# Accumulation of Immature Langerhans Cells in Human Lymph Nodes Draining Chronically Inflamed Skin

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## Abstract

The coordinated migration and maturation of dendritic cells (DCs) such as intraepithelial Langerhans cells (LCs) is considered critical for T cell priming in response to inflammation in the periphery. However, little is known about the role of inflammatory mediators for LC maturation and recruitment to lymph nodes in vivo. Here we show in human dermatopathic lymphadenitis (DL), which features an expanded population of LCs in one draining lymph node associated with inflammatory lesions in its tributary skin area, that the Langerin/CD207$^+$ LCs constitute a predominant population of immature DCs, which express CD1a, and CD68, but not CD83, CD86, and DC–lysosomal-associated membrane protein (LAMP)/CD208. Using LC-type cells generated in vitro in the presence of transforming growth factor (TGF)-β1, we further found that tumor necrosis factor (TNF)-α, as a prototype proinflammatory factor, and a variety of inflammatory stimuli and bacterial products, increase Langerin expression and Langerin dependent Birbeck granules formation in cell which nevertheless lack costimulatory molecules, DC–LAMP/CD208 and potent T cell stimulatory activity but express CCR7 and respond to the lymph node homing chemokines CCL19 and CCL21. This indicates that LC migration and maturation can be independently regulated events. We suggest that during DL, inflammatory stimuli in the skin increase the migration of LCs to the lymph node but without associated maturation. Immature LCs might regulate immune responses during chronic inflammation.

Key words: Langerhans cells • dendritic cells • inflammation • cell differentiation • migration

## Introduction

Dendritic cells (DCs)* sample antigen in the periphery, where they are present in an immature state as illustrated by Langerhans cells (LCs) in the skin, mucosae and in the lung, and migrate to lymphoid organs where mature DCs present antigen to T cells and can initiate immune responses (1, 2). Full activation by DCs of an antigen-specific T cell into an effector cell requires the expression of costimulatory molecules by DCs and the accumulation of MHC class II complexes, achieved during a complex process referred to as DC maturation (references 3 and 4, for reviews, see references 1 and 2). Migration to the lymph nodes and maturation of DCs are therefore crucial steps in the initiation of specific immune responses (1, 2). DC maturation has been characterized mostly in DCs cultured in vitro and may be induced by numerous inflammatory stimuli including the disruption of the epidermis (5, 6), proinflammatory cytokines (e.g., TNF-α and IL-1) (3, 4, 7, 8), necrotic cells (9, 10), and pathogen products including bacterial components (3, 4, 8). DC maturation has also been shown to result in vitro and in vivo from cognate CD4$^+$ T cell help mediated by CD40L (11–15). The migration of DCs from the periphery to draining lymph nodes involves inflammatory stimuli and cytokines (e.g., TNF-α and IL-1) that result most notably in spe-

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**Abbreviations used in this paper:* BG, Birbeck granule; DC, dendritic cell; DCi, interstitial-type dendritic cell; DL, dermatopathic lymphadenitis; LC, Langerhans cell.


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cific changes in chemokine receptor expression as well as in DC chemokine responsiveness (16–20). Those steps are coordinated in a number of models and DC migration frequently appears to result from exposure to the same stimuli as DC maturation. The role of inflammatory stimuli in the maturation of DCs needs however to be further investigated in vivo. Two major issues must be considered. First, many previous experiments have involved DC purification ex vivo, or culture in vitro, or in vivo perturbations that may alter DC maturation and function. A second issue is that immature DCs does not represent an homogenous population in vivo or in vitro (1, 2) and the maturation of different subsets may require distinct stimuli (21). This is important as mature DCs induce immunity, whereas the persistence of an immature phenotype could lead to a state of tolerance, e.g., due to the lack of costimulatory molecules (22–26).

As an in vivo model of DC mobilization with the LC phenotype, we chose to study dermatopathic lymphadenitis (DL), a reactive condition that features the enlargement of (usually) one draining LNs in reaction to inflammatory lesions in its tributary skin area (27–31). DL may be associated with a variety of skin disorders (31). Histologically, the enlarged LNs display a marked expansion of the paracortex, including pale-staining large cells identified as LCs on the basis of their morphology, their expression of CD1a, and the presence of Birbeck granules (BGs; references 28–30). The accumulation of BG-containing cells in draining LNs has also been observed following experimental skin lesion in rabbits (32). DL is thought to involve immigration of LCs from the skin because (a) the affected lymph nodes drain a site of skin disease, (b) because LCs can be observed in the afferent sinuses of the affected lymph nodes, and (c) such cells frequently contain melanin, which is likely to be transported from the skin in a TGF-β–dependent, LC-dependent manner (33). Nevertheless there is still no direct, formal proof that the LN LCs in DL derive from the epidermis rather than from other precursors.

In basal conditions, LCs are immature DCs (5, 6, 34), located above the basal layer of epithelial cells in the skin, oral, nasal, esophageal, pulmonary, vaginal, and rectal mucosae. LCs specifically express E-cadherin, CD1a, and the lectin Langerin/CD207, an endocytic receptor that induces BG formation (35, 36). Their emigration from the skin to the lymph node via afferent lymphatics has been well documented (1, 2, 29, 34) and the current paradigm of DC migration and maturation actually originates from observations on LCs.

In this study, we found that LCs present in T cell areas of skin-draining LNs of DL patients were largely immature, indicating that recruitment to the lymph node and maturation of LCs can be independently regulated events and questioning the role of inflammatory mediators in the maturation of LCs in vivo. In vitro–generated LCs further indicated that in the presence of TGF-β1, TNF-α, and other inflammatory stimuli-favored the maintenance of an immature phenotype in spite of the acquisition of CCR7 expression and responsiveness to LN homing chemokines. Altogether, our results suggest that immature LCs may play a role in secondary lymphoid organs during chronic inflammation.

## Materials and Methods

*Antibodies, Media, and Cytokine.* FITC-conjugated CD1a (clone BL1, mouse IgG1), MHC class I (MHC ABC, mouse IgG2a), MHC class II (DR, mouse IgG2), and CD83 (mouse IgG2b) were obtained from Immunotech. Uncoupled DC-LAMP/CD208 (104.G4, mouse IgG1), CD80 (mouse IgG1), and CD40 (mouse IgG1) were also purchased from Immunotech. PE-conjugated CD14 (Leu-M3, mouse IgG2b), CD1a, and CD86 (mouse IgG2b) were obtained from Becton Dickinson. mAb DCGM4 (mouse IgG1) to Langerin/CD207 has been described previously (36). Uncoupled anti–E-cadherin (HECD-1, mouse IgG1) was obtained from R&D Systems. Anti-CCR7 (clone 2H4 mouse IgM) was obtained from Becton Dickinson. The medium used for cell culture experiments (complete medium) was RPMI 1640 (GIBCO BRL) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% heat-inactivated FCS Myoclone (all from GIBCO BRL). Recombinant human GM-CSF was provided by Sandoz AG. Recombinant human IL-4, TGF-β1, TNF-α, IL-1β, LARC, ELC, and SLC were all purchased from R&D Systems. LPS from *Escherichia coli* 0127-B8 and 026-B6 were purchased from Sigma-Aldrich. Lysine fixable FITC-dextran, MW = 40,000 and heat-inactivated *E. coli* (K12) conjugated to Texas-red were purchased from Molecular Probes. BCG–GFP was a gift from C. Locht, INSERM, Institut Pasteur de Lille, Lille, France (37). Murine fibroblast cell lines transfected with human CD40L (LcCD40L) or CD32 (LcCD32) were provided by F. Brière (Schering-Plough; reference 38). Tetanus toxoid was a gift of F. Le Deist (Laboratoire d'Immunologie Clinique, Hopital Necker, Paris, France).

*Patients.* Fixed and frozen samples from lymph nodes removed for diagnostic purpose were retrieved from the files of the Necker-Enfants Malades and Henri Mondor Hopitals, Paris, France. DL is the reactive condition that realizes the enlargement of (usually) one draining LNs in reaction to inflammatory lesions in its tributary skin area (31). Diagnosis of dermatopathic lymphadenopathy was established at Necker-Enfants Malades and Henri Mondor Hopitals as the presence of a marked expansion of the paracortex by pale-staining histiocytic cells expressing CD1a, occasionally containing melanin, with the preservation of the B cell follicles and the absence of other specific lesion (31). The presence of a skin lesion in the tributary skin area of the involved LNs was documented in all the cases included in the study. Characteristics of the patients with DL are summarized in Table I.

