derived DC precursors that were different from those induced by MIP-3α (Fig. 2, A and B). In both the transwell and the Boyden-type assay, the cells that had been attracted by MIP-1α remained adherent to the membrane (Fig. 2, B and C). In contrast, the cells that had responded to MIP-3α detached from the lower side of the membrane in the transwell system (Fig. 2 A) and remained membrane bound only in the Boyden-type assay (Fig. 2 B). The proadhesive effect of MIP-1α was pronounced also when an additional migration-inducing stimulus (e.g., MIP-3α) was provided at the same time (Fig. 2 C).

To better characterize the cell populations that respond to chemokines by adhesion versus transmigration, CD1a+ and CD14+ DC precursor subsets were flow sorted on day 6, recultured overnight, and then tested in the transwell chemotaxis assay. In concordance with our results obtained with nonsorted DC precursor subsets, MIP-3α induced vigorous transmigration of sorted CD1a+ LC, but not of CD14+ non-LC DC precursors (Fig. 3 A). The enumeration of cells that migrated but did not detach from the transwell membranes revealed that only a minor part of the migrated CD1a+ LC precursors remained membrane bound, and that only very few CD14+ cells adhered to the filters in response to MIP-3α (Fig. 3 B). The argument that LC, rather than non-LC DC precursors, are the main MIP-3α-responsive cell population was further corroborated in the Boyden-type microchamber chemotaxis assay using flow-sorted CD1a+ and CD14+ DC precursors (data not shown). In another series of experiments, we used the same experimental set-up to study MIP-1α-induced migration of isolated DC precursors. However, unlike their nonsorted founder population, neither of the two sorted DC precursor subsets significantly migrated to MIP-1α either in the transwell or in the Boyden-type microchamber chemotaxis assay (data not shown). The reason for this discrepancy is not currently understood, but may be due to desensitization of MIP-1α receptors by chemokines (3, 4, 21) released after the sorting procedure. To still be able to identify the principal MIP-1α-responsive DC precursor, we measured MIP-1α-induced actin polymerization, a cellular event occurring early after chemokine receptor triggering (8). As revealed by filamentous (F)-actin and mAb co-staining experiments performed on nonsorted day 6 DC progenitor subsets, CD14+ non-LC DC precursors and, to a lesser extent, CD1a+ CD14+ cells, but no CD1a+ LC precursors, responded to MIP-1α (Fig. 3 C). In summary, our cumulative data indicate that MIP-1α (Fig. 2) and RANTES (not shown), which act on the same set of receptors, induce non-LC DCs rather than LC precursors to migrate and to firmly adhere to the substratum. It is possible that the differential responses of defined DC precursors to MIP-3α and MIP-1α may be important for the fine-tuning of migration- versus adhesion-dependent immobilization and, as a result, for the correct localization of the various types of DCs in the tissues.

LCs and non-LC DCs do not respond to chemokines that attract their precursors, but both respond to MIP-3β. When we compared the effect of chemokines on the migration of E-cad+CD11b-CD1a+ LCs and of E-cad+ CD11b+CD1a+ non-LC DCs between days 12 and 14, we observed that neither of the DC types responded to MIP-3α (Fig. 4 A). In fact, it appears that LCs have completely lost the MIP-3α reactivity characteristic of their precursor stage, and that the non-LC DCs did not acquire responsiveness to MIP-3α. The finding of a selective but transient
involvement of MIP-3α in the migration of defined LC precursors, but not of monocyte-related DC/DC precursors (Fig. 1 A and Fig. 4 A), together with the observation of a temporary appearance of MIP-3α-responsive cells in bulk progenies of cytokine-stimulated CD341 HPCs (18), suggests that MIP-3α could have a specific role in guiding LC precursors to their correct anatomical location in vivo.

The refractory state for chemokine-induced migration seen at day 12 of the cultures (Fig. 4, A–C) lasts for only a short time period, since at day 14 the LC/DC progeny displayed pronounced migratory responses to MIP-3β (Fig. 4 C). Data presented in Fig. 4 C also show that day 14 LCs and non-LC DCs display quantitatively comparable chemotactic responses to MIP-3β. Thus DCs show subtype-restricted chemokine responses at the committed precursor stage only.

