

The monoclonal antibody DCGM4 recognizes Langerin, a protein specific of Langerhans cells, and is rapidly internalized from the cell surface

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We generated monoclonal antibody (mAb) DCGM4 by immunization with human dendritic cells (DC) from CD34⁺ progenitors cultured with granulocyte-macrophage colony-stimulating factor and TNF- α . mAb DCGM4 was selected for its reactivity with a cell surface epitope present only on a subset of DC. Reactivity was strongly enhanced by the Langerhans cell (LC) differentiation factor TGF- β and down-regulated by CD40 ligation. mAb DCGM4 selectively stained LC, hence we propose that the antigen be termed Langerin. mAb DCGM4 also stained intracytoplasmically, but neither colocalized with MHC class II nor with lysosomal LAMP-1 markers. Notably, mAb DCGM4 was rapidly internalized at 37 °C, but did not gain access to MHC class II compartments. Finally, Langerin was immunoprecipitated as a 40-kDa protein with a pI of 5.2–5.5. mAb DCGM4 will be useful to further characterize Langerin, an LC-restricted molecule involved in routing of cell surface material in immature DC.

Key words: Monoclonal antibody / Langerhans cell / Internalization / MHC class II

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1 Introduction

Dendritic cells (DC) are APC that are required for initiation of a specific T cell-driven immune response [1]. As exemplified by Langerhans cells (LC), DC residing in non-lymphoid tissue are immature cells whose primary function is to capture antigen [2].

This function is achieved either through specialized surface membrane endocytic structures or through macropinocytosis [3]. Concomitant with processing of antigen in specialized organelles of the endocytic pathway, the DC migrate to secondary lymphoid tissue and mature into highly efficient APC.

The maturation process of LC includes loss of adhesion receptors such as E-cadherin [4] and disappearance of the characteristic Birbeck granules [5]. Conversely, upon acquisition of antigen presentation function, costimulatory receptors such as the CD80 and CD86 molecules are up-regulated to permit T cell activation [6]. Maturation events of DC can be reconstituted *in vitro* by TNF- α [7] and CD40 ligand (CD40L) [8] which mimic the response to pro-inflammatory cytokines following encounter with pathogen and contact with T cells in secondary lymphoid tissue, respectively.

The highly specialized and anatomically localized functions of DC are thus controlled by tight regulation of expression of a number of key molecules. Consequently, it appears of interest to search for molecules selectively expressed by different DC subpopulations.

Culture systems have become available to obtain large numbers of DC *in vitro*. DC can be obtained from cord blood CD34⁺ hematopoietic progenitor cells (HPC) cultured with TNF- α and GM-CSF (CD34-derived DC) [9], or

[1 19613]

Abbreviations: DC: Dendritic cell LC: Langerhans cell MR: Mannose receptor CD40L: CD40 ligand HPC: Hematopoietic progenitor cells MFI: Median fluorescence intensity

from peripheral blood monocytes with GM-CSF and IL-4 (monocyte-derived DC) [7, 10]. In addition, phenotypically and functionally distinct DC subpopulations can be isolated either *in vitro* [11] or *ex vivo* from various organs [12, 13]. These considerations made it attractive to raise mAb specifically reactive with DC subsets.

We have characterized mAb DCGM4, obtained following immunization of mice with CD34-derived DC. mAb DCGM4 reacts selectively with immature DC of the LC lineage. The antibody recognizes a 40-kDa cell surface and intracytoplasmic protein termed Langerin, which is subtly regulated by cytokines. mAb DCGM4 is rapidly internalized from the cell surface at 37 °C. The restricted distribution and the routing capacity of Langerin point to its importance in the physiology of LC.

2 Results

2.1 Selection of mAb DCGM4 reactive against Langerin

Supernatants from 854 hybridomas were screened for cell surface reactivity on DC obtained from CD34⁺ HPC cultured for 12 days in GM-CSF and TNF- α . Supernatant of one hybridoma, designated DCGM4, reacted only with a minor subset of DC and not with a panel of different hematopoietic-derived cell types (data not shown). mAb DCGM4 was purified and found to be of IgG1/ κ isotype as determined by ELISA. As the observed reactivity appeared of particular interest, mAb DCGM4 was selected for further studies. Finally, as early experiments indicated that LC were recognized by this mAb, we termed the antigen recognized by mAb DCGM4 “Langerin”.