*Immunohistochemistry and Immunofluorescence on Tissue Sections.* Immunohistochemical analysis of frozen lymph node 5 μm-thin section fixed in acetone was done after the streptavidin-biotin immunoperoxidase technique as described previously (21, 39). Double staining was performed according to published procedures (40). Fast Blue (Sigma-Aldrich) and AEC (Sigma-Aldrich) were used as substrates for alkaline phosphatase and peroxidase, respectively. For confocal microscopy, slides were rehydrated for 5 min in PBS with 2% pooled normal human AB serum (staining medium), and then incubated for 1 h at room temperature with mouse anti–DC-LAMP, anti-CD83, or anti-Langerin followed by goat anti–mouse Cy5, FITC-conjugated mAb to Langerin and to HLA-DR, PE-conjugated mAb to CD1a, CD14, CD86, and CD68. Slides were mounted with Fluoprep (Biomerieux SA, Marcy l'Etoile, France) and analyzed with a confocal laser system (LSM 510; Zeiss Laboratory).

*In Vitro Culture of LC-type Cells.* DCs differentiated from monocytes were cultured as described previously (21, 41) with the following modifications. Cells were cultured in complete me-

**Table I.** *Patients with Dermatopathic Lymphadenitis*

| Age/Sex | Diagnosis (Skin) | Skin lesions | Biopsied LN | Time between onset of skin lesions and LN biopsy |
|---|---|---|---|---|
| 25/F | Scabies | Back | Spinal | 1 mo |
| 57/M | Nummular dermatitis | Arms | Axillary | >1 mo |
| 54/M | Eczema-like | Localized, thorax | Cervical G | >1 mo |
| 45/M | Eczema | Generalized | Cervical D | Unknown |
| 33/M | Urticaria | Generalized | Axillary | 3 wk |
| 29/M | Acne | Arms and face | Axillary | >1 mo |
| 45/M | Hidrosadenitis | Axillary | Axillary | >1 mo |
| 24/F | T cell lymphoma (Mycosis Fungoides) | Arm | Axillary | Unknown |
| 14/M | T cell lymphoma (Mycosis Fungoides) | Arms, shoulders | Axillary | 1 y |
| 20/M | T cell lymphoma (Mycosis Fungoides) | Arms, shoulders | Axillary | 1 y |
| 55/M | T cell lymphoma (Mycosis Fungoides) | Arm, face | Axillary | unknown (>1 y) |
| 40/M | Skin B cell lymphoma | Arms, shoulders | Axillary | >1 mo |

dium supplemented at day 0 with 100 ng/ml GM-CSF, 10 ng/ml IL-4, and 10 ng /ml TGF-β1. At days 2 and 4, fresh medium, supplemented with GM-CSF and TGF-β1 (but not IL-4) was added. At days 5–7 of culture, cells were resuspended in 24-well tissue culture plates at a concentration of $5.10^5$ cell/ml in complete medium supplemented with the above-mentioned cytokines. Cells were then stimulated for 40 h with TNF-α, IL-1β, LPS, Texas-red–conjugated *E. coli* (Bioparticles; Molecular Probes) or BCG-GFP, at various doses or kept in medium alone. For analysis of CD40L-mediated activation, fibroblastic L-cells transfected with either CD40L (LcCD40L), or CD32 (LcCD32), as control, were irradiated at 80 Gy and added to the culture wells in a ratio of 2/10. BGG-GFP was grown in liquid culture at 37°C in Stauton medium containing 25 μg/ml of kanamycin, using stationary tissue culture flask. For infection experiment, fresh bacteria were collected at the end of the exponential phase (10E9 CFU/ml), centrifuged at 5,000 *g* for 30 min at 4°C, and washed twice with LPS-free RPMI 1640 (GIBCO BRL). Pellets were then suspended in RPMI 1640 without antibiotics to the appropriate dilution in order to obtain the required multiplicity of infection. BCG-GFP was cocultured with LC-type cells for up to 36 h at a multiplicity of infection of 20:1 to 50:1.

*Flow Cytometry and Confocal Microscopy Analysis of Cell Suspensions.* $3.10^5$ cells per point were incubated in 96-well plates (Becton Dickinson) for 15 min at 4°C in PBS, 2% human AB serum, and 0.01 M $NaN_3$, with mAbs at the appropriate concentration, or with control isotype-matched irrelevant mAbs at the same concentration. After washing, cells were incubated when appropriate with $F(ab')_2$ GAM-FITC or APC (Immunotech) for 15 min at 4°C in the same buffer and washed again. Propidium iodide was added to each sample before analysis in order to exclude dead cells. $5.10^4$ events were analyzed with a FACScalibur™ (Becton Dickinson) using CELLQuest™ software (Becton Dickinson). For confocal immunofluorescence microscopy examination we followed previously reported protocols (21, 35). In brief, cells were adhered to glass slides coated with 50 μg/ml poly-L-lysine (Sigma-Aldrich), fixed in 4% paraformaldehyde in $Ca^{2+}/Mg^{++}$-free PBS, and quenched with 0.1 M glycine. Cells were permeabilized in PBS/saponin (0.01%)/gelatin (0.25%)/NP-40 (0.1%), and sequentially incubated with FITC-conjugated mAb to Langerin or HLA DR, PE-conjugated mAb to CD68 and CD86, and unconjugated mAb to Langerin or DC–LAMP, revealed by anti–mouse-Cy5 (Jackson ImmunoResearch Laboratories). Mounted slides were analyzed with either confocal laser microscope systems at our disposal (Zeiss LSM 510, and CLSM SP1).

*Chemotaxis Assay.* Cell migration was evaluated using a chemotaxis microchamber technique (48-well Boyden microchamber; Neuroprobe) as described previously (16). In brief, human recombinant MIP-3α/LARC/CCL20 was diluted to 500 ng/ml, MIP-3β/ELC/CCL19 and 6Ckine/SLC/CCL21 were diluted to 100 ng/ml, respectively. Diluted chemokines were added to the bottom wells and $10^5$ cells per well were applied to the top wells of the chamber, with a standard 5-μm pore polycarbonate filter (Neuroprobe) separating the bottom wells. After 1 h and 15

