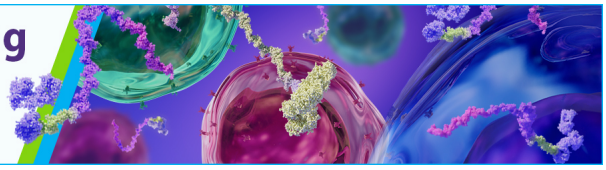

The Journal of Immunology

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J Immunol 2001; 167:741-748; ;
doi: 10.4049/jimmunol.167.2.741
http://www.jimmunol.org/content/167/2/741

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

The Dendritic Cell Populations of Mouse Lymph Nodes[1]

Sandrine Henri,[2]* David Vremec,* Arun Kamath,* Jason Waithman,* Stuart Williams,† Christophe Benoist,‡ Kim Burnham,§ Sem Saeland,¶ Emanuela Handman,* and Ken Shortman*

The dendritic cells (DC) of mouse lymph nodes (LN) were isolated, analyzed for surface markers, and compared with those of spleen. Low to moderate staining of LN DC for CD4 and low staining for CD8 was shown to be attributable to pickup of these markers from T cells. Excluding this artifact, five LN DC subsets could be delineated. They included the three populations found in spleen ($CD4^+8^-$DEC-205^-, $CD4^-8^-$DEC-205^-, $CD4^-8^+$DEC-205^+), although the CD4-expressing DC were of low incidence. LN DC included two additional populations, characterized by relatively low expression of CD8 but moderate or high expression of DEC-205. Both appeared among the DC migrating out of skin into LN, but only one was restricted to skin-draining LN and was identified as the mature form of epidermal Langerhans cells (LC). The putative LC-derived DC displayed the following properties: large size; high levels of class II MHC, which persisted to some extent even in CIITA null mice; expression of very high levels of DEC-205 and of CD40; expression of many myeloid surface markers; and no expression of CD4 and only low to moderate expression of CD8. The putative LC-derived DC among skin emigrants and in LN also showed strong intracellular staining of langerin. ***The Journal of Immunology,*** **2001, 167: 741–748.**

The Ag-presenting dendritic cells (DC)[3] of mouse and man are heterogeneous as judged by surface Ag markers. The incidence of the different DC subtypes varies with the tissue examined. We have delineated three main DC subtypes in mouse spleen (1). These are $CD4^+CD8^-$DEC-205^-CD11b^+, $CD4^-CD8^-$DEC-205^-CD11b^+, and $CD4^-CD8^+$DEC-205^+CD11b^-. The CD8 on the DC is in the form of an $\alpha\alpha$ homodimer rather than the $\alpha\beta$ heterodimer of T cells. Cell kinetic and other evidence indicated that these three mature spleen DC subtypes are the products of separate developmental streams, rather than being maturation steps within a single DC lineage (2). This is in line with earlier evidence that the cytokine requirements and transcription factors involved in DC development differ for the $CD8^+$ and the $CD8^-$ DC (3–6). In mouse thymus, the analysis of the DC populations is complicated by the pickup by the DC of Ags, including CD8$\alpha\beta$ and CD4, derived from T-lineage thymocytes (1). If such pickup is eliminated, thymic DC appear to be all $CD4^-$DEC-205^+CD11b^-, but do include major CD8$\alpha\alpha^+$ and minor $CD8^-$ subsets (1). Our earlier studies indicated that the $CD8^+$ DC of the thymus are of lymphoid-precursor rather than myeloid-precursor origin (reviewed in Refs. 7–9). Arguing mainly by analogy, we proposed that the $CD8^+$ DC of the spleen were also of lymphoid origin (7–9). However, recent evidence (L. Wu, unpublished observations, and Ref. 10) indicates that although some splenic $CD8^+$ DC derive from a lymphoid precursor, many appear to be of myeloid precursor origin. Therefore, CD8 expression is a poor indicator of the lymphoid precursor origin (5, 11–13). Nevertheless, CD8 marks a group of DC with a developmental history differing from that of $CD8^-$ DC.

The DC populations of the lymph nodes (LN) appeared to be still more complex than those of the spleen, although we had not assessed LN DC in the light of the additional populations we now distinguish in spleen and thymus nor considered the problems of Ag pickup. We previously had noted the presence in LN of a $CD8^-$DEC-205^+ subtype, which is largely absent from spleen (11). This may correspond to the minor DC subtype present in the thymus but obscured by the pickup of CD8$\alpha\beta$ from T cells. The s.c. LN (CLN), those draining the skin, would also be expected to include the mature form of epidermal Langerhans cells (LC; Ref. 14), absent from thymus, spleen, or mesenteric LN (MLN). It is not clear what proportion of CLN DC would be mature LC. These should be differentially labeled by painting the skin surface with a fluorescent dye (15, 16), but this may label dermal- as well as epidermal-derived DC (14, 17, 18). Studies by Anjuère et al. (16), Salomon et al. (15), and Ruedl et al. (18) suggested that the mature LC in unstimulated normal mouse CLN could be distinguished from other LN DC by their slow turnover, by being larger cells expressing the highest levels of class II MHC, and by the surprising marker combination $CD11b^+CD8^{int}$ (12, 16).

In this report, we compare the DC populations of LN with those of the spleen and delineate two additional DC subtypes present in LN, but not evident in spleen. We examine the extent of pickup of T cell-derived markers, which could obscure the characterization of LN DC. We also assess the phenotype of the putative mature LC in CLN, by comparison with the DC in MLN, by tracking skin-derived DC with a fluorescent dye, and by examining the DC that exit from cultured skin explants. The results suggest that more than one type of DC enter CLN from the skin. The mature LC in normal

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Received for publication February 13, 2001. Accepted for publication May 8, 2001.

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[1] This work was supported by the National Health and Medical Research Council, Australia and the United Nations Development Program/World Bank/World Health Organization Special Program for Research and Training in Tropical Diseases. A.K. was supported by a collaborative grant from Edward Jenner Institute for Vaccine Research.

