Mouse Lymphoid Tissue Contains Distinct Subsets of Langerin/CD207$^+$ Dendritic Cells, Only One of Which Represents Epidermal-Derived Langerhans Cells

Patrice Douillard,*[1] Patrizia Stoitzner,†[2] Christoph H. Tripp,† Valérie Clair-Moninot,* Smina Aït-Yahia,* Alex D. McLellan,‡ Andreas Eggert,§ Nikolaus Romani,† and Sem Saeland*[3]

*Laboratory for Immunological Research, Schering Plough, Dardilly, France; †Department of Dermatology, Innsbruck Medical University, Innsbruck, Austria; ‡Department of Microbiology/Immunology, University of Otago, Dunedin, New Zealand; §Department of Dermatology, University of Würzburg, Würzburg, Germany

Langerin/CD207 is a C-type lectin associated with formation of Birbeck granules (BG) in Langerhans cells (LC). Here, we describe a monoclonal antibody (mAb 205C1) recognizing the extracellular domain of mouse langerin. Cell-surface langerin was detected in all epidermal LC, which presented a uniform phenotype. Two subpopulations of langerin$^+$ cells were identified in peripheral lymph nodes (LN). One population (subset 1) was CD11c$^{low/+}$/CD8$\alpha^{-/low}$/CD11b$^+$/CD40$^+$/CD86$^+$. The other population (subset 2) was CD11c^{high}/CD8α^+/CD11b^{low}, and lacked CD40 and CD86. Only subset 1 was fluorescein 5-isothiocyanate (FITC$^+$) following painting onto epidermis, and the appearance of such FITC$^+$ cells in draining LN was inhibited by pertussis toxin. Mesenteric LN, spleen, and thymus contained only a single population of langerin$^+$ DC, corresponding to peripheral LN subset 2. Unexpectedly, BG were absent from spleen CD8α^+ DC despite expression of langerin, and these organelles were not induced by mAb 205C1. Collectively, we demonstrate that two langerin$^+$ DC populations (subsets 1 and 2) co-exist in mouse lymphoid tissue. Subset 1 unequivocally identifies epidermal LC-derived DC. The distribution of subset 2 indicates a non-LC origin of these langerin$^+$ cells. These findings should facilitate our understanding of the role played by langerin in lymphoid organ DC subsets.

Key words: dendritic cell subsets/Langerin/Langerhans cells
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Dendritic cells (DC) are highly specialized antigen-presenting cells (Banchereau and Steinman, 1998; Guermonprez *et al*, 2002). Sparsely distributed as sentinels in peripheral tissues, DC are bone marrow-derived leukocytes, with a considerable degree of heterogeneity (Vremec and Shortman, 1997; Anjuere *et al*, 1999; Banchereau *et al*, 2000; Ruedl *et al*, 2000; Ardavin *et al*, 2001; Henri *et al*, 2001; Shortman and Liu, 2002). In mice, the majority of DC express CD11c (integrin $\alpha x\beta 2$), with several distinct subpopulations including CD8α^+/CD11b (Mac-1, integrin $\alpha M\beta 2)^{low}$, and CD8α^-/CD11b^{high} cells (Shortman and Liu, 2002).

Langerhans cells (LC) represent a particular DC subset localized in basal and suprabasal layers of the epidermis, and in stratified mucosal epithelia (Romani *et al*, 2003). LC differ ultra-structurally from other DC by the presence of cytoplasmic organelles, known as Birbeck granules (BG), and consist of superimposed membranes separated by leaflets with periodic "zipper-like" striations (Birbeck *et al*, 1961; Wolff, 1967). LC are characterized by strong expression of the transmembrane type-II Ca^{2+}-dependent lectin langerin/CD207 (Valladeau *et al*, 2000, 2002). The extracellular portion of langerin consists of a membrane-proximal neck region, and a C-terminal carbohydrate recognition domain (CRD), with specificity for mannose, GlcNAc, and fucose (Valladeau *et al*, 2000; Stambach and Taylor, 2003), and recently reported to recognize Lewis x-related sequences (Galustian *et al*, 2004). Langerin is a potent promoter of BG formation in LC, and in unrelated cells following transfection of its coding sequence (Valladeau *et al*, 2000, 2002).

Because of their specialized location, LC constitute the first immune barrier to the external environment. LC transport antigens captured in skin and epithelia to draining lymph nodes (LN), although they undergo a maturation program that allows them to present processed antigens to naïve T cells, thus inducing specific immunity (Romani *et al*, 1989; Stoitzner *et al*, 2003). This scheme has provided a useful paradigm to study other DC subpopulations. The classical "LC paradigm," however, needs to be revisited (Serbina and Pamer, 2003; Wilson and Villadangos, 2004). For instance, recent reports indicate that LC may not prime

Abbreviations: BG, Birbeck granule; CRD, carbohydrate recognition domain; DC, dendritic cell; FITC, fluorescein isothiocyanate; LC, Langerhans cell; LN, lymph node; mAb, monoclonal antibody; MHC, major histocompatibility complex

[1]Present address: Eucodis Forschungs- und Entwicklungs GmbH, Brunner Strasse 59, A-1230 Vienna, Austria.
[2]Present address: Malaghan Institute of Medical Research, PO Box 7060, Wellington, New Zealand.
[3]Present address: INSERM U 503/IFR 128, 21 avenue Tony Garnier, F-69365 Lyon cedex 07, France.

T cells in the context of epidermal/epithelial antiviral immunity, a function that requires participation of other distinct DC subtypes (Allan *et al*, 2003; Zhao *et al*, 2003; Belz *et al*, 2004b). In addition, the capacity of LC to induce tolerance to self-antigens expressed in the skin is a matter of debate (Mayerova *et al*, 2004; Shibaki *et al*, 2004).

These considerations led us to develop a monoclonal antibody (mAb) to the extracellular domain of CD207. This allowed us to investigate whether langerin/CD207 could be used to subdivide DC subsets in mouse lymphoid tissue. We describe here the existence of two langerin$^+$ subsets, only one of which represents DC derived from skin LC, a finding that should be valuable for further defining the functional properties of this population within the LN.

Results

mAb 205C1 recognizes an epitope in the extracellular domain of mouse langerin/CD207 Unlike human langerin, no antibody that recognizes cell-surface expressed mouse langerin has, to our knowledge, been described. We therefore generated hybridomas with the goal of producing such an antibody. As shown in Fig 1*A*, this approach allowed us to select an antibody, mAb 205C1, of IgM isotype, reactive with a cell-surface epitope on the COP5 cells transfected with mouse langerin cDNA used for immunization (see Material and Methods). By contrast, mAb 205C1 gave only marginal background staining on COP5 cells transfected with mock plasmid, or with plasmid encoding human langerin. Human LC obtained from *in vitro* culture of CD34$^+$ cells with TGF-β1 were found to be unreactive with mAb 205C1 (data not shown). Binding of 205C1 antibody was blocked by pre-incubation of mouse langerin-transfected COP5 cells with mannan (Fig 1*B*), indicating that the epitope recognized by the mAb interferes with the extracellular CRD of the lectin. The reactivity pattern of 205C1 with modified constructs of mouse langerin confirmed its specificity for the extracellular portion of the molecule (data not shown). MAb 205C1 also stains LC in fixed epidermal sheets from naïve mice, as shown in Fig 1*C* (*left*) illustrating a double-staining with anti-major histocompatibility complex (MHC) class II (*red*) and 205C1 anti-langerin mAb (*green*), on acetone-fixed dorsal ear epidermis. Previously, we described mAb 929F3, which stains epidermal LC and is a valuable tool to follow their emigration into afferent lymphatic vessels after stimulation (Stoitzner *et al*, 2003). Anti-langerin mAb 929F3, is of rat origin, and gives a potent intracellular staining, including a stronger signal than mAb 205C1 in immunohistology (Fig 1*C*, *right*), but does not recognize the cell-surface form of the molecule. MAb 929F3 was used in this study to monitor intracellular langerin.

To summarize, we describe a novel antibody, mAb 205C1, specific for the extracellular domain of mouse langerin, thus allowing us to study cell-surface expression of langerin on DC.