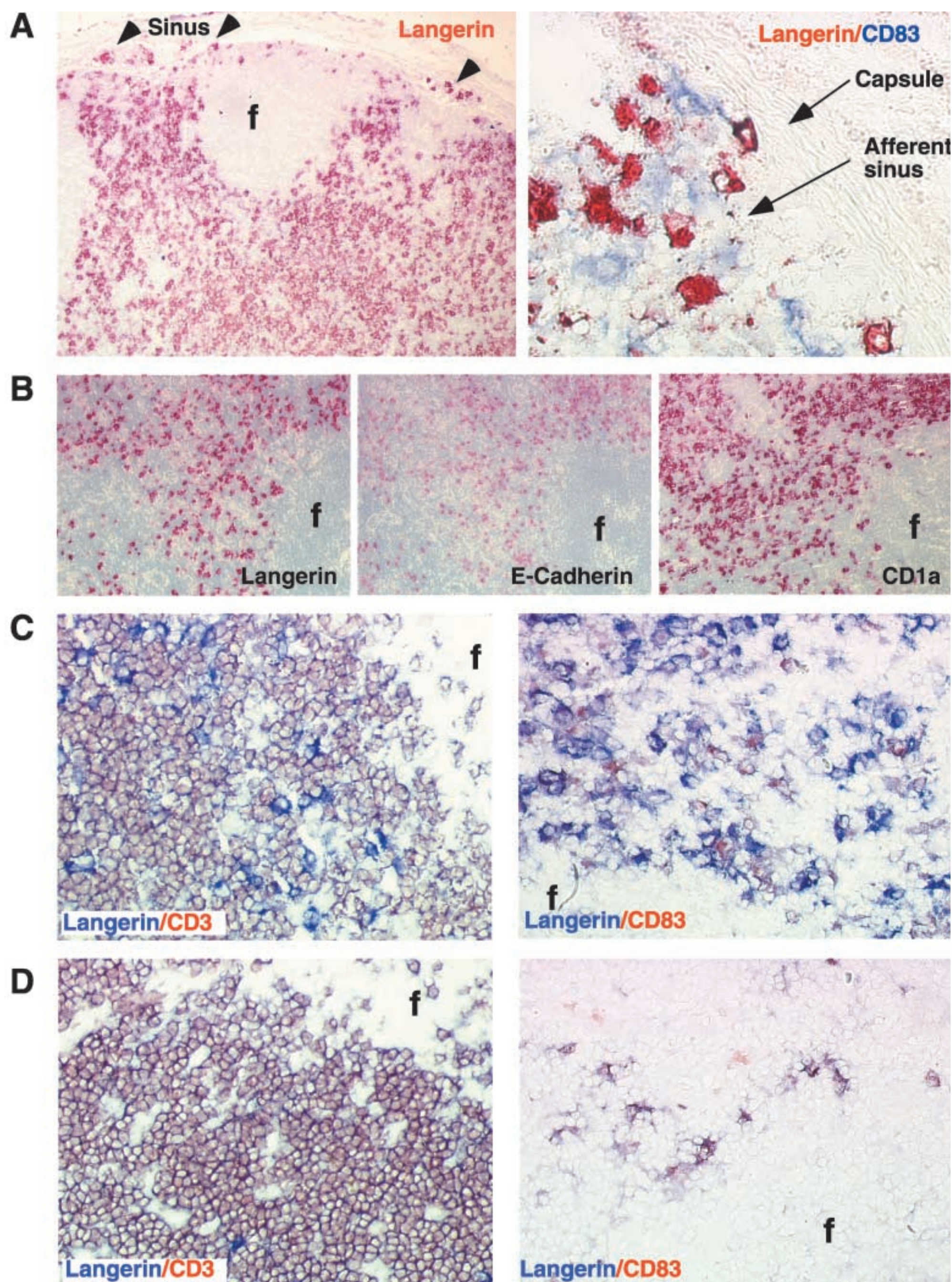



**Figure 1.** High numbers of LCs are recruited in afferent sinuses and T cell areas of inflammatory-skin draining LNs. LNs cryostat section (5 μm thick) from patients with DL were stained the indicated antibodies, representative sections are shown. (A, left) Langerin staining (in red, original magnification: ×100); (right) Langerin staining (in red) and anti-CD83 (in blue), original magnification: × 400. Arrowheads indicate the peripheral sinus. (B) Left and middle, Langerin, and E-cadherin staining (in red) on serial sections. Right CD1a staining, original magnification: ×100. "f" denotes B cell follicle. (C) Double immunostaining of DL sections using anti-Langerin (revealed in blue with alkaline phosphatase) and anti-CD83 (left), or anti-CD3 (right) (revealed with peroxidase in brownish). (D) Similar staining on sections from reactive LN draining normal skin.

min of incubation at 37°C, cells that had migrated to the underside of the filter were stained with Field's A and Field's B (BDH, Dorset, England) and counted in two randomly selected low-power fields (original magnification: ×20). Each assay was performed in duplicate and the results were expressed as the mean ±SD of migrating cells per two fields.

*Autologous Response to Tetanus Toxin.* T cell proliferative response to tetanus toxin was evaluated as described previously (21). In brief, DCs were collected, washed three times, pulsed for 40 h with tetanus toxin or medium alone, with or without TNF-α (10 ng/ml) or LcCD40L. Cells were washed twice in PBS, resuspended in RPMI 1640 with 10% human AB serum and added in triplicate at various concentrations to $10^5$ autologous T cells per well in 96-well tissue culture plates (Falcon; Becton Dickinson Labware). T cells were isolated by standard Ficoll-Paque method followed by magnetic depletion of non-T cells (MACS®; Miltenyi Biotec). $^3$[H]thymidine (Amersham Life Science) incorporation was measured in newly synthesized DNA over 18 h, using pulses initiated at day 4 or 5 of the culture with 1 μCi/well of $^3$[H]thymidine. Cells were then harvested with a 96-well Harvester (Amersham Pharmacia Biotech), collected on glass-fiber filter (Amersham Pharmacia Biotech) and the incorporation of thymidine was measured with a β-plate micro scintillation counter (LKB; Amersham Pharmacia Biotech).

Transmission electron microscopy and immunogold labeling. DCs were fixed with 2% glutaraldehyde in cacodylate buffer. Af-

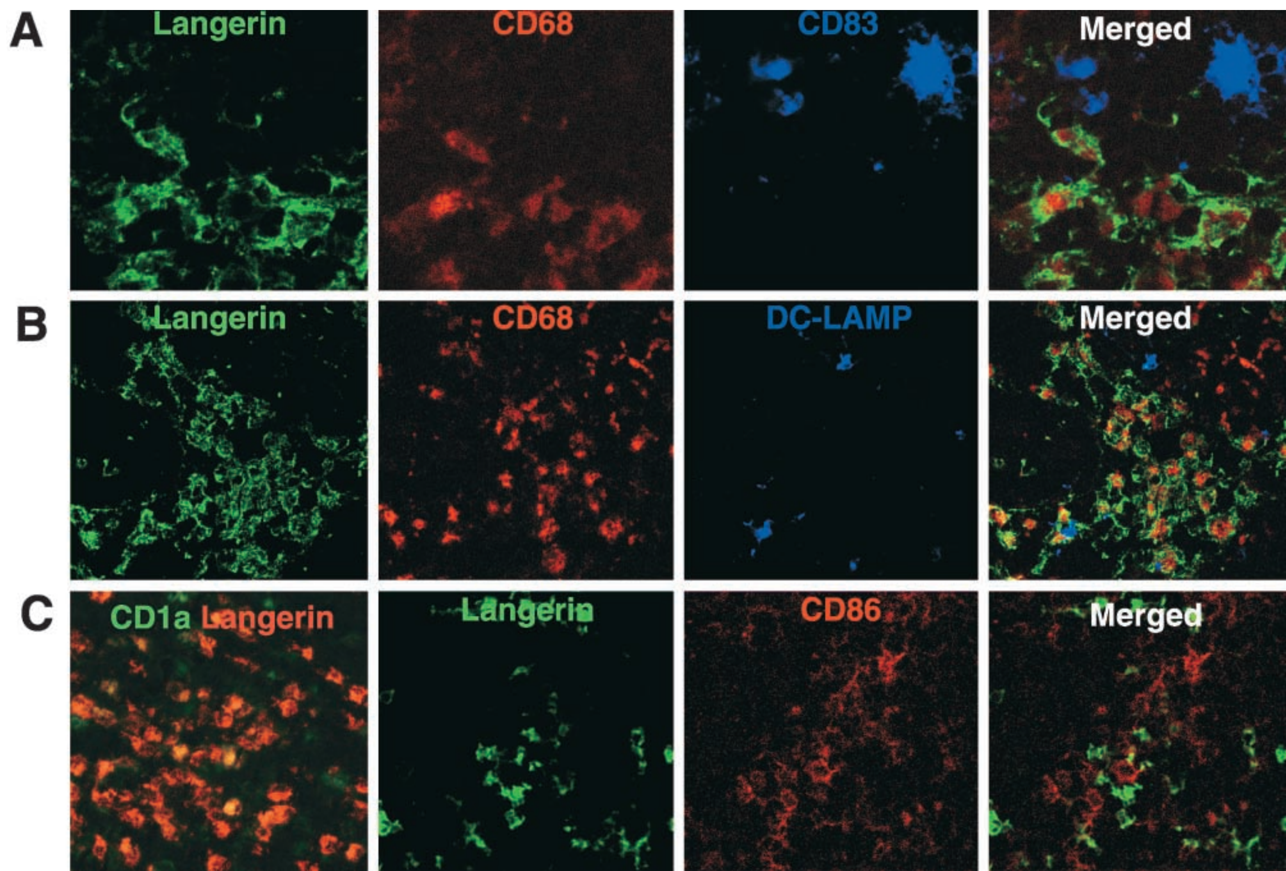
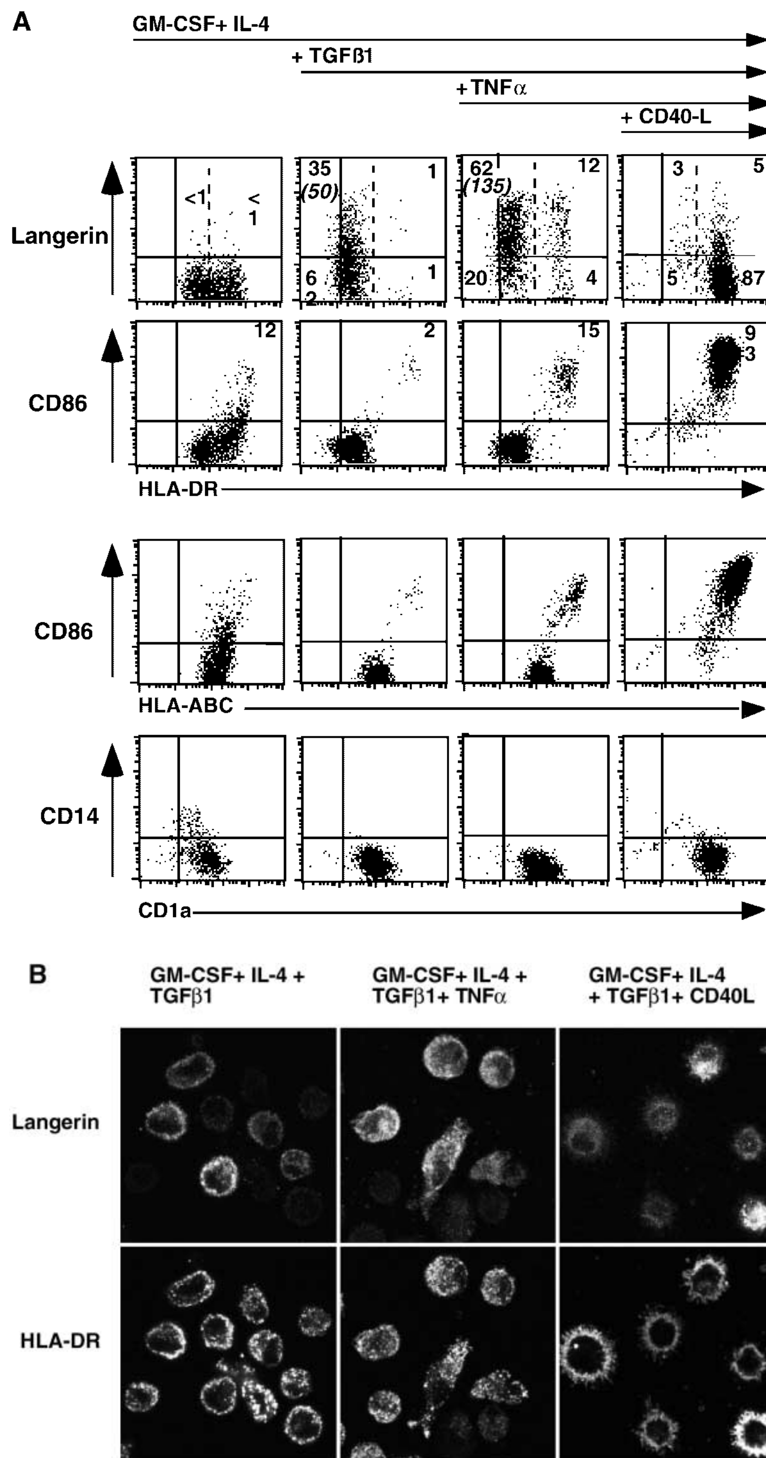



