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TGF-β1 Prevents the Noncognate Maturation of Human Dendritic Langerhans Cells[1]

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TGF-β1 is critical for differentiation of epithelial-associated dendritic Langerhans cells (LC). In accordance with the characteristics of in vivo LC, we show that LC obtained from human monocytes in vitro in the presence of TGF-β1 1) express almost exclusively intracellular class II Ags, low CD80, and no CD83 and CD86 Ags and 2) down-regulate TNF-RI (p55) and do not produce IL-10 after stimulation, in contrast to dermal dendritic cells and monocyte-derived dendritic cells. Surprisingly, while LC exhibit E-cadherin down-regulation upon exposure to TNF-α and IL-1, TGF-β1 prevents the final LC maturation in response to TNF-α, IL-1, and LPS with respect to Class II CD80, CD86, and CD83 Ag expression, loss of FITC-dextran uptake, production of IL-12, and Ag presentation. In sharp contrast, CD40 ligand cognate signal induces full maturation of LC and is not inhibited by TGF-β1. The presence of emigrated immature LCs in human reactive skin-draining lymph nodes provides in vivo evidence that LC migration and final maturation may be differentially regulated.

Therefore, due to the effects of TGF-β1, inflammatory stimuli may not be sufficient to induce full maturation of LC, thus avoiding potentially harmful immune responses. We conclude that TGF-β1 appears to be responsible for both the acquisition of LC phenotype, cytokine production pattern, and prevention of noncognate maturation. ***The Journal of Immunology*****, 1999, 162: 4567–4575.**

Dendritic cells (DC)[3] are the most potent APC for initiating primary and secondary immune responses (1, 2). They differentiate from their precursors into so-called "immature" DCs, which are present in most tissues, in a sentinel position (1). The best characterized immature DC is the Langerhans cell (LC), located above the basal layer of epithelial cells in the skin, oral, nasal, esophagal, pulmonary, vaginal, and rectal mucosae. Immature DCs are efficient in Ag uptake but need to mature and migrate into lymphoid organs before acquiring the capacity to prime T cells efficiently (1). Migration and maturation of DCs after capture of Ags are thus key events in the induction of immunity. Upon Ag exposure, DCs travel to the lymphoid tissues where they may complete their maturation (1). Mature DC express high levels of class I and II Ags, CD80, CD86, and CD83, produce IL-12, and can prime naive CD4-helper and CD8-cytotoxic T cells (1–3). The maturation process of DCs in the human was best studied on monocyte-derived DC in the presence of GM-CSF and IL-4 (4–8). In this model, the maturation process requires activation of the immature DC by various stimuli, including bacterial components (e.g., LPS), inflammatory cytokines (e.g., TNF-α and IL-1), and cognate $CD4^+$ T cell help, mediated by CD40L (5, 7, 8). Recent studies showed that stimulation of $CD8^+$ T cells by DCs was achieved after a two step process: first, DCs were induced to mature by Th cells via CD40/CD40L interaction or by viral infection, and second, this so-called "licensed" DC may directly stimulate cytotoxic T cells (3, 9, 10).

At this time it is difficult, however, to reconcile the ability of DCs to mature in response to both the T cell-dependent signal (CD40L) and nonspecific stimuli with the specificity of the cognate immune response, which requires $CD4^+$ T cell help for the response to most Ags. If inflammatory stimuli had the same effect as CD40L, LPS alone should be able to license DCs to activate killer T cells in vivo, bypassing the need for $CD4^+$ help. Also, LC from mucosal barriers, which are frequently challenged with LPS and inflammatory signals, should be continuously activated and should stimulate CD4 helper as well as CD8 killer T cells in the absence of actual danger (11).

However, monocyte-derived DCs do not behave like LC. Indeed, while TNF-α-induced maturation in GM-CSF + IL-4 monocyte-derived DC is mediated exclusively via TNFRIp55 (6), only TNFRIIp75 mediates effects of TNF-α in human LC (12). The cytokine TGF-β1, which is present in the mucosal barriers, is required for differentiation of epithelial-associated dendritic LC from their precursors, including the monocyte (13–16). We have previously shown that while GM-CSF- and IL-4-treated monocytes give rise to non-Langerhans DCs, the addition of TGF-β1 allows monocytes to differentiate toward dendritic LC (15).

We show here that TGF-β1, indeed, drastically changes the requirements for DC maturation in this model. First, cells grown in

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[3] Abbreviations used in this paper: DC, dendritic cell; LC, Langerhans cell; CD40L, ligand for CD40; TRITC, tetraethylrhodamine isothiocyanate; TT, tetanus toxin; CLA, cutaneous lymphocyte-associated Ag; LAMP, lysosome-associated membrane protein.

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the presence of TGF-β1 (LC) exhibit a more immature phenotype, lose TNFRIp55 expression, and do not produce IL-10 after stimulation. Second, and more important, TGF-β1 inhibits LC maturation in response to nonspecific signals such as LPS, TNF-α, and IL-1, but not to the cognate signal CD40L, while it does not inhibit down-regulation of E-cadherin expression upon exposure to TNF-α and IL-1. Furthermore, we have observed immature LC in human reactive skin-draining lymph nodes; this may reflect in vivo the relevance of our findings.

These results are consistent with the crucial role of CD40-mediated activation for the final maturation and licensing of DCs (3). By differentially regulating the differentiation, maturation, and functions of LC (and possibly of other DC subsets) in response to cognate T-dependent and nonspecific inflammatory signals, TGF-β1 appears to be physiologically involved in the fine tuning of the immune response by DCs.

Materials and Methods

Media, reagents, Abs, and cell lines

The medium used was RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% heat-inactivated FCS Myoclone (all from Life Technologies, Gaithersburg, MD), referred to below as complete medium. Recombinant human GM-CSF was provided by Sandoz (Bale, Switzerland), recombinant human IL-4 was purchased from Genzyme (Cambridge, MA), and recombinant human TGF-β1, TNF-α, and IL-1β were all purchased from R&D Systems (Minneapolis, MN). LPS from *Escherichia coli* 0127-B8 and 026-B6 were purchased from Sigma Immunochemicals (St. Louis, MO). Lysine-fixable FITC-dextran (M_r = 40,000) was purchased from Molecular Probes (Eugene, OR). Murine fibroblast cell lines transfected with human CD40L (LcCD40L) or CD32 (LcCD32) were kindly provided by Dr. J. Banchereau and Dr. F. Brière (Schering-Plough, Dardilly, France) (17). Tetanus toxoid was a kind gift of Dr. F. Le Deist (Laboratoire d'Immunologie Clinique, Necker, Paris, France). Anti-CD40 BB20-activating Ab (IgG1) was obtained from Diaclone (Besançon, France). FITC-conjugated CD1a (clone BL1, IgG1), MHC-I (MHC ABC, IgG2a), MHC-II (IgG2), CD83 (IgG2b), and uncoupled CD80 (IgG1) and CD40 (IgG1) were obtained from Immunotech (Marseille, France). Phycoerythrin (PE)-conjugated CD14 (Leu-M3, IgG2b) and CD86 (IgG2b) were obtained respectively from Becton Dickinson (Le Pont de Claix, France) and PharMingen (San Diego, CA). Uncoupled anti-E-cadherin (HECD-1, mouse IgG1) was obtained from R&D Systems. Lag Ab (mouse IgG1) was a kind gift of Dr F. Furukawa (Hamamastu University, Hamdacho, Japan). CD120a (anti-TNFRIp55, clone MR1–2) was obtained from Genzyme. Anti-DR (L-243, mouse IgG2a) was obtained from the American Type Culture Collection (Manassas, VA). Anti-lysosome-associated membrane protein-1 (LAMP-1) rabbit antiserum was kindly provided by Dr. S. Carlsson, Umeå University, Umeå, Sweden (18).

