Epidermal and Dermal Dendritic Cells Display Differential Activation and Migratory Behavior While Sharing the Ability to Stimulate CD4\(^+\) T Cell Proliferation In Vivo

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*J Immunol* 2008; 181:418-430; 
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Epidermal and Dermal Dendritic Cells Display Differential Activation and Migratory Behavior While Sharing the Ability to Stimulate CD4+ T Cell Proliferation In Vivo1,2

Elena Shklovskaya, Ben Roediger, and Barbara Fazekas de St. Groth3

Migrated Langerhans cells (m-LCs) have recently been shown to comprise only a minority of skin-derived dendritic cells (DCs) expressing Langerin in cutaneous lymph nodes. We have used BM chimeric mice that differ in CD45 and MHC class II alleles to unequivocally distinguish between radioresistant m-LCs and radiosensitive migrated dermal DCs (m-dDCs), to determine their phenotype, response to contact sensitization, and ability to activate naive CD4+ T cells in vivo. We have also characterized three subsets of dDCs and their migratory counterparts, as distinguished by expression of CD11b and Langerin. Each of the four subsets of skin DCs showed differential migration to draining LN in response to contact sensitizing agents. Migration of Langerin+/CD11b+ and Langerin+/CD11b+ dDCs peaked after 1 day, followed by Langerin+/CD11b+ dDCs at 2 days and Langerin+/LCs at 4 days. Moreover, while m-LCs and m-dDCs had similar surface phenotypes in the steady state, they displayed unexpectedly different activation responses to contact sensitization: m-dDCs markedly up-regulated CD80 and CD86 at day 1, whereas m-LCs up-regulated CD40, with delayed kinetics. Thus, m-dDCs are likely to be responsible for the initial response to skin immunization. However, when expression of cognate MHC class II was restricted to LCs and m-LCs, they were also capable of processing and presenting protein Ag to drive naive CD4 T cell proliferation in vivo. Thus, m-dDCs and m-LCs display distinct behavior in cutaneous lymph nodes while sharing the ability to interact specifically with T cells to control the immune response. The Journal of Immunology, 2008, 181: 418–430.

Two sources are believed to contribute to dendritic cell (DC)4 populations in cutaneous lymph nodes (cLN) (1). Blood-borne precursors enter via high endothelial venules (HEV) and give rise to subpopulations of CD11chighMHCII+ DCs that closely resemble those present in the spleen. Migratory DCs (m-DCs) leave the skin via afferent lymphatics and up-regulate MHCII that closely resemble those present in the spleen. Migratory DCs (m-DCs) represent a heterogeneous population with the phenotype Langerin+CD11b−. In the steady state, LCs preferentially migrated to the paracortical area of the LN and were distributed in a ring around the deeper T cell zone. In contrast, radiosensitive m-dDCs could be subdivided into three subsets on the basis of differential expression of Langerin and CD11b. The Langerin+/CD11b+ subset expressed high levels of MHCII, Langerin+/CD11b+, and Langerin+/CD11b+ m-dDC subsets corresponded to dDC subsets with the same phenotypes. More than half of the Langerin+ m-DCs in cLN were of dermal rather than epidermal origin. Skin painting with a contact-sensitizing agent revealed a complex regulation of skin DC migration to the draining cLN, with Langerin+/CD11b+ and Langerin+/CD11b+ dDC numbers peaking at 1 day, followed by Langerin+/CD11b+ dDCs at 2 days and Langerin+/LCs at 4 days. In addition, m-LCs and m-dDCs showed distinct patterns of costimulatory molecule expression in response to contact sensitization, with m-LCs up-regulating CD40 relatively
late, whereas m-dDCs rapidly up-regulated CD80 and CD86. Finally, by restricting expression of IE to LCs and m-LCs, we were able to show for the first time that they are able to process and present protein Ag in vivo to naive IE-restricted CD4+ T cells. Thus, multiple skin-derived DC subsets with differences in phenotype, migratory behavior, and anatomical distribution have the ability to interact specifically with naive T cells in draining cLN.

Materials and Methods

Mice

All mice were housed under specific pathogen-free conditions in the Centenary Institute (CI) Animal Facility. Ieα4 transgenic mouse line 107-1 on a C57Bl/6 CD45.2 background (hereafter termed IE') (14) expressed chimeric IeαIEββ molecules with a wild-type distribution while Ieα6 expression in line 36-2 (hereafter termed IE) (14) was restricted to thymic epithelia, making the mice tolerant of IE in the absence of expression on peripheral DCs or B cells. The IE' line was crossed with B6.SILPrpc1-H2d (H-2d CD45.1) mice to create an IE' CD45.1 homozygous line, while IE' mice were bred on rag1−/− mice on a C57Bl/6 background to create an IE'CD45.2rag1−/− homozygous line. TCR transgenic mice expressing the 5C.C7 TCR (Vα11 Vβ3) (15, 16) specific for the C0OH-terminal epitope of moth cytochrome c (MCC) in the context of Ieα' or Ieαββ' were bred on an IE'rag1−/− background. Approval for all animal experimentation was obtained from the Animal Ethics Committee at the University of Sydney.