Langerin/CD207 is expressed at the surface of epidermal LC Availability of mAb 205C1 prompted us to evaluate the pattern of langerin expression by epidermal LC. As shown in Fig 2*A* (*upper row*), virtually all freshly isolated LC (MHC-II^{high}) obtained after trypsinization of the ear epidermis displayed langerin on the cell surface as detected with the 205C1 mAb. In addition, the cell-surface langerin$^+$ LC also expressed CD11c. As further shown in Fig 2*A* (*upper row, right*), gating on the CD11c$^+$ cells (*R2 window*), indicated that 205C1$^+$ LC (*black dots*) expressed low levels of CD11b, but were CD8α negative. Lack of CD8α reactivity was confirmed on epidermal sheets (Fig S1). The freshly-isolated LC also displayed intracellular langerin as detected with mAb 929F3 (Fig 2*A*, *lower row*), in accordance with previously published distribution data (Valladeau *et al*, 2002). We observed that freshly isolated LC expressed both surface and intracellular langerin on the same cells, and no "single stained" population was detected (data not shown). Interestingly, when kept in culture over a 3 d period of time, epidermal LC downregulated both surface and intracellular langerin, with surface langerin disappearing more rapidly (Fig 2*B*, decrease of percentage of positive cells and mean fluorescence intensity). This disappearance was paralleled by upregulation of CD40 and CD86 (Fig 2*B*, increase of percentage of positive cells), as well as enhanced expression of MHC class II molecules on the cell surface (data not shown). No CD8α was detected on these matured LC (data not shown). In addition to the description of langerin expression, the reported phenotype of freshly isolated and cultured epidermal LC is entirely consistent with our previous experience, as compiled in Table S1.

In conclusion, we here demonstrate that mouse LC express a cell-surface form of langerin, similarly to their human counterparts.

Expression of langerin/CD207 by DC subsets in LN Langerin has previously been described in LN by Southern blot analysis of cDNA libraries from mouse tissues and immunohistology (Valladeau *et al*, 2002). Based on the newly available anti-langerin mAb, we explored the phenotype of these cells. As analyzed by flow cytometry, we found langerin-expressing cells in all LN we studied, but strikingly their phenotype considerably differed. In peripheral LN, i.e., a mix of retro-auricular, maxillar, inguinal, and popliteal LN, we found two different subsets of DC expressing the langerin molecule. Langerin was clearly detected at the cell surface, as revealed by mAb 205C1 (Fig 3*A*, *top row, left*), although a higher proportion of the CD11c$^+$ cells expressed langerin intracellularly, as detected with mAb 929F3 (Fig 3*A*, *top row, right*). Among the langerin$^+$ cells (*R2 window*), we found two different subsets (subsets 1 and 2) according to their expression of CD8α (Fig 3*A*, *top row black dots in R2*). In subset 1 (CD8α^{neg}) the DC expressing langerin did not express CD8α and were CD11c$^{low/+}$ (Fig 3*A*, *gray dots in R2*), and CD11b^{high} (not shown). In the experiment shown in Fig 3*A* (*top*), subset 1 cells constitute 47% (100–53) of cell surface langerin$^+$ cells, and 62.2% (100–37.8) of intracellular langerin$^+$ cells. In subset 2, langerin-expressing DC expressed CD8α and were CD11c^{high} (*black dots in R2*), and CD11b^{low} (not shown). In the representative experiment shown in Fig 3*A* (*top*), subset 2 cells constitute 53% of cell surface langerin$^+$ cells, and 37.8% of intracellular langerin$^+$ cells. Intracellular langerin was strongest on subset 1 cells, i.e., CD11c^{low} CD8α^{neg} (Fig 3*A*, *top right row*). When cells were analyzed from LN of

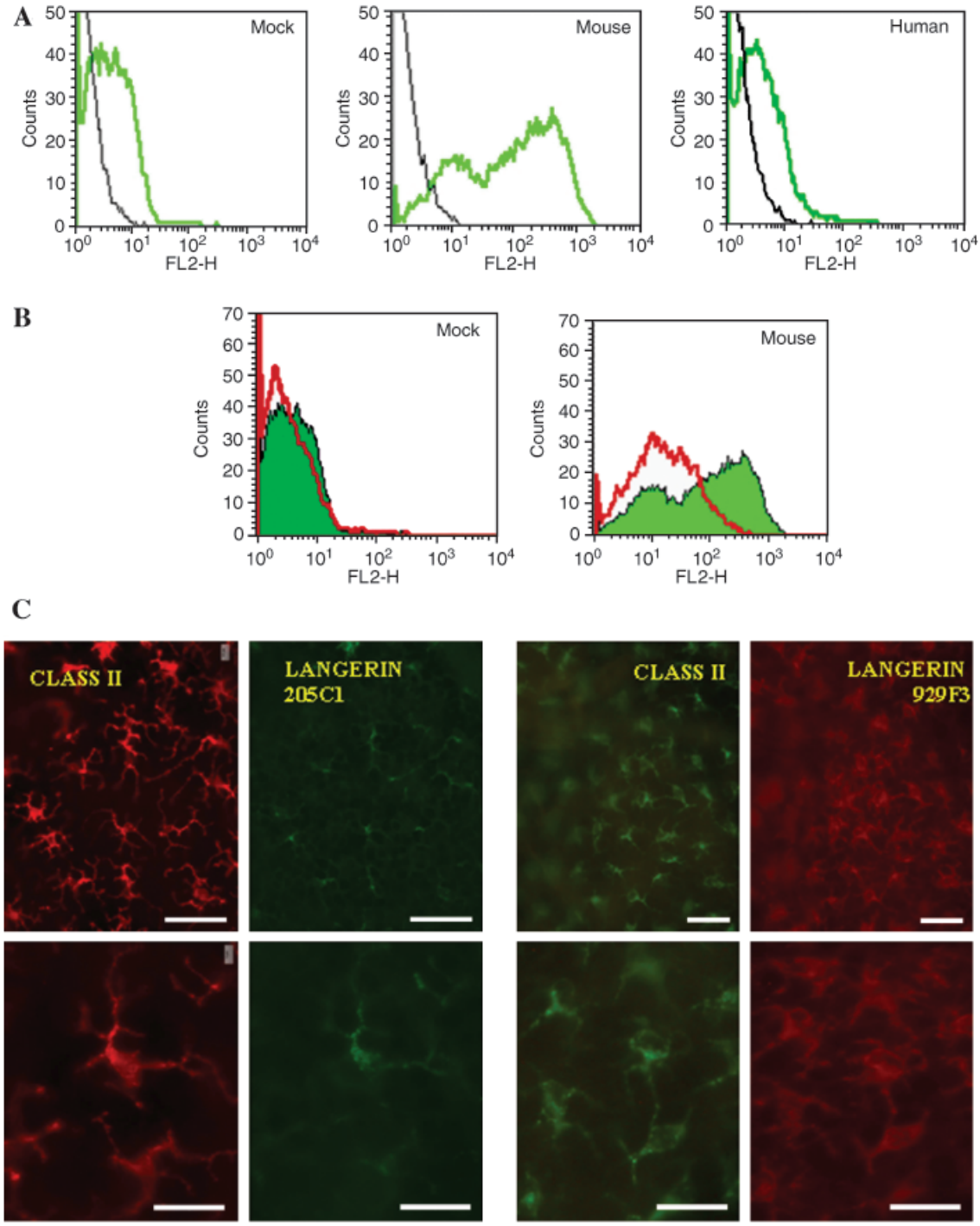


Figure 1
Characterization of monoclonal antibody (mAb) 205C1. (*A*) COP5 cells were transfected with an empty plasmid (mock) or a plasmid encoding for mouse (*middle panel*) or human langerin (*right panel*). Cells were stained with mAb 205C1 (*green bold line*), or with mouse IgM isotype control (*black thin line*). The secondary antibody was a RPE-labeled goat anti-mouse. (*B*) Twenty-four hours after transfection, COP5 cells were detached and incubated for 30 min with (*empty red histogram*) or without (*green histogram*) 1 mg per mL mannan in the presence of sodium azide before staining with 205C1 antibody. (*C*) 205C1 mAb stains epidermal Langerhans cells *in situ*. Ear epidermis was separated from dermis, fixed in acetone for 20 min at room temperature, and double-stained (i) left four pictures: with rat anti-class II (mAb 2G9) followed by goat anti-rat Alexa Fluor 594™ (red), and with Alexa Fluor 488™-coupled mAb 205C1 anti-langerin (green) or (ii) right four pictures: with mAb 929F3 anti-langerin revealed by anti-rat Ig-biotin/streptavidin–Texas Red, and with fluorescein 5-isothiocyanate (FITC)-coupled mAb 2G9. Top pictures are at $\times$ 400 (*left panels*) and $\times$ 300 (*right panels*) magnification, bottom ones at $\times$ 1000. Scale bars equal 20 μm in top panels and 10 μm in the bottom panels.

separate peripheral origins, a similar dichotomy of langerin$^+$ cells was obtained, based on expression levels of CD8α and CD11c (data not shown). Even though our mice were naïve, we found a proportion of langerin$^+$ cells in peripheral LN expressing the activation markers CD40 and CD86 (black dots in R2 window) (47.4% and 60.6%, respectively, in the experiment shown in Fig 3*A*, *bottom row*). Interestingly, these "activated" cells were those from subset 1, i.e., CD11c^{low} CD8α^{neg}, which furthermore expressed high levels of MHC class II (data not shown). By contrast,

