Differentiation of myeloid dendritic cells into CD8α-positive dendritic cells in vivo

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Bone marrow-derived dendritic cells (DC) represent a family of antigen-presenting cells (APC) with varying phenotypes. For example, in mice, CD8α− and CD8α+ DC are thought to represent cells of lymphoid and myeloid origin, respectively. Langerhans cells (LC) of the epidermis are typical myeloid DC; they do not express CD8α, but they do express high levels of myeloid antigens such as CD11b and FcγR. By contrast, thymic DC, which derive from a lymphoid-related progenitor, express CD8α but only low levels of myeloid antigens. CD8α+ DC are also found in the spleen and lymph nodes (LN), but the origin of these cells has not been determined. By activating and labeling CD8α+ epidermal LC in vivo, it was found that these cells expressed CD8α on migration to the draining LN. Similarly, CD8α+ LC generated in vitro from a CD8 wild-type mouse and injected into the skin of a CD8αKO mouse expressed CD8α when they reached the draining LN. The results also show that CD8α+ LC are potent APC. After migration from skin, they localized in the T-cell areas of LN, secreted high levels of interleukin-12, interferon-γ, and chemokine-attracting T cells, and they induced antigen-specific T-cell activation. These results demonstrate that myeloid DC in the periphery can express CD8α when they migrate to the draining LN. CD8α expression on these DC appears to reflect a state of activation, mobilization, or both, rather than lineage. (Blood. 2000;96:1865-1872)

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Materials and methods

Animals

Five- to 7-week-old female C57BL/6 and B10 BR mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN), and C57BL/6 CD8α knockout mice were obtained from Jackson Laboratory (Bar Harbor, ME). The mice were housed in our animal facility.

Cytokines and media

Recombinant murine stem cell factor (SCF), granulocyte macrophage–colony-stimulating factor (GM-CSF), tumor necrosis factor (TNF)-α, and interleukin (IL)-1β were purchased from Preprotech (Rocky Hill, NJ). Cells were cultured in vitro in the presence of complete medium (CM) that included RPMI supplemented with 10% fetal calf serum (FCS), penicillin (100 U/mL), and streptomycin (100 μg/mL). DC isolation procedures used phosphate-buffered saline (PBS) supplemented with 3% FCS and 5 mmol/L.
EDTA (PBS-EDTA-FCS). Cell staining and sorting were performed in the presence of PBS-EDTA-FCS and 0.1% azide.

**Immunofluorescence analysis and flow cytometry**

All cell preparations were preincubated with anti-CD3/16 to minimize nonspecific binding. Three-color analyses were performed on a FACScalibur (Becton Dickinson, Mountain View, CA), and sorting was performed on a FACSVantage (Becton Dickinson). Mouse monoclonal antibodies (mAb) to CD8α chain (Ly-2; IgG2a), CD11c (HL3; IgG1), IA-β (A6-120.1; IgG2a), Thy-1 (G7; IgG2c), Gr-1 (RB6-8C5; IgG2b), B-220 (RA3-6B2; IgG2a), CD11b (M1/70; IgG2b), CD3ε (145-2C11; IgG1), TCRβ chain (H57-597; IgG2), CD4 (GK1.5; IgG2b), CD86 (GL1; IgG2a), CD40 (HM40-3; IgM), CD16/CD122 (2.4G2; IgG2b), isotype controls, and the second-step antibodies (APC-conjugated streptavidin, phycoerythrin (PE)-conjugated goat antirabbit IgG, and a PE-conjugated antirat IgG) were purchased from Pharmingen (San Diego, CA). Mouse mAb to DEC-205 (NLDC-145; IgG2a) was purchased from Serotec (Raleigh, NC), and a mouse polyclonal antibody recognizing E-cadherin was kindly provided by Dr W. J. Nelson. A different clone of mAb recognizing CD8ε (CT-CD8αa; IgG2a) was purchased from Caltag (Burlingame, CA). In skin painting experiments, the DC-enriched population was stained with biotin-conjugated CD11c and PE-conjugated IA-β, and APC-streptavidin conjugate was used as a second step.