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[3] Abbreviations used in this paper: DC, dendritic cells; LN, lymph nodes; CLN, s.c. LN; MLN, mesenteric LN; LC, Langerhans cells; TRITC, tetramethylrhodamine-5-(and-6)-isothiocyanate; int, intermediate.

0022-1767/01/$02.00

CLN may be distinguished by a much higher expression of a number of typical DC activation markers by a very high level of class II MHC only partially controlled by the class II MHC regulating factor CIITA (19–21) and by the presence in the cytoplasm of residual langerin, a molecule associated with the Birbeck granules and the Ag-processing system of LC (22, 23).

Materials and Methods

Mice

Mice were bred under specific pathogen-free conditions at Walter and Eliza Hall Institute animal breeding facility. Most experiments used female C57BL/6J Wehi mice at 5–7 wk of age. For constructing bone marrow chimeras, the recipients were C57BL/6 Ly5.1 Pep^{3b} mice aged 8–12 wk. The bone marrow donors included the above strains, as well as CD8$\alpha^{-/-}$ C57BL/6 mice and $CD4^{-/-}$ C57BL/6 mice, the latter two strains originally obtained from T. Mak (Ontario Cancer Institute, Toronto, Canada). The $CIITA^{-/-}$ mice were obtained directly from the Institut de Génétique et de Biologie Moléculaire et Cellulaire (Strasbourg, France); their generation is described elsewhere (19, 21).

Bone marrow chimeric mice

Bone marrow chimeric mice were constructed as described previously (1). Briefly, 3×10^6 bone marrow cells from wild-type Ly5.1 mice together with 3×10^6 bone marrow cells from $CD8^{-/-}$ or $CD4^{-/-}$ Ly5.2 mice were injected into lethally irradiated Ly5.1 mice. For each such experimental group, a control group involving transfer of wild-type Ly5.1 bone marrow together with wild-type Ly5.2 bone marrow into the Ly5.1 recipients was set up. Six weeks later, the separate types of LN were taken from the pooled experimental or control groups and the DC isolated for analysis.

Lymphoid organs

The lymphoid organs used for DC isolation and comparative analysis were spleen, MLN, and CLN, the CNL being a pool of axillary, brachial, and inguinal LN. For experiments on the $CIITA^{-/-}$ mice, inguinal LN and brachial LN were studied separately. For experiments on DC migration after labeling the skin of the ears, auricular LN were used.

DC isolation procedure

The DC isolation procedure was based on that described recently (1, 2). Briefly, LN or spleen fragments from 8–16 mice were digested for 20 min at room temperature with collagenase-DNase and then treated for 5 min with EDTA to disrupt T cell-DC complexes. All subsequent procedures were at 0–4°C in a Ca^{2+}- and Mg^{2+}-free medium. Light-density cells were selected by centrifugation in a Nycodenz medium (Nycomed, Oslo, Norway). For LN DC, a density of 1.082 g/cm^3, 4°C, 310 milliosmolar was used, giving a slightly better yield with equivalent purity compared with the density of 1.077g/cm^3 optimal for spleen DC isolation. Cells not of the DC lineage were then depleted by incubating the cells with previously optimized amounts of anti-CD3 (KT3), anti-Thy1 (T24/31.7), anti- B220 (RA3-6B2), anti-Gr-1 (RB6-8C5) and anti-erythrocyte (TER-119) and then removing the Ab-binding cells with anti-rat Ig-coupled magnetic beads (Dynabeads; Dynal, Oslo, Norway). Note that the anti-CD4, anti-F4/80, and anti-CD11b, used in earlier versions of the procedure (24, 25), were all omitted from the depletion mix to avoid loss of the $CD4^+8^-$ DC population (1). Note also that anti-B220, omitted in error from a previous description (1), was included. The LN DC at this stage were ~90% pure. The preparation then usually was subjected to presorting to remove 5–10% autofluorescent cells (1), or used directly for immunofluorescent labeling and analysis by flow cytometry with gating to eliminate a low level of autofluorescent cells (1).

For isolation of DC from single LN, the initial light density selection step was omitted, but the immunomagnetic bead depletion was retained. Reduced medium volumes and small-sized tubes were used throughout. Presorting was omitted. The 10% DC in the enriched preparations then were gated as $CD11c^+$ cells.

DC migration from mouse ear skin on culture

The DC migration procedure was modified from that of Schuler and Steinman (26), with exit of DC from the skin enhanced by chemokine following the approach of Kellerman et al. (27). The ears were removed from 10–20 mice, cleared of hair, and briefly washed in 70% ethanol. The ears were placed ventral-side down and split, removing the dorsal skin from the cartilage. The dorsal skin was placed split-side down in 1 ml of modified mouse osmolarity RPMI 1640 culture medium containing 10% FCS (2, 25) for 2–4 h at 37°C in a humidified 10% CO_2-in-air incubator to eliminate the many non-DC initially released into the culture. The skin then was transferred onto another 1-ml culture medium, this time containing 0.1 μg of recombinant mouse 6Ckine (R&D Systems, Minneapolis MN) to enhance DC migration. After 24 h of further incubation at 37°C, the cells that had migrated into the culture medium were harvested and kept in cold medium. The skin was transferred onto fresh warm medium containing 6Ckine and then incubated a further 24 h at 37°C. The cells that migrated out of the skin over the first and second 24-h incubations then were pooled. The yield averaged $3–6 \times 10^4$ cells per ear. The cells then were immunofluorescent-stained and analyzed as for the LN DC preparation. For direct comparison with skin-derived DC, a CLN DC preparation was incubated 24 h at 37°C in the same 6Ckine-containing culture medium.