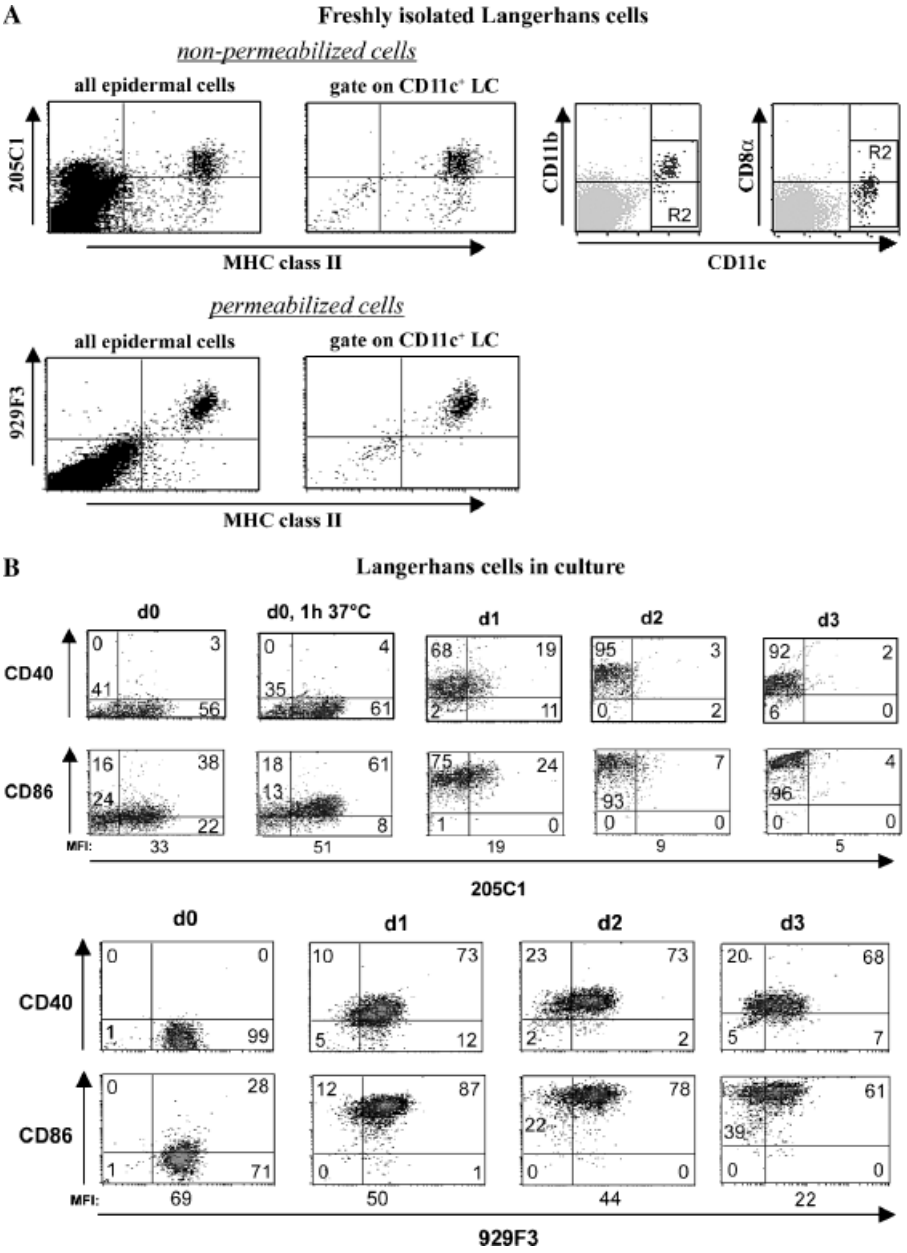
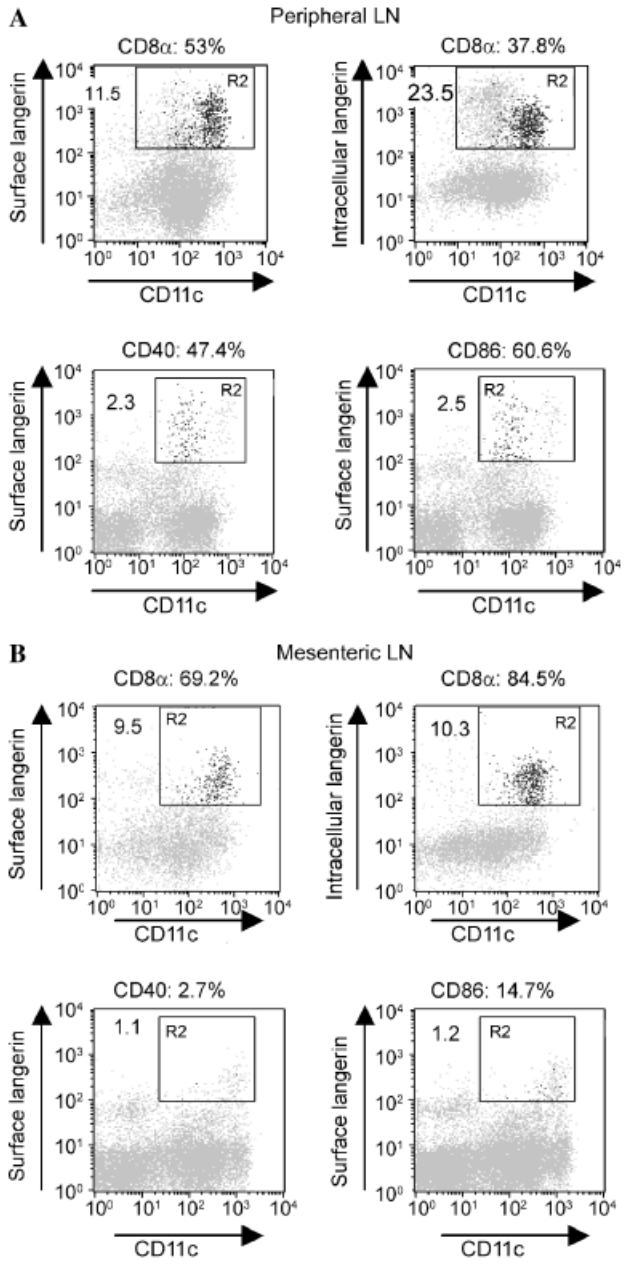


Figure 2
Flow cytometry analysis of langerin/CD207 expression by epidermal murine Langerhans cells. (*A*) Freshly-isolated Langerhans cells (LC). Cell-surface analysis (non-permeabilized cells, *upper row*): epidermal cells obtained after trypsinization of the ear epidermis were stained with anti-langerin (monoclonal antibody (mAb) 205C1), anti-major histocompatibility complex (MHC) class II, anti-CD11c, and anti-CD11b or anti-CD8α. Cells were first gated according to their FSC and SSC characteristics, and then analyzed for their co-expression of langerin and the above markers. Note that the LC are CD8α negative. In upper right dot-plots, R2 represents cells gated on CD11c expression, and black dots correspond to langerin$^+$ cells within this window. Intracellular analysis (permeabilized cells, *lower row*): cells were first stained on the surface with anti-MHC class II and anti-CD11c, then fixed and permeabilized for intracellular staining with 929F3 anti-langerin. (*B*) Langerin/CD207 expression by LC in culture. Isolated epidermal LC were cultured over a period of 3 d with granulocyte macrophage colony-stimulating factor (GM-CSF). Unenriched cell populations were subjected to 4-color flow cytometric analyses. Expression of cell surface (mAb 205C1) or intracytoplasmic (mAb 929F3) langerin, and anti-CD40 or anti-CD86 is depicted on cells gated for MHC class II and CD11c double-expression, i.e., Langerhans cells. Numbers shown inside the panels represent the percentages of cells in each quadrant, whereas numbers below the *x*-axes correspond to mean fluorescence intensity (MFI) of the langerin stainings. Note the rapid decrease of cell-surface langerin and absence of CD40 expression on freshly isolated cells.