**Isolation of epidermal Langerhans cells**

LC were obtained from epidermal sheets of mouse ears following a protocol modified from that of Schuler and Steinman. Briefly, ears were split with the aid of forceps into dorsal and ventral halves and incubated in a Petri dish containing 0.5% trypsin (Gibco BRL, Gaithersburg, MD) in PBS for 20 minutes at 37°C to allow the separation of epidermal sheets from the dermis. The epidermis was separated from the dermis with fine forceps. A suspension of epidermal cells was obtained by filtering the trypsinized epidermal sheets through a stainless steel sieve, followed by washing in PBS with 5% FCS. The resultant cell suspension was resuspended in CM and cultured in a 24-well plate in the presence of 10 ng/mL GM-CSF. Twenty-four hours later, nonadherent cells were collected and washed, and the cell concentration was adjusted to 4 × 10^6 cells/mL in PBS. Four microliters Nycoprep (Nycomed, Oslo, Norway) was gently layered under the cell suspension of epidermal cells and centrifuged for 20 minutes (1800 rpm) at 4°C. The low-density fraction contained more than 30% LC, as assessed by flow cytometry.

**Langerhans cell migration assay in vivo**

Mice were painted on the shaved abdomens and footpads with 400 Lo f4 mg/mL fluorescein isothiocyanate (FITC) (F-7250; Sigma, St. Louis, MO) dissolved in 1:1 acetone:dibutylphalate (D-2270; Sigma). Draining inguinal LN were harvested 24 hours later. The purified and sorted LC were obtained from epidermal sheets through a stainless steel sieve, followed by washing in PBS with 5% FCS. The resultant cell suspension was resuspended in CM containing 0.5% trypsin (Gibco BRL, Gaithersburg, MD) in PBS for 20 minutes at 37°C to allow the separation of epidermal sheets from the dermis. The epidermis was separated from the dermis with fine forceps. A suspension of epidermal cells was obtained by filtering the trypsinized epidermal sheets through a stainless steel sieve, followed by washing in PBS with 5% FCS. The resultant cell suspension was resuspended in CM and cultured in a 24-well plate in the presence of 10 ng/mL GM-CSF. Twenty-four hours later, nonadherent cells were collected and washed, and the cell concentration was adjusted to 4 × 10^6 cells/mL in PBS. Four microliters Nycoprep (Nycomed, Oslo, Norway) was gently layered under the cell suspension of epidermal cells and centrifuged for 20 minutes (1800 rpm) at 4°C. The low-density fraction contained more than 30% LC, as assessed by flow cytometry.

**Induction and measurement of cytokine secretion from migratory Langerhans cells and CD8α dendritic cells**

Populations of FITC ‘CD8αCD11c’ and FITC ‘CD8αCD11c’ populations were cultured in the presence of an ovalbumin-specific, MHC class I-restricted T-cell hybridoma (B3Z) for 24 hours at a ratio of 1 FITC ‘cell’ to 10 B3Z cells. IL-2 release from the hybridoma cells was analyzed by ELISA.

**Antigen capture and presentation assay**

Ovalbumin (grade VI, Sigma) mixed with complete Freund’s adjuvant (DIFCO, Detroit, MA) was injected intradermally into the footpads of mice. Two days later, the skin was sensitized as described above, and the draining lymph nodes were harvested 24 hours later. The purified and sorted FITC ‘CD8αCD11c’ and FITC ‘CD8αCD11c’ populations were cultured in the presence of an ovalbumin-specific, MHC class I-restricted T-cell hybridoma (B3Z) for 24 hours at a ratio of 1 FITC ‘cell’ to 10 B3Z cells. IL-2 release from the hybridoma cells was analyzed by ELISA.