Immunofluorescent labeling and flow cytometric analysis of DC preparations

The mAb, the fluorescent conjugates, the labeling procedure, and the flow cytometric analysis details all have been described previously (1, 2, 24, 25). The mAb HD24 recognizing an intracellular epitope of murine and human langerin was provided by Schering-Plough (Dardilly, France) and was conjugated to FITC in this laboratory. In most experiments, presorting before immunofluorescent labeling was used to eliminate 5–10% autofluorescent non-DC in the enriched DC preparations (1). In some experiments in which autofluorescence was minimal (<5%), autofluorescent cells were gated out along with dead cells in the propidium iodide channel. Propidium iodide was included in the final wash to label and exclude dead cells in all experiments except those involving langerin staining. In the analysis of the DC-enriched preparations, anti-class II MHC or anti-CD11c or both together were used to stain, define, and gate DC, along with gating for the high-side and forward light-scatter characteristics of DC. The other mAb stains then were used to analyze and subdivide these gated DC. To stain for the cytoplasmic domain of langerin, cells were fixed and permeabilized after surface staining as above. Cells were fixed in a 1% formaldehyde-2% glucose-5 mM sodium azide solution for 20 min at room temperature, washed, and then permeabilized with 0.1% saponin-2% FCS-EDTA-balanced salt solution. This permeabilization medium then was used as the subsequent medium for staining. The cells were centrifuged and then blocked with anti-FcR Ab (2.4G2) for 15 min. The FITC-conjugated mAb HD24 or FITC-conjugated isotype-matched control was added and incubated with the cells for 30 min before washing and analysis.

Fluorescent labeling of DC in ear skin

The approach was similar to that of Anjuère et al. (16) and Cumberbatch et al. (28). Tetramethylrhodamine-5- (and-6)-isothiocyanate (TRITC) and FITC were obtained from Molecular Probes (Eugene, OR). A 10% solution of TRITC or FITC was made in DMSO and then diluted to 1% in a solvent of acetone-dibutylphthalate, 1:1. The dorsal side of both ears of C57BL/6 mice was painted with 10 μl of either of these 1% fluorochrome solutions. After 24–48 h, the auricular LN were removed and DC isolated as described above.

Results

Comparison of spleen and LN DC

In previous studies by us (24, 25) and by others, selective loss of DC subpopulations and the complications caused by autofluorescent cells and pickup of material from T cells (1) would have distorted the immunofluorescent staining and the analysis of LN DC populations. Therefore, we compared the DC populations of CLN, MLN, and spleen using isolation and analysis conditions that avoid these problems (1). Presorting was used to remove any autofluorescent macrophage-like cells. The immunomagnetic bead cell depletion procedure adopted avoided the loss of the $CD4^+$ $F4/80^+$ DC population (1). Results were basically the same if no immunodepletion at all was used, but were then less crisp because of overlap with the fluorescence distribution of non-DC contaminants. The CD4, CD8, and DEC-205 staining of the LN DC was examined in detail, because those markers had proved particularly useful for segregating spleen DC (1, 2), as Salomon et al. (15) first noted staining for CD4 on murine DC when analyzing LN, and Anjuère et al. (12, 16) reported CD8 expression by mature LC in LN. The expression of a series of markers on LN DC is given in

Fig. 1, and an example of a comparative multiparameter analysis is given in Fig. 2.

All LN, like spleen, contained a CD8α^-DEC-205$^-$ population (Fig. 2, population 1). All LN, like spleen, contained a proportion of DC staining strongly for CD8α, but not for CD8β (Fig. 2, population 2). However, in contrast to spleen, LN DC and especially CLN DC included many more cells showing intermediate and low levels of CD8α staining (Figs. 1 and 2). LN of all types contained some DC staining brightly for CD4, but the proportion was much less than in spleen (Fig. 1). However, LN DC included many cells showing intermediate and low levels of CD4 staining (Fig. 1). The few CD4^{high} cells in LN were all CD8$^-$, as in spleen (data not shown). Because these CD4^{high} DC were a minority population in LN, as opposed to spleen (1), they are grouped together in Fig. 2 and elsewhere with the CD4$^-$8$^-$ DC that they most resemble.

LN of all types contained a higher proportion of DC expressing DEC-205 and high levels of class II MHC than did spleen (Figs. 1 and 2). As we have noted previously (11), DEC-205 staining did not correlate with CD8 staining in LN DC, in marked contrast to spleen DC. Although all CD8α^{high} DC were DEC-205$^+$, as in spleen (Fig. 2, population 2), LN contained additional major groups of DEC-205$^+$ DC, which stained negative to intermediate for CD8α (Fig. 2, populations 3 and 4).

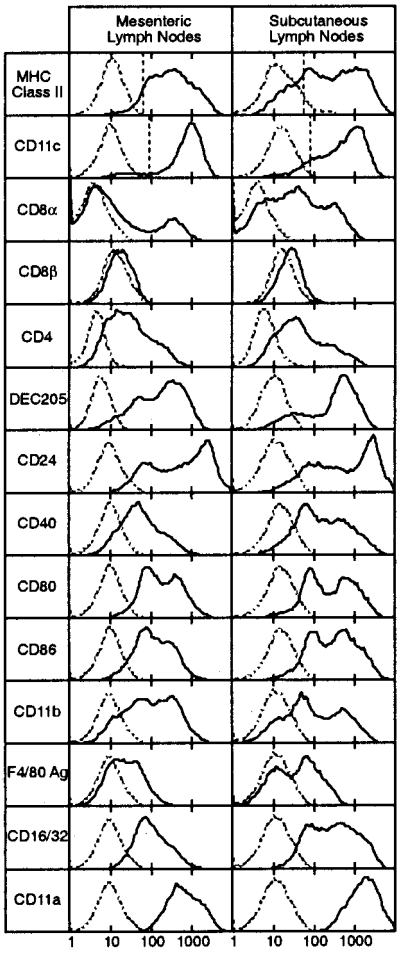

FIGURE 1. Comparison of the surface staining of the DC of MLN and CLN. DC were isolated from LN pooled from 8–16 mice. The DC preparations were stained for MHC class II and CD11c together with two other markers and then analyzed by four-color flow cytometry. Note the staining for class II MHC was very bright, but used a mAb of low fluorochrome conjugation to maintain on-scale fluorescence. The vertical broken lines indicate the typical gates set to define MHC class II$^+$ CD11c$^+$ DC in all subsequent analyses. The faint dotted line indicates the background fluorescence omitting only the relevant conjugated mAb. Details of the mAb and fluorochromes used are in *Materials and Methods*. Each histogram is typical of the results of 3–12 experiments.