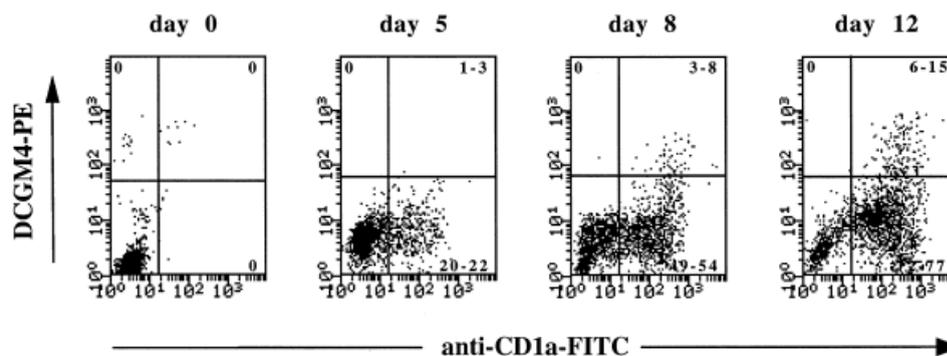


Figure 2. Expression of Langerin on DC derived from cord blood CD34⁺ HPC. Kinetics of CD1a and Langerin expression during culture of CD34⁺ HPC in GM-CSF plus TNF- α . At the time points indicated, cells were double-labeled using FITC-conjugated anti-CD1a and mAb DCGM4 plus PE-conjugated anti-mouse IgG-PE. Results are representative of more than five experiments.

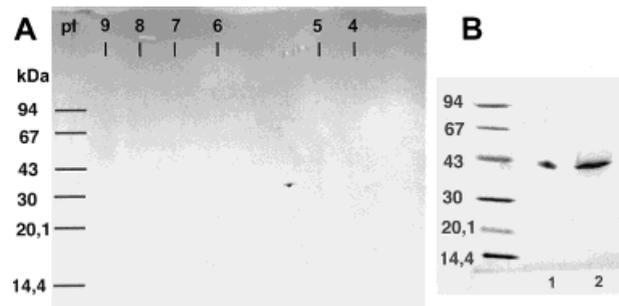


Figure 1. Biochemical analysis of Langerin. (A) Two-dimensional electrophoresis of immunopurified Langerin. (B) Analysis of immunopurified Langerin under non-reducing (lane 1) and reducing conditions (lane 2).

2.2 Langerin is a 40-kDa glycoprotein

Immunoprecipitation from DC extracts with mAb DCGM4, followed by two-dimensional electrophoresis, indicated that Langerin is a 40–42-kDa protein with a pI of 5.2–5.5 (Fig. 1A). If DTT was omitted during the purification steps, the profile was not modified on the gel, suggesting that Langerin occurs as a single chain or as a homodimer with non-covalent association (Fig. 1B). Finally, Langerin is a glycoprotein, as most of the carbohydrate constituents were removed by N-glycosidase treatment (data not shown).

2.3 Langerin is selectively expressed on Langerhans-type DC *in vitro*

CD34⁺ HPC cultured with a combination of GM-CSF and TNF- α for 12 days differentiate into CD1a⁺ DC [9]. We examined expression of Langerin during such cultures. As illustrated in Fig. 2, no staining with mAb DCGM4 was

detected at day 0 or day 5, indicating that CD34⁺ HPC and their immediate progeny do not express Langerin. The antigen appeared at day 8 on a small subset of the CD1a⁺ cells. Between days 8 and 12, Langerin expression reached a maximum, with mAb DCGM4 staining between 15 and 35 % of the CD1a⁺ cells (Fig. 2). Caux et al. [11] have shown that CD34⁺ HPC differentiate along two independent pathways from distinct precursor subsets, identified by early and mutually exclusive expression of CD1a and CD14. When such precursor subsets were separated by cell sorting at day 6 and cultured with GM-CSF and TNF- α for another 6 days, Langerin was largely expressed on the CD1a-derived DC which display features of LC [11]. By contrast to CD34-derived DC, mAb DCGM4 did not react with DC from peripheral blood monocytes cultured for 6 days with GM-CSF and IL-4, further confirming the restriction of Langerin expression (data not shown). Studies *in vitro* and *in vivo*