langerin$^+$ DC from subset 2 (i.e., CD11c^{high} CD8α$^+$) did not express CD40 and only a few of them were CD86$^+$. The subset 2 cells were also MHC class II^{low} (data not shown). The phenotypes of subsets 1 and 2 are summarized in Table S1, as a side-by-side comparison with *in situ*, freshly isolated, and cultured epidermal LC. Antigen-uptake and migration to transport antigens from the skin to draining LN is an important functional feature of LC (Romani *et al*, 2001). In order to determine the relationship between epidermal LC and the subsets of langerin$^+$ cells observed within draining LN, we painted mouse ears with fluorescein 5-isothiocyanate (FITC) and harvested retro-auricular draining LN. Cells containing FITC were observed in the draining LN, with a peak on days 2–3 post-painting. These included CD11c$^+$cells, as well as cells lacking CD11c, the identity of the latter population remaining to be defined (Fig 4, *second row*). Markedly, only a proportion of langerin$^+$ cells (24% in the representative experiment presented in Fig 4, *middle row*) were FITC$^+$. We investigated CD8α expression on CD11c$^+$ cells in relation to langerin and FITC. As shown in Fig 4 (*penultimate row*), virtually all langerin$^+$/CD11c$^+$/FITC$^+$cells lacked CD8α. These cells correspond to subset 1, in accordance with their expression of CD40, CD86, and high levels of MHC class II (data not shown), and display all features of emigrant epidermal LC. By contrast, the vast majority of subset 2 cells, i.e., langerin$^+$/CD8α$^+$, remarkably lacked FITC (Fig 4, *penultimate row*). Finally, in addition to subset 1, FITC was also readily detected among CD11c$^+$/langerin$^-$/CD8α$^-$ cells (Fig 4, *bottom row*), presumably representing dermal DC. We next sought to confirm that FITC had been captured by DC prior to their migration from the skin, and not by resident DC within the lymphatic system. Indeed, combined intradermal and epicutaneous treatment of ear skin with pertussis toxin, an inhibitor of G-protein coupled receptors (e.g., chemokine receptors) led to inhibition of DC migration to the LN in

response to topical application of FITC. Thus, the percentages of FITC-bearing, epidermal-derived LC, as defined by their CD11c$^+$/langerin$^+$/CD8α^- phenotype was reduced by about 40% in three independent experiments (33.6% *vs* 19.5%, 35.7% *vs* 15.3%, and 17.7% *vs* 10.5% in LN draining FITC-treated skin, with and without additional pertussis toxin treatment, respectively) (Fig 4). Finally, we compared langerin expression in mesenteric LN to that observed in peripheral LN. As shown in Fig 3*B*, we observed cells expressing langerin (R2 window), both at the cell surface, and intracellularly (top row right) within mesenteric LN. Strikingly, virtually all langerin$^+$ cells displayed a phenotype corresponding to peripheral LN subset 2, i.e., CD11c^{high} and mostly CD8α^+ (Fig 3*B*, *experiment shown in top row*). Moreover, the activation markers CD40 and CD86 were almost completely absent from the langerin$^+$ cells (Fig 3*B*, *experiment shown in bottom row, black dots in R2*).



Taken together, our results demonstrate that in naïve mice, two distinct subsets of langerin$^+$ cells co-exist in peripheral LN, whereas only one is present in mesenteric LN. Only one of the peripheral LN subsets (subset 1) represents epidermal LC immigrants.

Expression of langerin/CD207 by DC subsets in other lymphoid organs In order to extend our characterization, we analyzed by flow cytometry the expression of langerin in other lymphoid organs. We found subset 2 langerin$^+$ DC both in spleen and thymus, quite similar to subset 2 in peripheral and mesenteric LN. Thus, virtually all cell-surface (mAb 205C1) langerin-expressing cells (R2 gate: 9.8% and 7.2% in spleen and thymus, respectively, in the experiment shown) were CD11c^{high} (Fig 5, *upper row*). In addition, the vast majority of the langerin$^+$ cells (90% in spleen and 78.6% in thymus, respectively) expressed CD8α (Fig 5, *upper row, black dots in R2 window*), DEC205$^+$ and CD11b^{low} (not shown). The same result was obtained by intracellular staining of langerin with mAb 929F3 (Fig 5, *bottom row*). As in the LN, we detected more cells with intracellular langerin than with the molecule at the cell surface. Both in spleen and thymus, langerin-expressing cells were negative for the activation markers CD40 and CD86, and displayed low levels of MHC class II on their surface (data not shown). We thus failed to detect subset 1 DC in these organs. In accordance with previous Southern blot analysis of cDNA libraries (Valladeau *et al*, 2002), we were unable to detect any langerin$^+$ cells in Peyer's patches. Finally, no langerin-expressing cells were found in freshly collected bone marrow (data not shown), although some cells are able to differentiate into langerin$^+$ cells upon culture with granulocyte macrophage colony-stimulating factor (GM-CSF) and TGF-β1 (data not shown and (Valladeau *et al*, 2002)).

Thus, our results demonstrate that langerin is not restricted to LN, but that the phenotype of spleen and thymus

Figure 3
Langerin/CD207 expression in lymph node (LN) dendritic cell (DC) subsets. (*A*) Peripheral LN. A mix of inguinal, popliteal, retro-auricular, and maxillar LN were meshed through a stainless sieve, enriched for CD11c, and processed for 4-color flow-cytometry analysis. Cells were first gated according to their FSC and SSC characteristics (not shown), and then analyzed for their co-expression of CD11c and cell-surface (monoclonal antibody (mAb) 205C1) or intracytoplasmic (mAb 929F3) (*A*; *top row right*) langerin. R2 illustrates the gate selected for langerin$^+$ cells. Black dots in top row represent the CD8α^+ cells within R2, and the percentage of langerin$^+$/CD8α^+ cells is reported above each window. Bottom row shows CD40$^+$ (*left*) and CD86$^+$ (*right*) cells, depicted in black within the langerin$^+$ R2 gate, and percentages of langerin$^+$/CD40$^+$ or langerin$^+$/CD86$^+$ cells are reported above each window. Note the two distinct CD11c$^+$/langerin$^+$ populations. This staining has been performed five times, featuring quantitative but no significant qualitative variability with respect to the phenotypes considered. (*B*) Mesenteric LN. As above, cells were analyzed for their co-expression of CD11c and surface or intracellular (*B*; *top row right*) langerin. R2 illustrates the gate selected for langerin$^+$ cells. Black dots in top row represent the CD8α^+ cells, and the percentage of langerin$^+$/CD8α^+ cells is reported above each window. Bottom row shows virtual absence of CD40$^+$ (*left*) and CD86$^+$ (*right*) cells (black dots in R2), and the percentages of langerin$^+$/CD40$^+$ or langerin$^+$/CD86$^+$ cells is reported above each window. Note the single CD11c$^+$/langerin$^+$ population. This staining has been performed three times. Both in panels *A* and *B*, the dot plots showing CD40 and CD86 are from a different experiment than the CD11c/langerin/CD8α dot plots, thus explaining different percentages of langerin$^+$ cells (R2).