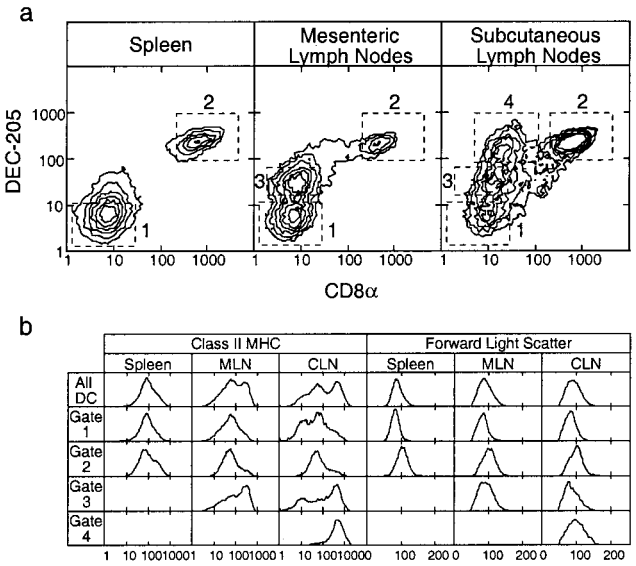

FIGURE 2. Multiparameter analysis of the surface phenotype of DC in spleen, MLN, and CLN. DC were isolated and stained as in Fig. 1 and then gated on CD11c$^+$ cells to define DC. *a*, DEC-205 vs CD8α distribution for the CD11c$^+$-gated DC. The broken lines define populations 1, 2, 3, and 4 in subsequent analyses. Note that population 1 includes CD4$^+$ and CD4$^-$ DC, as described elsewhere (1), and so combines two distinct DC subsets. *b*, Distribution of class II MHC and DC size, as revealed by forward light scatter, on the populations gated as in *a*. Results are typical of over 12 experiments on spleen DC and 4 experiments on LN DC.

Differences between DC in MLN and CLN

It was evident that as well as differences between spleen DC and LN DC in general, there also were particular differences between the DC found in CLN compared with MLN. Because the CLN might be expected to contain skin-derived DC not present in MLN, a more extensive comparison of the surface markers was undertaken (Figs. 1 and 2).

The DC showing high levels of CD8α staining did not differ significantly between MLN and CLN, when many samples were compared. The DC showing intermediate levels of CD8α staining were variable in incidence, but usually more numerous in CLN. Importantly, neither LN group showed bright or intermediate staining for CD8β, even though the anti-CD8β mAb used gave bright staining of T cells; thus, both CD8^{high} and CD8^{int} cells were CD8$\alpha\alpha$, as for spleen DC (1, 24). However, low-level staining was seen for both CD8α and CD8β, suggesting these weakly staining DC had CD8$\alpha\beta$ on their surface.

The level of class II MHC expressed by LN DC was in general higher than that on spleen DC, but CLN contained in addition a group of around 25% of DC with exceptionally high surface class II MHC levels, higher than on MLN (Figs. 1 and 2*b*). Note that staining for class II MHC deliberately used a low fluorochrome-to-protein conjugation ratio on the mAb to keep the fluorescence distribution on-scale and allow accurate color compensation; thus, the actual level compared with other markers is much higher than indicated. These class II MHC^{high} DC also were relatively large in size, as judged by the forward light-scatter profiles (Fig. 2*b*). Such a high expression of class II MHC on larger-sized cells has been considered a marker of LC-derived DC in CLN (15).

CLN also contained a distinct population of DC showing only moderate staining with anti-CD8, but expressing DEC-205 at levels as high or higher than on CD8^{high} DC. This population was seen only at marginal levels in MLN and not detected in spleen (Figs. 1 and 2, population 4). This distinct group of DEC-205^{high} CD8^{low} DC corresponded to the large-sized MHC class II^{high} cells and so were putative LC-derived DC. CLN also contained some DC expressing very high levels of the activation markers CD40, CD80, and CD86, levels higher than seen on spleen or MLN DC (Fig. 1). In agreement with the results of Anjuère et al. (16) a high expression of CD11a also was observed (Fig. 1). Cross-correlation studies (data not shown) indicated that these DC also corresponded to the DEC-205^{high} CD8^{low} class II MHC^{high} group (Fig. 2, population 4).

Not all markers characteristic of CLN mapped to the same DC population. CLN contained more DC staining especially strongly for the low-affinity Fc receptor CD16/32 (Fig. 1). However, only part of these CD16/32^{high} cells overlapped population 4 (Fig. 2), the remainder being DC expressing lower levels of class II MHC and DEC-205. CLN also contained more DC expressing intermediate rather than high levels of CD11c (Fig. 1). However, these did not correlate with population 4 (Fig. 2), and such DC have been suggested to be monocyte-derived rather than LC-derived (17).

Overall, these results suggested that the mature forms of skin-derived DC, including mature LC, are likely to be within the CLN DC expressing especially high levels of class II MHC, DEC-205, CD40, CD80, CD86, and CD11a but staining only weakly for CD8α and CD4.

Tests for authentic CD4 and CD8 expression by LN DC

The low to moderate levels of staining for CD4 and CD8 among LN DC recalled the situation in the thymus where such staining was attributable to pickup of Ags from T lineage thymocytes (1). The finding that at least the low-level staining for CD8 involved both CD8α and CD8β on the DC surface was indirect evidence that this was attributable to pickup from CD8$\alpha\beta$ T cells. To distinguish authentic CD4 or CD8 expression by the DC themselves from staining attributable to pickup of Ag from associated T cells, bone marrow chimeras were constructed. Irradiated mice were reconstituted with mixes of wild-type and CD4$^{-/-}$ bone marrow, or mixes of wild-type and CD8$\alpha^{-/-}$ bone marrow, using a Ly5 allotype difference to distinguish DC derived from the normal or from the gene-knockout bone marrow. If staining was lost from the gene-knockout DC, despite the presence of T cells expressing CD4 and CD8, the staining of the wild-type DC was considered to be authentic; if staining persisted on DC lacking the relevant gene, it must have been attributable to material picked up from wild-type T cells.

