Brief Definitive Report

Transforming Growth Factor β1, in the Presence of Granulocyte/Macrophage Colony-stimulating Factor and Interleukin 4, Induces Differentiation of Human Peripheral Blood Monocytes into Dendritic Langerhans Cells

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Summary

Langerhans cells (LCs) are dendritic cells (DCs) that are present in the epidermis, bronchi, and mucosae. Although LCs originate in bone marrow, little is known about their lineage of origin. In this study, we demonstrate that in vitro LCs may originate from monocytes. Adult peripheral blood $CD14^+$ monocytes differentiate into LCs ($CD1a^+$, E-cadherin$^+$, cutaneous lymphocyte-associated antigen$^+$, Birbeck granules$^+$, Lag$^+$) in the presence of granulocyte/macrophage colony-stimulating factor, interleukin 4, and transforming growth factor β1 (TGF-β1). This process occurs with virtually no cell proliferation and is not impaired by 30 Gy irradiation. Selection of monocyte subpopulations is ruled out since monocyte-derived DCs can further differentiate into LCs. Our data suggest that in vivo LC differentiation may be induced peripherally, from a nonproliferating myeloid precursor, i.e., the monocyte, in response to a TGF-β1–rich microenvironment, as found in the skin and epithelia. Therefore, the monocyte may represent a circulating precursor critical to the immune response in vivo.

Dendritic cells (DCs) constitute a family of APCs defined by their morphology and their excellent capacity to initiate a primary immune response (1–3). Langerhans cells (LCs) are paradigmatic DCs that are present within epithelial cells in the epidermis, bronchi, and mucosae (4). After an antigenic challenge, LCs migrate into the T cell areas of lymph nodes where they act as professional APCs (2). Although LCs originate in bone marrow, little is known about their lineage of origin.

DCs can be generated in vitro from $CD34^+$ cord blood or bone marrow progenitors in the presence of GM-CSF and TNF-α (5–9), as well as from peripheral blood mononuclear cells in the presence of GM-CSF and IL-4 (1, 10–12). However, LCs represent a discrete population among DCs, and the current hypothesis is that LCs arise from a specific precursor distinct from monocyte precursors (7, 9). Human LCs share CD1a antigen expression with DCs generated in vitro (1, 7, 10, 11). However, LCs specifically express the epithelial antigen E-cadherin (13) and the skin homing antigen (cutaneous lymphocyte-associated antigen, CLA) (14, 15), both relevant to their unique localization within epithelial cells, and they exhibit Birbeck granules (BGs) and their associated Lag antigen (16, 17).

In this context, recent attention has focused on TGF-β1, which is synthesized at high level by epithelial cells, and whose presence is apparently required for the development of LCs in vivo (18). We therefore investigated the effects of TGF-β1 on DC differentiation at the peripheral level.

Materials and Methods

Media and Reagents. The medium used was RPMI 1640 supplemented with 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FCS (all from GIBCO BRL, Gaithersburg, MD). Recombinant human GM-CSF was provided by Sandoz AG (Bale, Switzerland), recombinant human IL-4 was purchased from Genzyme Corp. (Cambridge, MA), and recombinant human TGF-β1, polyclonal chicken anti–human TGF-β1 neutralizing antibodies, and control chicken Ig were all from R&D Systems (Minneapolis, MN).

Culture of Peripheral Blood Monocytes. $CD14^+$ monocytes were isolated from peripheral blood mononuclear cells of healthy volunteers by negative magnetic depletion using hapten-conjugated CD3, CD7, CD19, CD45RA, CD56, and anti-IgE antibodies (monocyte isolation kit, MACS; Miltenyi Biotec, Berglsch Gladbach, Germany) and a magnetic cell separator (MACS) according to the manufacturer's instructions, routinely resulting in ⩾95% purity of $CD14^+$ cells.

Cells were cultured in 6- or 24-well tissue culture plates (Costar Corp., Cambridge, MA) in fresh complete medium supple-

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mented with 250 ng/ml GM-CSF + 100 ng/ml IL-4. TGF-β1 was added at the beginning or, in some experiments, at day 2, 4, or 6 of the culture. At days 2 and 4, half of the medium was removed and an equivalent volume of fresh medium, supplemented with the above mentioned cytokines, was added.