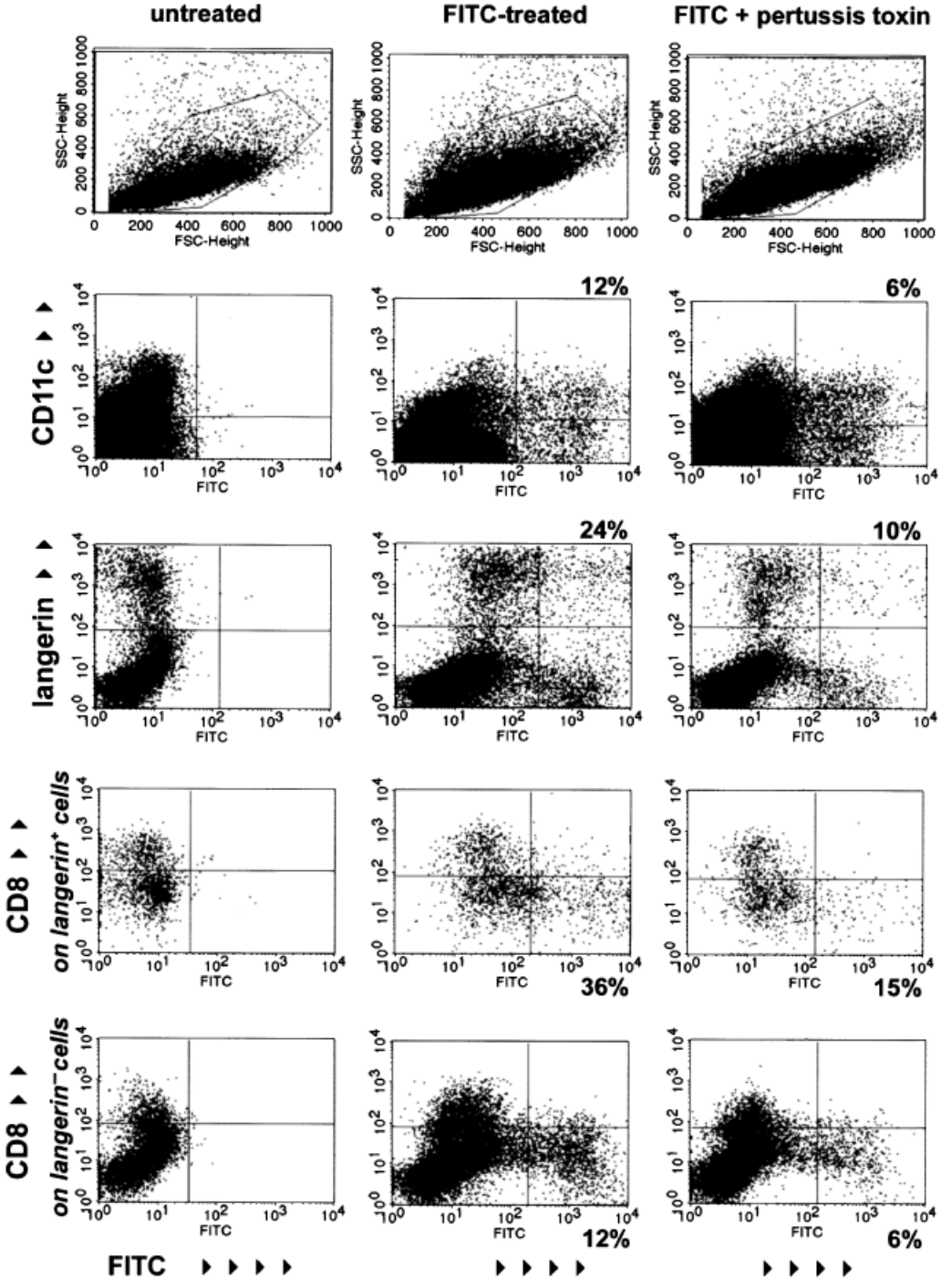


Figure 4
Fluorescein 5-isothiocyanate (FITC)- bearing cells in skin-draining lymph nodes (LN). Unenriched LN cell suspensions were analyzed by 4-color flow-cytometry. Treatment of mice is indicated on top of each column. Top row shows the typical forward/side scatter plots of the investigated populations. The second row depicts CD11c *versus* FITC fluorescence. In the middle row, CD11c$^+$ cells were selected by gating and their expression of langerin and FITC tracer is plotted. Both langerin$^+$ cells and langerin$^-$ cells carry FITC. In the two bottom rows, cells were additionally selected by gating for langerin$^+$ cells (*penultimate row*) and langerin$^-$ cells, i.e., dermal/interstitial and LN-resident dendritic cells (DC) (*bottom row*). Note that only the CD8α^- populations, i.e., the presumptive skin-derived DC, have taken up FITC. The CD8α^+ populations carry virtually no FITC. Also note the reduction of FITC-bearing, CD8α^- cells in response to pertussis toxin treatment (*middle vs right-hand panels*). Percentages from top to bottom correspond to FITC$^+$ of total CD11c$^+$, FITC$^+$ of total langerin$^+$, FITC$^+$ of total langerin$^+$/CD8α^-, and FITC$^+$ of total langerin$^-$/CD8α^- cells. This figure is representative of three independent experiments.

langerin-expressing cells is homogenous, unlike the heterogeneity observed in peripheral LN.

Spleen langerin/CD207$^+$ DC lack BG In the spleen, langerin was detected both at the cell surface (mAb 205C1) and intracellularly (mAb 929F3) in the CD11c$^+$ CD8α^+ DC subset (Fig 5). As it has been shown that langerin is involved in the formation of BG (Valladeau *et al*, 2000), we speculated that the spleen langerin-expressing DC would contain such cytoplasmic organelles. In order to address this question, we FACS-purified the CD11c$^+$ CD8α^+ DC subset from spleen (Fig 6*A*), and fixed the cells for electron microscopy analysis. The purity of the sorted cells (gate R3 in Fig 6*A*) was typically in the range of 95%–98% (data not shown). We chose not to use mAb 205C1 for FACS-sorting in order to avoid potential *ex vivo* triggering of langerin. Surprisingly, as shown in Fig 6*C–F*, we did not detect any BG in the cytoplasm of the sorted spleen CD8α^+ DC, despite their expression of langerin. By contrast, BG were readily observed in a preparation of epidermal LC processed similarly for electron microscopy analysis (Fig 6*B*). *Ex vivo* treatment of human epidermal LC with anti-langerin mAb enhances BG formation (Valladeau *et al*, 2000). We thus incubated FACS-purified spleen CD11c$^+$/CD8α^+ DC (1 h at 37°C) with mAb 205C1 as a surrogate langerin ligand, prior to processing the cells for electron microscopy. This

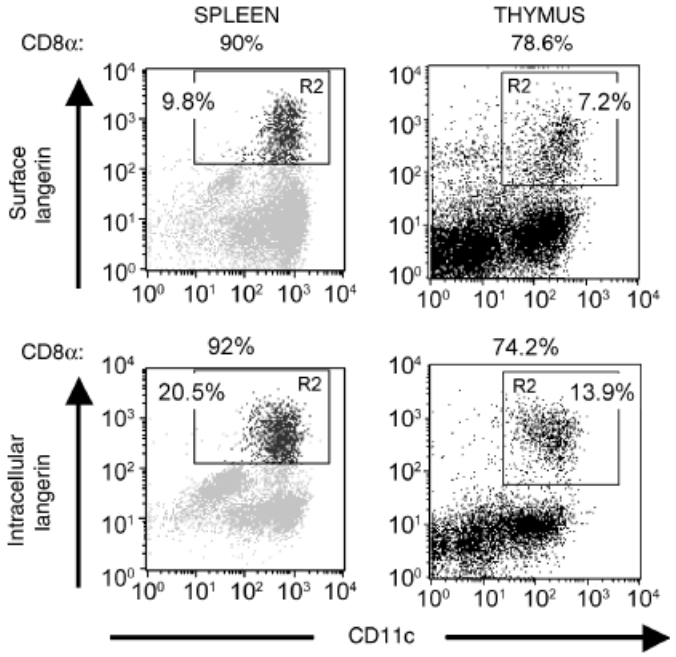


Figure 5
Langerin/CD207 expression in spleen and thymus. Spleen and thymus were digested with collagenase and cells were processed for flow-cytometric analysis. Cells were first gated according to their FSC and SSC characteristics (not shown), and then analyzed for their co-expression of CD11c and cell-surface (monoclonal antibody (mAb) 205C1) (*top row*) or intracytoplasmic (mAb 929F3) (*bottom row*) langerin. Langerin$^+$ cells were further gated (R2), with the proportion of positive cells indicated in the R2 window. The percentage of langerin$^+$/CD8α^+ cells is reported above each window. CD8α^+ cells are illustrated as black dots within R2. Stainings have been performed at least three times, with some quantitative but no significant qualitative variability.

treatment, however, did not result in the induction of BG (data not shown), as the cells presented an ultra-structure similar to that of spleen DC not exposed to anti-langerin mAb. Our results thus show that cell-surface expression of langerin does not necessarily correlate with the presence of BG.

Discussion

Langerin/CD207 is a type-II transmembrane protein containing an extracellular lectin domain. We have generated a mAb, mAb 205C1, which recognizes an extracellular epitope of mouse langerin either within or in close proximity to its mannose-binding site (Fig 1). To our knowledge, 205C1 is the first such described antibody, allowing us to establish that murine LC do express cell-surface langerin (Fig 2, and Kissenpfennig *et al* (2005a), similar to their human counterparts (Valladeau *et al*, 2000). The previously described anti-langerin mAb 929F3 (Stoitzner *et al*, 2003) exclusively recognizes the intracellular form, thus combining use of the two mAb conveniently permits to determine the localization of langerin.

Freshly isolated LC uniformly expressed MHC class-II, CD11c, CD11b, and DEC205/CD205, but neither maturation markers such as CD40 and CD86, nor CD8α. When cultured, LC upregulated surface MHC class-II, CD40 and CD86, but remained CD8α^{neg} (Fig 2 and Table S1). In addition, the cultured LC downregulated cell-surface langerin (Fig 2), an observation that parallels the human molecule (Valladeau *et al*, 1999), and reflects the maturation process associated with emigration from epidermis towards draining LN. It also parallels the disappearance of BG during the typical 3 d maturation period of mouse epidermal LC

