Definition of Dendritic Cell Subpopulations Present in the Spleen, Peyer’s Patches, Lymph Nodes, and Skin of the Mouse

By Fabienne Anjuère, Pilar Martín, Isabel Ferrero, Marta López Fraga, Gloria Martínez del Hoyo, Natalia Wright, and Carlos Ardavín

DENDRITIC CELLS (DC) ARE antigen-presenting cells (APC) with a key function in the immune system as initiators of T-cell responses against microbial pathogens and tumors. Although during the past few years our understanding of DC biology has remarkably increased, a precise characterization of the different DC subpopulations remains to be achieved with regard to their phenotype and lineage relationships. In this report, we have extensively studied the DC subpopulations present in the thymus, spleen, Peyer’s patches, lymph nodes (LN) and skin of the mouse. Thymus DC and 60% spleen DC have a lymphoid DC phenotype, ie, CD8⁺ DEC-205high Mac-1low, whereas 40% spleen DC have a myeloid DC phenotype, ie, CD8⁻ DEC-205low Mac-1high. Both CD8⁺ and CD8⁻ DC are leukocyte function-associated antigen-1 (LFA-1)high and highly adherent. Within Peyer’s patches the majority of DC correspond to the CD8⁺ DEC-205high Mac-1low lymphoid category. In the LN, together with CD8⁺ and CD8⁻ DC, an additional nonadherent CD8⁻ LFA-1⁻ subpopulation with lymphoid DC characteristics is described. Finally, in the skin both epidermal Langerhans cells (LC) and dermal DC are CD8⁻ DEC-205high Mac-1high, and do not express LFA-1. Interestingly, LC migration experiments indicate that LC underwent the upregulation of CD8 and LFA-1 upon migration to the LN, supporting the hypothesis that LC belong to the CD8⁻ lymphoid lineage.

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MATERIALS AND METHODS

Animals. BALB/c and C57BL/6 mice were purchased from IFFA Credo (L’Arbresle, France). In all experiments 5- to 7-week-old female mice were used.

Isolation of DC from the thymus, spleen, Peyer’s patches, and lymph nodes. DC were purified from thymus, spleen, Peyer’s patches, and mesenteric and peripheral (auricular, axillar, and inguinal) LN, following an isolation protocol modified from our previously described method that avoids DC culture.11,12 Organs were cut into small fragments and then digested with collagenase A (0.5 mg/mL; Boehringer-
keratinocytes were depleted by treating the recovered low-density cells for 50 minutes at 4°C with a monoclonal antibody (MoAb) mixture including anti-CD3 (clone KT3-1.1), anti-CD4 (clone GK1.5), anti-IL-2Rα (clone PC61.5), antimacrophage antigen F4/80 (clone C1A3-1) and anti-granulocyte antigen Gr1 (clone RB6-8C5). The unwanted cells were then removed magnetically after incubation for 30 minutes at 4°C with a 1:1 mixture of antismouse Ig and antirat Ig coated magnetic beads, (Dynabeads, Dynal, Oslo, Norway) at a 7.1 bead-to-cell ratio. Flow cytometry analysis of the DC-enriched preparations obtained by this isolation method showed that they had a purity greater than 75% for the thymus, spleen, and Peyer’s patch DC preparations, and greater than 90% for the mesenteric and peripheral LN DC preparations, as assessed by their CD11c expression (data not shown). Subsequent phenotypic analysis of the different DC subsets was performed after gating for CD11c+ cells.

**LC migration assay.** BALB/c mice received 10 µL of 1% fluorescein isothiocyanate (FITC) (Sigma) dissolved in 1:1 acetone: dibutylylphosphate (ADBP), on the dorsum of both ears following the protocol described by Cumberbatch et al. After 18 hours, 48 hours, or 5 days, the draining auricular LN DC were isolated as described.