LCs Display a Restricted Set of Chemokine Receptors. To see (a) whether a correlation exists between the chemokine expression pattern and the migratory properties of different types of DCs, and (b) whether our findings are relevant to the in vivo situation, we analyzed the chemokine receptor profile of in vitro-generated LCs and freshly isolated epidermal LCs. As shown in Fig. 5 B, epidermal LCs express mRNAs encoding CCR6, CCR7, and CXCR4, which are the receptors for MIP-3α, MIP-3β/SLC, and SDF-1, respectively. In contrast, these cells are virtually devoid of CCR1, CCR3, and CCR5 transcripts, and express only limited amounts of CXCR2 mRNA. Although a similarly restricted set of chemokine receptor mRNAs was expressed by in vitro-generated LCs (Fig. 5 A), other types of immature DCs and DC precursors, i.e., the progeny of cytokine-stimulated CD34+ HPCs at day 6 (Fig. 5 D), blood DCs (Fig. 5 E), and monocyte and/or mdDCs (10, 13, 31), express mRNA for a wide array of different chemokine receptors, including CCR1, CCR2, CCR3, CCR5, and CXCR4. These two different patterns were mirrored by the FACS® data of chemokine receptor expression on the surface of in vitro-generated LC versus non-LC DC precursors (Fig. 6 A). CCR6 was the only chemokine receptor among those studied that was present on the surface of CD1a1 LC precursors (Fig. 6 A). Curiously, CXCR4 was not detected on the surface of these cells, but was found in their cytoplasm (Fig. 6 A; data not shown). In contrast, CD14+ DC precursors expressed several chemokine receptors, including CCR1 and CCR5, thus suggesting that the migratory responses to MIP-1α seen in Fig. 2 are indeed confined to this subset.

Immunohistochemistry using both mouse and rabbit Abs demonstrated strong in situ CCR6 expression by dendritic epidermal cells of normal human skin (Fig. 6 B; data not shown). In serial sections, these cells were weakly HLA-DR+, and thus represent LCs in their nonactivated state.
Selective Role of MIP-3α in LC Migration

In most experiments we also observed a weak, albeit distinct, granular anti-CCR6 reactivity in keratinocytes of the Malpighian layer (Fig. 6 B). Our additional finding of CCR6 mRNA in purified keratinocytes (data not shown) is indicative of receptor synthesis by these cells. CCR6 surface expression by a major subset of freshly isolated HLA-DR<sup>1</sup>CD1a<sup>1</sup> LCs was also demonstrated by FACS® (Fig. 6 C). In vitro culture of these LCs under maturation-promoting conditions resulted in a rapid loss of anti-CCR6 surface immunoreactivity that even preceded the maturation-related upregulation of the costimulatory molecule B7-2 (Fig. 6 C). These data suggest that epidermal LCs, although CCR6<sup>+</sup> in their nascent state, downregulate this receptor during the process of in situ maturation. In correlation with these results and the maturation-dependent responses of LCs to either MIP-3α or MIP-3β (Figs. 1 and 4), we observed a complete loss of CCR6 mRNA production with continued synthesis of CCR7 mRNA during cytokine-mediated maturation of epidermal LCs (Fig. 5 C). Similarly to CCR6 mRNA, CCR2 transcripts disappeared during the in vitro maturation of epidermal LCs whereas the expression of CXCR4 mRNA remained unchanged (Fig. 5 C). In conclusion, it appears that immature LCs ex vivo and those generated in vitro display a similar and restricted set of chemokine receptors, and that mature cells no longer produce chemokine receptor mRNAs, with the notable exception of CCR7 and CXCR4.

In view of data published previously by Zaitseva et al. (32), the apparent lack of CCR5 mRNA expression by epidermal LCs appears surprising. Although unlikely, it is conceivable that LCs express CCR5 mRNA at levels too low to be detected by our RT-PCR assay. It is important to note that the LC-purification strategy employed here strictly excluded CD2<sup>+</sup> and/or CD3<sup>+</sup> skin T cells that ex-
press CCR5 (data not shown). Moreover, using two different mAb clones and RT-PCR, we also failed to detect CCR5 protein and mRNA expression by in vitro-generated LCs (Fig. 5 A; data not shown), but could amplify CCR5-specific transcripts from day 6 cytokine-stimulated HPCs (Fig. 5 D) and from freshly isolated and cytokine-stimulated PB-DCs (Fig. 5 E; data not shown).