Cell Proliferation and Apoptosis Studies. For immunocytochemical detection of cell cycle–associated antigens, harvested cells were cytocentrifugated using a Cytospin 3 device (Shandon SA, Eragny, France). Slides were air-dried, fixed in acetone, and stained with anti-Ki67 antibodies (clone Mib-1, IgG1; Immunotech, Marseille, France) with an avidin-biotin-peroxidase protocol (19) revealed by 3-3′ diaminobenzidine as chromogen (Vectasin ABC kit, Vector, CA). [^{3}H]Thymidine (Amersham Life Science, Buckinghamshire, UK) incorporation was measured in newly synthesized DNA over 48 h, using pulses initiated at day 0, 2, or 4 of the culture with 1 μCi/well of [^{3}H]thymidine. Cells were then harvested with a 96-well Harvester (Pharmacia, St. Quentin, France), collected on glass-fiber filter (Pharmacia) and the incorporation of thymidine was measured with a Beta-plate microscintillation counter (LKB, Pharmacia). For apoptosis analysis by flow cytometry (20) and cell cycle analysis, 5×10^5 cells for each point were resuspended in 500 μl PBS containing 0.1% triton and 50 μg/ml propidium iodide (PI) (Sigma Chemical Co., St. Louis, MO), and incubated for 20 min at 37°C. Analysis of the DNA content of 3×10^4 cells was performed immediately using a flow cytometer FACScan® (Becton Dickinson, Mountain View, CA) with the Lysis II software (Becton Dickinson). Allogeneic mixed leukocyte reaction was performed as previously described (1).

Flow Cytometry and Cytological and Immunocytological Analysis of PBMCs and PBMC-derived Cells. For single- and two-color flow cytometry, 1.5×10^5 cells were incubated for 30 min at 4°C in PBS, 2% human AB serum, and 0.01 M NaN_3, with FITC-conjugated CD1a (clone BL1, IgG1; Immunotech), CD80 (IgG1; Immunotech), CD40 (IgG1; Immunotech), HLA-I (HLA ABC, IgG2a; Immunotech), HLA-II (IgG1; Becton Dickinson), and/or PE-conjugated CD14 (Leu-M3, IgG2b; Becton Dickinson) and CD11b (IgG2a; Becton-Dickinson) mAbs at the appropriate concentration, or with control isotype-matched irrelevant mAbs at the same concentration. After washing, cells were analyzed with a FACScalibur® (Becton Dickinson) using CellQuest software (Becton Dickinson). For cytological examination, harvested cells were processed as described above and stained using the May-Grunwald-Giemsa technique (RAL, Rieux, France). For immunochemistry, slides were stained with CD1a (BL1, IgG1; Immunotech), E-cadherin (HECD-1, IgG1; R&D Systems), Lag (mouse IgG1; gift of Dr F. Furukawa, Hamamastu University, Hamdacho, Japan), and CLA (HECA-452, rat IgM; gift of Dr. L.J. Picker, University of Texas, Dallas, TX).

Ultrastructural Study. Cells to be processed for electron microscopy were fixed overnight at 4°C in 2.5% glutaraldehyde (Sigma Chemical Co.) in 0.1 M cacodylate buffer, pH 7.4, followed by postfixation for 1–2 h in 0.1 M cacodylate-buffered 2% osmium tetraoxide (Merck, Darmstadt, Germany). After dehydration in a series of graded ethanol and propylene oxide solutions, cells were embedded in epoxy resin (Epon 812; TAAB, Janning, Vanves, France). Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a JEOL.1200 EX2 electron microscope (JEOL, Croissy sur Seine, France).

Results and Discussion

DCs with Phenotypic and Ultrastructural Features of LCs Are Generated from Human Peripheral Blood Monocytes in the Presence of TGF-β1, GM-CSF, and IL-4. When cultured for 5–7 d in the presence of TGF-β1, GM-CSF, and IL-4, $CD14^+$ monocytes (≥95% pure) generated large numbers of dendritic shaped cells (70–80% of the initial population). These cells expressed CD1a, E-cadherin, and CLA (Fig. 1, *b*, *d*, *f*, *h*, and Table 1). Typical BGs, i.e., tennis racket–like structures with rodlike profiles, electron-opaque central lamella, and rounded ends were identified by electron microscopy in 26–33% of cells in three out of three experiments (Fig. 1 *i*). Although rods were relatively short as compared with those observed in some epidermal LCs, 20–40% of cells stained strongly for the BG-associated Lag antigen, emphasizing that these monocyte-derived cells are LCs (Fig. 1 *j*, Table 1). As previously described (1, 10), monocyte-derived DCs were also generated from the same monocytes in presence of GM-CSF and IL-4, and expressed CD1a, but not CLA, BGs or Lag, and only marginal levels of E-cadherin in 10% of cells (Fig. 1 *a*, *c*, *e*, *g*, and Table 1). Both monocyte-derived DCs and LCs were highly efficient in stimulating proliferation of allogenous lymphocytes; as few as 50 DCs were sufficient to trigger T cell proliferation (data not shown). In vivo, among DCs,