**Figure 2.** LN Langerin+ LCs express CD1α and CD68 but not CD83 DC–LAMP and CD86. (A) LNs cryostat section from patients with DL were stained with anti-CD83 antibody revealed with Cy5 (blue), FITC-anti-Langerin (green), and PE-anti CD68 (red) and analyzed by confocal microscopy. Representative sections are shown. Original magnification: ×400; (B) triple immunostaining on DL section using FITC-conjugated Langerin (green), anti-DC–LAMP antibody revealed with Cy5 (blue), and PE-anti CD68 (red) and analyzed by confocal microscopy. Magnification: ×100. (C) Double immunostaining on DL section using FITC anti-CD1a (green) and anti-Langerin revealed with Cy3 (red), and FITC-anti-Langerin (green), and PE-anti CD86 (red) and analyzed by confocal microscopy. Representative sections are shown. Original magnification: ×100.

ter rinsing in cacodylate buffer with sucrose for 12 h, the cells were processed for transmission electron microscopy. Cells were postfixed with an aqueous solution of 1% osmium tetroxide in cacodylate buffer with sucrose and embedded in epoxy medium after dehydration through a graded series of ethanols. Ultrathin sections were stained with lead citrate and uranyl acetate and examined with a JEOL 1200EX electron microscope (CMEABG, Universite de Lyon, France). For Langerin cross-linking and immunogold labeling, DC suspension were incubated for 1 h at 4°C with either DCGM4 mAb at 2 μg/ml in PBS 2% BSA (Sigma-Aldrich) or with control mouse IgG at the same concentration and then washed at 4°C and incubated for various time periods with goat anti–mouse Igs conjugated to 5-nm gold granules (Amersham Pharmacia Biotech). Cells were then washed and processed as above.

## Results

*LCs Selectively Accumulate in LNs Draining Inflammatory Skin.* To investigate the differentiation state of human DCs after skin lesions, we examined axillary and cervical LNs draining pruritic skin from patients with DL ($n = 12$, Table I) in comparison with control reactive LNs (RL, $n = 10$) without history of skin lesion. Patients with DL presented either eczema ($n = 3$), acne, hidrosadenitis, urticaria, or scabies infection ($n = 1$ each) or cutaneous lymphoma ($n = 5$). Control samples were obtained from patients without skin lesion and with reactive axillary and cervical LNs of miscellaneous etiology. Strikingly very large numbers of cells expressing Langerin (CD207), a LC-specific lectin (35, 36), were present in the T cells areas and in the afferent sinuses of all DL LNs (Fig. 1, A–C). In contrast, Langerin+ cells were very rare or not detectable in inflamed peripheral LNs in the absence of skin lesions (Fig. 1 D). The Langerin+ cells coexpressed CD1a (Fig. 1 B, see also Fig. 2 C) and the LC adhesion molecule E-cadherin, albeit at low levels (Fig. 1 B). Langerin+ cells were localized in close contact with T cells, as evaluated by anti-CD3 staining (Fig. 1 C). Altogether, these features characterize a selective and prominent accumulation of LCs in DL LNs. These features were similar in all 12 DL LNs examined. The predominance of Langerin+ cells in the LN in the presence of skin lesions (Fig. 1, C and D), their location in the afferent sinuses (Fig. 1 A) and the presence of Langerin+ cells containing melanin (unpub-



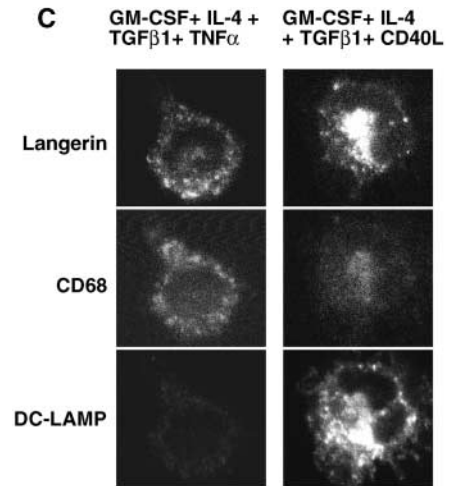




**Figure 3.** In vitro differentiation of LC-type cells. (A) TNF-α upregulates membrane Langerin expression but not MHC class I, class II, and CD86. Cells were cultured for 5–6 d in the presence of the indicated cytokines as indicated in Materials and Methods. 10 ng/ml IL-4 was added at day 0 only. Cells were analyzed by flow cytometry for membrane expression of HLA-ABC, HLA-DR, Langerin, and CD1a and CD86. Dot plots are gated on viable cells. (B) CD40L but not TNF-α induces internalization of Langerin, upregulation of membrane HLA-DR and acquisition of a Mature dendritic shape. Cells cultured as in A are permeabilized and analyzed by confocal microscopy for expression of Langerin (top panel) and DR (bottom panel). Left panels represent cells cultured for 5–6 d in GM-CSF, IL-4 (added at day 0 only), and TGF-β1, middle panels represent cells cultured for 5–6 d in GM-CSF, IL-4 (added at day 0 only), TGF-β1, and TNF-α (10 ng/ml for the last 40 h of culture). The right panels represent cells cultured for 5–6 d in GM-CSF, IL-4 (added at day 0 only), TGF-β1, with CD40L transfected fibroblasts (for the last 40h of culture). Incubation with control fibroblasts (LcCD32) as described in Materials and Methods does not results in the activation of cells (unpublished data). Original magnification: × 400. (C) TNF-α treated LC-type cells express CD68 while CD40L treated LC-type cells express DC-LAMP. Cells cultured and processed as in B were analyzed for expression of Langerin, DC-LAMP and CD68 by three-color confocal microscopy. Left panels represent cells cultured for 5–6 d in GM-CSF, IL-4 (added at day 0 only), TGF-β1 and TNF-α (10 ng/ml for the last 40 h of culture). Right panels represent cells cultured for 5–6 d in GM-CSF, IL-4 (added at day 0 only), TGF-β1, with CD40L transfected fibroblasts (for the last 40 h of culture). Incubation with control fibroblasts (LcCD32) as described in Materials and Methods does not result in the activation of DCs (unpublished data and reference 26).

lished data), suggest that these LCs originate from the skin. Yet, we cannot be exclude that they derive from other, e.g., circulating, precursors.