have shown that TGF- β plays an essential role in LC development [14, 15]. We evaluated the effect of TGF- β on Langerin expression by *in vitro* derived DC. TGF- β strongly up-regulated the expression of Langerin and other LC-specific markers such as E-cadherin on CD34-derived DC (Fig. 3). As further shown in Fig. 3, Langerin was also detected intracellularly following membrane permeabilization. Moreover, reactivity with anti-Lag, another marker of LC [16], was enhanced by TGF- β in permeabilized cells but never observed at the cell surface (Fig. 3). Taken together, the above data demonstrate that Langerin expression is restricted to LC-like cells *in vitro*.

2.4 Analysis of Langerin expression *in situ* and on *ex vivo* isolated DC

In situ distribution of Langerin was examined by immunohistological analysis. Within the epidermis, Langerin was only found in LC, which were also stained with anti-Lag and anti-CD1a antibodies (Fig. 4a–c). In tonsil (Fig. 4d–f), Langerin⁺ cells were strikingly found in the epithelium (Fig. 4f). A few cells were occasionally stained in the T cell areas, but never observed in germinal centers (Fig. 4d–e). Langerin⁺ cells were also present in lung epithelium (Fig. 4g–i). Notably, Langerin was neither detected in lymph node or thymus sections nor in *ex vivo* purified DC isolated from peripheral blood (data not shown). To further confirm the expression of Langerin on LC, we performed electron microscopy on epidermal suspensions. Immunogold labeling with mAb DCGM4 confirmed that Langerin was clearly associated with the LC cytomembrane (Fig. 5a). No labeling was observed on keratinocytes or melanocytes in the epidermal cell suspensions (Fig. 5b, c). Taken together, these data confirm that the expression of Langerin is restricted to LC.

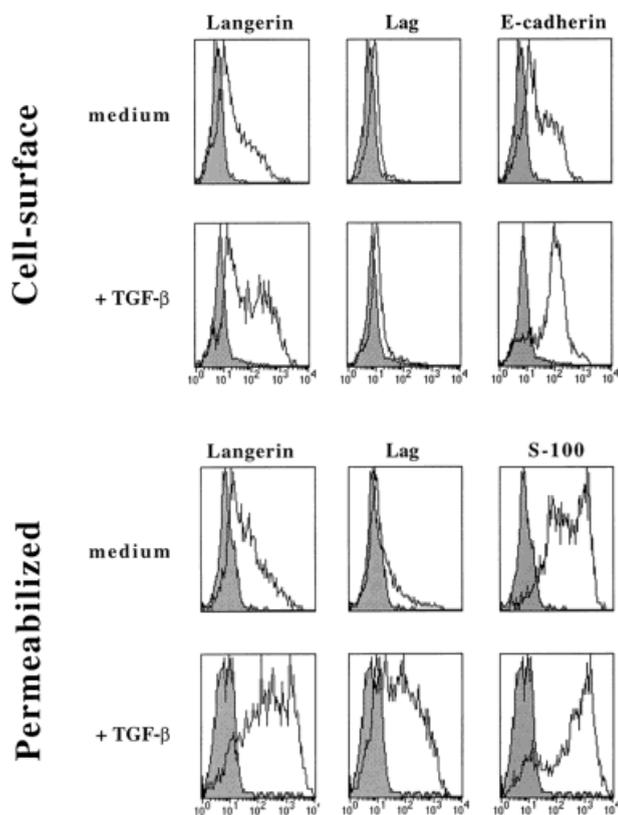


Figure 3. Langerin expression is up-regulated by TGF- β . Cord blood CD34⁺ HPC were cultured for 12 days in GM-CSF and TNF- α in the absence or presence of 1 ng/ml TGF- β from day 7 to day 12. Cells were processed for staining without (top) or with pretreatment with 0.3 % saponin (bottom), using mAb revealed by FITC-conjugated anti-mouse Ig. Grey histograms show isotype-matched controls. Results are representative of more than five experiments.