**Mixed lymphocyte reaction**

Graded numbers of a purified population of FITC ‘CD8αCD11c’ or FITC ‘CD8αCD11c’ cells were irradiated (30 Gy) and added to allogeneic (B10.BR) T cells (2 × 10^6/well) in a final volume of 0.2 mL in 96-well flat-bottom plates (Corning, Corning, NY). Cell proliferation was measured by adding 1 μCi 1H-thymidine/well after 3 days. Cells were harvested 18 hours later, and the incorporated 1H-thymidine was counted in a β-plate counter (Wallac, Gaithersburg, MD). Results are presented as the mean of triplicate cultures.
ELISA

Cytochrome secretion was quantified by ELISA adapted from Pharmingen protocols. In brief, ELISA plates (Corning) were coated overnight at 4° with 50 μL antimouse IL-2, IL-12, and IFN-γ antibodies (Pharmingen) diluted in 0.1 mol/L NaHCO3, to a concentration of 2 μg/mL. The plates were then blocked with PBS–5% FCS for 3 to 4 hours and were washed several times with PBS/0.1% Tween 20. Serial dilutions of the standard were made at a starting concentration of 5 ng/mL for IL-2 (R&D, Minneapolis, MN) and 50 ng/mL for IL-12 and IFN-γ (Prepotech). Fifty microliters of the sample or the standard was added to the plate at room temperature. After 4 hours of incubation, the plates were washed, and biotinylated detection antibody (Pharmingen) was added at a concentration of 2 μg/mL. Three hours later, the plates were washed and incubated with streptavidin-horseradish peroxidase (Vector Laboratories, Burlingame, CA) diluted 1:2000. One hour later, the plates were washed and the bound peroxidase was detected with TMB substrate (Zymed, San Francisco, CA). The amount of reaction product was assessed on an ELISA plate reader (Bio-Rad, Hercules, CA) at an optical density of 650 nm. The detection limit was 50 pg/mL.

Reverse transcription–polymerase chain reaction

Total RNA was extracted from purified FITC+CD8+CD11c+ cells, FITC+CD8−CD11c+ cells, FITC−CD8+CD11c+ (resident DC), and total LN cells using an RNeasy kit (Qiagen, Valencia, CA) and treated with DNase. RNA was quantified by spectrophotometry. First-strand cDNA was synthesized from total RNA using the Superscript II RNase H-reverse transcriptase (Gibco BRL) with random primers, as described by the manufacturer. For all samples, synthesis of cDNA was controlled by reverse transcription–polymerase chain reaction (RT-PCR) using β-actin primers for 30 cycles. CD8α chain mRNA was examined by using the primers (sense primer, caggaataataagtacgttctcacc; antisense primer, atgtaaatatcacaggcgaagtcca) and CD3α by using the following primers: gaagaattcacagcttagcaga (sense) and tggagagtgttagaacatcag (antisense). CCR6 mRNA was examined by using the primers ctgcaagcttaggagtctcag (sense) and gctatcaactcataattggt (antisense). CCR7 mRNA was examined by using the primers acagcggcctccaga (sense) and tgacgtcataggcaatgttgagctg (antisense). Thymus and activation-regulated chemokine (TARC) mRNA was examined by using the primers caggaattgcaaggtctgtagatc (sense) and ttgagctgagtgtagagtc (antisense). Monocyte-derived chemokine (MDC) mRNA was examined using the primers gttgctctgctgctttcgc (sense) and ggacagttagctgtagattc (antisense).

Results

Migratory Langerhans cells express CD8α mRNA but not CD3 mRNA

To determine whether CD8α was synthesized by the FITC+ DC or was acquired passively from T cells that might have shed CD8 molecules, the presence of the CD8α chain message in sorted FITC+ DC (more than 90% purity) was assessed by RT-PCR analysis. As shown in Figure 2 panel B, the sorted FITC+CD8+CD11c+ DC expressed a clear CD8α chain message but no CD3 message. The production by DC of CD8 mRNA makes it highly unlikely that CD8 antigens detected by flow cytometry were passively acquired, and the absence of the CD3 message confirms that the sorted cells were not contaminated by T cells.