*LCs in Human LNs that Drain Inflammatory Skin Are in an Immature State.* We further determined the differentiation stage of Langerin$^{+}$ cells within DL LNs. Cells expressing CD83, a marker of mature DCs, were present in comparable numbers in the T cell zone of both skin reactive and control LNs (Fig. 1, C and D); however, the abundant Langerin$^{+}$ cells appeared to be largely devoid of CD83 in both the T cell zone and in the afferent sinuses of DL LN (Fig. 1, A and C), suggesting that the accumulated LCs are not fully mature DCs. The expression of CD68 a lysosomal protein expressed in macrophages and immature DCs (42), DC–LAMP, a lysosomal protein restricted to mature DC (43), CD83, MHC class

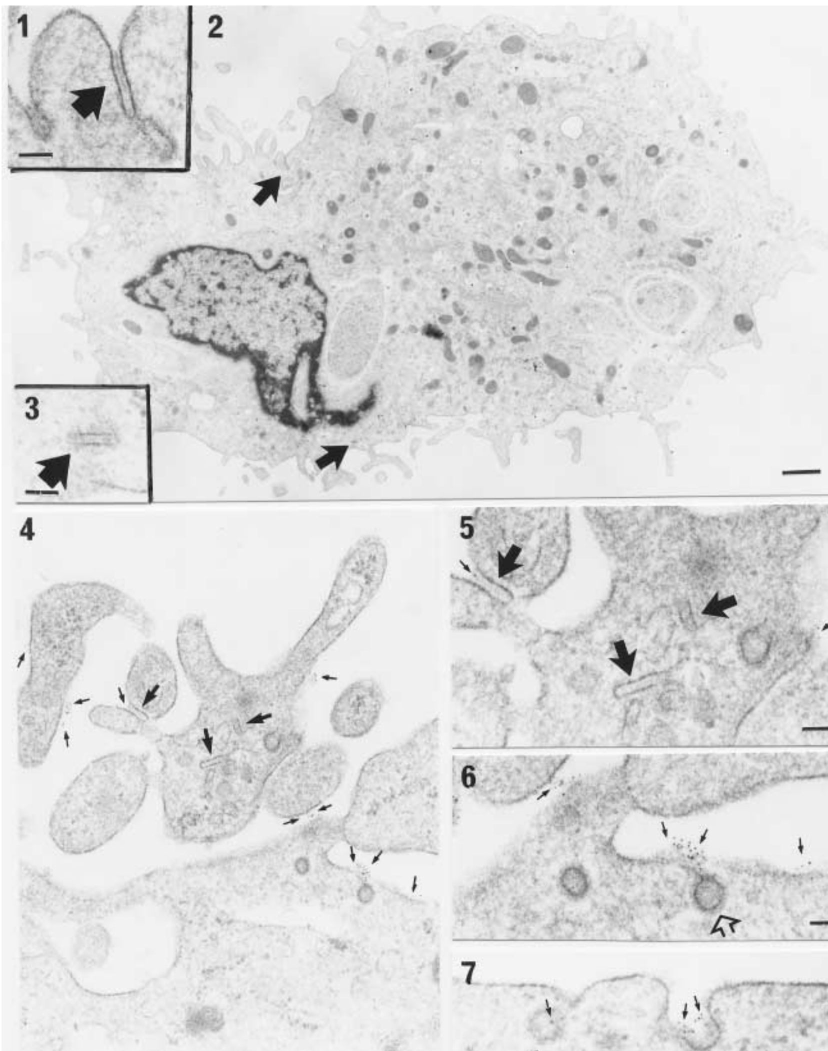



**Figure 4.** Cross-linking of Langerin induces the formation of BGs in TNF-α treated LC-type cells. (1–3) LC type-cells were cultured in the presence of GM-CSF, IL-4 (added at day 0 only), TGF-β1 and TNF-α (added at 10 ng/ml for the last 40 h of culture). Inset (1 and 3) show magnification of BGs. Bar = 100 nm on panels 1 and 3 and 1 p.m. on panel 2. (4–7) Cells were cultured as for panels 1–3, and Langerin was cross-linked with mouse anti-Langerin Ab and gold-labeled goat anti–mouse Ig for various times. Panel 4 shows the formation of large numbers of gold labeled BGs and coated pits after a very short time (<1 min). Bar = 100 nm. Panels 5–7 show BGs and coated pits at higher magnification. In the absence of TGF-β1 however, TNF-α did not induce the expression of Langerin, nor the formation of BGs (unpublished data). Bar = 100 nm.

I and II antigens, CD1a, and the costimulatory molecule CD86 was determined by confocal immunofluorescence microscopy.

Analysis of triple-stained sections demonstrated that most Langerin$^+$ cells were positive for CD68, but negative for CD83 and DC–LAMP, whereas CD83$^+$ cells were negative for Langerin and CD68 and positive for DC–LAMP (Fig. 2, A and B). Consistently, Langerin$^+$ cells were mostly CD1a$^+$ and CD86$^-$ (Fig. 2 C). Langerin$^+$ cells expressed both class I and II antigens, with a predominant intracellular expression of class II antigens (unpublished data). These features were similar in all 12 DL LNs examined. These data demonstrate that LCs recruited in the T cell area of inflammatory skin draining LNs can have a predominant immature phenotype.

These results also show the coexistence of immature LCs and mature DCs in inflammatory skin draining LNs. CD83$^+$ dendritic can be observed within the afferent sinuses (Fig. 1 A), and are present in inflamed LNs in the absence of skin lesions (Fig. 1 D), suggesting that the Langerin$^+$, CD68$^+$, CD83$^-$ DC–LAMP$^-$ CD86$^-$ subset corresponds to immature LCs and the CD83$^+$ DC–LAMP$^+$ DC subset correspond to interstitial DCs (DCi) emigrating from the dermis (44) more likely than to mature Langerin$^-$ LCs. Our results thus suggest that inflammatory signals could differentially affect the maturation of Langerhans and non-Langerhans DCs in vivo.