Culture of peripheral blood monocytes

DC and LC were prepared as previously described (15). Fresh $CD14^+$ monocytes were isolated from PBMC of healthy volunteers obtained by the standard Ficoll-Paque method and immediately separated by negative magnetic depletion using hapten-conjugated CD3, CD7, CD19, CD45RA, CD56, and anti-IgE Abs (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) (19) and a magnetic cell separator (MACS) according to the manufacturer's instructions, routinely resulting in >95% purity of $CD14^+$ cells. Cells were cultured in flasks, or in 6- or 24-well tissue culture plates (Costar, Cambridge, MA) for 5 to 7 days in complete medium supplemented with 250 ng/ml GM-CSF and 100 ng/ml IL-4, resulting in their differentiation into $CD1a^+$ DCs, or 250 ng/ml GM-CSF, 100 ng/ml IL-4, and 10 ng/ml TGF-β1, resulting in their differentiation into $CD1a^+$, E-cadherin$^+$, cutaneous lymphocyte-associated Ag $(CLA)^+$, Lag, and Birbeck$^+$ dendritic LC. At days 2 and 4, fresh medium, supplemented with the above-mentioned cytokines, was added. FCS was absolutely required to obtain reproducibly homogeneous populations of $CD1a^+CD14^-CD83^-CD86^-$ DC (but not LC) in the presence of GM-CSF and IL-4. However, we have determined, in a previous report (15), that concentration of TGF-β1 found in FCS-supplemented medium was below 0.1 ng/ml, which is 100-fold less than required for acquisition of the LC phenotype by monocytes. The present study was done with a single FCS batch, but several differents batches were tested with the same efficiency to generate DC and LC.

Stimulation of DC and LC

DC and LC were collected at days 5–7 of culture, washed three times in complete medium at 37°, and resuspended in 24-well tissue culture plates at a concentration of 5×10^5 cell/ml in complete medium supplemented with 250 ng/ml GM-CSF and 100 ng/ml IL-4 with or without TGF-β1 (10 ng/ml) for stimulation. TNF-α, IL-1β, LPS, or medium alone was added at various doses for 40 h for stimulation with inflammatory cytokines and LPS. For study of CD40L-mediated activation, fibroblastic L cells transfected with either CD40L, or CD32 as control, were irradiated at 80 Gy and added to the culture wells in a proportion of 1/10. Alternatively, DC and LC were cocultured with fibroblastic L cells transfected with CD32 (FcγRII) together with increasing concentrations of activating anti-CD40 Abs. In all conditions, cells and supernatants were collected after 40 h of activation. LC grown in the presence of TGF-β1 were either stimulated in the absence of TGF-β1 (and referred to as T−), or stimulated in the presence of TGF-β1 (and referred to as T+).

Flow cytometry analysis of PBMCs and PBMC-derived cells

For single- and two-color flow cytometry, 3×10^5 cells 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, mAbs at the appropriate concentration, or with control isotype-matched irrelevant mAbs at the same concentration. After washing, cells were incubated when appropriate with $F(ab')_2$ goat anti-mouse (GAM)-FITC (Immunotech) for 15 min at 4°C in the same buffer, washed again, and then 10^4 events were analyzed with a FACScalibur (Becton Dickinson) using CellQuest software (Becton Dickinson).

Quantitation of endocytosis in single cell by FACS analysis

FITC-dextran uptake of DCs was assessed as previously described (6). Cells were resuspended in complete medium and incubated at 37°C with 5% CO_2. FITC-dextran was added at a final concentration of 1 mg/ml. The cells were washed four times with cold PBS, 2% human AB serum, and 0.01 M NaN_3 and were analyzed with a FACScalibur (Becton Dickinson) using CellQuest software (Becton Dickinson).

Confocal microscopy

Cells were adhered to glass slides coated with 50 μg/ml polyL-lysine (Sigma), fixed in 4% paraformaldehyde in Ca^{2+}/Mg^{2+}-free PBS, and quenched with 0.1 M glycine. Cells were permeabilized in PBS/saponin (0.01%)/gelatin (0.25%)/Nonidet P-40 (0.1%) and sequentially incubated with mAb L243 and anti-mouse FITC-conjugated secondary Abs, anti-LAMP-1 rabbit serum and TRITC-conjugated secondary Ab, or appropriate controls. Mounted slides were analyzed with a confocal laser microscope system attached to a microscope.

Quantitation of cytokine production by ELISA

Supernatants were stored at −70°C until cytokine measurements. Production of IL-10 and bioactive IL-12 p70 were measured in duplicate using ELISA Quantikine Kits (R&D Systems) according to the manufacturer's instructions. Sensitivity of IL-10 and IL-12 detection was, respectively, 1.5 pg/ml and 0.5 pg/ml.

Autologous response to TT

DCs were collected, washed three times, pulsed for 48 h with TT or medium alone, with or without LPS (10 ng/ml) or LcCD40L. Cells were then washed two times in PBS, and half of the cells were fixed with 0.001% glutaraldehyde for 20 min on ice. Cells were washed again two times in PBS, resuspended in RPMI with 10% human AB serum, and added in triplicate at various concentrations to 10^5 autologous T cells/well in 96-well tissue cultue plates (Falcon, Oxnard, CA). T cells were isolated by the standard Ficoll-Paque method followed by magnetic depletion of non-T cells (MACS; Miltenyi Biotec). [^{3}H]Thymidine (Amersham Life Science, Buckinghamshire, U.K.) incorporation was measured in newly synthesized DNA over 18 h, using pulses initiated at days 4 or 5 of the culture with 1 mCi/well of [^{3}H]thymidine. Cells were then harvested with a 96-well Harvester (Pharmacia, St. Quentin, France) and collected on glass-fiber filters (Pharmacia); the incorporation of thymidine was measured with a β-plate microscintillation counter (LKB, Pharmacia).