Bone marrow-derived Langerhans cells injected into CD8α knockout mice express CD8α when they reach the lymph nodes

BM-LC were generated from C57BL/6 mice and injected subcutaneously into the footpads of syngeneic CD8α knockout mice.
mice. As described in “Materials and methods,” these cells were generated in vitro from lineage-negative HPC cultured in the presence of cytokines, including GM-CSF, TGF-β, and SCF. Cells obtained at day 13 were typical monocytes, as evidenced by their morphology and endocytic activity (data not shown). After 3 additional days of culture in the presence of GM-CSF, TNF-α, and IL-4, most cells differentiated into DC. They expressed high levels of MHC class II molecules, CD11c and CD11b antigens, the epithelial marker E-cadherin, and costimulatory molecules, whereas a small subset of the cells (less than 10%) differentiated into macrophages (data not shown). None of these cells expressed CD8 or TCR molecules (Figure 3).

Purified CD8<sup>+</sup> BM-LC were injected subcutaneously into the footpads of CD8<sup>−/−</sup> knockout mice. Twenty-four hours after skin sensitization with FITC, the draining LN were harvested and analyzed by immunohistochemistry. Frozen sections of the LN were stained to identify the T-cell zone and examined by confocal microscopy. As shown in Figure 5A, most FITC<sup>+</sup> cells in the LN expressed CD8α antigen and were located in the T-cell zone and not in the follicles. Using an RT-PCR assay, we then analyzed the expression of chemokine and chemokine receptor mRNA. RNA was extracted from a sorted population of FITC<sup>+</sup> CD8<sup>+</sup> cells isolated from the LN and from LC freshly isolated from the skin. As shown in Figure 6, FITC<sup>+</sup>CD8<sup>+</sup> LC expressed CCR6 and CCR7 and the T-cell attracting chemokines MDC and TARC. In contrast, freshly isolated LC from the skin express CCR6 but not CCR7, MDC, or TARC.

To generate an immune response, antigen-loaded DC must rapidly interact with antigen-specific T cells in lymphoid organs. Mechanisms that would be expected to increase encounters between DC and T cells include the localization of migratory DC to T-cell areas of the LN or their production of T-cell attracting chemokines. To address these possibilities, we analyzed the geographic localization, chemokine receptors, and chemokine expression of FITC<sup>+</sup>CD8<sup>+</sup> cells present in the LN and freshly isolated LC from the skin. Twenty-four hours after skin sensitization with FITC, the draining LN were harvested and analyzed by immunohistochemistry. Frozen sections of the LN were stained to identify the T-cell zone and examined by confocal microscopy. As shown in Figure 5A, most FITC<sup>+</sup> cells in the LN expressed CD8α antigen and were located in the T-cell zone and not in the follicles. Using an RT-PCR assay, we then analyzed the expression of chemokine and chemokine receptor mRNA. RNA was extracted from a sorted population of FITC<sup>+</sup> CD8<sup>+</sup> cells isolated from the LN and from LC freshly isolated from the skin. As shown in Figure 6, FITC<sup>+</sup>CD8<sup>+</sup> LC expressed CCR6 and CCR7 and the T-cell attracting chemokines MDC and TARC. In contrast, freshly isolated LC from the skin express CCR6 but not CCR7, MDC, or TARC.

CD8<sup>+</sup> Langerhans cells capture, process, and present antigens to T cells

To assess the capacity of migratory LC to stimulate allogeneic T cells, we isolated FITC<sup>+</sup>CD8<sup>−</sup>CD11c<sup>+</sup> and FITC<sup>+</sup>CD8<sup>−</sup>CD11c<sup>+</sup> cells from the LN of painted mice and cocultured them with freshly isolated allogeneic T cells. As shown in Figure 7, panel A, the
CD8\(^+\) LC stimulated allogeneic T cells to proliferate vigorously in the mixed lymphocyte reaction. To assess the capacity of migratory LC to process and present external antigen in association to MHC class I molecules, we injected ovalbumin mixed with complete Freund's adjuvant intradermally into the footpads of mice and subsequently painted the mice with FITC. Twenty-four hours later, FITC\(^+\)CD8\(^+\)CD11c\(^+\) and FITC\(^-\)CD8\(^-\)CD11c\(^+\) cells isolated from the draining LN were cultured with the ovalbumin-specific, MHC class I-restricted T-cell hybridoma, B3Z. As shown in Figure 7A,B, in the presence of CD8\(^+\) LC, the B3Z cells secreted high levels of IL-2. However, FITC\(^-\)CD8\(^-\)CD11c\(^+\) cells failed to stimulate either B3Z hybridoma cells or allogeneic T cells (Figure 7A,B).