**Flow cytometry.** Phenotypic analysis of DC subpopulations was performed after triple staining with FITC-conjugated anti-CD11c (clone N418, hamster IgG), phycoerythrin (PE)-conjugated anti-CD8a (clone CT-CD8a, rat IgG2a; Caltag, San Francisco, CA) and biotin-conjugated anti-Mac-1 (clone M1/70, rat IgG2b), anti-DEC-205 (clone N418, rat IgG2b) antibodies and streptavidin-tricolor (Caltag). Analysis of the phenotype of CD11c+ cells in LC-migration assays was performed after double staining with PE-conjugated anti-CD8α and biotin-conjugated anti-CD11c, anti–Mac-1, anti–DEC-205, or anti–LFA-1 followed by streptavidin-tricolor Ig isotype-matched control antibody stainings for rat IgG2a MoAbs were performed with anti-B220 (clone RA3-6B2, rat IgG2a) and shown in Fig 2. Equivalent background staining profiles were obtained with the nonreactive MoAbs anti-CD69 (clone H.1.2F3, hamster IgG) and anti–TCR-Vα11 (clone RR8.1, rat IgG2b), used as isotype-matched control antibodies for hamster IgG and rat IgG2b MoAbs, respectively (data not shown). All the staining steps were performed at 0° to 4°C in PBS containing 5 mM/L EDTA and 2% FCS. Analysis was performed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) at the Flow Cytometry Laboratories of the Faculty of Biology and the Fundación Jiménez Díaz (Madrid, Spain), using Lyssys II and PC-Lyssys softwares (Becton Dickinson).

**RESULTS**

DC have been characterized on the basis of their antigen presentation potential and their phenotype in a variety of lymphoid and nonlymphoid organs, using sophisticated isolation strategies. Although different DC subsets display specific functional and phenotypic features, they share a characteristic phenotypical profile: in the mouse, DC are considered to express MHC II+, CD11c+, B7.2+, CD40+, HSA+, CD3−, CD4−, B220−, Igα, Igβ, Gr1−. In addition, as shown in Fig 1 the differential expression of other cell surface molecules, such as CD8, Mac-1, DEC-205, and LFA-1 allows a precise definition of DC subpopulations in the thymus, spleen, Peyer’s patches, LN, and skin of BALB/c mice. Equivalent DC subpopulations can be defined in C57BL/6 mice, although minor differences in the proportion of the different splenic and LN DC subpopulations were observed (data not shown).
Spleen DC. Spleen DC can be subdivided in two splenic DC subpopulations on the basis of CD8 expression (A and B, Fig 1). CD8$^+$ and CD8$^-$ subsets represent approximately 60% and 40% of total splenic DC, respectively. CD8$^+$ splenic DC have the same phenotype than thymic DC (not shown), i.e., they are CD8$^+$, express the endocytic receptor DEC-205 recognized by the MoAb NLDC-145, display low levels of the myeloid marker Mac-1, and have high levels of LFA-1, FcR, B7-2, and CD40 on their surface (Fig 2). On the other hand, CD8$^-$ splenic DC are DEC-205 low Mac-1 high, display high levels of LFA-1 and FcR, and express the costimulatory molecules B7-2 and CD40 at lower levels than CD8$^+$ splenic DC. Our results concerning a differential CD40 expression by splenic CD8$^+$ and CD8$^-$ DC are in agreement with Vremec and Shortman, although in this report the level of CD40 expression by both splenic DC subsets was lower than that described here, probably due to differences in the reagents used to detect this molecule by flow cytometry.

As discussed below, on the basis of their phenotypic, developmental, and functional characteristics, CD8$^+$ and CD8$^-$ DC have been defined as lymphoid and myeloid DC, respectively.

Peyer's patch DC. The majority (around 70%) of DC isolated from mouse Peyer's patches belong to the CD8$^+$ lymphoid category, and consequently express DEC-205 and...
LFA-1 at high levels and low levels of Mac-1 (Fig 1). The rest of Peyer’s patch DC includes around 10% of CD8⁺ myeloid DC and around 20% of DC displaying intermediate levels of CD8. These CD8⁺⁺ DC are similar to those found in the peripheral LN (see below).