Immunohistochemistry

Serial cryostat sections of skin-draining reactive lymph node biopsies from three patients with dermatopathic lymphadenopathy were stained with CD1a, CD80, CD83, or CD86 mouse primary Abs and then labeled with a goat anti-mouse alkaline phosphatase-conjugated Ab. Double-stainings

were performed using peroxidase and alkaline phosphatase-antialkaline phosphatase (APAAP) protocols according to published procedures (20–22). Fast Blue (Sigma) and 3 amino-9 ethylcarbazole (Sigma) were used as substrates for alkaline phosphatase and peroxidase, respectively.

Results

Monocyte-derived LC behave as fully immature LC

As previously described by us, freshly isolated CD14$^+$ monocytes from healthy donors differentiate toward E-cadherin$^+$, Lag$^+$, CLA$^+$, Birbeck-granules$^+$ LC in the presence of TGF-β1, GM-CSF, and IL-4, while, in the absence of TGF-β1, they differentiate into non-Langerhans monocyte-derived DCs (15). We further studied expression of MHC Ags, costimulatory and activation molecules (CD80 and CD86, CD83) by DC and LC. Flow-cytometry showed that, in comparison with DC, LC expressed similar levels of membrane MHC class I but a 10-fold lower level of membrane MHC class II Ags (Fig. 1, *c-f*). LC also exhibited a lower expression of CD80 than DC (Fig. 1, *g* and *h*). CD83 and CD86 were negative (Fig. 1, *i-l*). TGF-β1 thus induced down regulation of membrane MHC II and CD80. Monocyte-derived LC stably retained this immature phenotype in culture in the presence of TGF-β1 from day 5 to days 12–15, when cell death occurs.

We then investigated MHC-II and lysosomal Ag localization at the cellular level in LC and DC by confocal microscopy. Cells were fixed, permeabilized, stained with Abs to mature class II molecules and LAMP-1 (present in the lysosomal compartment), and examined by confocal microscopy. As shown in Fig. 2*a*, mature class II molecules in DC were present on the cell surface and in an extensive cellular compartment also containing LAMP-1, as previously described (6). However, in LC derived from the same donors, in the presence of TGF-β1, MHC class II Ags were almost totally intracellular and mostly colocalized with the LAMP lysosomal staining (yellow structures in Fig. 2, *a* and *b*). LC grown in the presence of TGF-β1 thus exhibited features of early DC (23).

Monocyte-derived LC down-regulate TNFRI and do not produce IL-10 after stimulation

It has been shown that LC in vivo do not express TNFRIp55 and do not produce IL-10 (24–27). We observed that the TNFRIp55 receptor CD120a was also down-regulated on monocyte-derived LC to a low to negative level, in comparison with DC (Fig. 1, *m* and *n*). Moreover, as shown in Fig. 3 (*upper panel*) monocyte-derived LC, in contrast to monocyte-derived non-Langerhans DC ($p < 0.001$), do not produce IL-10 after stimulation with either LPS, TNF and IL-1 or CD40L.

Therefore, TGF-β1 is responsible for loss of TNFRIp55 expression and IL-10 production by LC in vitro. The monocyte-derived LC represent a stable and homogeneous immature LC population exhibiting phenotypical and functional features of epidermal LC in vivo.

TGF-β1 inhibits LPS, TNF-α, and IL-1, but not CD40L-induced maturation of LC

In addition to being necessary for in vivo and in vitro differentiation of LC (13, 28), TGF-β1 elicits diverse cellular responses, including modulation of numerous immune and inflammatory responses (29, 30). To investigate the effect of TGF-β1 on DC maturation, cells cultured for 6 days in the presence of GM-CSF and IL-4 (DC) or in the presence of GM-CSF, IL-4, and TGF-β1 (LC) were collected, washed, cultured for 40 h in the presence of LPS, TNF-α, IL-1, both TNF-α and IL-1, or murine fibroblasts transfected with either human CD32 or CD40L and then analyzed for MHC II and costimulation molecule expression by flow cytometry and confocal microscopy, macropinocytosis activity, IL-12 production, and Ag presentation.

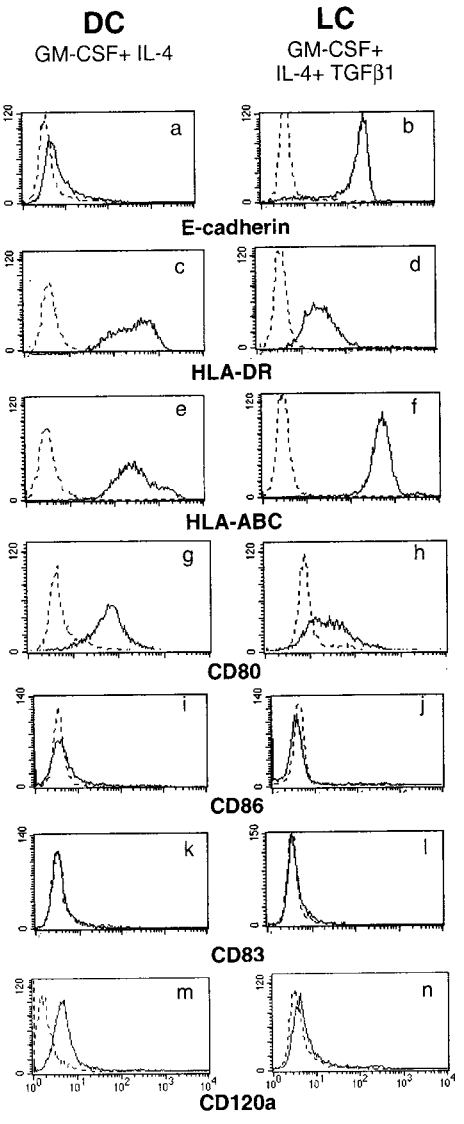

FIGURE 1. Flow cytometry analysis of MHC, costimulatory, and activation Ags on DC and LC. Day 6 DC cultured in the absence of TGF-β1 (*a, c, e, g, i, k, m,* and *o*) and LC cultured in the presence of TGF-β1 (*b, d, f, h, j, l, n,* and *p*) were stained with anti E-cadherin (*a* and *b*), MHC-DR (*c* and *d*), MHC-ABC (*e* and *f*), CD80 (*g* and *h*), CD86 (*i* and *j*), CD83 (*k* and *l*), and anti-CD120a (TNFRIp55) (*m* and *n*) mAbs, and 10^4 events were analyzed with a FACScalibur (Becton Dickinson) using CellQuest software (Becton Dickinson). Dashed histograms represent isotypic controls. Data are representative of 10 experiments on different donors.

TGF-β1 inhibits LPS, TNF-α, and IL-1, but not CD40L-induced phenotypic maturation of LC. Stimulation with 10 ng/ml of LPS, TNF-α, IL-1, and both TNF-α and IL-1 induced up-regulation of class II Ags and CD86 on DC (cultured in the absence of exogenous TGF-β1) (Fig. 4) as previously described (5, 8). However, strikingly, less than 20% of TGF-β1-treated LC were induced to up-regulate class II Ags and CD86 expression after a 40-h exposure to 10 ng/ml LPS, TNF-α, or IL-1, and only 25–40% of LC (vs 95% of DC) were induced to mature even after exposure to 10 ng/ml TNF-α and 10 ng/ml IL-1β in combination (Fig. 4).