*TNF-α Potentiates Expression of Langerin and Formation of BGs in Immature Monocyte-derived LC-type Cells.* To explore the effect of inflammatory cytokines on LC differentiation and migration, we cultured LC-type cells in the presence of TNF-α. LCs may be derived from CD34$^+$ hemopoietic progenitor-cells (45–48). However, these cells represent a wide range of maturation stages. To synchronize the maturation stages of LCs in the cultures we used mainly LC-type cells derived from human monocytes, grown in the presence of GM-CSF, IL-4, and TGF-β1 (21, 41). This further allowed us to compare LCs with DCi derived from the same monocytes differentiated in the presence of GM-CSF and IL-4 (7, 21, 49). When cultured in the absence of TNF-α approximately one-third of the monocyte-derived LC-type cells expressed significant surface levels of Langerin (Fig. 3 A). Remarkably, the addition of TNF-α at days 5 or 6 of the culture strongly in-

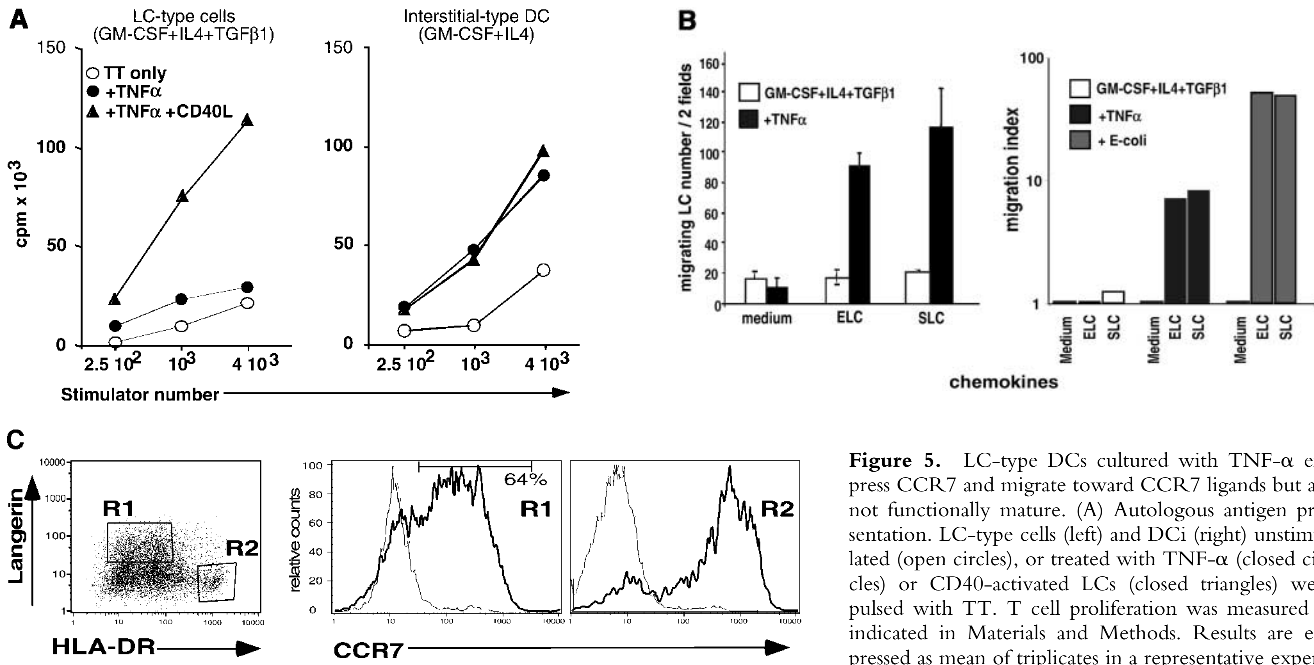



**Figure 5.** LC-type DCs cultured with TNF-α express CCR7 and migrate toward CCR7 ligands but are not functionally mature. (A) Autologous antigen presentation. LC-type cells (left) and DCi (right) unstimulated (open circles), or treated with TNF-α (closed circles) or CD40-activated LCs (closed triangles) were pulsed with TT. T cell proliferation was measured as indicated in Materials and Methods. Results are expressed as mean of triplicates in a representative experiment. SD were <20%. (B) Both SLC and ELC trigger migration of TNF-α and *E. coli* treated, but not control unstimulated LC-type cells. Day 6, LCs were treated for 48 h with TNF-α (10 ng/ml) or with heat-inactivated *E. coli* (50:1 ratio, right). Then, samples were recovered and cells were tested for their capacity to migrate in response to ELC (100 ng/ml) and to SLC (100 ng/ml). Migration assays were performed in Boyden microchambers. (Left) Results are expressed as number of migrating LCs per two low power fields (original magnification: ×20). (Right) Results are expressed as migration index compared with medium alone (without chemokine). Results are representative of more than three independent experiments. (C) CCR7 expression. Day 6, LC-type and DC-type cells were treated for 48 h with 10 ng/ml TNF-α. Cells were washed and stained with PE-conjugated anti-Langerin, FITC-anti-DR and anti-CCR7 or with isotype-matched controls antibodies and analyzed by flow cytometry. In the left panel, the dot-plot represent langerin and HLA-DR expression of LC-type cells treated with TNF-α. On the center and right panels histograms represent CCR7 expression on gated-cell populations (R1:Langerin1 DRlo; R2: Langerin-, DRhi). Thick lines represent the labeling obtained with CCR7 antibody and thin lines represent labeling with the isotype-matched control. Results are representative of two independent experiments.

creased the proportion of Langerin$^+$ cells (Fig. 3 A). Langerin$^+$ cells increased from 33 ± 12% to 75 ± 15% of total cells ($n = 6$ independent experiments), with a considerable higher MFI (from 66 ± 20 to 250 ± 130). However, the presence of TGF-β1 was mandatory for the induction of Langerin and BGs by TNF-α (unpublished data) demonstrating that TNF-α acts in synergy with TGF-β1 to promote LC differentiation.

In contrast to the up regulation of Langerin in the vast majority of the cells, TNF-α treatment increased surface expression of MHC class II and CD86 molecules only in a minor proportion of Langerin$^+$ cells (Fig. 3 A), in accordance with our previous results (21). While the presence of TGF-β1 is absolutely required during the first 6 d of culture to generate LCs, the presence of TGF-β1 contribute but was dispensable to maintain the immature LC phenotype upon addition of proinflammatory signals (21, and unpublished data). Confocal microscopy further confirmed that TNF-α treated LC-type cells expressed CD68 and intracellular MHC class II but not DC–LAMP (Fig. 3, B and C). In striking contrast to TNF-α, CD40L induced a full maturation of LC-type cells in vitro, as illustrated by the strong upregulation of MHC class II and CD86 (Fig. 3, A and B), the loss of CD68 and the induction of DC–LAMP (Fig. 3 C). Although CD40L stimulation resulted in the translocation of class II molecules to the cell surface and the loss of surface Langerin expression, Langerin remained detectable within intracellular compartments for at least 48 h (Fig. 3 B).

Electron microscopy confirmed that TNF-α–treated cells have typical LC features as demonstrated by the presence of BGs (Fig. 4, panels 1–3). To assess whether Langerin is functional in TNF-α–treated cells, we monitored the formation of BGs after Langerin cross-linking. LC-type cells exposed to mouse anti-Langerin Ab and gold-labeled goat anti–mouse Ig developed very rapidly (<1 min) high numbers of gold-labeled BGs (Fig. 4, panels 4–7). These data further emphasize that TNF-α induces LCs to express functional Langerin in conjunction with TGF-β1.

*TNF-α Induces Functionally Immature LCs, which Express CCR7 and Respond to LN Homing Chemokines.* Next, we investigated other functional properties of LC-type cells activated by TNF-α such as their Ag presentation capacity and their ability to migrate toward the LN environment. First, in accordance with our previous results (21) we found that treatment with TNF-α did not enable LC-type cells to stimulate the proliferation of autologous antigen specific memory T cells in vitro (Fig. 5 A), confirming the immature state of the TNF-α–treated LC-type cells. In contrast, monocyte-derived DCi generated in the absence of TGF-β1 became