Bone marrow (BM) chimeras

To create IE→IE' BM chimera mice, IE' CD45.2 hosts were irradiated with 1200Gy split-dose irradiation (2 doses 600Gy each, 3 h apart) and 24 h later received 5 × 10⁶ BM cells from IE' CD45.1 donors. All chimeric mice were allowed to reconstitute for at least 3 mo before use in experiments. At that time, chimeras contained fewer than 1% host B cells, 6% host CD8+ T cells, and 10% host CD4+ T cells. The NK cell, granulocyte, monocyte, and erythroid lineages showed 100% donor chimerism. To create IE→IE chimera mice, IE' CD45.1 hosts were sublethally irradiated with 600Gy or 700Gy and received a BM graft (5 × 10⁶ cells) consisting of IE'CD45.2rag1−/− BM mixed with host-type BM. The percentage of IE' donor BM varied from 20 to 40% in different experiments. Resulting chimeras had normal CD4+, CD8+, and B cell counts in the peripheral lymphoid organs and, as expected, IE' B cells were not present. In 40% IE'→IE' chimeras, CD45.2 myeloid reconstitution was on average 42% (range, 30–65%).

DC isolation

Spleens and LNs were digested with collagenase/DNase as described previously (17), using 0.3 mg/ml collagenase/disapase (Roche Diagnostics) and 0.02 mg/ml DNase (Sigma-Aldrich). Epidermal and dermal sheets were obtained from ears split into dorsal and ventral halves and incubated for 35 min at 37°C (Boehringer Mannheim). Epidermal and dermal sheets were then washed extensively in PBS before digestion with 0.3 mg/ml collagenase/disapase for 40 min (epidermal sheets) or 1.5 h (dermal sheets) at 37°C. Single-cell suspensions were prepared by passing the digestion product through an 80-gauge stainless steel mesh. Density gradient DC enrichment steps were omitted to avoid cell loss.

FACS analysis

Eight- and nine-color FACS analysis of single-cell suspensions was performed using an LSRII digital flow cytometer (BD Biosciences) for data acquisition and FlowJo software (Tree Star) for data analysis. All staining with mAbs was performed in FACS buffer (PBS containing 5% FCS, 10 mmol EDTA, 0.02% sodium azide) after blocking non-specific staining due to FcR binding with anti-CD16/32 (clone 2.4G2; CI). mAbs against CD11c, B220, CD45.1, CD45.2, CD4 (clone RM4–5), CD8 (clone 53-6.7). With the exception of anti-CD205, which was detected with anti-rat Ab conjugated with FITC (Jackson ImmunoResearch Laboratories), mAbs were directly conjugated with either FITC, PE, PerCP, cyanin conjugates PE-Cy7 and PerCP-Cy5.5, allophycocyanin, Al-
density gradient centrifugation (Histopaque-1083; Sigma-Aldrich). CD11b+ monocytes were then purified by magnetic selection using CD11b-FITC and anti-FITC MACS beads (Miltenyi Biotech). CD11b+ cells (35.5 \times 10^6 and 17.3 \times 10^6 cells per recipient for BM and blood, respectively) were injected i.v. into SFU-pretreated IE+ recipients painted with FITC 16 h earlier. Draining LNs (axillary, brachial, and inguinal) and spleens were collected 32 h later (48 h after FITC painting) and analyzed for the presence of IE+ DCs by FACS.

**Results**

Four distinct skin-derived m-DC populations in the cLNs of BM chimeric mice

To provide an unequivocal distinction between donor- and host-derived DCS in BM chimeric mice, we costained for two independent cell surface markers, CD45 and MHCII.
mouse strains with differential expression of a transgenic IEα-chain on a C57BL/6 (IEα−) background (14) were used to restrict IE expression to host- or donor-derived cells.

IE−→IE+ chimeras were made by replacing BM in lethally irradiated IE−CD45.1−CD45.2− hosts with BM from congenic IE−CD45.1−CD45.2− donors (Fig. 1A). Retention of host phenotype DCs in the epidermis was confirmed by flow cytometric analysis of skin DCs (Fig. 1, B and C). DCs with the host phenotype (IE−CD45.1−) constituted 95.7 ± 0.5% of epidermal DCs and on average 16.0 ± 2.7% of dDCs, consistent with the radioresistant dDC population identified by Bogunovic et al. (24) (Fig. 1C). In agreement with previous studies (4), host phenotype DCs were found exclusively in the MHCIIhighCD11cint (migratory) DC subset of cLNs and not in the MHCIIlowCD11chigh (blood-borne) DC subpopulation of LN or spleen (Fig. 1, D and E). Analysis of LNs, spleen, and thymus from IE−→IE+ chimeric mice showed that only LN draining skin contained significant numbers of host phenotype DCs (Fig. 2). The highest frequency of m-DCs was found in the inguinal and popliteal LNs (16% of all cLN-DCs) followed by auricular (9.3%), axillary (8.2%), brachial (5.5%), cervical (3.6%), and para-aortic LNs (0.9%). Fewer than 1% of DCs in pancreatic and mesenteric LNs and <0.1% in spleen and thymus expressed IE.