Table 1. *Effects of TGF-β1 on CD1a, E-cadherin, CLA, and Lag Expression*

		day 6		
	day 0	GM-CSF + IL-4	GM-CSF + IL-4 + anti–TGF-β	GM-CSF + IL-4 + TGF-β1
Cell number ($\times 10^5$/ml	10	7.225 ± 0.6	6.35 ± 0.8	7.25 ± 0.5
% CD1a-positive cells ± SD	<1	89.25 ± 3	91 ± 3	93 ± 4
% E-cad-positive cells ± SD	<1	11.1 ± 6	6.2 ± 5	82 ± 8
% CLA-positive cells	ND	<10	<10	80–90
% Lag-positive cells	0	0	ND	20–40

Anti–TGF-β1 blocking antibody was used at a concentration (20 μg/ml) that inhibits as high as 10 ng of recombinant TGF-β1. CD1a and E-cadherin expressions were determined by flow cytometry analysis; CLA and Lag expressions were determined by immunocytochemistry. Results are the mean (±SD) of four experiments.

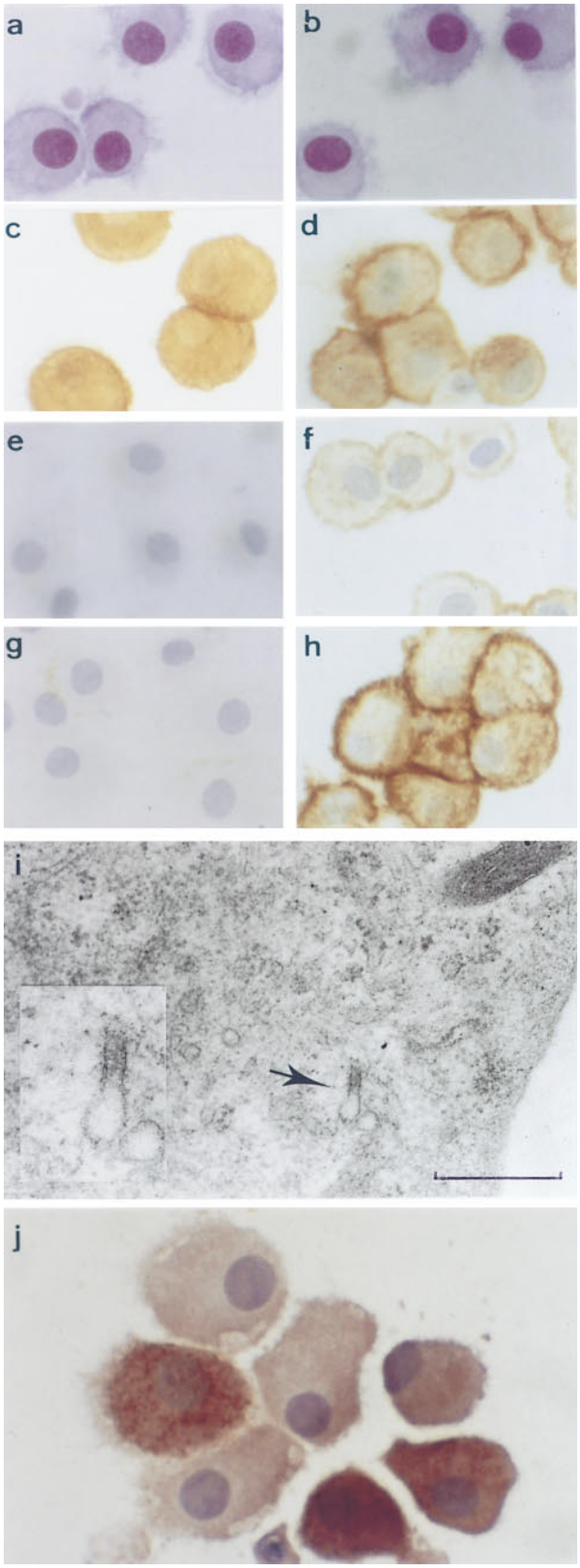

Figure 1. Cytological, immunocytological, and ultrastructural analysis of purified monocytes cultured for 6 d in the presence of GM-CSF and IL-4 alone (*a*, *c*, *e*, and *g*), or GM-CSF, IL-4, and TGF-β1 (*b*, *d*, *f*, *h*, *i*, and *j*). May-Grunwald-Giemsa staining (*a* and *b*) showed typical large cells, with round to oval-shaped or coffee-bean nuclei, and a large pale cytoplasm with cytoplasmic expansions. DCs cultured in the presence of GM-CSF and IL-4 alone are stained with CD1a (*c*) but neither E-cadherin (*e*) nor CLA (*g*). In contrast, cells cultured in presence of TGF-β1, GM-CSF, and IL-4 are stained with CD1a (*d*), E-cadherin (*f*), and CLA (*h*). (*i*) Typical BGs (*arrow* and *inset*) were observed in 26–33% of cells. For both culture conditions, 15 cells were carefully examined in each of three separate experiments. Original magnification: ×12,000; bar represents 0.5 μm. *Inset*: original magnification: ×25,000. (*j*) 20–40% of cells in GM-CSF, IL-4, and TGF-β1 supplemented culture displayed strong reactivity for the Lag antigen. No staining was observed in monocyte-derived DC cultures in the absence of TGF-β1.