**CD8\(^+\) LC secrete a higher level of Th1 cytokines than CD8\(^-\) DC**

To characterize the cytokine profile of the migratory LC, FITC\(^+\)CD8\(^+\)CD11c\(^+\), FITC\(^-\)CD8\(^-\)CD11c\(^+\), and FITC\(^-\)CD8\(^-\)CD11c\(^+\) cells were purified from LN and stimulated in vitro overnight with lipopolysaccharide or IL-12. As shown in Figure 8, significant amounts of Th1 cytokines, such as IFN-\(\gamma\) and IL-12, were secreted by CD8\(^+\) DC. The amount of IFN-\(\gamma\) and IL-12 secreted by CD8\(^+\) DC was greater than that secreted by CD8\(^-\) DC.

**Discussion**

Although the lymphoid origin of CD8\(^+\) thymic DC has been conclusively demonstrated (reviewed in Sprent et al\(^a\); also see Ardavin et al\(^b\)), the lineage of CD8\(^-\) DC in peripheral lymphoid organs has not yet been determined. CD8\(^+\) DC were initially identified by Ardavin et al in the thymus of mice, in which they appear to develop from an endogenous precursor rather than to arrive preformed from the circulation.\(^a\) On transfer to irradiated mice, this precursor gave rise to T, B, and natural killer cells and to CD8\(^-\) DC.\(^b\) In a subsequent study the same investigators identified CD8\(^-\) DC in the spleen and the LN of mice.\(^2\) This observation led to the concept of a second CD8\(^+\) DC lineage that comprises subsets of DC in peripheral lymphoid organs and in the thymus. However, despite their phenotypic similarity, these 2 populations can be distinguished from one another on other grounds. For example, thymic CD8\(^+\) DC express BP-1, a glutamyl aminopeptidase not expressed by splenic or LN CD8\(^-\) DC.\(^17\) Thymic DC express other lymphoid markers, such as Thy-1, that are not present on CD8\(^-\) DC in lymphoid organs.\(^17\) Functionally, thymic DC induce the negative selection of thymocytes but do not have a role in positive selection.\(^18\) In contrast, peripheral CD8\(^-\) DC are potent stimulators of T-cell proliferation and induce the secretion of high levels of Th1 cytokines.\(^19,20\) These differences suggest that peripheral and thymic CD8\(^+\) DC represent developmentally distinct cell types.

We show here that freshly isolated LC and LC derived from HPC do not express the CD8 antigen. However, when LC are activated in vivo with a skin sensitizer that results in their migration to the draining LN, CD8 expression is induced on the surfaces of the cells. In our studies CD8 expression on migratory LC was not caused by the presence of contaminating cells or the passive absorption of CD8 molecules shed by T cells because migratory LC in the LN did not express CD3 or TCR antigen. Moreover, these cells expressed CD8 mRNA, showing that they are able to synthesize CD8 despite their lack of any detectable CD3 mRNA. Finally, we showed that CD8\(^-\) LC generated in vitro from the bone marrow cells of CD8 wild-type mice and injected into syngeneic CD8\(^-\) knockout mice\(^22\) expressed CD8e when they reached the draining LN. Because the LC were injected into mice that had been rendered incapable of expressing CD8, the CD8 expression detected in the draining LN could only have been expressed by the injected cells. These results show clearly that classical myeloid DC, such as LC, can express CD8e when they migrate to draining LN.
The results presented here also demonstrate that CD8+ LC are uniquely equipped to attract and interact with T cells. They express the chemokine receptors CCR6 and CCR7, facilitating their recruitment in the periphery and their migration to the T-cell zones of secondary lymphoid organs. CCR6 is the receptor for MIP-3α,22,23 a chemokine induced by inflammatory stimuli and expressed mainly by keratinocytes, fibroblasts, and endothelial cells.24,25 CCR6 is also the only known receptor of β defensins, a family of small peptides expressed by keratinocytes and released on microbial invasion.26 The presence of CCR6 on LC allows their recruitment to sites of inflammation in response to danger signals, thereby enhancing the role of these cells in innate and adaptive immunity. The expression of CCR6 by migratory DC was assessed 18 hours after FITC activation, and it would be expected to decline at later time points.25 CCR7 is the receptor of SLC,27 a chemokine expressed by stromal cells in vessels31,32 and naive T cells,29 suggesting that it may have a role in the T-cell areas of LN, high endothelial venules, and lymphatic vessels.28,29 SLC attracts both activated DC30 in the lymphatic vessels31,32 and naive T cells,29 suggesting that it may have a role in the colocalization of migratory CD8+ LC and naive T cells. Our studies also showed that CD8+ LC in LN produce MDC33,34 and TARC,35 2 recently discovered chemokines involved in T-cell attraction. The expression of CCR7 and T-cell–attractant chemokines by CD8+ LC may explain their exclusive localization in T-cell areas of LN.