**LN DC.** As illustrated in Fig 1 and 2, mesenteric and peripheral LN DCs can be subdivided in three DC subpopulations. Apart from a CD8⁺ DEC-205high Mac-1low lymphoid DC subpopulation and a CD8⁺⁻ DEC-205low Mac-1high myeloid DC subpopulation equivalent to those described in the spleen, a third DC subpopulation displaying high levels of DEC-205, and from low to intermediate levels of CD8, can be defined (C, Fig 1). The expression of CD8 and DEC-205 by the CD8⁺⁺ LN DC subset supports the view that it belongs to the lymphoid category. In support of this hypothesis, in a recent report Inaba et al. have shown that LN DC displaying intermediate levels of CD8 are functionally equivalent to the CD8⁺⁺ splenic DC.

CD8⁺⁺, CD8⁺⁻, and CD8⁻ LN DC subpopulations represent approximately 20%, 60%, and 20% respectively in the mesenteric LN, and 20%, 65%, and 15% in the peripheral LN. Forward scatter analysis showed that lymphoid DC, ie, CD8⁺⁺ DC from the thymus, spleen, Peyer’s patches, and LN, and as well as CD8⁺⁻ from LN, are bigger than myeloid DC, ie, CD8⁻ DC from the spleen and LN (Fig 2).

The comparative analysis of CD8⁺⁻ DC from mesenteric and peripheral LN (Fig 1 and 2, Table 1) showed that mesenteric LN CD8⁺⁻ DC comprise two subpopulations defined on the basis of the CD8 versus LFA-1 expression (C1 and C2 in Fig 1). Around 50% are CD8⁺⁻high LFA-1low (C1 cells) whereas the remaining 50% are CD8⁺⁻low LFA-1high (C2 cells). Both C1 and C2 cells are Mac-1low FcR⁺⁺. On the other hand, peripheral LN CD8⁺⁻ DC constitute a single population of CD8⁺⁻low LFA-1high cells (C2’ cells), similar to the C2 population described in the mesenteric LN, although in contrast to the latter, peripheral LN CD8⁺⁻ DC
display intermediate to high levels of Mac-1 and FcR. With regard to C1 and C2 CD8\textsuperscript{int} DC subpopulations described in the mesenteric LNs, their functional significance is currently being analyzed.

Interestingly, as shown in Fig 3, the analysis of the adherence capacity of DC purified from mesenteric or peripheral LN showed that both the CD8\textsuperscript{1} and CD8\textsuperscript{2} DC subsets, which display high LFA-1 levels, were strongly adherent. On the other hand, CD8\textsuperscript{int} DC from mesenteric LN (including the previously described C1 and C2 subpopulations), as well as CD8\textsuperscript{int} DC from peripheral LN, were nonadherent. Interestingly, these CD8\textsuperscript{int} nonadherent DC displayed low to intermediate levels of LFA-1. Additional adherence assays have shown that thymic DC, and both CD8\textsuperscript{1} and CD8\textsuperscript{2} spleen DC, are adherent cells, whereas skin DC are nonadherent (data not shown).

**Skin DC**. Epidermal DC, known as epidermal LC, constitute a single population with a distinctive phenotype (Fig 1). LC are CD11c\textsuperscript{1}, express neither CD8 nor LFA-1, and display high levels of Mac-1 and FcR, but intermediate levels of B7-2 and CD40 (Fig 2). In addition after isolation, a majority of LC express low levels of DEC-205, but around 30% are DEC-205\textsuperscript{high}. As previously reported\textsuperscript{16} and as indicated in Materials and Methods, DEC-205 was partially degraded by the trypsin treatment employed during the isolation process. However, after overnight culture at 37°C, LC underwent an upregulation of DEC-205, and all LC became DEC-205\textsuperscript{high}, displaying this marker at an expression level comparable to that found on CD8\textsuperscript{+} DC from the thymus, spleen, or LN (Fig 1), according to Inaba et al.\textsuperscript{16} Importantly, trypsin treatment did not affect either CD8 or LFA-1 expression, as shown by trypsin treatment assays performed in the same conditions on mesenteric LN and by the fact that some lymphocytes present in the epidermal LC preparations obtained after trypsin treatment, displayed CD8 and LFA-1 expression levels comparable to that of untreated peripheral LN lymphocytes (data not shown).