In sharp contrast, after a 40-h coculture with CD40L-transfected fibroblasts, both DC and LC presented a similar up-regulation of class II Ags and CD86 (Fig. 4).

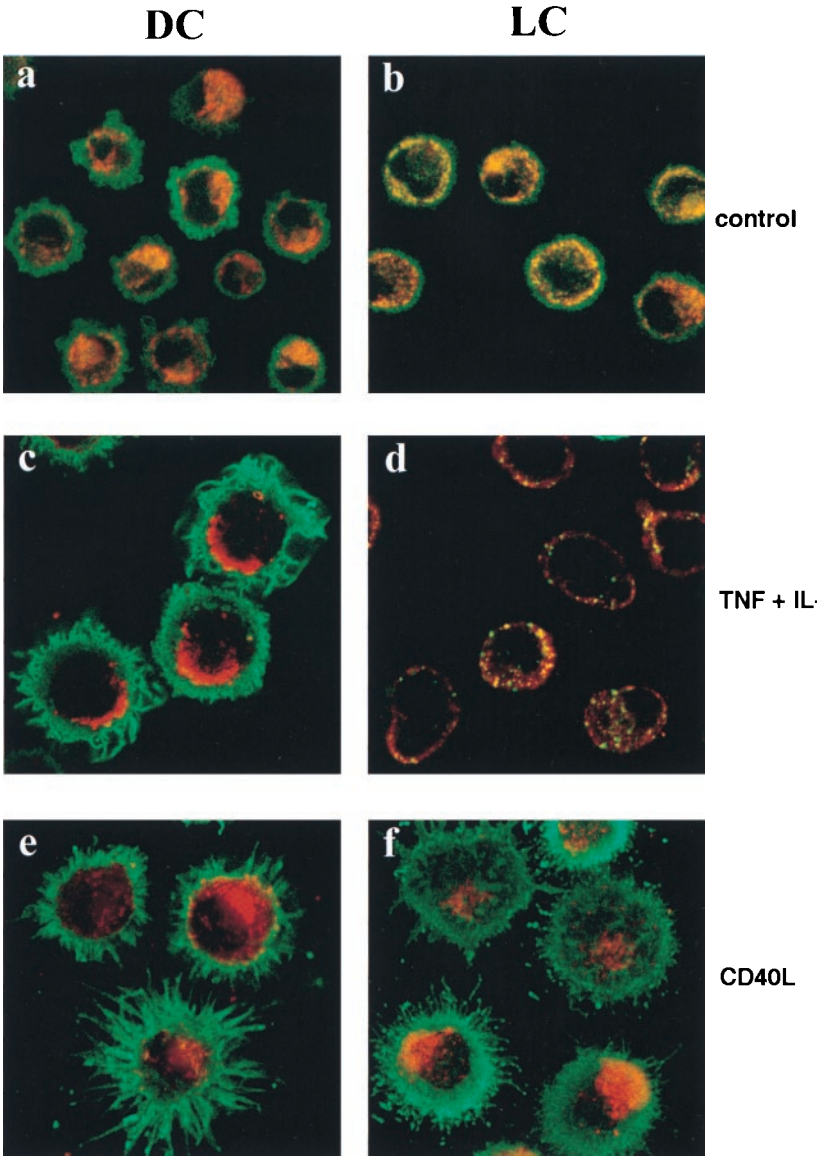

FIGURE 2. Confocal microscopy analysis of DC and LC. Unstimulated DC (*a*), and LC (*b*), TNF-α and IL-1β-stimulated (40 h) DC (*c*), and LC (*d*), CD40L-stimulated (40 h) DC (*e*), and LC (*f*) were fixed, permeabilized, and stained with mouse L-243 Ab to mature class II molecules (revealed in green by FITC-conjugated secondary Ab) and rabbit anti-LAMP-1 antiserum (revealed in red by TRITC-conjugated secondary Ab). When red and green staining overlap, the compartment appears yellow. Original magnification ×1000; data representative of three independent experiments.

Similar results were obtained for CD83 (Fig. 5) and CD80 (not shown). Thus, up-regulation of MHC II, costimulatory and activation-associated Ags is differentially regulated in DC and LC, the latter appearing to be less responsive to nonspecific signals. To further investigate this phenomenon, we examined MHC II cellular location in stimulated cells by confocal microscopy.

Confocal microscopy analysis showed that DC stimulated with TNF-α and IL-1 displayed nice dendritic cytoplasmic expansions and that mature class II molecules were exclusively present on the cell surface, and no longer in the LAMP-1$^+$ lysosomal compartment (Fig. 2*c*). In contrast, in a large proportion of LC (>50%), MHC class II Ags remained almost totally intracellular, while often not colocalizing with the LAMP lysosomal staining (Fig. 2*d*), thus resembling the intermediate DC phenotype (23).

Stimulation with CD40L, however, induced similar acquisition of the dendritic-shaped morphology and class II expression at the cell surface in both DC and LC (Fig. 2, *e* and *f*).

Dose response experiments (Fig. 5) further indicated that, while ≥90% of DC were induced to express CD86 and CD83 in response to LPS in a dose-dependent manner, with a maximal effect for 1 ng/ml LPS, ≥90% of LC stimulated in the presence of TGF-β1 (LC T+) did not, even in response to doses as high as 1 μg/ml LPS. Similarly, below a concentration of 10 ng/ml of both TNF-α and IL-1, only a very low percentage of LC (<20%) was induced to mature, while ≥90% of DC responded to much lower doses.

To investigate whether unresponsiveness of LC to inflammatory stimuli was due to their stage of differentiation or to the presence of TGF-β1 during activation, cells were stimulated after retrieval of TGF-β1 (LC T−). When TGF-β1 was withdrawn, the amounts of LPS (Fig. 5, LC T−) and TNF-α and IL-1 (Fig. 5, LC T−) needed to activate LC were significantly lower, but remained at least 2 to 3 logs higher than those required for DC maturation. Similar results were obtained when 20 μg/ml of blocking anti-TGF-β Ab (15) was added to ensure the absence of active TGF-β1.

For comparison, activation via CD40 was studied in a dose-dependent manner by cocultivating day 6 DC and LC with CD32 (human FcgRII)-transfected fibroblasts and increasing amounts of stimulating anti-CD40 Ab for 40 h (Fig. 5). As expected, induction of CD83 and CD86 expression followed similar kinetics for DC and LC. Similar results were obtained for MHC-II Ag expression, although they were already expressed at a relatively high level on DC (Figs. 1 and 4).