CD8+ and CD8− DC share a number of properties, including dendritic morphology and elevated surface expression of MHC class II. However, there are significant differences between these 2 populations. Although CD8− DC are found in nonlymphoid tissues and in lymphoid organs, CD8+ DC are found only in lymphoid organs. More important, CD8+ DC are present only in the T-cell areas of the spleen and LN.36 However, CD8− DC are absent from the thymus and are present outside the T-cell zones of secondary lymphoid organs.36 CD8+ DC express higher amounts of peptide MHC complexes on their surfaces,37,38 secrete higher levels of IL-12 when stimulated with Staphylococcus aureus Cowan I strain or IFN-γ, induce T-cell stimulation, and drive Th1 differentiation.20,21 In contrast, CD8− DC express lower levels of MHC peptide complexes, secrete higher levels of IL-10, and drive Th2 differentiation.20 In our studies, freshly emigrant CD8+ DC secreted 3 times more IL-12 and 6 times
Forty-eight hours later, the cytokine profile of each population was determined by secretion or in the presence of lipopolysaccharide to induce IL-12 secretion. More recently, Ohteke et al. showed that CD8\(^{+}\) DC, but not CD8\(^{-}\) DC, were capable of secreting significantly more IFN-\(\gamma\) than natural killer cells at an early stage of Listeria monocytogenes infection or after IL-12 administration.

The findings presented here, together with previous results, suggest that CD8\(^{+}\) DC in the periphery and outside the T-cell zones of secondary lymphoid organs are ordinarily immunologically inactive. These cells capture and process antigens in the environment and, in the presence of a danger signal, migrate to the T-cell zones of lymphoid organs, where they present the antigen to specific T cells. CD8 is expressed on DC either during migration or on their arrival in LN. Although the trigger for CD8 expression remains unknown, the presence of this molecule appears to reflect a stage of differentiation or activation rather than lineage.

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**Figure 8. CD8\(^{+}\) DC secrete higher levels of IL-12 and IFN-\(\gamma\) than CD8\(^{-}\) DC. Sorted populations of 1 \times 10\(^{5}\) FITC-CD8\(^{+}\)CD11c\(^{+}\), FITC-CD8\(^{-}\)CD11c\(^{+}\), and FITC-CD8\(^{-}\)CD11c\(^{-}\) DC were cultured in the presence of IL-12 to induce IFN-\(\gamma\) secretion or in the presence of lipopolysaccharide to induce IL-12 secretion.**

**More IFN-\(\gamma\) than CD8\(^{-}\) DC. These findings suggest that CD8\(^{+}\) LC may play a fundamental role in the induction of immunity by priming Th1 responses. Thus, Reis e Sousa et al. showed that after in vivo challenge with Toxoplasma gondii, CD8\(^{+}\) DC were the predominant APC producing IL-12, suggesting an important role for these cells in microbial immunity. More recently, Ohteke et al. showed that CD8\(^{+}\) DC, but not CD8\(^{-}\) DC, were capable of secreting significantly more IFN-\(\gamma\) than natural killer cells at an early stage of Listeria monocytogenes infection or after IL-12 administration.**