2.5 Langerin is down-regulated after CD40L activation

Removal of TNF- α , a cytokine known to induce DC maturation [7], for the last 3 days of culture up-regulated Langerin expression (data not shown). In line with this result, loss of Langerin surface membrane expression associated with increased HLA-DR was observed following activation through CD40 ligation, a signal which triggers maturation of DC [8] (Fig. 6). Moreover, LC obtained from epidermal explants and cultured for 2 days lost detectable Langerin expression (data not shown). These results confirm the above distribution data in that TGF- β , which induces an LC phenotype, up-regulates Langerin expression, whereas signals that trig-

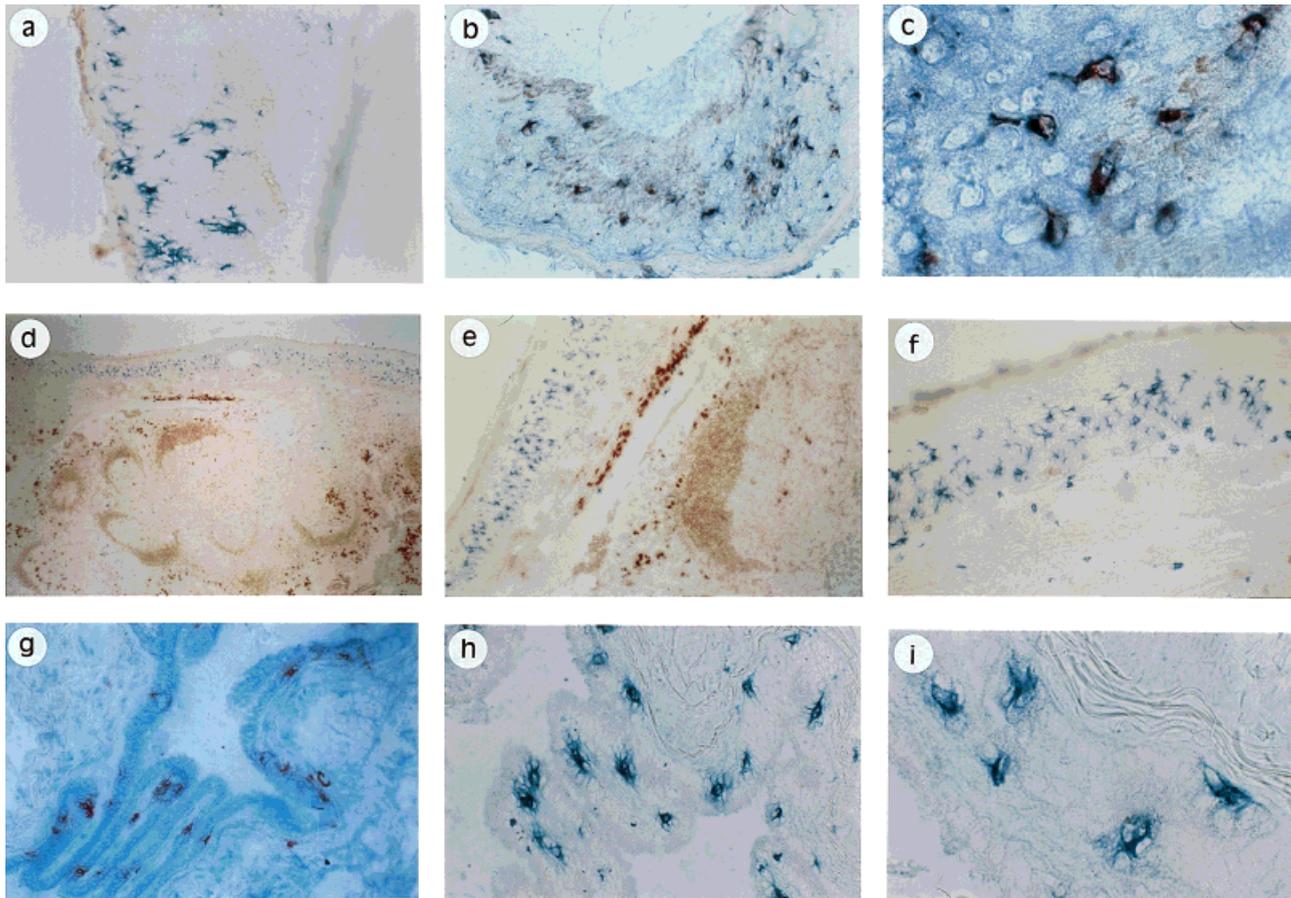


Figure 4. Immunohistological analysis of Langerin expression. In skin, staining with mAb DCGM4 (a, magnification $\times 400$), mAb DCGM4 with anti-Lag or anti-CD1a (b, magnification $\times 400$ and c, magnification $\times 1000$, respectively) shows Langerin expression by LC. In tonsil, mAb DCGM4 and anti-sIgD (follicular mantle B cells) (red) reveal Langerin⁺ DC (blue) in epithelium (d, magnification $\times 100$, e, magnification $\times 200$ and f, magnification $\times 400$). In lung, mAb DCGM4 and counterstaining with hematoxylin shows LC-like Langerin⁺ cells (red) only in bronchiolar epithelium (g, magnification $\times 200$). At higher magnification, these Langerin⁺ DC (blue) show dendritic morphology (h, $\times 400$, i, $\times 1000$). No staining was detected with control mAb (not shown).