LC have been shown to represent immature DC that differentiate into mature DC when they migrate to the T-cell areas of the draining lymph nodes after an antigenic stimulation.\textsuperscript{20} Because most LN DC express intermediate to high levels of CD8 and are positive for DEC-205, it can be speculated that LC might represent immature DC of the lymphoid type, which acquire a mature lymphoid DC phenotype upon stimulation and migration. To test this hypothesis, we analyzed the phenotype of LC migrating to the peripheral draining LN after exposure to contact-sensitizing chemicals. For this purpose, BALB/c mice received 10 µL of 1% FITC in ADBP on the dorsum of both ears, following the method described by Cumberbatch et al.\textsuperscript{18} After skin sensitization, the phenotype of the FITC\textsuperscript{+} LC that had migrated to the draining auricular LN was analyzed by purifying the DC from the draining LN. As illustrated in Fig 4, showing the phenotype of purified auricular LN DC 5 days after skin sensitization, FITC\textsuperscript{+} cells were exclusively found within

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Table 1. Phenotypic Characteristics of CD8\textsuperscript{int} DC Present in the Mesenteric and Peripheral Lymph Nodes

Fig 3. Phenotype of adherent and nonadherent DC subsets from mesenteric and peripheral LN. Mesenteric and peripheral LN DC were purified as described, incubated for 90 minutes at 37°C, and the nonadherent and adherent fractions collected and analyzed for the expression of CD8 and LFA-1. The contour plots show the LFA-1 versus CD8 profiles of adherent and nonadherent DC after gating for CD11c\textsuperscript{+} cells. The dotted lines mark the lower limit defining the expression at high levels of the corresponding marker. These data are representative of four experiments with similar results.
the CD8\textsuperscript{int} DC subpopulation and represent around 20% of total DC from the draining LN. Similar results were obtained when the LN were analyzed 18 hours or 48 hours after skin sensitization (data not shown). FITC\textsuperscript{1} DC had an almost identical phenotype than FITC\textsuperscript{2} CD8\textsuperscript{int} DC present in the auricular LN. No FITC\textsuperscript{1} cells were found within the auricular T-cell or B-cell populations (data not shown). Consequently, these data suggest that at least some peripheral LN CD8\textsuperscript{int} DC derive from LC that have migrated to the draining LN from the epidermis, and that LC migration was accompanied by the upregulation of CD8 and LFA-1, as illustrated in Fig 5.

However, because a population of DC located in the dermis has been described,\textsuperscript{17} together with epidermal LC, within the human and mouse skin, in our experiments dermal DC could have also been induced to migrate after skin sensitization and could contribute to the FITC\textsuperscript{+} DC population found in the draining LN. In fact, it could be speculated that FITC\textsuperscript{+} CD8\textsuperscript{int} DC derive from dermal DC, and not from epidermal LC as we have proposed above. Nevertheless, as it has been shown that skin sensitization induces epidermal LC migration to the LN,\textsuperscript{18,21-23} if only dermal DC but not epidermal LC were the precursors of FITC\textsuperscript{+} CD8\textsuperscript{int} cells found in the LN, we would also expect to find FITC\textsuperscript{+} CD8\textsuperscript{+} DC corresponding to migrating FITC\textsuperscript{+} LC in the draining LN. However, our results clearly
show that FITC+ DC found in the draining LN constitute a well-defined homogenous CD8int population. In addition, the compared phenotypic analysis of epidermal and dermal DC shown in Fig 6 indicates that in the mouse, both populations have an almost identical phenotype, according to Lenz et al.,17 suggesting a close lineage relationship between them. More importantly, our data indicate that both skin DC subsets did not express either CD8 or LFA-1, and therefore they strongly support the rationale that CD8 and LFA-1 intermediate levels found on at least a proportion of FITC+ LN DC in LC migration experiments are the result of CD8 and LFA-1 upregulation by LC.