Analysis of Langerin/CD207 expression confirmed that IE+ cells in the cLNs were Langerin+, as expected, but more than half the Langerin− cells did not express IE (Fig. 1, F and G), as recently reported (8, 12, 13). Langerin expression was present in both IE− m-DC and IE+ m-dDC fractions of the MHCIIhigh CD11cint m-DC populations (Fig. 1H, left panel). Very few Langerin-expressing blood-borne DCs could be found in cLN (Fig. 1H, right panel). Further phenotypic analysis of cLN (Fig. 1I) revealed that Langerin+IE− m-DCs were CD11bhigh and CD103low (αεβ7 integrinlow), as recently reported (8). Interestingly, IE− m-dDCs could be divided into three subsets on the basis of Langerin and CD11b expression: a major Langerin+CD11b−CD103low subset, a Langerin+CD11blowCD103low subset and a Langerin−CD11blow subset that was CD103+ rather than universally CD103+ as reported by Bursch et al. (8). To determine whether the
three subsets were also present in the dermis, expression of CD11b and Langerin was measured on DCs from freshly isolated dermal sheets and compared with expression by fresh LCs (Fig. 1). Three dermal subsets could be identified (Langerin CID11b, Langerin CD11b low, and Langerin+CD11b bright), corresponding to the three m-dDC subsets in cLN (compare Fig. 1, J and I). Fresh LCs were uniformly Langerin CID11b.

We also examined reverse IE+→IE BM chimeras to confirm the presence of the three dDC subsets. To set up a paired IE+→IE vs IE→IE chimera model that would be suitable for functional studies, the relatively low percentage of IE+ m-DCs and the lack of IE expression by B cells in IE→IE BM chimeras were matched by transferring IE+CD45.1+BM, to produce DCs but no B cells, mixed with host-type IE BM.

FIGURE 3. BM-derived IE+ dDCs and m-dDCs in IE→IE chimeric mice. A, IE CD45.1 hosts were sublethally irradiated (700R), reconstituted with a mixture of BM cells from IE+CD45.2 BM donors and host-type BM cells (2 × 10^6 and 3 × 10^6 cells, respectively), and analyzed for donor DC chimerism 3 mo later. Donor-type DCs were gated as IE+CD45.1+CD45.2-. B, Donor DC chimerism in epidermis (top panel) and dermis (bottom panel) in a representative experiment using skin samples pooled from four to five animals. Skin DCs were gated as pan-CD45 MHCI CD11c cells (left panels). C, Skin DC chimerism in seven independent experiments. Mean value is shown as a bar and individual measurements as dots. D, Donor-type IE+ DCs in the spleen. B220- cells were excluded before gating DCs as shown in the left panel. E, Donor-type IE+ DCs were found in the migratory (gate R1) and blood-borne (gate R2) DC subsets of cLN. DCs were gated within B220- cells as shown in the left panel. F, In spleen and cLN, DCs were fixed, permeabilized, and stained for intracellular Langerin. Representative profiles are shown. G, A representative profile of IE and Langerin expression in the migratory (gate R1) and blood-borne (gate R2) subsets of the DCs in cLN. DCs were gated as shown in E. H, Representative profiles of CD11b, Langerin, and CD103 expression by m-DCs in cLN. m-DCs gated as shown in E were further subdivided into IE+ (left panels) and IE- (right panels) subsets. I, IE+ m-dDCs in cLN (H, top left panel) were analyzed for the frequency of CD11b+ Langerin-, CD11b+ Langerin-, and Langerin+ subsets in seven independent experiments. Mean value is shown as a bar and individual measurements as dots. J, A representative analysis of CD11b and Langerin expression by DCs in freshly isolated epidermal and dermal skin sheets. DCs were gated as shown in B.
CD45.1<sup>+</sup>CD45.2<sup>−</sup> BM into sublethally irradiated hosts (Fig. 3A). In the experiment shown in Fig. 3, 40% of the donor BM was IE<sup>−</sup>CD45.1<sup>−</sup>CD45.2<sup>+</sup> rag<sup>−/−</sup>, giving rise to a mixed chimera termed 40% IE<sup>−</sup>→IE<sup>+</sup>. Skin analysis (Fig. 3, B and C) demonstrated that rag<sup>−/−</sup> BM-derived IE<sup>−</sup>CD45.1<sup>−</sup> DCs were present in the dermis (36.5 ± 3.4%) but very few were found in the epidermis (3.4 ± 0.6%).