Therefore, LC maturation in response to inflammatory stimuli

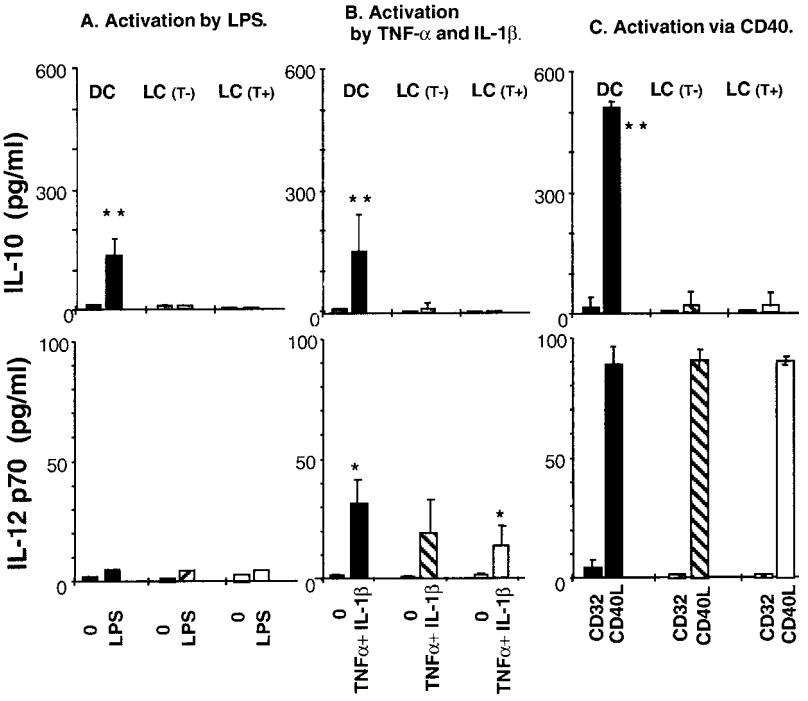

FIGURE 3. Cytokine production by LC and DC in response to LPS, TNF-α, and IL-1 and CD40L. LC do not produce IL-10 upon stimulation with either LPS (*A*), TNF-α and IL-1 (*B*), or CD40L (*C*) (**, $p < 0.001$ for comparison with LC). This was observed when TGF-β1 was present at the time of differentiation and was not influenced by subsequent retrieval of TGF-β1 at the time of stimulation. Production of IL-12 by LC is down-regulated by TGF-β1 after stimulation with TNF-α and IL-1 (*, $p < 0.05$ for comparison between DC and TGF-β1-treated LC) (*B*), but not with CD40L (*C*). Day 6 DC and LC were incubated for 40 h with 10 ng/ml LPS, 10 ng/ml TNF-α and IL-1, either CD40L-transfected fibroblasts or CD32-transfected fibroblasts as control, in the presence (T+, open bars) or absence (T−, hatched bars) of 10 ng/ml TGF-β1. Supernatants were then collected and were analyzed for IL-10 p70 (*upper panel*) and IL-12 (*lower panel*) production using sensitive ELISA techniques (R&D Systems). Results are mean and SD of at least three experiments, except for IL-12 p70 production after LPS-stimulation, where a representative experiment is shown.

was inhibited, and the presence of TGF-β1 at the time of stimulation further increased the unresponsiveness of LC.

TGF-β1 may also regulate maturation of DC generated in the absence of exogenous TGF-β1 in response to inflammatory cytokines. Addition of TGF-β1 at the time of stimulation partially inhibits up-regulation of membrane class II and CD86 expression on DC, since 30–40% of DC stimulated with 10 ng/ml of both TNF-α and IL-1 remained $CD86^-$ with intermediate level of surface class II Ags (data not shown). However, maturation (i.e., up-regulation of membrane class II and CD86 expression) of DC induced by 10 ng/ml LPS is not inhibited by TGF-β1, since ≥95% of LPS-stimulated DC became DR^{high} $CD86^{high}$, whether or not TGF-β1 (10 ng/ml) was added at the time of stimulation (data not shown). Therefore, with respect to stimulation with LPS, TGF-β1 might act specifically on LC while it acts on both DC and LC with respect to cytokines.

TNF-α and IL-1 or LPS-treated LC maintain their pinocytic activity in the presence of TGF-β1. Macropinocytosis activity was described to be correlated to the immature stage of DCs (6). We thus investigated whether the apparent absence of LC maturation after exposure to inflammatory stimuli described above was associated with changes in their pinocytic activity. As shown in Fig. 6, while FITC-dextran intake was almost abolished in DC after exposure to LPS, little or no change was detectable in LC, although basal FITC-dextran intake is slightly lower than DC. Comparable results were obtained after activation with TNF-α and IL-1 (not shown), while CD40L-stimulated DC and LC displayed a similar decrease of pinocytic activity (Fig. 6).

Down-regulation of IL-12 production in LC in response to LPS, TNF-α, and IL-1, but not CD40L. Mature DCs produce IL-12, which plays a major role in cross-talk with lymphocytes. We thus checked cytokine production of immature and activated DC and LC, using ELISA, in culture supernatants after nonspecific or CD40-mediated activation (Fig. 3, *lower panels*). Activation with LPS (Fig. 3*A*) did not reproducibly result in significant production of IL-12 by DC and never resulted in the production of IL-12 by LC. When stimulated by either TNF-α and IL-1 (Fig. 3*B*) or CD40L (Fig. 3*C*), DC produced significant levels of bioactive IL-12 p70. In contrast, while CD40L-activated DC or LC produced identical amounts of IL-12 p70, TNF-α and IL-1-stimulated LC produced 50% less IL-12 than DC ($p < 0.05$). This was reproducible in four separate experiments, although levels of IL-12 production in response to TNF-α and IL-1 varied among donors. As it was observed for CD83 and CD86 Ag expression, retrieval of TGF-β1 at the time of stimulation partially restored IL-12 production by LC.

Therefore, LC grown and stimulated in the presence of TGF-β1 appeared to mature poorly in response to noncognate signals, while activation via CD40 appeared to induce similar maturation in comparison with DC.

Proliferative response to TT is dependent on LC activation via CD40L or T lymphocytes. Recent data showed that CD40L, which is mainly expressed by activated $CD4^+$ helper T cells (31), provides the Th signal to induce the final maturation of DCs in vivo (3, 9, 10). We thus investigated the functional maturation of LC in response to inflammatory stimuli, CD40L, and Ag-specific T cells by using the autologous response to TT assays.

DC and LC pulsed with TT were able to stimulate autologous T cell proliferative responses to TT in immune individuals. However, while incubation of DC with LPS increased the Ag-specific proliferative response, incubation of LC with LPS had no effect ($p < 0.05$) (Fig. 7*A*).

Moreover, fixation of unstimulated or LPS-treated pulsed LC before the coculture with autologous T cells abolished the proliferative response of T lymphocytes ($p < 0.05$) (cpm were ≤ 300 and identical to control cultures in which TT was omitted) (Fig. 7*B*). This indicated that LC have to mature before becoming able to stimulate T cells and that LPS alone with TT was not able to induce such maturation, while, in contrast, LC-Th cell crosstalk induced LC maturation, resulting in T cell proliferation.