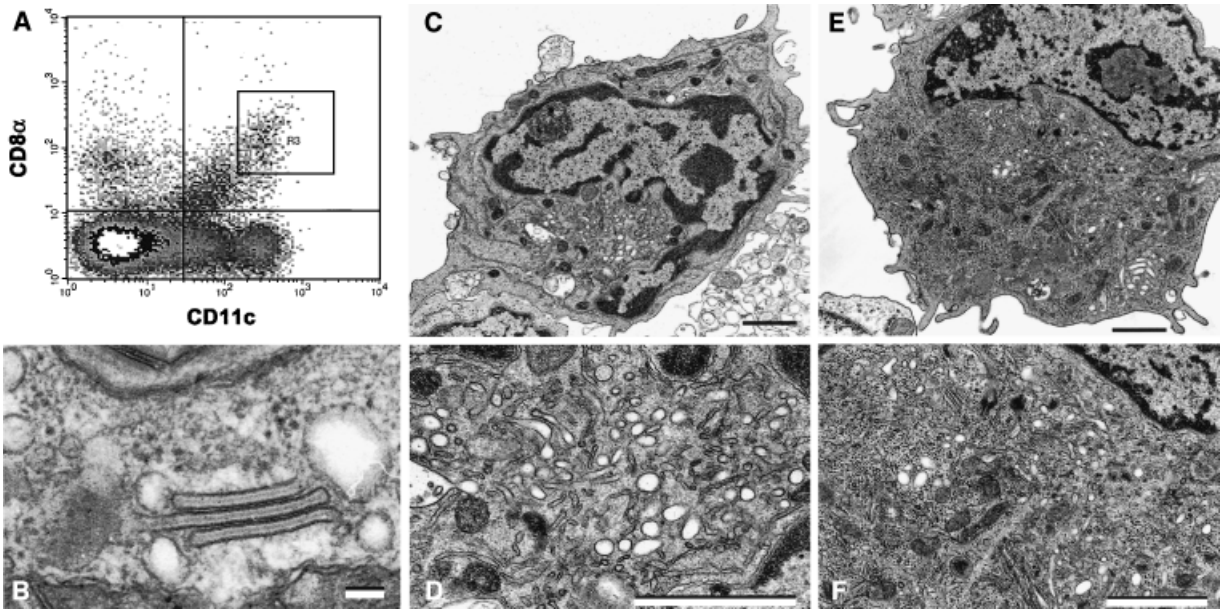


Figure 6
Ultrastructure of CD8α$^+$ spleen dendritic cells (DC). CD8α$^+$ DC were highly purified by FACS cell-sorting based on the rigorously set sorting gate (R3), as illustrated in panel *A*, processed for electron microscopy, and analyzed for the presence of Birbeck granules (BG). Virtually none were found in some 270 cell profiles from three different experiments (i.e., cell sorts). Two examples of normally appearing spleen DC are depicted at low (*C* and *E*) and high (*D* and *F*) magnification. For comparison, a stack of typical BG from a Langerhans cell in murine skin is shown in panel *B*. *Scale bars* equal 0.1 μm in *B* and 1 μm in *C–F*.

(Schuler and Steinman, 1985). There is ample evidence that epidermal LC do not express CD8α, either *in situ*, freshly isolated, short-term cultured (i.e., overnight), or cultured for 2–3 d (Romani *et al*, 1985; Witmer-Pack *et al*, 1987; Romani *et al*, 1991). Similarly, it has been shown repeatedly, that CD11b/C3bi receptor/Mac-1 antigen is expressed by LC *in situ* and that its surface expression does not markedly change upon culturing for 2–3 d. A compilation of the phenotype of LC is presented in Table S1, as based on our experience.

Mouse langerin transcripts were originally reported in tissues other than epidermis or mucosa (Valladeau *et al*, 2002). Taking advantage of the mAb recognizing cell-surface and intracellular forms of the molecule, we analyzed langerin expression in lymphoid organs of BALB/c mice. Surprisingly, we found two DC subpopulations expressing langerin in peripheral LN (Fig 3). Both subpopulations (subsets 1 and 2) reacted with mAb 205C1 and 929F3, demonstrating the presence of cell-surface and intracellular langerin.

The first peripheral LN population (subset 1) displays a phenotype similar to that of epidermal LC, but with an "activated" profile, i.e., high levels of MHC class II, as well as expression of CD40 and CD86 (Figs 3 and 7, and Table S1). This langerin$^+$/CD11c$^+$/CD11b$^+$/CD8α$^{-/low}$ subset corresponds to "mature LC" (Henri *et al*, 2001; Allan *et al*, 2003; Wilson and Villadangos, 2004). It was recently reported that epidermal LC migrate in the steady state to draining LN in a CCR7-dependent fashion (Ohl *et al*, 2004). The somewhat higher expression of CD11c described on the immigrant cells in the latter study could be because of mouse strain differences (C57BL/6 *vs* BALB/c). We unequivocally demonstrate the epidermal origin of subset 1, as this population contained all the langerin$^+$ cells harboring FITC in draining retro-auricular LN following painting of this tracer onto ear skin (Fig 4). In line with Ohl *et al* (2004), the appearance of langerin$^+$/FITC$^+$ cells was inhibited by the G-coupled protein receptor antagonist pertussis toxin, consistent with a chemokine-mediated event (Fig 4). Finally, the FITC$^+$ cells clearly remained CD8α$^{-/low}$, in contrast to a study by Merad *et al* (2000) in which migratory LC acquired CD8α following FITC sensitization. Taken together, our data indicate that the phenotype of the LC-derived cells that immigrate to draining LN is not grossly different in the steady state as compared with a situation of epidermal disturbance in response to a chemical sensitizer. It has been reported that DC expressing low amounts of CD8α produce less IL12p70 than CD8α$^+$ DC from skin-draining LN upon stimulation with CD40-ligand and lipopolysaccharide (Hochrein *et al*, 2001), a finding which may have consequences for T cell priming by LC-derived DC.

We unexpectedly found another langerin$^+$ DC subpopulation (subset 2) in peripheral LN. Subset 2 was clearly distinguishable from the LC-derived langerin$^+$ subset 1, by virtue of its expression of CD8α, and low levels of MHC class II and CD11b. Moreover, subset 2 cells did not express CD40 and CD86 (Figs 3 and 7, and Table S1). The phenotype of this novel langerin$^+$ subset surprisingly corresponds to the "blood-derived" CD8α$^+$ DC population (Ruedl *et al*, 2000; Shortman and Liu, 2002; Wilson and Villadangos, 2004). The functions of such CD8α$^+$ DC include cross-presentation in the MHC class I pathway (den Haan *et al*, 2000), and cross-tolerance with deletion of self-reactive CD8$^+$ T cells (Belz *et al*, 2002). In addition, CD8α$^+$ DC capture dying cells (Iyoda *et al*, 2002), and appear to be specialized in priming CTL immunity to viruses (Allan *et al*, 2003; Belz *et al*, 2004a, b). It has been suggested that LC transfer their antigen cargo captured in epidermis to skin-draining LN-resident CD8α$^+$ DC, which accomplish the T cell priming (Allan *et al*, 2003). Our data demonstrating that both the mature form of LC and LN-resident CD8α$^+$ DC express cell-surface langerin raise the question of whether the molecule might be involved in this process by virtue of its sugar-recognition, and its endocytic properties (Valladeau *et al*, 1999). Yet, disruption of the *langerin* gene neither abrogates cross-presentation of ovalbumin by CD8α$^+$ splenic DC, nor a number of key LC functions tested (Kissenpfennig *et al*, 2005a). The latter results, however, do not rule out a specialization of langerin for certain antigens.

Our data imply that the presence of langerin alone cannot be used to discriminate peripheral LN DC of skin origin. Nevertheless, subtle differences with resident LN cells were observed, as only a fraction of the langerin$^+$ LC-derived subset 1 cells displayed langerin at their cell-surface, consistent with the downregulation observed in epidermal cultures. In contrast, all subset 2 cells exhibited both cell-surface and intracellular forms of langerin. Finally, subset 1 included a fraction of cells with much higher intracellular langerin than was the case for subset 2.

Of interest, the phenotype of the langerin$^+$ cells in mesenteric LN (Fig 3), spleen, and thymus (Fig 5) is remarkably similar to that of subset 2 in peripheral LN. Our findings corroborate the detection of langerin transcripts in these tissues (Valladeau *et al*, 2002), and a report describing langerin protein in CD8α$^+$ spleen DC (McLellan *et al*, 2002). The presence of cell-surface langerin in these tissues prompted us to evaluate the presence of BG, as these organelles are formed by engagement of this lectin (Valladeau *et al*, 2000, 2003). Because of technical difficulties of *ex vivo* purifying sufficient cells for electron microscopy, we focused our analysis on spleen. Strikingly, FACS-sorted spleen CD11c$^+$/CD8α$^+$ DC, which do express cell-surface langerin (Fig 5), displayed no BG in their cytoplasm (Fig 6). Likewise, pre-incubation with mAb 205C1 antibody failed to induce the formation of these organelles (data not shown), by contrast to an anti-human langerin mAb (DCGM4) in human LC (Valladeau *et al*, 2000). Our findings demonstrate that langerin expression is not a sufficient condition to induce BG. Previously, human monocyte-derived DC cultured in IL-15, and characterized by strong expression of cell-surface langerin were found to lack BG (Mohamadzadeh *et al*, 2001).