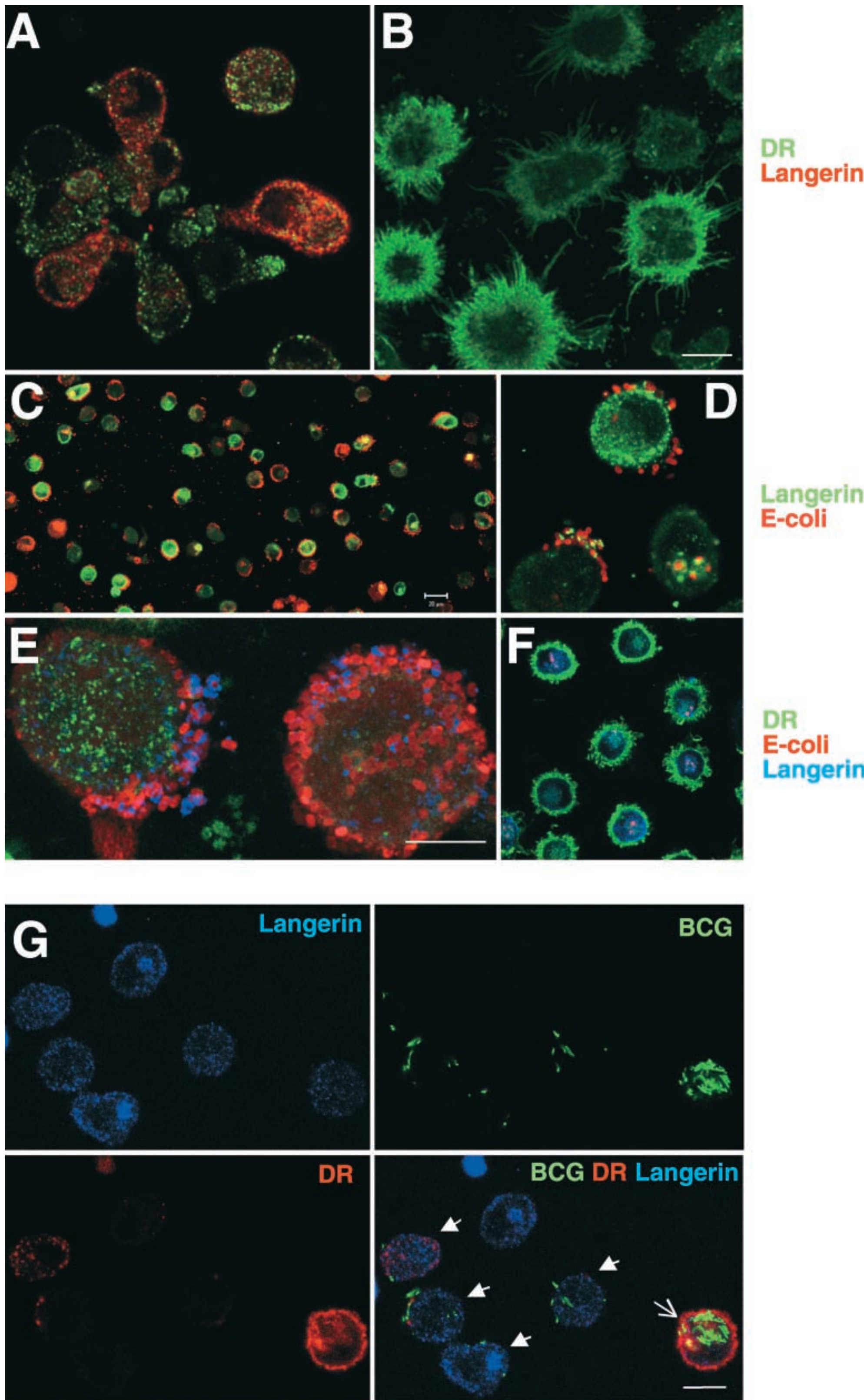



**Figure 6.** LPS and bacteria induce the maturation of DCi but not LC-type cells. Interstitial-type and LC-type DC were cultured for 5–6 d as indicated in Materials and Methods and incubated with either LPS, heat-inactivated *E. coli*, or BCG-GFP. (A and B) Coculture with LPS induces the maturation of interstitial type, but not LC-type DCs. LC-type (A) and (B) DCi treated with LPS were fixed, permeabilized, stained with FITC-conjugated anti–HLA-DR antibody and anti-Langerin antibody revealed with anti–mouse Cy3 (in red), and analyzed by confocal microscopy. Original magnification: ×400. (C–E) Coculture with heat inactivated *E. coli* does not induce the maturation of LC-type DC. LC-type DCs were cultured for 40 h in the presence of Texas-red conjugated heat-inactivated *E. coli* at a 50/1 ratio (C-E) or LcCD40L (F) and then collected, washed, fixed, permeabilized and stained with either FITC-conjugated anti-Langerin antibody (C and D) or FITC-conjugated anti–HLA-DR antibody and anti-Langerin antibody revealed with anti–mouse Cy5 (in blue) (E and F), and analyzed by confocal microscopy. Coculture of DCi induced their maturation (unpublished data). Panel G LC-type DC were cultured for 36 h in the presence of BCG-GFP and then collected, washed, fixed, permeabilized, stained with PE-conjugated anti-HLA-DR antibody and anti-Langerin antibody revealed with anti mouse Cy5 (in blue), and analyzed by confocal microscopy. Arrow indicates a Langerin$^{-}$ DR$^{hi}$ DC and arrowheads Langerin$^{+}$ DR$^{lo}$ DCs. Coculture of DCi with BCG-GFP induced their maturation (unpublished data). Bar = 10 μm, unless otherwise indicated.

mature after exposure to TNF-α and thereafter, efficiently stimulated autologous antigen specific T cells (Fig. 5 A).

On the other hand, the presence of immature LCs within the afferent sinuses in DL LNs suggests that immature LCs are able to migrate toward the LNs. This migration has been shown to involve downregulation of E-cadherin expression (50), and acquisition of the chemokine receptor CCR7 allowing migration in response to the che-

mokines SLC (CCL19) and ELC (CCL21) (16–20). We have previously shown that TNF-α downregulates E-cadherin expression on immature LC-type cells (21). Therefore, we evaluated the chemotactic activity of the CCR7 ligands, SLC (CCL21) and ELC (CCL19) on the monocyte-derived LCs. Both chemokines remarkably induced the migration of TNF-α–treated, but not control LC-type cells generated in the absence of TNF-α (Fig. 5 B). In accordance with this finding, TNF-α increased the amounts of CCR7 mRNA by 32–64-fold as evaluated by real-time quantitative PCR (unpublished data). To rule out that the expression of CCR7 was due to the small fraction of mature LC-type cells present in the cultures, we then studied the expression of CCR7 on TNF-α–treated MHC class $II^{Lo}$ LC-type cells (Fig. 5 C). A high percentage (64%) of "immature" MHC class $II^{Lo}$ cells expressed high levels of CCR7. Altogether, these data indicate that, even in the absence of maturation, TGF-β1 and TNF-α can induce the chemotactic machinery required for the emigration of LCs toward the draining LNs.

*Bacterial Products also Induce Langerin Expression, Maintenance of an Immature LC Phenotype and Migration toward CCR7 Ligands.* We further investigated the effects of exposure to bacterial products, likely to occur in an inflammatory skin, mucosa, or lung, on the maturation of monocyte-derived LCs. Exposure to LPS was found to induce Langerin expression on LC-type DCs (Fig. 6 A). This induction resulted in a twofold increase in $Langerin^+$ cells (from 28 to 52% of cells as counted on wide-field micrographs) but LPS induced neither DC–LAMP expression, nor the translocation of MHC class II molecules to the cell surface (Fig. 6 A). In contrast to LC-type cells, LPS induced the maturation of DCi used as controls without significant induction of Langerin expression (Fig. 6 B).

We speculated that, in contrast to purified LPS, whole bacteria corpses might promote both the recruitment and the maturation of LCs. Therefore, we cultured LC-type cells for up to 40 h in presence of heat-inactivated *E. coli*–conjugated to Texas-red (at a 50:1 bacteria to cell ratio). Exposure to *E. coli* led to an increase in both the percentage of Langerin-positive cells (from 28 to 58% as counted on Fig. 6 C), and the intensity of Langerin staining (data not shown). Interestingly, most of the $Langerin^+$ cells interacted with bacteria on their cell surface (Fig. 6 D), whereas Langerin-negative cells internalized the bacterial corpses. MHC class II staining remained mostly intracellular and typical of immature DCs in these cells (Fig. 6 E). Analogous to TNF-α treatment, the exposure to *E. coli* triggered also the migration of LC-type cells toward CCR7 ligands (Fig. 5 B). In contrast to LCs cultured in the presence of heat-inactivated *E. coli* alone, subsequent exposure to CD40L induced LC maturation with a typical MHC class II surface staining and intracellular langerin staining (Fig. 6 F). Unlike LCs, DCi cultured with heat-inactivated *E. coli* rapidly and fully matured as assessed by translocation of MHC class II to the cell surface and DC–LAMP expression (unpublished data).

Finally, we wished to determine whether exposure to live bacteria would necessarily induces LC maturation in vitro. For this purpose, we used the slow growing live *Mycobacterium bovis* (BCG) expressing the (BCG-GFP), instead of the impractical rapidly-dividing *E. coli*. As shown in Fig. 6 G, most $Langerin^+$ LCs also remained immature after culture in the presence of GFP-BCG expressing intracellular MHC class II, although they bound mycobacteria. These data demonstrate that in vitro cultured LC-type cells remain in an immature state despite exposure to live bacteria. Taken together, our results show that TGF-β1 cooperates with a wide range of inflammatory stimuli-including TNF-α, LPS, and bacteria- likely to be encountered in inflammatory skin as well as in mucosae and lung to promote the differentiation of LCs, which exhibit an immature phenotype but are prone to migrate to draining LNs.