MIP-3\(\alpha\) is the prime candidate for recruiting LC precursors into the epidermis. To get information about the expression of chemokines in normal human skin and to correlate it with the chemokine receptor pattern of LC precursors, we performed immunohistochemistry and RT-PCR on frozen skin sections and FACSPurified skin cells, respectively. Keratinocytes were found to express MIP-3\(\alpha\) mRNA (Fig. 7 I); accordingly, MIP-3\(\alpha\) immunoreactivity was seen in the basal and suprabasal layers of the epidermis (Fig. 7 A). RT-PCR revealed no MIP-3b mRNA in isolated keratinocytes (Fig. 7 I). Together with our finding of only sparse and focal epidermal MIP-3b immunoreactivity (not shown), this suggests that MIP-3\(\beta\), unlike MIP-3\(\alpha\), is not constitutively produced in the epidermis. Conversely, we observed strongly MIP-3b-immunoreactive cells throughout the dermis (Fig. 7 D). SDF-1\(\alpha\) immunoreactivity was seen in the epidermis but, in contrast to MIP-3\(\alpha\), its expression was strictly confined to the basal keratinocyte layer (Fig. 7 C). As shown in Fig. 7 E, normal human epidermis is devoid of MCP-1 immunoreactivity, an observation that finds support in a previous publication (33). Also, no MIP-1\(\alpha\), MIP-1\(\beta\), or RANTES immunoreactivity was seen in normal epidermal keratinocytes (data not shown).

Thus, it appears that two chemokines, MIP-3\(\alpha\) and SDF-1\(\alpha\), are produced constitutively by human keratinocytes, and therefore could both be involved in LC homing to the epidermis. Our findings that SDF-1\(\alpha\) does not attract LC precursors (Fig. 1 C) and that these cells also fail to express CCR4 on their surface (Fig. 6 A) speak against a role of SDF-1\(\alpha\) in LC homing. Since ex vivo–purified LCs express CCR2 transcripts (Fig. 5 B), one could argue that this receptor is involved in the attraction of LCs into the epidermis. This is unlikely because (a) resting keratinocytes do not express the CCR2 ligand MCP-1 at the protein level (33; Fig. 7 E), and (b) LC precursors do not express CCR2 on their surface (Fig. 6 A), and do not migrate in response to MCP-1 (Fig. 1 B). In this context, it is noteworthy that mice genetically manipulated to express MCP-1 in the epidermis have close to normal LC numbers while accumulating dermal DCs and macrophage-like cells (34).

In conclusion, among all the chemokine–chemokine receptor pairs studied, only MIP-3\(\alpha\)–CCR6 fulfills the spatial and temporal expression, as well as function requirements, for a ligand–receptor pair responsible for LC homing to the epidermis. This is evidenced by (a) the constitutive expression of MIP-3\(\alpha\) in normal human epidermis (Fig. 7 A and I); (b) the selective in vitro chemotactic responses of CD1\(\alpha\) LC precursors to MIP-3\(\alpha\) (Figs. 1 A and 3 A); (c) the expression of CCR6 by in vitro–generated LC/LC precursors (Figs. 5 A and 6 A), by skin-derived LCs (Figs. 5 B and 6 C), and by LCs in situ (Fig. 6 B); (d) the absence of CCR6 from non-LC DCs, e.g., PB-DCs (Fig. 5 E), mdDCs (17), and dermal DCs (data not shown); and (e) the rapid loss of CCR6 surface expression during LC maturation, as illustrated by a reverse regulation of CCR6 and B7-2 expression during LC culture (Fig. 6 C), and the lack of CCR6 expression by cytokine-matured epidermal LCs (Fig. 5 C).

MIP-3\(\alpha\), although absent from spleen and bone marrow, is also expressed in appendix, thymus, and tonsils as well as in fetal liver and lung (35–39). Curiously, in tonsils (18), skin (this study), and perhaps also in the other organs containing epithelial cells, MIP-3\(\alpha\) is expressed constitutively by ectodermal rather than bone marrow–derived cells. Because keratinocytes produce MIP-3\(\alpha\) (Fig. 7, A and I), and at the same time apparently also express CCR6 (Fig. 6 B; data not shown), it is possible that, in addition to driving LC homing, this chemokine plays a previously unrecognized autocrine role in keratinocyte homeostasis.