Note that the phenotype of epidermal LC performed on LC isolated from epidermal sheets after culture, basically coincides with the analysis performed on freshly isolated LC presented in Fig 2. However, in agreement with previous reports15,24,25 because the isolation method employed to isolate in parallel both skin DC subsets involves a 24-hour incubation step at 37°C, upon culture LC underwent the upregulation of CD40, B7-2, and MHC II and the downregulation of Mac-1 and FcR, but their expression of CD8 and LFA-1 remained unchanged (see Fig 2 and 6; data corresponding to B7-2 and MHC II are not shown).

**DISCUSSION**

Although a wide variety of DC subsets have been described in different lymphoid and nonlymphoid organs of the mouse,26 two main DC categories, ie, lymphoid and myeloid DC, can be characterized on the basis of their origin, phenotypic profile, and physiological properties. Lymphoid DC, such as thymic DC or CD8+ splenic DC, are defined as CD8+, DEC-205high, Mac-1low, whereas myeloid DC, such as CD8– splenic DC are CD8–, DEC-205low, Mac-1high. Evidence of the lymphoid origin of CD8+ DC derives from thymus reconstitution experiments showing that mouse thymic DC originate intrathymically from the CD4low lymphoid precursor population that has no myeloid potential.13 Later, Wu et al5 reported that CD8+ but not CD8– splenic DC are generated after intravenous transfer of CD4low lymphoid precursors. Additional experiments have shown the differential capacity of CD8+ and CD8– spleen DC to induce the stimulation of CD4+ and CD8+ peripheral T cells, to phagocytose zymosan particles, and to secrete IL-12.7,10 Importantly, attempts to generate in vitro CD8+ splenic DC from CD8– splenic DC have been unsuccessful so far, suggesting that the two splenic DC subpopulations belong, as proposed previously,5 to different cell lineages and do not represent different differentiation or maturation stages of a unique DC population. In this sense, it has been recently reported that in mice with a deletion at the C terminus of the Ikaros gene (Ikaros CΔC mice),12 CD8+ but not CD8– splenic DC were generated. Interestingly, these mice displayed some T-cell differentiation and had a normal development of myeloid cells but lacked B and NK cells, providing further evidence that CD8+ DCs are related to the T-cell lineage. Besides, these data might indicate that CD8+ DC do not belong to the myeloid lineage but rather share some differentiation requirements with B and NK cells.

The data derived from skin sensitization-mediated LC-migration experiments showed that some CD8int DC present in the peripheral LN derive from LC that have migrated from the epidermis, and that LC migration involves CD8 and LFA-1 upregulation. In this sense, CD8int DC may represent recent immigrants reaching the LN as a consequence of an antigenic stimulation and/or a recirculation process. In this sense, differences in Mac-1 and FcR expression between mesenteric and peripheral LN CD8int-low DC may reflect their respective provenance, because mesenteric LN are known to drain the spleen and intestinal mucosa whereas peripheral LN drain essentially the skin. With regard to Mac-1 expression, our data illustrate that upon migration LC partially downregulated this molecule, although they still expressed Mac-1 at high levels, comparable to that expressed by CD8int FITC+ DC found in the auricular LN of the FITC-treated mice (Fig 4), or by CD8int DC from control peripheral LN (Fig 1). Therefore, the expression of Mac-1 by CD8int DC from the peripheral LN most likely reflects the fact that at least a proportion of these cells derive from skin LC that express high levels of Mac-1.