As expected, IE<sup>+</sup> DCs were present at a similar frequency in the spleen and the blood-borne DC subset of the cLN (Fig. 3, D and E). Within the MHCII<sup>high</sup> m-DC LN subset, IE<sup>+</sup> DCs were slightly less frequent, reflecting “dilution” by host-type IE<sup>−</sup> m-LCs (Fig. 3E). As in IE<sup>−</sup>→IE<sup>+</sup> chimeras, Langerin expression was not detected in the spleen or blood-borne LN DCs (Fig. 3G). When IE<sup>−</sup> and IE<sup>+</sup> m-DC subsets were analyzed for the expression of CD11b, Langerin, and CD103, IE<sup>−</sup> BM-derived m-DCs could be divided into the same three subsets that were present within the IE<sup>−</sup> BM-derived m-DCs in

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**Figura 4.** Distribution and cell surface phenotype of m-LC and m-dDC subsets in cLN in the steady state. A. Right panels: Popliteal LNs from IE<sup>−</sup>→IE<sup>+</sup> and 40% IE<sup>−</sup>→IE<sup>+</sup> mice were analyzed for IE expression (red) by immunofluorescence. Left panels: DAPI and B cell staining was performed on serial sections to identify anatomical structures within the LN (B, B cell follicle; M, medulla; T, T cell zone). Images shown are representative of at least five animals. B and C, Comparison of cell surface phenotype of m-LCs (IE<sup>−</sup> m-DCs in pooled auricular, axillary, brachial, and inguinal LNs from five IE<sup>−</sup>→IE<sup>+</sup> mice) and m-dDCs (IE<sup>−</sup> m-DCs in pooled auricular, axillary, brachial, and inguinal LNs from five IE<sup>−</sup>→IE<sup>+</sup> mice). B, Representative histograms showing expression of twelve markers by m-LCs (red filled histograms) and m-dDCs (bold blue lines). Markers are indicated along the x-axis. Expression of the relevant marker by splenic DCs (gray filled histograms) and blood-borne MHCII<sup>high</sup>CD11c<sup>++</sup> LN DCs (bold dashed line) is shown for comparison. Note that expression of CD11b, CD8 and CD205 was analyzed separately in the CD8<sup>+</sup> and CD8<sup>−</sup> splenic DC subsets. For CD207/Langerin, expression in freshly isolated epidermal LCs is shown for comparison. C, Mean fluorescence intensity (MFI) values were calculated for the histograms shown in B. Markers were grouped according to whether they were preferentially expressed by m-LCs or m-dDCs, and expression by blood-borne DCs is also shown for comparison. Blue diamonds, m-dDCs; red triangles, m-LCs; open circles, blood-borne LN-DCs; gray squares, splenic DCs (note that expression of CD205 and CD8 by blood-borne splenic and LN-DCs is shown for the CD8<sup>+</sup> DC subset only, and expression of CD11b by blood-borne and m-dDCs is shown for the CD11b<sup>+</sup> DC subset only). Data is representative of three independent experiments.
FIGURE 5. Contact sensitizer-induced migration of skin DC subsets to draining LNs. A 0.5% solution of FITC in dibutyl phthalate/acetone was painted onto a large area of abdominal skin of IE\(^{+}\)/H11002 and 20% IE\(^{-}\)/H11001 BM-chimeric mice. Groups of mice were painted on successive days so that the response on days 1, 2, 3, 4, 5, and 7 could be analyzed on a single day. Draining inguinal, brachial, and axillary LN were pooled from three to four animals before staining. 

A, Migration kinetics of m-DC subsets in IE\(^{+}\)/H11002 mice. DCs were gated as shown on the left. Boxes indicate gates for IE\(^{+}\)/H11001 Langerin\(^{+}\) m-LCs (solid lines) and IE\(^{+}\)/H11002 Langerin\(^{+}\) m-dDCs (dashed lines).

B, Migration kinetics of m-DC subsets in IE\(^{+}\)/H11001 mice. Top row, IE\(^{+}\) m-dDCs were gated as shown in the left panel and analyzed for the expression of CD11b and Langerin. The frequency of Langerin\(^{+}\) CD11b\(^{+}\), Langerin\(^{+}\) CD11b\(^{low}\), and
IE⁻→IE⁺ chimeras (compare Fig. 3H, left panel with Fig. 1I, right panel). The relative frequencies of the three m-dDC subsets were Langerin⁺CD11b⁺ 33.9 ± 4.8%, Langerin⁻CD11blow 31.9 ± 1.4%, and Langerin⁺CD11blow 26.6 ± 3.0% (Fig. 3I). Although CD11b and Langerin were both expressed bimodally in the IE⁺ m-DC population (a mixture of radiosensitive m-LCs and host BM-derived m-dDCs), Langerin⁺ m-dDC could not be clearly distinguished from Langerin⁺ m-LCs. The three subsets of IE⁻ dDCs were also identified in fresh skin samples (Fig. 3J).