Recently, an alternative spliced isoform (ΔE3) of mouse langerin has been described (Riedl *et al*, 2004). The ΔE3 isoform lacks the membrane-proximal neck-domain, is unable to bind mannan (presumably because of lack of trimerization mediated by the neck domain), and its transient expression in mouse fibroblasts fails to induce BG formation (Riedl *et al*, 2004). Notably, however, ΔE3 displays the extracellular lectin CRD domain recognized by mAb 205C1. Thus, lack of BG in spleen langerin$^+$ DC could be because of predominance of the ΔE3 isoform, an issue of interest for

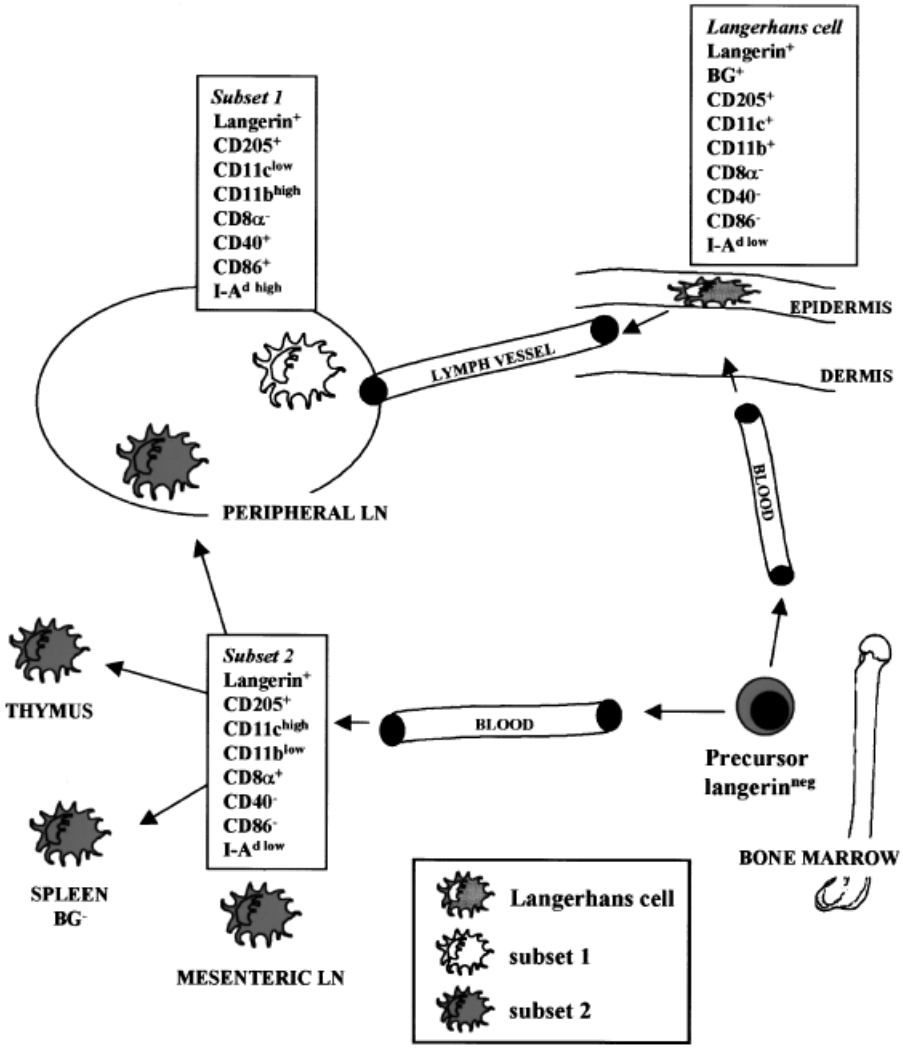


Figure 7
A model summarizing the distribution of Langerhans cells (LC) and langerin/CD207$^+$ dendritic cells (DC) in BALB/c mouse lymphoid tissue. Epidermal LC migrate from skin to draining lymph nodes (LN), where they represent langerin$^+$ subset 1, present in steady state or following sensitization. In addition, peripheral LN contain a distinctive langerin$^+$ subset 2, presumably blood-derived, that resemble mesenteric LN, spleen, and thymic langerin$^+$ DC. Note that despite their phenotypic similarity, the model does not imply that the latter langerin$^+$ cells are directly derived from a common precursor.

further studies. Another possibility is that, under physiological conditions, the formation of BG is distinctly regulated in LC and other langerin$^+$ DC, perhaps through differential availability of intracellular adaptor molecules.

In conclusion, our data unexpectedly demonstrate that lymphoid tissue langerin$^+$ DC segregate into distinct subsets, only one of which represents the epidermal LC-derived component. In this context, mice with genetically disrupted Id2 transcription factor neither have epidermal LC nor CD11c$^+$/CD11b$^-$/CD8α^+ spleen DC, linking the development of progenitors of these two DC subtypes (Hacker *et al*, 2003). We hypothesize that the langerin$^+$ DC subsets acquire their differentiation markers, i.e., CD8α or CD11b, according to their local environment. We propose a model summarizing the distribution and phenotype of mouse LC and langerin$^+$ DC in lymphoid organs (Fig 7). It is noteworthy that our data with anti-langerin mAb largely corroborate the very recently described distribution of fluorescence in a transgenic mouse expressing EGFP under control of the *langerin* gene promoter (Kissenpfennig *et al*, 2005b). Our findings should facilitate further dissection of the respective roles of LC-derived and LN-resident DC in directing productive immunity and peripheral tolerance.

Material and Methods

Mice BALB/c AnN mice were purchased from Charles River (St Germain-sur-l'Arbresle, France), and used at 8–12 wk of age. All experiments involving mice were conducted according to University, National, and EU Institutional Guidelines.

Generation of 205C1 antibody mAb 205C1 against cell-surface mouse langerin was developed in the laboratory (Schering-Plough, Dardilly, France), as follows: BALB/c mice were immunized with COP5 fibroblasts transfected with mouse langerin cDNA. Thus, 10×10^6 cells were i.p. injected twice at 3 wk intervals, followed by an i.v. boost with 50×10^6 cells 3 d before fusion. Splenocytes were fused (1:5 ratio) with the murine myeloma cell line SP_2O, using polyethylene glycol 1000 (Sigma-Aldrich, St Louis, Missouri). Hybrid cells were distributed in 96-well plates and fed with DMEM F12 (Life Technologies, Gaithersburg, Maryland) supplemented with 10% (vol/vol) horse serum (Life Technologies), 2 mM L-glutamine (Life Technologies), 80 μg per mL gentamycin (Schering-Plough), 1% (vol/vol) culture medium additive (EFS, Lyon, France), 10^{-5} M azaserine (Sigma-Aldrich), and 5×10^{-5} M hypoxanthine (Sigma-Aldrich). Supernatant fluids were screened for reactivity with murine langerin-transfected COP5 cells. Selected hybridomas were cloned by limiting dilution. The selected hybridoma, named 205C1, was further grown in DMEM F12 supplemented with 10% (vol/vol) horse serum, 2 mM L-glutamine, and 80 μg per mL

gentamycin (Schering-Plough). Ascites was produced in BALB/c mice, and the IgM antibody was purified by size-exclusion chromatography. MAb 205C1 was either used uncoupled, or coupled with Alexa Fluor 488™ (Molecular Probes, Eugene, Oregon), according to the manufacturer's recommendations.

Media and reagents *Ex vivo*-derived cells were cultured in RPMI-1640 (Life Technologies), supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS) (Life Technologies), 2 mM L-glutamine, 5×10^{-5} M 2β-mercaptoethanol (Sigma-Aldrich), 10 mM Hepes (Life Technologies), 1 mM sodium pyruvate (Sigma-Aldrich), non-essential amino acids (Life Technologies), and 40 μg per mL gentamycin, hereafter referred to as complete medium. Other reagents used for cell cultures were recombinant mouse GM-CSF (Schering-Plough), and recombinant TGF-β1 (R&D Systems Inc., Minneapolis, Minnesota). Transient expressions were performed in the murine fibroblastic cell line COP5 (Tyndall *et al*, 1981). Cells were washed twice in phosphate-buffered saline (PBS) and 6×10^6 cells were electroporated with 15 μg of a pCEV4 plasmid (Takebe *et al*, 1988) containing mouse or human langerin cDNA (Valladeau *et al*, 2002), or with an empty vector (mock control). Cells were harvested and processed after 24 h in culture in complete medium. In addition to mAb 205C1, the following antibodies were used for phenotypic analysis: (i) mAb 929F3 (rat IgG2a), produced in the laboratory, for intracytoplasmic staining of mouse langerin/CD207 (Stoitzner *et al*, 2003); (ii) commercial antibodies against mouse antigens (BD-Pharmingen, San Diego, California), including anti-CD3 complex (clone 17A2), anti-CD4 (clone GK1.5), anti-CD8α (clone 53-6.7), anti-CD11b (clone M1/70), anti-CD11c (clone HL3), anti-CD40 (clone 3/23), anti-CD80 (clone 16-10A1), anti-CD86 (clone GL1), anti-I-A/I-$E^{diverse}$ (clone 2G9), anti-I-A/I-E (clone M5/114.15.2), anti-I-A^d (clone AMS-32.1); (iii) control isotypes including rat IgG2a (clone R35-95), rat IgG2b (clone A95-1), hamster IgG (clone A19-3), and mouse IgM (clone G155-228).