Indeed, pulsed LC stimulated with CD40L, either fixed or unfixed, give rise to comparable proliferative responses (Fig. 7*B*), indicating that CD40L is sufficient to induce maturation of LC.

Therefore, maturation of LC, necessary to induce a T cell proliferative response to TT, may be achieved by coculturing LC with either Ag-specific T cells, or with CD40L, but not by incubating

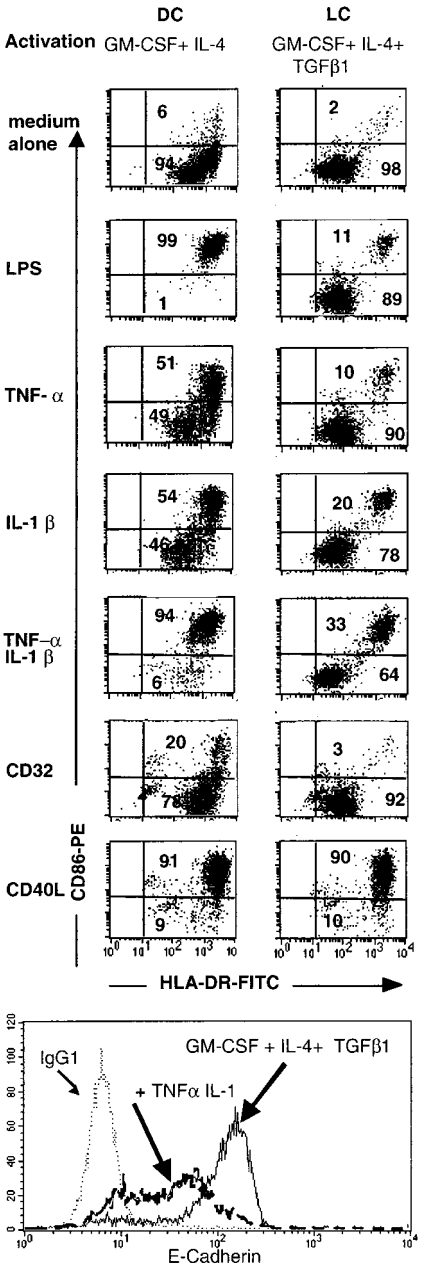

FIGURE 4. E-cadherin down-regulation is induced after exposure to TNF-α and IL-1, but up-regulation of class II and CD86 requires stimulation of LC via CD40. DC cultured in the presence of GM-CSF and IL-4 (*left panel*) and LC cultured in the presence of GM-CSF, IL-4, and TGF-β1 (*right panel*) were incubated for 40 h with 10 ng/ml of LPS, TNF-α, IL-1, both TNF-α and IL-1, CD32-transfected fibroblasts, or CD40L-transfected fibroblasts. Cells were then analyzed by flow cytometry using anti-MHC-DR Ab coupled to FITC and CD86 Ab coupled to phycoerythrin. For the study of E-cadherin expression, only LC were studied since DC do not or poorly express E-cadherin. Data representative of six independent experiments on different donors.

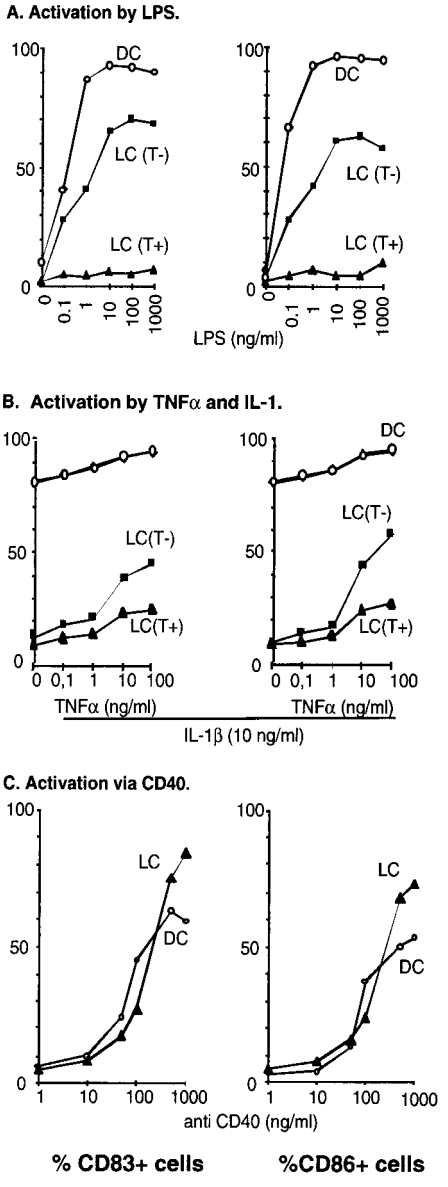

FIGURE 5. Inhibition of LPS and TNF-α/IL-1-, but not CD40L-, induced phenotypic maturation of LC. Effect of withdrawal of TGF-β1. Day 6 DC and LC were incubated with increasing amounts of LPS from *E. coli* 0127-B8 (*A*), 10 ng/ml IL-1 and increasing amounts of TNF-α (*B*), or with fibroblastic L cells transfected with CD32 together with increasing concentrations of activating anti-CD40 Abs (*C*). Cells were cultured in the presence of GM-CSF and IL-4, with (T+) or without (T−) 10 ng/ml TGF-β1 for 40 h. Cells were then analyzed by flow cytometry, and percentages of $CD83^+$ and $CD86^+$ cells were plotted against LPS, cytokines, and anti-CD40 Ab concentration. Data representative of three independent experiments.

them with LPS. This provides functional evidence to support our results analyzing class II Ags and activation marker expression, macropinocytosis activity, and IL-12 production.

In vivo and in vitro evidence that LC migration and maturation are independently regulated events

However, the maturation process of LC includes their migration toward the draining lymph node (32). We thus investigated whether migration of LC may occur before they fully mature in phenotype or function. TNF-α and IL-1 were demonstrated to contribute to LC emigration from the epithelia (33), at least in part by reducing membrane expression of E-cadherin (34–36). We thus investigated regulation of E-cadherin expression on monocyte-derived LC. As shown in Fig. 4, addition of 10 ng/ml of TNF-α and IL-1 induced significant down-regulation of E-cadherin expression on monocyte-derived LCs in the presence of GM-CSF, IL-4, and TGF-β1.