Thus, four distinct subsets of skin-derived DCs can be identified in the cLN of BM chimeras: Langerin⁺CD11bint m-LCs, Langerin⁺CD11b⁺ m-dDCs, Langerin⁻CD11b⁺ m-dDCs, and Langerin⁺CD11blow m-dDCs.

Microanatomical distribution and cell surface phenotype of m-DC subsets in the steady state

Having confirmed that IE⁺ MHCII⁺CD11c⁻ DCs in the cLN of IE⁻→IE⁺ and 40% IE⁺→IE⁻ chimeras represent m-LCs and m-dDCs, respectively, we compared the location and cell surface...
phenotype of m-LCs and m-dDCs in the steady state (Fig. 4). IE+ m-LCs comprising 13% of total LN DCs were distributed in the outer paracortical area of the node, in a ring surrounding the deeper T cell area (Fig. 4A). In contrast, the mixture of m-dDCs and blood-borne DCs in 40% IE+/H110013 IE chimeras was scattered throughout the interfollicular, outer paracortical, and deep paracortical zones.

We next measured the expression of a number of cell surface markers, including MHCII and costimulatory molecules, in the steady state (Fig. 4, B and C). m-LCs expressed slightly higher levels of CD40 and CD205, and lower levels of CD134L/OX40L and CD80 than m-dDCs, while expression of non-transgenic MHCII (IAb), CD8, CD86, CD153, CD54, and CD70 was similar for all m-DCs. Expression of these molecules by m-dDCs was unimodal, in contrast to CD11b and Langerin, indicating that all three subsets of m-dDCs expressed them at similar levels in the steady state.

Differential migration of four subsets of skin DCs to the draining LN in response to FITC skin painting

To compare migration patterns of m-LCs and m-dDCs in response to a contact sensitizer, we painted a large area of abdominal skin of IE+/H11001 IE+/H11002 and IE+/H11001 IE mice with 0.5% FITC in acetone:dibutyl phthalate. Migration of IE+ DCs was measured over the course of the next 7 days (Fig. 5). The frequency of Langerin+ m-LCs decreased dramatically in the first 24 – 48 h after skin painting, followed by an increase to above the pretreatment value on day 4 (Fig. 5A, boxes with bold line, and Fig. 5B, bottom row, circled). The frequency of Langerin+ m-dDCs decreased ~2-fold in the first 24–48 h after painting but returned to baseline thereafter (Fig. 5A, boxed with dashed line and Fig. 5B, top row). The initial decrease in the frequency of Langerin+ m-LCs and m-dDCs was due to the dramatic increase in the two Langerin+ m-dDC subsets. Numbers of Langerin+ CD11b+ m-dDCs increased 14.4-fold with a peak at 24 h, while Langerin+CD11b-low m-dDCs increased 14.3-fold by 24 h and peaked at 48 h (15.9-fold increase) (Figs. 5C and 5D lower panel). These two subsets accounted for the majority of the 10-fold increase in m-DC within 24 h, from 1.6 ± 0.3 × 10^5 cells to 15.3 ± 0.3 × 10^5 cells (Fig. 5C). The Langerin+ dDCs also mobilized quickly and reached a maximum at 24 h, but showed only a 4.6-fold increase to 2.1 ± 0.4 × 10^5 cells. In contrast, LCs started to arrive in the draining cLN only 48 h after skin painting and peaked at day 4, when m-LCs represented 35–40% of all MHCII+ m-DCs (Fig. 5A and 5C, right panel). The peak number of m-LCs, 1.9 ± 0.6 × 10^5 cells, represented a 6.7-fold increase over baseline. Total Langerin+ DCs, representing a mixture of the epidermal and dermal Langerin+ DC subsets, peaked on day 3 (4.1-fold increase, or 3.1 ± 0.2 × 10^5 cells), consistent with previous findings in Langerin-GFP mice (9) (Fig. 5C, right panel).

Taken together, these results demonstrate that although all three dDC subsets are mobilized rapidly, the CD11b+Langerin+ and CD11b-lowLangerin+ dDC subsets predominate in the draining LN 24–48 h after skin painting, at the peak of the DC response. Although
Langerin+ dDCs are also mobilized rapidly, this subset represents only 10–13% of all MHCIImhigh m-DCs at 24–48 h. LCs arrive in the LN as total numbers of MHCIImhigh m-DCs are declining, and represent the majority of Langerin+ cells only on day 4, when they comprise slightly less than half of all MHCIImhigh m-DCs.