On the other hand, as discussed above, the FITC+ CD8int DC population obtained in the draining LN after skin sensitization may be result of the migration of both epidermal LC and dermal DC. The relative contribution of LC to the FITC+ CD8int DC

![Fig 6. Comparative phenotypic analysis of epidermal LC and dermal DC. The histograms show the expression of the indicated markers by epidermal LC and dermal DC isolated from epidermal and dermal sheets respectively, after 24-hour culture as described. Note that [] represent the FSC. These data are representative of three experiments with similar results.](image-url)
population would most likely be more important than that of dermal DC because our experiments of skin DC purification, performed in parallel with epidermal and dermal sheets, indicate that epidermal LC and dermal DC are present in the adult mouse ear skin at a 5:1 ratio. In support of this rationale, it has been recently reported that TNF-α or oxazolone-induced LC migration to the draining LN was blocked by antibodies against the α6 integrin subunit, which is expressed by epidermal LC but not by LN DC.23

Our data strongly suggest that at least a proportion of FITC+/CD8⁺ LN DC derive from epidermal LC, and therefore that LC undergo the upregulation of CD8 and LFA-1 upon migration. Therefore, LC acquire a lymphoid DC-like phenotype upon migration to the LN, suggesting that they belong to the lymphoid CD8⁺ DC lineage. This hypothesis is supported by the fact that LC have been shown to migrate to the T-cell areas of the lymphoid organs on antigen stimulation,26 and that CD8⁺ DC have been shown to be located in the T-cell areas of the spleen and LN.10,19

Concerning LC lineage, classically, LC have been considered to be of myeloid origin,1 although this hypothesis has not been formally shown. In fact, DC were also globally considered as myeloid precursor-derived, until it was shown in the mouse that the CD8⁻ DC category derived from lymphoid precursors.13 In this sense, the origin and precursors of the so-called myeloid DC, not expressing CD8 in the mouse, also remain undefined. Similarly, the immediate precursors of LC have not yet been identified. In the first instance, the observation that in mice homozygous for a dominant-negative mutation in the Ikaros gene (Ikaros DN⁻/⁻ mice), T cells, B cells and splenic DC did not develop, whereas LC and myeloid cells were produced,27 suggested a correlation between LC and the myeloid lineage. However, the analysis of bone marrow chimeric mice reconstituted with Ikaros DN⁻/⁻ precursor cells12 showed that Ikaros DN⁻/⁻ mice might have an intrinsic defect in LC/DC differentiation, the deficiency in the generation of T and B cells being accompanied by a blockade in DC differentiation at an immature LC stage. Thus the data derived from Ikaros-deficient mice have not provided an explanation so far for the correlation between DC and LC lineages. Therefore further experiments are needed to conclusively determine the origin of epidermal LC.

In conclusion, our results support the view that lymphoid DC comprise thymus DC, CD8⁺ spleen DC, CD8⁺/high and CD8⁺/low Peyer’s patch and LN DC as well as LC, whereas CD8⁻ DC from spleen and LN represent the so-called myeloid DC. Although, as mentioned before, functional differences between mouse CD8⁺ and CD8⁻ splenic DC have been reported,7,8 a precise analysis of the APC capacity of the different DC subsets has to be achieved, and importantly, their respective precursors remain to be characterized. In this sense, although thymus DC have been shown to derive from thymic CD4⁺/low precursors,13 the progenitors of both CD8⁺ and CD8⁻ peripheral DC continue to be largely unknown. The definition of DC precursors might be specially relevant to define the best source of DC to obtain in vitro the large number of DC required for antitumor therapeutic purposes. On the other hand, it is important to take into account that DC differentiated in vitro may differ in their phenotype and APC function depending on the culture conditions and on their origin.1 Therefore, the study of the precursors and function of the different DC subtypes is crucial to fully exploit the DC tumor immunotherapy potential, because it can provide the information required to define the most adequate experimental conditions to induce an optimal DC-mediated T-cell antitumor response.

ACKNOWLEDGMENT

The authors thank Anton Rolink for the anti-CD40 hybridoma FGK45 (Basel Institute for Immunology, Basel, Switzerland), and the Flow Cytometry facility of the Fundación Jiménez Díaz for making it possible to perform FACS analysis after hours.

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