ger DC maturation decrease Langerin expression. Unlike cell surface expression, CD40 ligation did not result in complete disappearance of intracellular Langerin. This prompted us to analyze whether engagement of CD40 results in a shift in the intracellular localization of Langerin. Confocal microscopy on DC supplemented with TGF- β demonstrated that Langerin colocalized neither with class II HLA-DR (Fig. 7a) nor with the lysosomal marker LAMP-1 (Fig. 7b). While inducing translocation of HLA-DR to the cell surface, as expected during DC maturation (Fig. 7c), CD40 ligation did not result in redistribution of Langerin to Lamp-1⁺ compartments (Fig. 7d). These findings demonstrate that CD40-mediated maturation induces loss of cell surface Langerin, but no detectable redistribution of the molecule into lysosomes.

2.6 Langerin mediates rapid internalization of mAb DCGM4

As LC represent immature DC specialized in capture of antigen, we examined the role of Langerin in endocytosis. Thus, we analyzed the capacity of Langerin to endocytose mAb DCGM4 as a ligand. We found that the antibody was quickly internalized by DC (Fig. 8a), with kinetics as rapid as an anti-mannose receptor (MR) mAb internalized via receptor-mediated endocytosis (Fig. 8b). Finally, as illustrated by confocal microscopy (Fig. 8B), mAb DCGM4 did not colocalize with HLA-DR following internalization (20 min at 37 °C). These results demonstrate that Langerin is implicated in rapid endocytosis that does not appear to intersect with routing of MHC class II molecules.