The differential response of the three dDC subsets to skin painting may reflect differences in the absolute numbers of dermal resident DCs (which would be expected to limit the number of DC that can be mobilized). However it does not preclude de novo DC differentiation induced by skin painting. In particular, monocytes have recently been suggested to contribute to DC responses by differentiating into skin DCs (25). To test the contribution of monocytes to dDC responses to FITC skin painting, IE+ mice were myeloablated with 5FU (to reduce recruitment of endogenous monocytes to the inflammatory site), painted with FITC, and adoptively transferred 16 h later with 35.5 × 106 or 17.3 × 106 CD11b+ monocytes purified from BM or blood, respectively, of IE+ mice. Then, 32 h after monocyte transfer (48 h after FITC painting), dLNs and spleens were isolated and analyzed for the presence of IE+ DCs (Fig. 6). Some monocyte-to-DC differentiation was observed in monocyte recipient mice (Fig. 6C, III and IV) but not in control mice that did not receive monocytes (Fig. 6C, I and II). Despite the high number of transferred cells, monocyte-derived DCs comprised fewer than 1% of the m-DC in the draining LN, a smaller proportion than the 1.5–2% of DCs in the spleen. Lack of FITC dye uptake by the monocyte-derived IE+ DCs suggests that IE+ DCs developed from monocytes in situ rather than via tissue migration. In two additional experiments in which significantly lower numbers of highly purified monocytes were adoptively transferred (4–5 × 105 of Gr1high and Gr1low cells/mouse), we failed to observe any monocyte-to-DC differentiation.

In conclusion, our results demonstrate that although monocytes can differentiate into DCs within 32 h, this mechanism does not contribute to the massive influx of skin DCs into the LNs in response to skin painting. Thus, the BM-derived IE+ DCs migrating to the cLNs of IE+–IE+ chimeric mice in response to skin painting with contact sensitizer were true dermal-resident DCs.

**Differential activation of LCs and dDCs in response to skin painting**

The surface phenotype of LCs and dDCs migrating to the LN in response to FITC skin painting was compared (Fig. 7). DC activation was not associated with FITC dye uptake, because activation patterns of FITC+ and FITC− DC subsets were identical, and similar changes were observed after painting with the vehicle (acetone:dibutyl phthalate) in the absence of FITC (not shown). Expression of CD80 and CD86 by m-DCs increased dramatically (3- to 5-fold) within 24 h of skin painting, coinciding with the peak of dDC migration to cLN (Fig. 7). In contrast, expression of CD80 by m-LCs always remained below the steady-state m-DC level and showed only a minor increase at 48 h. Expression of CD86 by m-LCs was increased 2.7-fold at 48 h. However, these transient increases did not coincide with peak LC migration and, thus, are most likely to represent bystander activation. In contrast, expression of CD40 steadily increased on m-LCs while the level on m-dDCs remained unchanged. There was no change in expression of CD54/ICAM1 following skin painting.

**Processing and presentation of Ag by m-DC subsets**

The effect of m-DCs on the response of T cells in the draining LN is dependent not only on cell surface phenotype and migratory kinetics, but also on their ability to process and present Ag.
compare the potency of Ag presentation by IE+DCs in IE→IE+ and IE+→IE− chimeras, we matched the number of IE+ DCs in the cLN of chimeric mice using 25% IE−→IE+ chimeras. 5C.C7 TCR transgenic CD4+ T cells with specificity for MCC in association with IE were used to detect processing and presentation of recombinant HELMCC protein (19, 26). Chimeric mice received a small cohort of naive 5C.C7 T cells labeled with CFSE, and were immunized s.c. with HELMCC emulsified in CFA the next day (Fig. 8A). The T cell response was assessed on day 3 after immunization by calculating recruitment into cell division and the number of divided T cells. Both m-LCs and m-dDCs could process HELMCC and recruit 5C.C7 T cells into division (Fig. 8, B and C). m-dDCs were more efficient in T cell priming than m-LCs, as both recruitment of T cells into division and the absolute number of 5C.C7 T cells 3 days after Ag challenge was higher in IE+→IE− mice as compared with IE−→IE+ mice (recruitment, 18.4 ± 3.4% and 33.5 ± 1.4%, respectively; number of 5C.C7 cells/mouse, 35 ± 8 × 10^3 and 109 ± 6 × 10^3, respectively). Similar results were obtained on day 5 (not shown) and after percutaneous immunization, measuring T cell proliferation on days 3 and 5 (not shown). The parallel kinetics of the responses to presentation by m-LCs and m-dDCs, despite the marked differences in their migration kinetics (Fig. 5), suggests that the CD4 T cell response was initially driven by processing and presentation of free Ag in the draining LN, rather than by migration of Ag-presenting DCs from the skin.