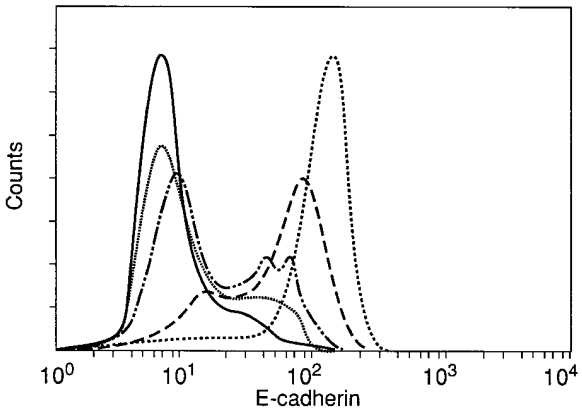

Figure 2. Dose-dependent induction of E-cadherin expression by TGF-β1, and inhibition of TGF-β1 effects by anti–TGF-β antibodies. In the absence of exogenously added TGF-β1 (*small dotted line*), only 11% of day 6 DCs expressed low levels of E-cadherin. Increasing amounts of TGF-β1 (0.1 ng/ml, *dotted and dashed line*, 1 ng/ml, *dashed line*; 10 ng/ml, *large dotted line*) induced increasing expression of E-cadherin. Addition of anti–TGF-β antibodies (TGF-β1 10 ng/ml + anti–TGF-β antibodies 20 μg/ml, *solid line*) completely abolished the effects of TGF-β1 on E-cadherin expression.

CD1a is expressed only by LCs and no equivalent to the in vitro monocyte-derived DCs has been found. Such monocyte-derived DC may therefore represent an intermediate level of differentiation between monocytes and LCs.

Monocyte-derived LCs are nonactivated (immature) LCs since they do not express CD83 and CD86. However, a 10-fold increase of HLA class II membrane expression, as well as the induction of CD83 and CD86 expression and the loss of E-cadherin expression, were observed after addition of TNF-α and IL-1 (data not shown).

Induction of LC Differentiation Is Dependent on TGF-β1 in a Dose-dependent Manner. As shown in Fig. 2, increasing amounts of recombinant TGF-β1 induced increasing E-cadherin expression by monocyte-derived LCs. Neutralizing anti-TGF-β antibodies completely inhibited E-cadherin expression induced by recombinant TGF-β1, as well as the low level of spontaneous E-cadherin expression on GM-CSF + IL-4 monocyte-derived DCs that could be attributed to a low level of endogenous active TGF-β1.