As shown in Fig. 3*a*, staining for both high and intermediate levels of CD8α disappeared from CLN and MLN DC when they were derived from CD8$\alpha^{-/-}$ mice, indicating authentic expression by the DC themselves. However, the shoulder of low-level CD8α staining persisted in the CD8$\alpha^{-/-}$ DC, so this lowest level of staining appeared to reflect Ag pickup. This pickup was more than previously seen from spleen DC, but less than seen with thymic DC (1). It was observed consistently that the DC expressing intermediate levels of CD8$\alpha\alpha$ were less frequent in the CLN of these reconstituted animals than in normal mice, suggesting that these DC were slow to be replenished from bone marrow.

As shown in Fig. 3*b*, the highest level of CD4 staining on a small population of LN DC disappeared from the DC derived from CD4$^{-/-}$ mice, indicating these few DC displayed authentic CD4 expression. However, much of the CD4 staining, at intermediate to low levels above the background, persisted in the DC derived from CD4$^{-/-}$ mice, and so had been derived from associated T cells. This level of pickup of CD4 was far more than seen with splenic DC, was greater than for CD8α on the LN DC, and it resembled the CD4 pickup among thymic DC (1).

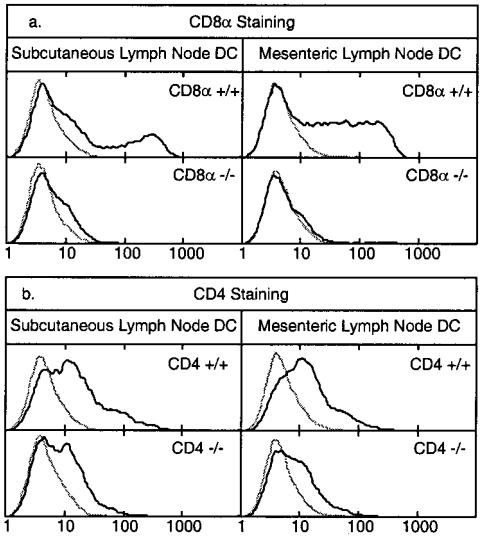

FIGURE 3. Tests for authentic CD8α and CD4 expression on LN DC. Bone marrow chimeric mice were constructed by injecting irradiated Ly5.1 mice with a mixture of bone marrow cells from Ly5.1 wild-type mice and bone marrow cells from Ly5.2 mice that were either CD8α null (*a*) or CD4 null (*b*). LN DC were isolated from these bone-marrow chimeras and stained for CD11c to define DC, for CD8α or CD4, and for Ly5.2 expression to gate for the null (−/−) vs wild-type (+/+) bone marrow-derived DC. A loss of CD4 or CD8α staining in the respective gene-deficient DC indicated that the staining of the wild-type DC represented authentic expression by the DC rather than pickup from associated T cells. Each result represents a single experiment, with a second experiment giving equivalent results.

The effect of the CIITA regulatory factor on class II MHC expression by LN DC

Because the surface levels of class II MHC seemed particularly high among CLN DC, the role of CIITA, a regulator of class II MHC expression, was examined (19). In a recent reassessment, such CIITA$^{-/-}$ mice were found to have a population of DC in certain LN, but not spleen, which retained class II MHC expression, albeit at a lower level (19). To determine which DC retained the class II MHC expression in CIITA$^{-/-}$ mice, DC in the inguinal LN, brachial LN, and MLN were isolated and analyzed. Because only a few CIITA$^{-/-}$ mice were available, the isolation procedures were modified to allow DC isolation from single LN. The expression of CD11c was used as the criterion to gate for DC in the partially enriched LN preparations, although this still allowed some non-DC contaminants to appear in the analysis. The gated DC then were analyzed for CD8α, DEC-205, and class II MHC, as shown in Fig. 4, as well as for CD40 (data not shown).

A small proportion of DC retaining clear class II MHC expression in CIITA$^{-/-}$ mice were present in inguinal LN (Fig. 4) and at a slightly reduced incidence in brachial LN (not shown), but were not present at significant levels in MLN (Fig. 4). This agreed with earlier immunofluorescent staining of tissue sections (data not shown). The class II MHC^{+} DC in CIITA$^{-/-}$ mouse inguinal (and brachial) LN were among the DC in these nodes staining brightest for DEC-205 (Fig. 4) and for CD40 (data not shown). Most of these class II MHC^{+} DC also showed intermediate staining for CD8α but none showed high staining for CD8α (data not shown).

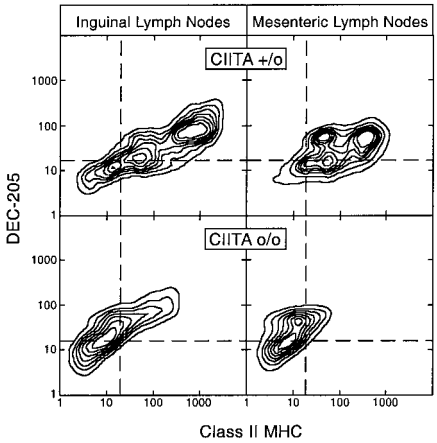

FIGURE 4. The effect of CIITA gene deletion on class II MHC expression by LN DC. DC were isolated from inguinal LN and MLN of CIITA null (0/0) or CIITA heterogeneous (+/0) mice and stained for CD11c (to define DC), for DEC-205 and for class II MHC. The results show the reduced but positive class II MHC staining seen in DEC-205$^+$ DC from the inguinal LN. Similar results were obtained in two other experiments.

Although these class II MHC$^+$ DC were those showing the highest staining for DEC-205 and CD40 in these CIITA$^{-/-}$ mice, the actual level of DEC-205 staining and of CD40 staining was below that of the corresponding bright staining DC in the control CIITA$^{+/-}$ mice. This suggested that the surface expression of many markers was (directly or indirectly) influenced by the absence of the CIITA gene. In addition, the DC numbers were ~2-fold lower in the CIITA$^{-/-}$ mice. Despite this difference in numbers and absolute staining levels, the results suggested that the DC retaining class II MHC expression corresponded to the class II MHC^{high} DEC-205^{high} CD40^{high} DC in CLN (Fig. 2, population 4), the DC suspected of being skin-derived LC.