Discussion

LCs within skin draining LN have generally been identified by expression of Langerin together with high levels of CD205 and CD40 (9, 27, 28). By using reciprocal BM chimeras differing by two allelic markers, CD45 and MHCII IE, we have defined the surface phenotype of m-LCs and m-dDCs, showing that m-LCs cannot be unequivocally distinguished from m-dDCs using any of these markers (Fig. 4). Our results indicate that the majority of Langerin-expressing m-DCs in cLN s are of donor BM origin and derived from the dermis rather than the epidermis, consistent with three recently published reports using Langerin-diphtheria toxin receptor and Langerin-diphtheria toxin receptor→wild-type BM chimeric mice to study reconstitution and migration of Langerin+ dDCs after diphtheria toxin-mediated ablation (8, 12, 13). We have extended those studies by using the BM chimera approach to compare the migration kinetics of Langerin+ m-dDCs and m-LCs, and to show for the first time that m-dDCs and m-LCs have distinct activation responses. In addition, we have used differential expression of Ag-presenting MHC alleles to directly demonstrate that m-LCs can support proliferation of naive CD4 T cells in the absence of presentation by m-dDCs or resident blood-derived DCs.

For the first time, our study also demonstrates the existence of two distinct subsets of Langerin-negative dDCs and m-dDCs, distinguished by their pattern of CD11b expression, and by migratory behavior. The three dDC subsets, comprising Langerin+CD11bhigh, Langerin−CD11b−, and Langerin+CD11blow cells, are present in murine dermis and cLN in similar proportions. They may correspond to the recently described DC subsets located within human dermis: CD14+CD1a−“Langerin−”, a novel CD14+CD1a+“Langerin−” subset, and CD14+CD1a+“Langerin+” cells. The authors believed to represent epidermal LCs migrating via the dermis but which in fact may be true dDCs (29). It is likely that the mouse monocyte-associated marker CD11b defines a dDC subset analogous to CD14+DCs in humans; Langerin+CD11b−DCs in mice may be equivalent to human CD1a+Langerin−DCs.

In the steady state, m-LCs were distributed in the outer paracortical area of the node, in a ring surrounding the deeper T cell area (Fig. 4A). This suggests that m-LCs are located at or close to the cortical ridge of the node (30) where they are perfectly positioned to scan naive T cells arriving via HEVs situated in the same anatomical area. It is likely that migration of CCR7+ LCs to the cortical ridge is driven by CCL21 produced by LN stromal cells (31). Alferink et al. (32) also observed a “ring” of GFP+ cells under steady-state conditions in the cLNs of CCL17-GFP knockin mice. In situ production of CCL17 by Ag-loaded m-LCs in the cortical ridge would attract T cells arriving through HEVs. The ring-like distribution of m-LCs was not apparent in the previous study of Kissenpennig et al. (9) in which Langerin-GFP+ DCs were scattered throughout the cLNs, similar to the distribution we observed in IE−→IE+ chimeric mice (Fig. 4 A). Our results show that several DC subsets could have contributed to the scattered distribution of Langerin-GFP+ cells (9), namely Langerin+ m-LCs, Langerin− m-dDCs, and the CD8α subset of blood-borne DCs (9, 11).

Upon activation in response to skin painting, the migratory responses of LCs and the three subsets of dDCs were remarkably different (Fig. 5). Migration of dDCs to the LN peaked early (24–48 h), while the peak of LC migration was delayed until day 4. In our hands, the behavior of FITC+ and FITC− skin-derived DCs in cLN was indistinguishable, and both populations were included in our analysis of induced migratory capacity. This allowed us for the first time to calculate the relative and absolute contribution of each of the four skin-derived DC populations to the cLN response to skin painting. Due to the very large numbers of incoming m-dDCs, the proportion of m-LCs dropped to <3% of all LN m-DCs in the first 24 h. All three subsets of m-dDCs were represented during this early migration, with preference for the two Langerin− m-dDC subsets, which showed a 10- to 15-fold increase within the first 24–48 h. Migration of Langerin+ m-dDCs also peaked at 24 h but produced only a 5-fold increase in absolute number. This is in contrast to the recent report of Bursch et al. (8), who started their analysis of Langerin+ m-DC migration at 48 h. The peak of LC migration on day 4 also represented an ~5-fold increase over baseline. Only at this late time point did m-LCs represent a significant proportion of cLN DCs. In previous studies, the migratory response of Langerin+ cells, assumed to be LCs, was shown to peak at day 4 (9, 33). By using a BM chimera model, we were able for the first time to separate the early migration peak of Langerin+ dDCs from the late LC peak.