LCs Differentiate from Peripheral Blood CD14$^+$ Monocytes, without Progenitor Proliferation or Selection of a Distinct Monocyte Subpopulation. The appearance of LCs in cultures of purified monocytes correlated with the disappearance of

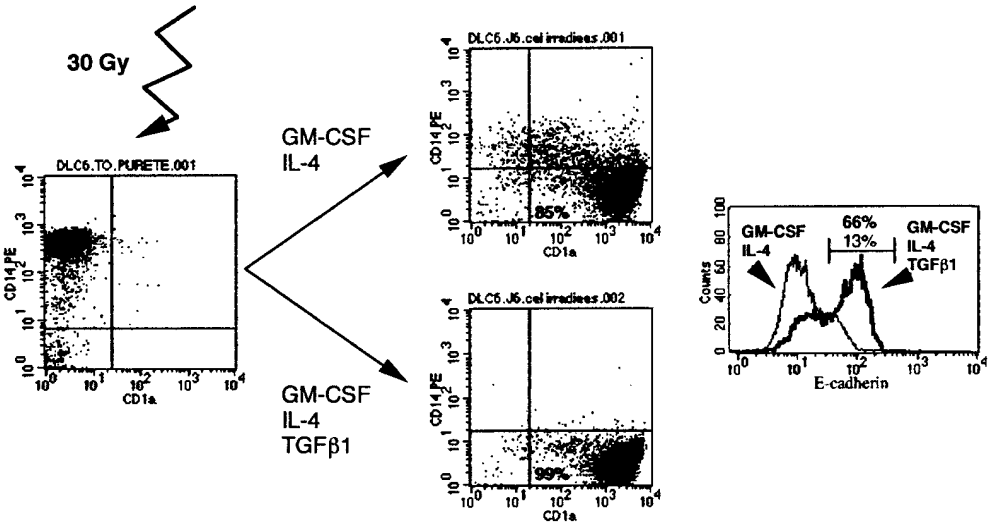

Figure 3. Differentation of 30 Gy irradiated monocytes. Freshly purified (>95% $CD14^+$) cells were irradiated (30 Gy), and then cultured for 6 d with either GM-CSF and IL-4 alone or GM-CSF, IL-4, and TGF-β1. Dot plots represent double immunostaining with CD1a and CD14 antibodies, histogram plots represent E-cadherin expression in the absence (*thin line*) or presence (*thick line*) of TGF-β1. Differentiation observed was similar to the differentiation of unirradiated monocytes. One representative experiment out of three is shown.

the monocytes as judged by cytological and immunophenotypic examination. Two-color flow cytometric analysis showed that on day 2, $CD14^+/CD1a^+$ cells appeared at the same time $CD14^+/CD1a^-$ cells disappeared, whereas at days 4 and 5, $CD14^-/CD1a^+$ DCs arose simultaneously to the disappearance of $CD14^+/CD1a^+$ cells (data not shown). LCs and DCs could not have been generated from proliferating progenitors because CD34 expression at day 0 was negative, the proliferative rate evaluated by PI incorporation was ⩽1% of cells on days 2, 4, and 6 of culture, and Ki67 antibody stained <5% of cells throughout culture. In addition, thymidine uptake during 48-h pulses initiated at day 0, 2, or 4 of culture was very low (<380 cpm, data not shown) and 30 Gy irradiation did not alter either DC or LC differentiation from purified monocytes (Fig. 3). Selection of distinct monocyte subpopulations was also ruled