However, in vivo migration of LC does not rely only on E-cadherin expression. We thus investigated the in vivo phenotype of LC that have emigrated from the skin to the draining lymph node

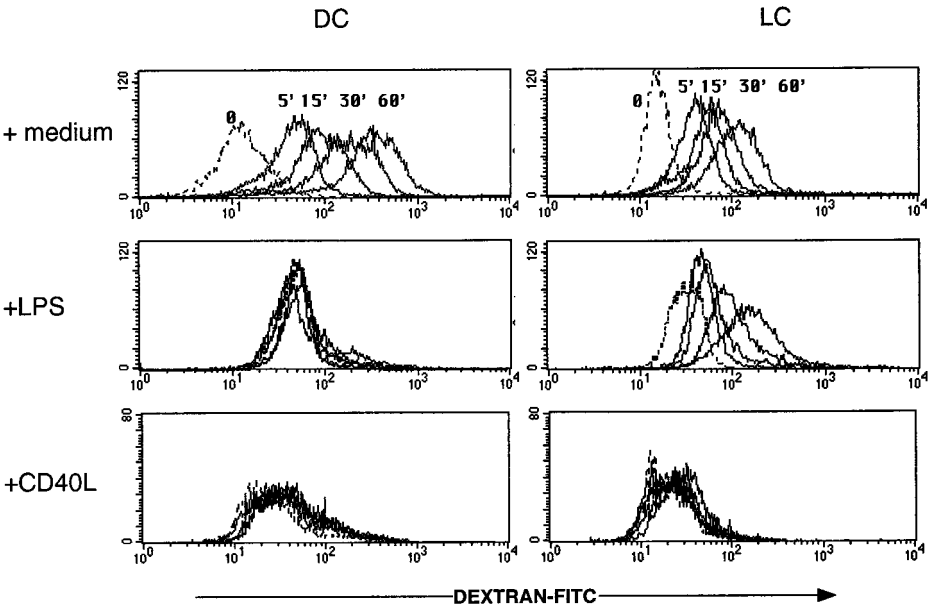

FIGURE 6. FITC-dextran uptake by DC and LC: TGF-β1 maintain fluid phase intake in LPS-treated LC. Cells were resuspended in complete medium and incubated at 37°C with 5% CO_2. FITC-dextran was added at a final concentration of 1 mg/ml for 5, 15, 30 and 60 min. Cells were then washed four times with cold PBS, 2% human AB serum, and 0.01 M NaN_3 and were analyzed with a FACScalibur (Becton Dickinson) using CellQuest software (Becton Dickinson). *Upper panel*, DC, cultured in the presence of GM-CSF and IL-4 (*left*), LC cultured in the presence of GM-CSF, IL-4, and TGF-β1 (*right*). *Middle panel*, DC (*left*) and LC (*right*) stimulated by 10 ng/ml LPS. *Lower panel*, DC (*left*) and LC (*right*) stimulated by CD40L-transfected fibroblasts. Results are representative of three experiments.

on three patients with dermatopathic lymphadenopathy, a benign reactive condition in which skin-draining lymph nodes are enlarged, due to the accumulation of LC in T cell zones. In three of three patients, immunohistochemical staining on serial sections showed that a large number of cells in the T cell zones expressed high levels of CD1a and are thus poorly mature LC; most of these cells did not express detectable levels of CD83, CD86, and CD80. A double immunostaining using CD1a and CD83 Abs is shown in Fig. 8; $CD1a^+CD83^{low/-}$ are numerous in the T cell zone (closed arrowhead), while some $CD1a^+CD83^+$ cells are also present (arrow). $CD1a^-CD83^+$ (open arrowhead) cells are mainly found in the B cell follicle (F). This result suggests that phenotypically immature LCs can migrate into the draining lymph node in response to an inflammatory signal.

Therefore, TGF-β1, which allows differentiation of immature LCs from monocytes, may not inhibit migration of LC to the paracortical area of draining lymph nodes in response to inflammatory stimuli such as TNF-α and IL-1. Rather, our results suggest that TGF-β1 prevents the functional maturation of these cells unless a cognate signal such as CD40L is provided.

Discussion

We have previously shown that TGF-β1, in the presence of GM-CSF and IL-4, induces differentiation of human peripheral blood monocytes into $CD1a^+$, E-cadherin$^+$, CLA^+, and Lag^+ dendritic LC expressing Birbeck granules (15). In the present study, we further extend this finding and show that monocyte-derived LCs represent a stable population of immature LCs, with intracellular class II Ags, which do not produce IL-10 after stimulation and do not express the TNFRIp55.

These results further indicate that monocyte-derived LC behave as do in vivo LC in many aspects. Indeed, it has been shown that in vivo LC only express TNFRIIp75 (24) and respond to TNF-α via TNFRIIp75 and not TNFRIp55 (12) while, in contrast, monocyte-derived DC respond to TNF-α by TNFRIp55 and not TNFRIIp75 (6). Also, in vivo LC and in vitro $CD34^+$-derived LC do not produce IL-10 (25–27), while dermal $CD11b^+$ macrophages/DCs in vivo (25, 26), in vitro CD34-derived (27), and monocyte-derived non-Langerhans DC produce IL-10 upon stimulation. These findings distinguish LC from dermal DC, which resemble more closely the monocyte-derived DCs generated in the presence of GM-CSF and IL-4 without exogenous TGF-β1, while the two DC subsets (dermal DC and LC) may be closely related (37).

More important, the present study also shows that maturation of LC is differentially regulated by TGF-β1: 1) TGF-β1 allows down-regulation of E-cadherin expression by the whole LC population in response to TNF-α and IL-1 but inhibits LC maturation after exposure to LPS, TNF, and IL-1, with respect to Class II, CD80, CD86, and CD83 Ag expression, loss of FITC-dextran uptake, production of IL-12, and Ag presentation; 2) in contrast, cognate T cell-dependent stimuli (i.e., CD40-mediated) induce full maturation of LC and are not influenced by TGF-β1; 3) in vivo maturation (i.e., induction of CD80, CD86, and CD83 Ag expression) does not necessarily precede migration, and, in accordance, it has been shown that LC do not need to be fully mature in phenotype or function before they leave the skin (32).

These results are consistent with the crucial role of CD40-mediated activation for the final maturation and "licensing" of DCs (3, 9, 10). LC are the DCs of the epithelial barriers, including the skin and oral, nasal, esophagal, pulmonary, vaginal, and rectal mucosae. These cells are thus challenged frequently with numerous pathogens and traumatic events and reside in epithelia where TNF-α and IL-1 are produced at relatively high levels (28). LC represent the main population of APC in the mucosal barriers, and the main source of IL-12 after stimulation, but do not produce IL-10 and drive Th1 responses such as delayed-contact hypersensitivity. It would be dangerous for the host if inflammatory stimuli were able to "license" LC in a way that enable them to stimulate an effector response. Our results indicate that TGF-β1 may be responsible for the cognate T cell dependence of LC maturation. By dampering the effect of inflammatory cytokines and LPS on the functional maturation of LC, TGF-β1 may prevent the noncognate maturation of LC via bystander inflammatory cytokines present in epithelia, thereby avoiding potentially harmful immune responses.