The nature of the DC that migrate out of cultured skin explants

To help distinguish the LC and other skin-derived DC in CLN, the cells that migrated out of the mouse ear skin explants in culture were isolated, immunofluorescent stained, and analyzed. These DC recently derived from skin might be expected to be initially less mature than their equivalents in CLN. However, the procedure involves incubation of migrating cells in culture medium containing 6Ckine at 37°C, which could itself induce further maturation. Accordingly, these skin-derived DC were compared with a side-by-side sample of CLN DC, which had been incubated in the same culture medium for the same average time as the cells exiting from the skin. The comparison is shown in Fig. 5.

The cells migrating out of skin included a small proportion of class II MHC$^-$CD11c$^-$ cells, which were gated out of the analysis as non-DC. The level of class II MHC on the migrating skin DC was high, but no higher than on incubated CLN DC overall. This was mainly because most of the CLN DC expressing lower levels of class II MHC (Fig. 3) had, on incubation, changed to expressing high levels on the cell surface. Likewise, CLN DC expressing these lower levels of CD40, CD80, and CD86 (Fig. 1) had all up-regulated these markers to a higher level on incubation. However, the migrating DC expressed still higher levels of these activation markers, indicating that the skin-derived DC already possess or readily acquire a very activated phenotype.

The DC migrating from skin expressed CD11b, F4/80 and CD32/16 at moderate levels, although much lower than the levels typical of macrophages. They were all clearly negative for CD4 and CD8α. Thus they expressed low levels of characteristic myeloid markers, but did not express characteristic lymphoid markers.

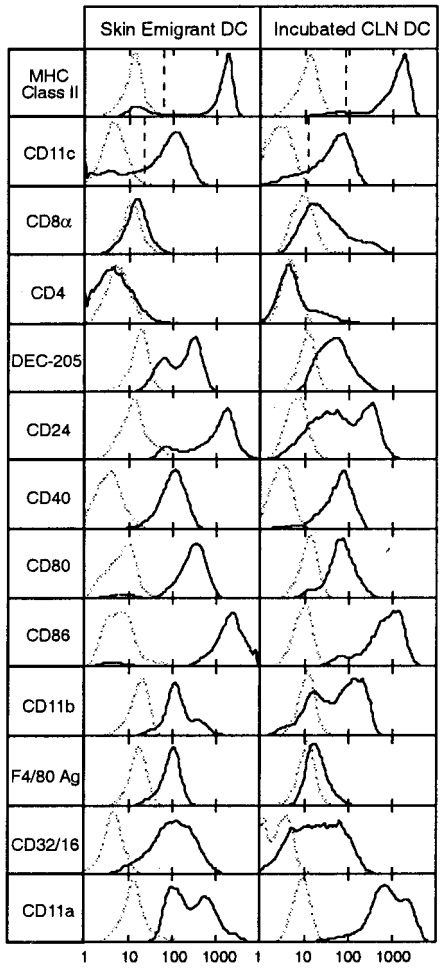

FIGURE 5. The surface phenotype of the DC that migrate out of mouse ear skin in culture. Split ear skin from 10–20 mice was incubated for 2 days in medium containing 6Ckine, and the cells migrating into the medium were collected in separate 1-day pools and then combined for staining and analysis as detailed in Fig. 1. For direct comparison, a preparation of CLN DC was incubated for 24 h in the 6Ckine-containing medium. Each histogram is typical of results from three to six experiments.

The DC migrating out of the skin expressed high levels of CD24, higher than on most incubated CLN DC. The migrating DC also were all positive for DEC-205, but always displayed two peaks of low and high DEC-205 expression. Two distinct peaks of DEC-205 fluorescence also were seen if the cells were fixed and permeabilized before staining, showing that the distinction reflected total DEC-205 levels and not just a difference in the proportion on the DC surface (data not shown). The migrating DC that were DEC-205^{high} surface-stained more brightly than the majority of incubated CLN DC, with only 20% of incubated CLN DC showing this high level of staining. This reinforces the picture from Figs. 1 and 2, suggesting that very high DEC-205 expression is a useful marker of one type of skin-derived DC.

Tracking skin-derived DC into LN

To mark in a more direct way LN DC that originated from skin, mouse ear skin was painted with FITC or TRITC and the fluorescent DC was tracked in the draining auricular LN. A discrete population of FITC or TRITC-labeled DC could be sorted from unlabeled LN DC and from autofluorescent contaminants by using

two fluorescence channels. An example with TRITC labeling is shown in Fig. 6. The sorted fluorescence positive DC and the negative DC then were stained in the other fluorescence colors for other DC markers and analyzed. The results were similar from days 1–3 after painting the ears, after which the FITC or TRITC fluorescence declined. Fig. 6 shows the results after day 1, when the label is more likely to be associated with the original migrating DC. These results were the same when only the cells with the brightest FITC or TRITC fluorescence were selected, rather than the total fluorescent population being gated as in Fig. 6. Again this is an argument against the label being passed from the original migrating DC into secondary LN DC. The label was found only in the draining auricular LN, with the adjacent cervical LN being negative. The TRITC-positive DC were analyzed and compared with the total DC of the auricular LN as shown in Fig. 6. Note that the unlabeled DC also should contain unlabeled skin-derived DC that arrived in the LN before painting.