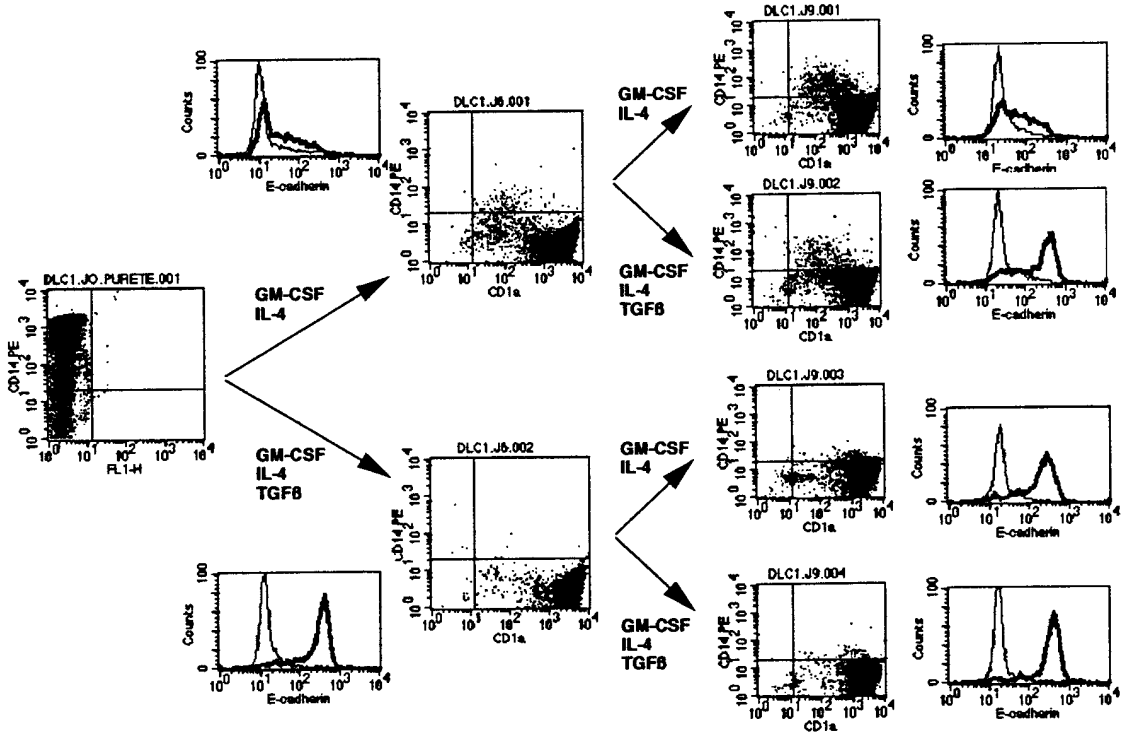

Figure 4. Both fresh monocytes and non-Langerhans DCs differentiate toward either DCs or LCs depending on the absence or presence of TGF-β1, and retrieval of TGF-β1 did not result in loss of the Langerhans phenotype. Dot plots represent double immunostaining with CD1a and CD14 antibodies at days 0, 6, and 9 of culture; histogram plots represent E-cadherin expression at the same time (*thick lines*); and thin lines represent isotypic control staining. Only TGF-β1–supplemented cultures gave rise to E-cadherin+ DCs. Late addition of TGF-β1 in day 6 DC cultures also resulted in expression of E-cadherin. Removal of TGF-β1 at day 6 did not result in downregulation of E-cadherin expression. Data are representative of six experiments.

out, since, in addition to low cell mortality throughout the culture period (trypan blue exclusion ≤6%) and a low rate of apoptosis (≤5% as measured by PI incorporation), monocyte derived-DCs obtained in the presence of GM-CSF and IL-4 could be further induced to differentiate into E-cadherin$^+$ and CLA$^+$, and Birbeck$^+$ LCs in the presence of GM-CSF, IL-4, and TGF-β1 (Fig. 4), thus confirming that LCs and DCs originate from the same population of CD14$^+$ monocytes.

Taken together, these data indicate that TGF-β1, in the presence of GM-CSF and IL-4, drives in vitro differentiation of human monocytes into dendritic LCs. This suggests that distinct phenotypes of myeloid DCs may represent different stages of differentiation rather than distinct lineages, and that, in addition to early precursors, circulating monocytes may be recruited to differentiate into LCs at the peripheral level, i.e., in mucosae, in response to TGF-β1. TGF-β1 is synthesized at high levels by keratinocytes and may play a role in the in situ regulation of the skin-associated immune system, since it positively regulates the E-cadherin ligand CD103, a costimulatory molecule expressed on intraepithelial T lymphocytes (21, 22). Moreover, the skin of TGF-$\beta^{-/-}$ mice is devoid of LCs (18), whereas irradiated TGF-$\beta^{+/+}$ recipient mice when grafted with TGF-$\beta^{-/-}$ bone marrow had donor-origin LCs (23). The monocyte therefore appears to be a highly plastic cell, and may represent a multipotent circulating precursor in the immune and hematopoietic systems in vivo, able to differentiate into macrophages in the presence of M-CSF (11), osteoclast-like multinucleated giant cells in the presence of M-CSF and IL-4 (12), and LCs in the presence of GM-CSF, IL-4, and TGF-β1.

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