After Ag exposure, TNF-α and IL-1 contribute to LC emigration from the epithelia (33, 34), at least in part by reducing membrane expression of E-cadherin (35, 36). Our results are in accordance with these data; however, we propose that these stimuli are not sufficient to induce final maturation of DC, due to the presence of

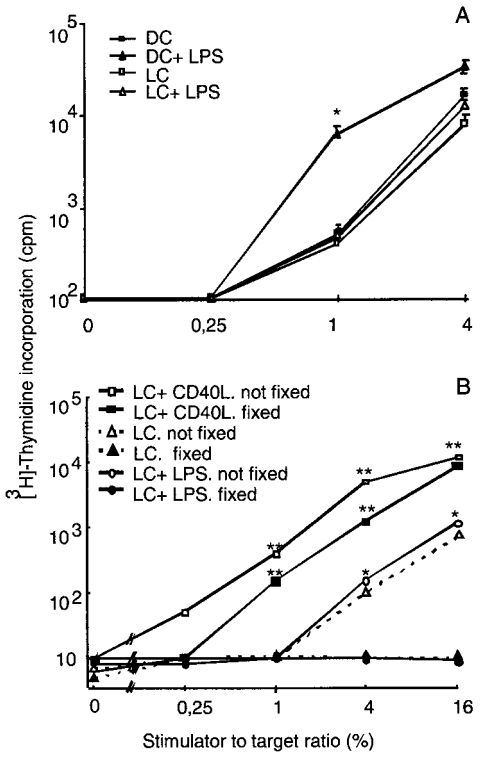

FIGURE 7. CD40L but not LPS induces Ag presentation by LC. *A*, While incubation of DC with LPS increased the Ag-specific proliferative response (*, $p < 0.01$ compared with unstimulated DC and with unstimulated and LPS-stimulated LC), incubation of LC with LPS had no effect. Day 6 DC (closed squares and closed triangles) and LC (open squares and open triangles) were pulsed for 48 h with TT or medium alone, left unstimulated (squares) or cultured with LPS (10 ng/ml) (triangle), washed three times, 30-Gy-irradiated, and added to 10^5 T cells/well from the same donor, in 96-well tissue culture plates. T cell proliferation was measured as indicated in *Materials and Methods*. Background thymidine incorporation, in the absence of pulse with TT, was subtracted; results are mean of triplicate experiments. *B*, T cell proliferative response to TT may be achieved by coculturing LC with either Ag-specific T cells, or with CD40L, but not by incubating them with LPS. Day 6 LC were pulsed for 48 h with TT or medium alone, left unstimulated or cultured with LPS (10 ng/ml) or CD40L-Lc (1 CD40L-Lc for 10 LC), then fixed with 0.001% glutaraldehyde or mock fixed and cocultured with T cells from the same donor as indicated in *Materials and Methods*. LC pulsed with TT, unstimulated and unfixed (open triangles) but not fixed (closed triangles) stimulate T cell proliferation (*, $p < 0.05$ for comparison between unfixed and fixed cells). Similar response was obtained for LPS-treated LC (not fixed, open circle; fixed, closed circle) (*, $p < 0.05$ for comparison between unfixed and fixed cells). CD40-activated LC induced strong T cell proliferation whether unfixed (open squares) or fixed (closed squares) (**, $p < 0.05$ for comparison between CD40L-stimulated cells (whether fixed or unfixed) and other conditions). Results are means of triplicate experiments; SD was always ≤ 20%. Background thymidine incorporation, in the absence of pulse with TT, was either ≤10% of total cpm of the corresponding TT-pulsed LC cultures or <300 cpm and was subtracted.

TGF-β1. Once in lymphoid organs, LC interact with naive Ag-specific helper T cells. This contact, which may be facilitated by attracting chemokines, can be sustained for 20 h in certain circumstances (38). If cognate recognition occurs, this crosstalk may lead to activation of both the helper T cell and the LC, thus initiating a helper $CD4^+$ immune response and "licensing" mature LC (so-called interdigitating DCs) to stimulate cytotoxic T cells (3). It could be tempting to further speculate that this crosstalk in the T cell area of lymph nodes may also result in tolerance.

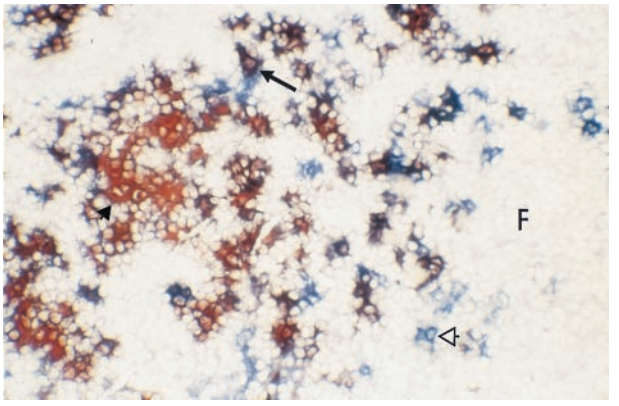

FIGURE 8. Emigrated immature LCs are observed in human reactive skin-draining lymph nodes. Skin-draining reactive lymph node biopsies from three patients with dermatopathic lymphadenopathy were studied. Cryostat sections were stained with CD1a (revealed in red using AEC as a substrate for peroxidase) and CD83 (revealed in blue with fast blue as a substrate for alkaline phosphatase). Many $CD1a^+CD83^{low/-}$ immature LCs are visualized in red in the T cell zone (closed arrowhead). $CD1a^-CD83^+$ mature DC and/or activated B cells are visualized in blue (open arrowhead) and are present in B cell follicle (F). $CD1a^+CD83^+$ double positive cells are visualized in dark blue-black (arrow). Micrograph from one representative experiment of three, original magnification ×200.

Uncontrolled maturation of LC by nonspecific bystander signals and consecutive inappropriate T cell activation might break tolerance. However, DCs grown in the absence of TGF-β1, which may correspond to dermal (39) and some nonmucosal DCs, become mature in response to cytokines or LPS and produce IL-10. These cells have been proposed to mediate humoral rather than cellular immune responses (40). In addition, LPS or TNF-α, away from the periphery, e.g. in the spleen, represent "danger" (e.g., septicemia). Conversely, production of TGF-β1 by tumoral cells may locally hamper inflammatory-induced maturation of DC and reduce antitumoral immune responses.

We have shown here that several DC activation pathways may be inhibited by TGF-β1, while another (i.e., CD40L) remains unaffected. Although a common ceramide-mediated signaling pathway was described for CD40L, TNF-α, and IL-1 in DC (41), it is clearly not affected in TGF-β1-treated LC, since CD40L-induced activation and Ag presentation are not inhibited. CD14, the main known LPS receptor, was barely detectable in both DC and LC (15). We have shown in this study that TGF-β1 down-regulates membrane TNFRIp55 on LC vs DC. However, stimulation via CD40 induces IL-12 production in both DC and LC at similar levels, but LC do not produce IL-10, while DC produce large amounts of this cytokine. Therefore, differential regulation of receptor expression is not the only mechanism involved in the alternative responses to external stimuli by DC and LC. CD40 may engage different downstream signaling molecules in the two cell types.

In conclusion, TGF-β1 appears to be a major cytokine in DC biology, responsible for the acquisition of the LC phenotype and the prevention of noncognate maturation.

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