The TRITC$^+$ DC were entirely among the larger DC and among those expressing the highest levels of class II MHC, CD40, and CD32/16, in agreement with the properties of DC migrating out of skin explants in culture. No TRITC$^+$ DC expressed high levels of CD8α, even 3 days after painting the ears. A very low-level CD8 staining on most cells was obtained, similar to the pickup level of Fig. 3*a*, together with a small population of the TRITC$^+$ DC showing intermediate CD8 staining. The TRITC$^+$ DC differed somewhat from the DC from skin explant cultures (Fig. 5) in showing reduced levels of DC with high expression of CD24 and CD11a. It is not clear whether this was attributable to down-regulation after the migrating DC reached the LN or to an artificial up-regulation of these markers during the culture required to collect the migrating DC. Overall, the putative skin-derived TRITC$^+$ LN DC represented a restricted subgroup of LN DC, but were not homogeneous, resembling a mix of populations 3 and 4 of CLN (Fig. 2). They resembled by most markers the DC migrating out of cultured skin explants.

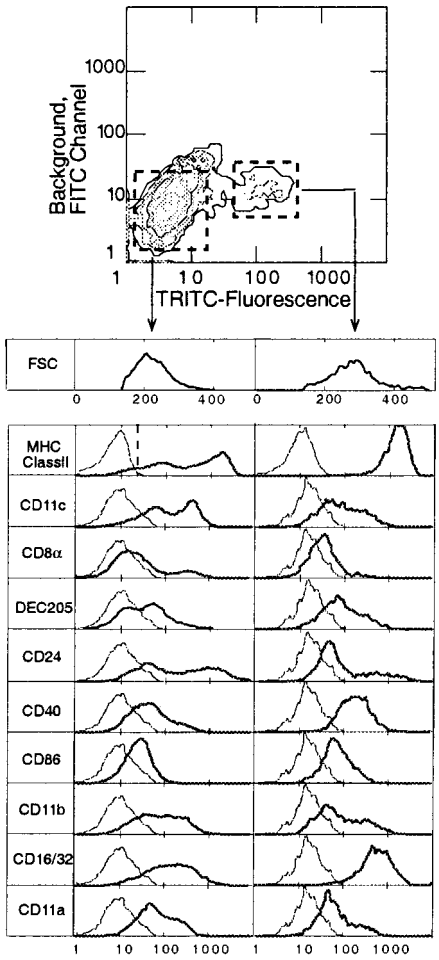

FIGURE 6. The surface phenotype of the TRITC-labeled DC found in draining LN after painting ear skin with TRITC. One day after painting the skin of both ears of mice, the auricular LN were removed and then the DC were isolated and the fluorescent cells sorted and separated from any autofluorescent cells, using the gates shown in the upper panel. The sorted cells then were immunofluorescent-labeled, analyzed, and gated for MHC class II$^+$ DC as in Fig. 1. Each histogram is typical of three separate experiments. Similar findings were obtained with FITC to paint the ear skin, but it then was necessary to avoid FITC-conjugated mAb.

Detecting LC-derived DC with langerin as a marker

It seemed likely that the DC migrating into LN from skin consisted of maturing forms of LC derived from the epidermis and dermal DC resembling the interstitial DC common to many tissues. To selectively label LC-derived DC, the DC were permeabilized and stained for cytoplasmic langerin, a marker of the Ag-processing system of LC (22, 23). Because Ag processing decreases as LC mature into T cell-stimulating DC, there was no guarantee that the langerin marker would persist. However, DC containing high levels of langerin were detected in both the DC migrating out of ear skin and in CLN as shown in Fig. 7. Surprisingly, a shoulder of moderate staining for langerin was obtained in DC from other tissues, including spleen and MLN (Fig. 7), and an above background shoulder of staining was seen in B cells (Fig. 7) and T cells (data not shown). The basis and specificity of this low-level staining was not established. It was concluded that only high-level expression of langerin could be used as a LC marker.

Of the DC migrating out of the skin explants, only the DEC-205^{high} subset stained strongly for langerin, marking them as LC

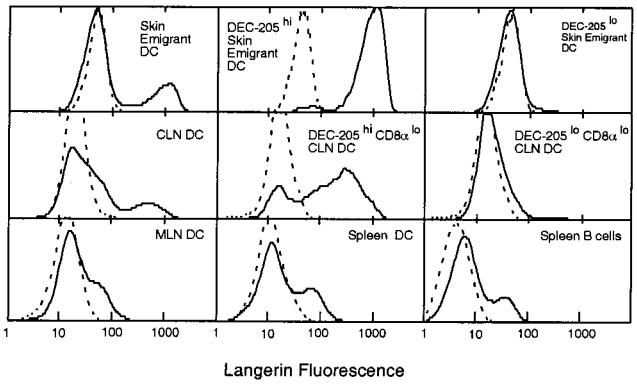

FIGURE 7. Cytoplasmic staining for langerin in DC from different sources. DC from spleen and LN were isolated and surface stained as in Figs. 1 and 2. DC migrating out of cultured ear skin were isolated and surface stained as in Fig. 5. The DC then were fixed, permeabilized, and stained for intracellular langerin with FITC-conjugated HD24 mAb (solid line) or with a FITC-conjugated isotype matched control Ab (broken line). Isolated splenic B cells were likewise stained as a control. The cells then were analyzed by flow cytometry, gating total DC as class II MHC$^+$, subdividing the skin emigrant DC into DEC-205^{high} and DEC-205^{low} as in Fig. 5, or gating the CLN DC into DEC-205^{high}CD8^{low} (fraction 5) or DEC-205^{low}CD8^{low} (fraction 1) as in Fig. 2. Only the high-level langerin staining was considered specific for LC-related DC. Results represent one of two separate experiments with FITC-conjugated HD24 mAb. Similar results, but with lower fluorescence staining, also were obtained with nonconjugated mAb in a two-stage staining procedure.

derived. The langerin$^-$ DEC-205^{low} population presumably represented dermal DC. Within CLN, very bright cytoplasmic staining for langerin was seen only in those DC showing the highest staining for class II MHC and DEC-205, but only lower staining for CD8α, marking population 4 of Fig. 2 as LC-derived or LC-related DC (Fig. 7), in confirmation of the previous conclusions.