## Discussion

Our study addressed the question of the regulation of LC maturation in response to inflammation in vivo and in vitro. We show here an expanded population of immature LCs in skin draining LNs of DL patients. The $Langerin^+$ $CD1a^+$ $E\text{-}cadherin^+$ cells observed in the T cell areas of DL LNs are likely to derive from skin LCs, as suggested by their presence in the afferent sinuses. Nevertheless, there is no formal direct proof that they do not derive from other (e.g., circulating) precursors. However, irrespective of their origin, our data demonstrate that recruitment to the LNs and maturation of LCs can be dissociated in response to inflammatory signals in vivo.

Although surprising, our observations in DL LNs are consistent with several other observations. Takahashi et al. have observed that the majority of the very rare $CD1a^+$ DCs present in peripheral human LNs, express neither CD86 nor CD83 and poorly stimulate alloreactive T cells, but are able to mature after in vitro culture (51). We have reported that even in inflammatory condition, $CD1a^+$ $CD83^-$ cells are numerous in the draining LNs (21). More recently, Ruedl et al. have shown that mouse DCs that have emigrated from the skin into the LNs after cutaneous challenge with acetone, dibutyl-phtalate, and LPS, are still able to internalize and process antigen (52) a property associated with immature DCs. Finally, LCs recovered from various tissues in patients with LC histiocytosis, a pediatric disease characterized by the accumulation of LCs in lymphoid and nonlymphoid tissues, are phenotypically and functionally immature, although able to mature in vitro in response to CD40 ligation (53).

As an in vitro model of LC mobilization in response to proinflammatory mediators, we studied the effects of inflammatory stimuli such as TNF-α and bacterial products on the functional activation of LCs differentiated from monocyte cultures in the presence of TGF-β1. We found that such monocyte derived LC-type cells can be stimulated by proinflammatory signals and (a) express high levels of Langerin which upon cross-linking is internalized into BGs, (b) express CCR7 and accordingly acquired the migration properties in response to LN chemoattractant

CCR7 ligands, and (c) do not acquire a mature phenotype (i.e., do not express CD86, DC–LAMP, are membrane MHC class II$^{lo}$, and do not stimulate T cells efficiently). This immature stage is presumably reinforced by the presence of TGF-β1. Of note, the αvβ6 integrin binds and activates latent TGF-β1, produced by epithelial cells such as keratinocytes, a mechanism triggered by inflammation (54). Based on these data, one can hypothesize that, in vivo, TGF-β1 may synergize with inflammatory stimuli such as TNF-α to enhance the differentiation of LCs from their precursors, antigen uptake via Langerin expression, and emigration of these LCs in an immature state to the draining LNs due to CCR7 expression. Further studies are however required to delineate the molecular mechanisms, which could differentially trigger LC migration and LC maturation.

CD40L, TNF-α, LPS, and bacteria induce the maturation of monocyte-derived DCi, while only CD40L induced the maturation of LCs cultured from the same monocytes in the presence of TGF-β1. Dissection of the effect of TGF-β1 on the cellular activation pathways downstream to CD40 and TNF-R, and on the regulation of chemokine-receptors expression and function may help to clarify the molecular basis for the split control of LC migration and LC maturation in response to inflammatory stimuli, as well as their differential effects on DCi and LCs. In this study, we formally identified Langerin$^+$ LCs and characterized their maturation stage during an ongoing physiologic chronic cutaneous inflammatory process. It is remarkable that immature LCs represent the majority of DCs in LNs draining these chronic lesions of various etiologies. This suggests that, at a given time, lymphocytes in T cell areas of DL LNs may mostly encounter immature LCs, whether or not individual LCs will ultimately become mature. Indeed, the fact that LCs recruited to the T cell areas of the draining LNs exhibit a typical immature phenotype, raises questions about the regulation of LC maturation, and importantly about the role of LCs in the regulation of immune responses since immature DCs have been proposed to mediate tolerance (1, 2, 22–25) (for a review, see reference 26).

The physiologic function of DCs in the steady-state in vivo has only begun to be determined. Recently, DCs have been shown to induce tolerance in vivo (22–25). Current evidence thus suggests that DCs that migrate constitutively from peripheral tissues in the absence of any overt antigenic or inflammatory stimuli (25, 55) do not mature and may mediate tolerance, while DCs that migrate as a result of inflammatory stimuli undergo maturation, due to the inflammatory signal, and therefore would trigger an immune response. It is still difficult to understand how autoimmune responses are avoided in inflammatory conditions. Based on our results we would like to hypothetize that, in contrast to DCi, migrating immature LCs most likely cannot deliver an activation signal to antigen-specific T cells and may therefore induce tolerance. It is conceivable that the recruitment of immature LCs to LNs in response to skin or mucosal inflammation might be a mechanism to prevent immunization against the self (epithelium) in inflammatory conditions, when abundant cell death may lead to the presentation of self-antigens. DCs efficiently carry out the exogenous pathway for MHC class I presentation (56–59) and immature LCs could therefore directly be involved in the maintenance of T cell peripheral tolerance because of a lack of costimulatory molecules. Alternatively, tolerance to self antigens needs not be direct, and immature LCs, upon reaching the LN, might transfer tissue-derived peptides to a subset of bystander DCs somehow specialized in tolerance, although direct evidence for this DCs subset remains elusive (25, 60). Another possibility, as stated above, is the recruitment of LC-precursor from the blood through HEV directly in the LN draining inflamed skin. In this case, "blood-derived LCs" could also directly be involved in the uptake of self-antigens and the maintainance of T cell peripheral tolerance.

The function of LN LCs could not be directly assessed here. However, patients with DL do not present skin depigmentation (vitiligo) or other skin autoimmune disease. This indicates that despite the likely presence of epithelial antigens in the LNs (melanin could be detected in some LN LCs in our study, unpublished data) the immune response does not lead to the recruitment or activation of cytotoxic anti-self effector T cells in the skin.

However, LCs may also display other functions within the LNs. They may store, or even acquire, antigen inside the T cell areas. LCs would probably also be prone to eventually undergo maturation within the T cell areas in response to further signals such as CD40L. The lack of a potent effect of bacteria on LC maturation in vitro does not rule out the activation of LCs in vivo in some circumstances, for example through T cell help due to CD40L$^+$ T cells that would recognize bacterial antigens presented by LCs. Therefore, LCs may be both involved in peripheral tolerance and the initiation of specific immune response. Although these questions cannot be addressed here, it will be of interest to determine the respective role and fate of immature LCs and of DCi within LNs. Conditional cell depletion, and cell-type specific knock-out experiments in mouse models will be useful to explore these points.

Finally, we do not know if the mature CD83$^+$ Langerin$^-$ DCs that also enter the afferent sinuses of DL LNs may correspond either to DCi or to mature LCs that would have lost Langerin expression. However, since (a) the CD83$^+$ DCs can be seen in the afferent sinuses (Fig. 1), and (b) Langerin expression persisted at high levels in intracellular compartments after in vitro maturation (at least for 48 h, Fig. 3 C, and reference 30), and (c) since DCi are peculiar by their quick differentiation from circulating monocytes and maturation upon inflammatory stimuli in vivo (44), the mature DCs observed in the LN sinuses are more likely to represent DCi rather than LCs. Monocyte-derived DCi cultured in the absence of TGF-β1 mature in vitro in response to inflammatory stimuli. It is possible that the Langerin$^-$ DCs that are observed in the sinuses and T cell areas of inflamed LNs represent the in vivo equivalent to

monocyte-derived DCi, emphasizing the heterogeneity of DC subsets.

In conclusion, we have shown here that inflammatory and bacterial stimuli contribute to the differentiation of immature LCs by upregulating Langerin expression in vitro, and allow their recruitment as immature DCs into the T cell areas of LNs in vivo. LCs may transport self-antigens, and possibly pathogens, to the lymphoid organs and then, depending on further signals received in the LN, either contribute to tolerance, or after maturation, initiate a productive immune response.

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