If MIP-3\(\alpha\) is expressed in several different organs, why do immature LCs appear only in stratified epithelium? It is conceivable that additional factors, and not MIP-3\(\alpha\) expression alone, determine whether LC precursors can populate a given organ. A prerequisite for homing into the epidermis is the emigration of LC precursors from the circulation, which, by analogy with other leukocytes, is likely to be mediated by a cascade of discrete multistep interactions with ECs (40). Organ-specific differential expression of endothelial adhesion molecules may provide an important additional source for the selectivity of LC homing into skin. The multistep adhesion paradigm may also explain how the lack of expression of leukocyte adhesion molecules, e.g., cutaneous lymphocyte-associated antigen (CLA), may be responsible for the fact that not all CCR6-bearing blood leukocytes (28) can enter the skin in response to MIP-3\(\alpha\).

We argued above that MIP-3\(\alpha\) is ideally positioned to guide the migration of LC precursors into the epidermis, but can it also drive their emigration from blood? To do this, MIP-3\(\alpha\) would have to be associated with dermal ECs and appear on their luminal surface (41, 42). This could be achieved either by EC binding and transcytosis of keratinocyte-derived MIP-3\(\alpha\), as has been demonstrated previously for IL-8 and RANTES (42), or by ECs themselves producing MIP-3\(\alpha\), as was shown for other chemokines (43). Postcapillary and small venular ECs in normal human skin display MIP-3\(\alpha\) immunoreactivity (Fig. 7 H), and isolated DMECs produce MIP-3\(\alpha\) mRNA (Fig. 7 I), showing that, in addition to keratinocytes, dermal ECs also produce this chemokine under normal conditions. The lack of MIP-3\(\alpha\) mRNA in HUVECs (Fig. 7 I) underlines the tissue-specific differences among ECs. Cumulatively, these findings suggest that, in addition to navigating LCs towards the epidermis, MIP-3\(\alpha\) may be the chemokine responsible for the transendothelial migration of LC precursors.

Additionally, the immunohistochemical studies revealed MIP-3\(\alpha\) and MIP-3\(\beta\) immunoreactivity to be associated with the ECs of afferent lymphatics (Fig. 7, F and G). This observation is in line with our previous in situ binding studies, which demonstrated the presence of saturable, broad specificity binding sites for CC (MCP-1, MCP-3, RANTES)
but not CXC chemokines on the lymphatic ECs in human dermis (44). Additionally, SLC immunoreactivity was shown to be associated with lymphatic vessels (45). The functional significance of chemokine binding to this microanatomical location is not yet clear. It is possible that chemokines immobilized on lymphatic ECs facilitate the entry of LCs into the lymphatics or, alternatively, promote their movement within the lymphatic channels toward the draining lymph node. In both cases, the primary role is likely to be played by CCR7 ligands, since LCs at this stage of their maturation downregulate CCR6 expression.

In this study, we show that LCs display a chemokine response pattern that is much narrower than perhaps anticipated. Thus, the restricted set of chemokine receptors expressed by constitutively trafficking LCs stands in striking contrast to the very broad chemokine receptor repertoire of DCs that
appear under inflammatory conditions (5, 10, 13, 31; this study). These findings suggest a scenario where the differential behavior of two ontogenetically different DC lineages is encoded by their differential expression of chemokine receptors. One cell type, the LC, follows only one tissue-derived constitutive signal, MIP-3α, and is remarkably ignorant to inflammation-related chemokine stimuli. As a result, it resides in the skin and plays a role in the homeostatic host defense. Another cell type, exemplified by the PB-DC, lacks CCR6 but displays a broad repertoire of chemokine receptors and, consequently, can home to any inflammatory site where it perpetuates or modulates the inflammatory tissue response. It also appears that the ultimate step in the DC odyssey, i.e., their migration into draining lymph nodes, follows one pattern common to all DC subtypes: LCs, non-LC DCs (this study; 18, 45), and monocyte-derived DCs (10, 13, 19, 20) start to express CCR7 and respond to MIP-3β upon receipt of maturation-mediating stimuli. This chemokine, and probably also the alternative CCR7 ligand SLC (22, 45), may guide DCs into T cell areas of draining lymph nodes.

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