Discussion

This study has confirmed that the DC population in LN is more complex than that in spleen or thymus. As well as the CD4$^+$8$^-$DEC-205$^-$, the CD4$^-$8$^-$DEC-205$^-$ and the CD4$^-$8$^+$ DEC-205$^+$ subsets, there are two additional DC subsets that lack CD4 and express at most only moderate levels of CD8α, but which clearly express DEC-205 and express high surface levels of class II MHC. One subset, common to all LN, expresses moderate levels of DEC-205 and may represent interstitial tissue-derived DC, monocyte-derived DC, or an activated version of the splenic CD4$^-$8$^-$DC. These are not mutually exclusive descriptions. A second more distinctive DC subtype, largely restricted to CLN, expresses higher levels of DEC-205 and appears to represent mature forms of LC. Both of these DC subtypes are present among the DC that migrate out of the skin into LN, as revealed either by catching the DC that emerge from skin explants before locating in LN or by tracking skin-derived DC after painting the skin with a fluorescent dye. As others have suggested (16–18), these two DC subtypes may derive from dermal DC and epidermal LC, respectively. All five DC subsets found in LN are capable of stimulating naive T cells in a mixed leukocyte culture system (our unpublished data). Other biological properties such as cytokine production are currently being investigated.

One objective of this study was to identify the mature form of LC among CLN DC and to determine what proportion of CLN DC are LC-derived in a normal mouse with noninflamed skin. A DC population representing at most 25% of CLN DC, and absent from spleen, thymus, or MLN, appeared to be LC derived. This points to a normal steady-state migration of some epidermal LC into CLN, even in the absence of obvious "danger" signals. This migration would be enhanced by inflammatory stimuli such as painting the skin with a solvent containing a fluorescent dye (18). An alternative explanation is that putative LC that fail to find a vacant niche in the epidermis lodge in the draining LN without ever serving as sentinel LC in the epidermis.

The putative LC-derived DC were identified in our study by a range of surface markers, none LC specific but differing sufficiently in level of expression to distinguish this population. Several of these marker combinations have been used previously by others to segregate LC-derived DC (15–18). Some of the LC-derived DC markers, such as large size, high CD40, and very high surface class II MHC, are markers of fully activated DC. Because other DC in the same LN are not in this extreme state of activation, the LC lineage must either be hypersensitive to activation stimuli or inherently of this phenotype. The persistence of some class II MHC on these DC even in CIITA-null mice suggests the regulation of class II MHC expression differs between the LC lineage and other DC. However, an alternative view is that CIITA simply acts as a 10- to 30-fold amplifier of class II MHC expression, and on its removal, only cells with an initially very high class II MHC expression are then detectable by immunofluorescence. We have found the high level of expression of DEC-205 to be an especially useful marker of the LC DC lineage, provided the CD8α^{high} DC that also show high DEC-205 expression are first gated out. These CD8$^+$ DC, common to all lymphoid tissues, cannot be confused with LC, because in addition to the much higher CD8α expression, they lack the myeloid markers CD11b, F4/80, and CD16/32.

The one marker we expected to be specific for the LC DC lineage was langerin, because it is associated with the Ag-processing system and the Birbeck granules of LC (22, 23). Indeed, if high level cytoplasmic staining was the criterion, this appeared to mark the LC-derived DC among skin emigrant DC and within CLN and confirmed the conclusions based on surface staining. The significance of lower-level cytoplasmic staining of DC in spleen, MLN, and other tissues is uncertain at present.

The possible expression of CD4 and CD8 on LN DC and on the LC lineage has been a source of confusion and controversy. We find no significant expression of CD4 on LC-derived DC and only a low incidence of true high CD4-expressing DC in LN generally. This is ironic because it was the study of Salomon et al. (15), who found that CD4$^+$ DC in LN, which prompted our finding of a major CD4$^+$ DC population in spleen (1). We now show that most of the low and medium staining of CD4 on LN DC is attributable to pickup of this Ag from T lymphocytes. The much higher pickup of CD4 compared with CD8 is presumably because CD4 T cells outnumber CD8 T cells in peripheral lymphoid organs. The lowest level of staining for CD8 on LN DC, visualized as a shift from the background rather than a clear population, also seems to be attributable to low-level pickup from T cells. However, both the high-level CD8α staining seen on a subset of LN and spleen DC and the intermediate CD8α staining characteristic of LN DC including some LC-derived DC appears to represent authentic expression by the DC themselves.

Various interpretations have been made of the expression of CD8α on DC derived from LC. Both Anjuère et al. (12, 16) and Merad et al. (13) have shown that although LC in the epidermis or those migrating from skin do not express CD8α, they can express this marker after entry into LN, at least if activated. We also find that LC exiting the skin do not express CD8α, but then find only general low level "pickup" staining and only a modest proportion of cells with medium level expression among the putative DC of LC origin in the LN. The few LC-related DC that could be classed as moderate expressors of CD8α are clearly distinguished from the CD8α^{high} DC population found in spleen, thymus, and LN. Therefore, our results are in line with those of Ruedl et al. (18), who found little CD8α expression by putative LC-derived DC. But given that mature LC can, under some circumstances, express CD8α, does this imply that they are of lymphoid origin, as originally proposed by Anjuère et al. (12, 16, 29)? Or does it imply that CD8α is a poor marker of precursor origin, as proposed by Merad et al. (13)? Recent studies have rather suggested that populations of bone marrow precursors considered as lymphoid-committed or myeloid-committed retain considerable developmental flexibility and that both are capable of forming CD8α^+ DC (L. Wu, unpublished observations, and Ref. 10). Conversely, in contrast to our initial conclusions (30), we (5, 11) and Martin et al. (31) have found that thymic lymphoid precursors can generate both CD8α^+ and CD8α^- DC. It appears CD8α is induced on developing DC at some stage downstream of early hemopoietic precursors. Induction of CD8α on DC generated in vitro can be achieved, although only on those DC generated by particular cytokines (32). Although CD8α no longer seems a reliable marker of the original precursor origin, its expression at high levels nevertheless seems to mark a functionally distinct DC state that in all lymphoid tissues has the highest capacity to produce the bioactive form of IL-12 (33). At least in spleen, it also marks a subtype of DC originating from a separate developmental stream (2). The branch point of this stream and the precise factors inducing CD8α expression on DC remain to be determined.

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