DC preparations

Epidermal LC Epidermal cell suspensions were obtained with porcine trypsin in Hanks' Balanced Salt Solution (Sigma-Aldrich) (Koch *et al*, 2001). Cells were analyzed by flow cytometry immediately after isolation, or after a culture period of 3 d in complete medium supplemented with 100 ng per mL GM-CSF. No further enrichment step was carried out with the suspensions, as LC are readily distinguishable by their MHC class-II and CD11c expression.

DC from lymphoid organs Spleen, thymus, mesenteric, popliteal, inguinal, and retro-auricular LN were collected from CO2-sacrificed mice. Organs were cut in small pieces, incubated at 37°C for 30 min in RPMI 1640 supplemented with 5% FBS, 10 mM Hepes, 1 mg per mL collagenase type IV (Sigma-Aldrich), and 40 μg per mL DNAse I (Sigma-Aldrich). EDTA 5 mM was added for the last 5 min. Digested fragments were filtered through a stainless-steel sieve, and cell suspensions washed twice in PBS supplemented with 5% FBS, 5 mM EDTA, and 5 μg per mL DNAse I. Spleen cell suspensions were re-suspended for 3 min RT in ammonium chloride to lyse red cells. Lymphoid tissue cells were washed two more times, and either directly analyzed by flow cytometry, or submitted to an additional enrichment step because of their notable low proportion of DC. Thus, for enrichment, cells were re-suspended in PBS/bovine serum albumin (BSA) 1% /ethylene diamine tetraaacetic acid (EDTA) 5 mM buffer, and magnetically separated by MACS (Miltenyi Biotec, Bergisch Gladbach, Germany), using CD11c (N418) MicroBeads according to the manufacturer's instructions. This enrichment procedure yielded populations consisting of 40%–80% $CD11c^+$ cells. Alternatively, for the purpose of FACS-sorting highly-purified $CD8\alpha^+/CD11c^+$ cells for electron microscopy studies, low-density spleen DC were enriched by density gradient centrifugation using the method of Vremec *et al* (1992), modified by McLellan *et al* (2002). Thus, spleens were teased out, by tearing the splenic capsule at the thick end, keeping the rest of the capsule intact, and gently squeezing out the splenic contents. Fragmented spleens were incubated with 1 mg per mL type I collagenase (Worthington, Lakewood, New Jersey) and DNase I (10 mg per mL) in 25 mM IMDM with 2% vol/vol FBS for 30 min at 37°C with gentle shaking. For the last 5 min, 10 mM EDTA was added and spleen fragments were pressed through a metal tea-sieve and collected into cold 5 mM EDTA, 10 mg per mL DNase I, 1% BSA in PBS. Cells were filtered through 70 μm cell strainers and washed twice in cold PBS/EDTA/BSA/DNase I. The digest from every three spleens was re-suspended in 5 mL of ice cold, iso-osmotic 14.1% Nycodenz (Nycomed Pharma, Oslo, Norway) in a 15 mL tube, overlaid with 2 mL mouse iso-osmotic buffer, and centrifuged for 25 min at 600*g*. Typically 10^7 low-density cells with a CD11c DC content of 20%–40% were obtained from each spleen. Low-density spleen cells were further FACS-sorted as follows: 5×10^7 cells per mL in PBS plus 1 mM EDTA were labelled with mAb N418 (hamster anti-mouse CD11c) and mAb 53–6.7 (rat IgG2a anti-mouse CD8α). After washing, cells were incubated with multiple species Ig-adsorbed FITC-anti-hamster Ig (Dianova, Hamburg, Germany) and PE anti-rat Ig (Dianova), and the $CD11c^+/CD8\alpha^+$ subset was sorted using a FACS Vantage cell sorter (Becton Dickinson, Heidelberg, Germany). Purity of the FACS-sorted fractions exceeded 90% as determined by CD11c expression.

Immunostaining

For flow-cytometry analysis Cell-surface staining was performed in a PBS buffer with 1% BSA, 0.02% sodium azide. In some experiments, COP5-transfected cells were pre-incubated with mannan (1 mg per mL; Sigma-Aldrich) for 15 min before staining. For intracytoplasmic staining, cells were permeabilized with saponin (0.1% vol/vol) for 5 min, and subsequent steps were performed in the same buffer, except the last wash which was performed in PBS. For surface plus intracellular staining, either Fix&Perm™ (An der Grub Bio Research, Kaumberg, Austria) or Cytofix/Cytoperm™ (BD-PharMingen) kits were used according to the manufacturers' recommendations. Incubations with the antibodies lasted at least 30 min, followed by two washes. When required, second steps were performed the same way. Flow-cytometric analysis was performed on a FACSCalibur™ (BD Biosciences, Mountain View, California) with the CellQuest™ software. Most of the experiments shown represent 4-color flow-cytometry analysis. Fluorochromes typically used in such experiments were Alexa 488™ or FITC, phycoerythrin (PE), PerCP-Cy5, and allophycocyanin (APC).

For immunohistochemistry Ears were separated with strong forceps into dorsal and ventral leaflets. Dorsal halves were incubated for 30 min at room temperature on NH_4SCN 0.5 M, and epidermis and dermis were separated from each other with forceps. Epidermal sheets were fixed with acetone for 20 min at room temperature, incubated with Alexa Fluor 488™-coupled anti-langerin mAb 205C1 and rat mAb 2G9 anti-mouse MHC class II. After three washes, sheets were incubated for 30 min with goat anti-rat IgG-Alexa Fluor 594™ (Molecular Probes). Alternatively, sheets were incubated with anti-langerin mAb 929F3 followed by biotinylated anti-rat IgG/streptavidin Texas-Red™, and with mAb 2G9-FITC. Isotype-matched mAb were included as negative controls. After three washes, sheets were placed on a slide and mounted under coverslips with one drop of Fluoromount G (Electron Microscopy Sciences, Fort Washington, Pennsylvania). Specimens were viewed on a Zeiss Axioscop® epifluorescence microscope. Pictures were taken using an Optronics MagnaFire® Digital Camera (Optronics, Goleta, California).

FITC-induced *in vivo* migration of LC Mice were painted on both sides of the ears with 25 μL of FITC (5 mg per mL) diluted in dibutylphthalate (Sigma-Aldrich):acetone (1:1). After indicated times, mice were sacrificed for collection of ear-draining LN (retro-auricular). LN were subsequently processed as described above. Pertussis toxin was purchased from Sigma-Aldrich (P7208). Intra-dermal injection of the toxin, as described by Itano *et al* (2003), did not inhibit

migration of LC as opposed to that of dermal DC, leading to the conclusion that toxin may not have reached the LC in effective concentrations. We therefore modified the protocol in the following manner. Pertussis toxin (10 μg per mL in 50 μL of PBS) was injected into the pinna of the ear 45 min before application of FITC (25 μL per dorsal ear half; 8 mg per mL in a 1:1 mixture of dibutyl-phthalate:acetone). Sixty minutes thereafter, another pertussis toxin treatment was performed epicutaneously (25 μL at a concentration of 10–20 μg per mL in a 1:1 glycerol:water mixture). Draining LN were analyzed 48 h after FITC treatment. Both unenriched LN cell suspensions (digested by means of collagenase P in the presence of DNAse for 25 min at 37°C followed by thorough washing in PBS containing EDTA), and suspensions enriched in DC by Nycodenz gradient centrifugation (McLellan *et al*, 2002), were immunolabeled and analyzed by flow-cytometry with similar results. Four-color staining was performed, using anti-CD11c-APC (BD), biotinylated anti-CD8α (BD), followed by streptavidin-PerCP (BD), and anti-langerin-PE, in addition to the cell tracer FITC. MAb 929F3 was used for the detection of langerin in permeabilized (Fix&Perm, An der Grub Bio Research, Kaumberg, Austria) cell suspensions. This antibody was visualized with an anti-rat-Ig conjugated to phycoerythrin (Jackson Immunoresearch, Avondale, Pennsylvania).

Electron microscopy FACS-sorted spleen cell suspensions were fixed with Karnovsky's half-strength formaldehyde-glutaraldehyde fixative, post-fixed in 3% aqueous osmium tetroxide, followed by en bloc contrasting in veronal-buffered 1% uranyl acetate. After dehydration in a graded series of ethanols, specimens were infiltrated and embedded in Epon 812 resin. Ultrathin sections were inspected with a Philips EM400 (Fei Company, Eindhoven, The Netherlands) at an operating voltage of 80 kV.

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Supplementary Material

The following material is available on line for this article.
Figure S1,
Table S1

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Address correspondence to: Sem Saeland, PhD, INSERM U 503/IFR 128, 21 avenue Tony Garnier, 69365 Lyon cedex 07, France. Email: semsaeland@yahoo.fr

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