The under-representation of the Langerin+ m-dDC subset in cLN 24–48 h after skin painting could have been due to a relatively poor chemotactic response of Langerin+ dDCs to CCR7 ligands driving the migration of skin DCs, as was suggested by Poulin et al. (13) on the basis of in vitro observations. A differential chemotactic response of human DC subsets to CCR7 ligands has also been described, although in that case it was the CD14+CD1a−“Langerin−” subset that displayed poor chemotaxis (29). An alternative explanation for the relative under-representation of Langerin+ m-dDCs is that circulating DC precursors may have made a substantial contribution to the influx of Langerin− m-dDCs into the draining LN. In particular, monocytes are a readily available source of DC precursors that can enter inflamed tissues, differentiate into DCs (25) and contribute to T cell responses (25, 34). Such inflammation-driven monocyte-DC differentiation has been observed in vivo as early as 18–72 h after initiation of inflammation (25, 35), although the size of the monocyte contribution to the tissue DC pool remains unclear. In addition, whether monocyte differentiation into DCs requires recirculation through peripheral tissues or can result from direct entry into an
inflamed LN from blood is not yet established (35). Our experimental evidence did not support the notion of monocyte-Dicer differentiation contributing to the early influx of DCs after skin painting; as we could detect fewer than 1% monocyte-derived DCs in the draining LN, despite adoptive transfer of a large number of monocytes (Fig. 6). Not only was this percentage lower than that in the spleen, but none of the monocyte-derived DCs had acquired FITC from the skin. Thus, our data suggest that the DC migratory response to skin painting is maintained by dermal resident DCs in the early phase (24–48 h), and later by migrating LCs (4 days). However, we have not ruled out a contribution of monocytes to the DC response at later stages.

An important physiological consequence of the migration of dDCs and LCs to cLN is Ag presentation to T cells. Our chimeric model uniquely allows the measurement of CD4+ T cell responses to a specific protein Ag that is processed and presented by radioresistant DCs in the draining LN, despite adoptive transfer of a large number of monocytes (Fig. 6). Not only was this percentage lower than that in the spleen, but none of the monocyte-derived DCs had acquired FITC from the skin. Thus, our data suggest that the DC migratory response to skin painting is maintained by dermal resident DCs in the early phase (24–48 h), and later by migrating LCs (4 days). However, we have not ruled out a contribution of monocytes to the DC response at later stages.

An important physiological consequence of the migration of dDCs and LCs to cLN is Ag presentation to T cells. Our chimeric model uniquely allows the measurement of CD4+ T cell responses to a specific protein Ag that is processed and presented by radioresistant IE+ DCs in IE→IE+ mice or radiosensitive donor BM-derived DCs in IE+→IE+ mice. Presentation by IE+ DCs is absolutely required for SC.C7 T cell responses in our experimental model (19, 26). The data presented here unequivocally demonstrate for the first time that radioresistant m-LCs can initiate CD4+ T cell responses to s.c. Ag in the absence of presentation by BM-derived DCs (Fig. 8), although BM-derived DCs were superior Ag presenters as demonstrated by an ~2-fold larger SC.C7 T cell response in IE+→IE+ mice vs IE→IE+ mice. It is of particular interest that m-LCs could process and present protein Ag to naive CD4+ T cells in vivo in the absence of m-DCs, as the role of LCs in Ag presentation has been questioned in a number of recent publications (6, 9, 36, 37). In contrast, the kinetics of LC migration, particularly their late arrival in the LN draining the inflammatory site, in conjunction with relatively inefficient induction of the primary costimulatory molecules CD80 and CD86 (Fig. 7), suggests a modulatory/regulatory role for this DC subset. In support of such a role, activation of m-LCs by skin painting was quite distinct from that of m-DCs, which rapidly up-regulated expression of CD80 and CD86, consistent with their role in driving skin immune responses (28). In contrast, the major change in m-LC phenotype was an increase in CD40 expression on days 4–5, corresponding to the peak of LC migration. The small increase in CD86 on day 2 preceded LC migration and may have been due to local cytokine effects in the draining LN. Although previous studies have noted up-regulation of CD80 and CD40 by mDCs on day 1 after skin painting (27), our demonstration that epidermal and dermal-derived DCs express distinct activation programs over the first 7 days of the response to skin painting is an important new insight into their in vivo roles.

In summary, we have provided evidence of four m-DC subsets in mouse LN draining the skin. m-LCs and the three subsets of m-DCs defined by differential expression of CD11b and Langerin all expressed the range of molecules required for naive T cell activation in cLN. Both m-DC and m-LC subsets processed protein Ag and induced a primary CD4 T cell response in vivo. In addition, migrating dDCs, but not LCs, showed dramatic up-regulation of the B7 molecules CD80 and CD86. Thus, m-DCs and m-LCs display differential behavior in cLN while sharing the ability to interact specifically with T cells to control the immune response.

Acknowledgments

We thank Emma Gilchrist for assistance with flow staining of LN samples, Mary Mouawad for assistance with flow staining of skin samples, and the staff of the Centenary Institute Flow Cytometry and Animal Facilities for expert assistance.

Disclosures

The authors have no financial conflict of interest.

References