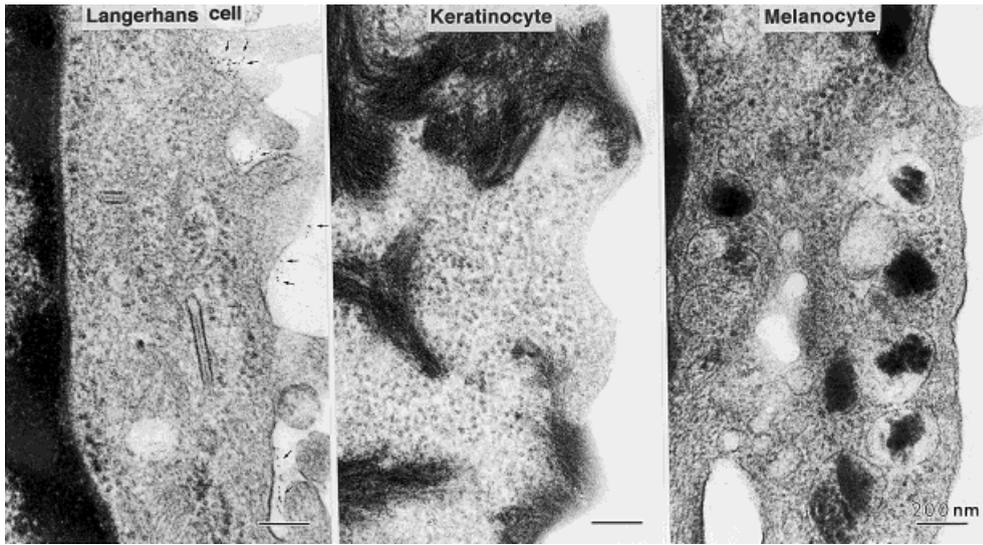


Figure 5. Langerin is expressed at the surface membrane of LC. Epidermal cell suspensions were obtained and processed for electron microscopy as described in Sect. 4.7. Staining with mAb DCGM4 is visualized (thin arrows) by 5-nm gold-labeled goat anti-mouse IgG1. No staining by mAb DCGM4 could be detected on keratinocytes or melanocytes. Observations are representative of three experiments on different skin samples.

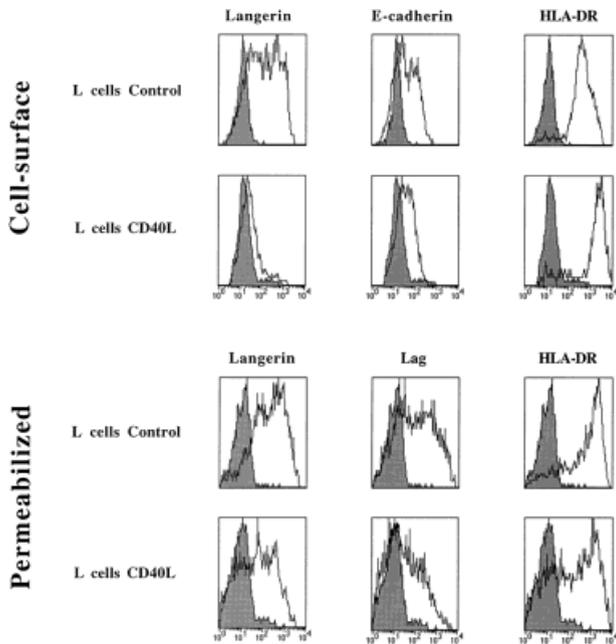


Figure 6. CD40 ligation decreases Langerin expression. CD34-derived DC were supplemented with 1 ng/ml TGF- β from day 7 to day 9 of culture. Cells were cultured for another 2 days with irradiated fibroblastic L cells transfected with CD40L, or with control L cells. Cells were processed for staining and FCM analysis without (top) or with permeabilization with 0.1 % saponin (bottom), using mAb revealed by FITC-conjugated anti-mouse Ig. Grey histograms show isotype-matched antibody controls. Results are representative of more than five experiments.

3 Discussion

DC represent a heterogenous cell population, present at trace levels in all tissues. Because of the diversity and corresponding functional specialization of DC subsets, the search for new DC molecules is a widely pursued objective. In the present study, we report the characterization of mAb DCGM4, which recognizes Langerin, a surface membrane and intracellular glycoprotein expressed by LC-type immature DC.

Langerin is expressed by LC in skin and in epithelia, such as lung bronchiolae, revealing a new link between DC lining the skin and airway. Langerin is distinct from other markers of LC such as E-cadherin or the CD1-molecules [4, 17]. A characteristic feature of LC is the presence of Birbeck granules, a unique cytoplasmic organelle of yet unknown function. LC react with anti-Lag antibody, directed against an intracellular protein associated with Birbeck granules [16]. mAb DCGM4 displays a distinct reactivity, as anti-Lag staining was never detected at the plasma membrane.

TGF- β 1, a cytokine produced by epithelial cells, strongly up-regulated expression of Langerin on CD34-derived DC. This is consistent with the LC distribution of the antigen, as TGF- β plays a key role in LC development both *in vitro* and in TGF- β -deficient mice [14, 15]. DC from peripheral blood did not react with mAb DCGM4. As blood DC include immature cells [13], representing pre-

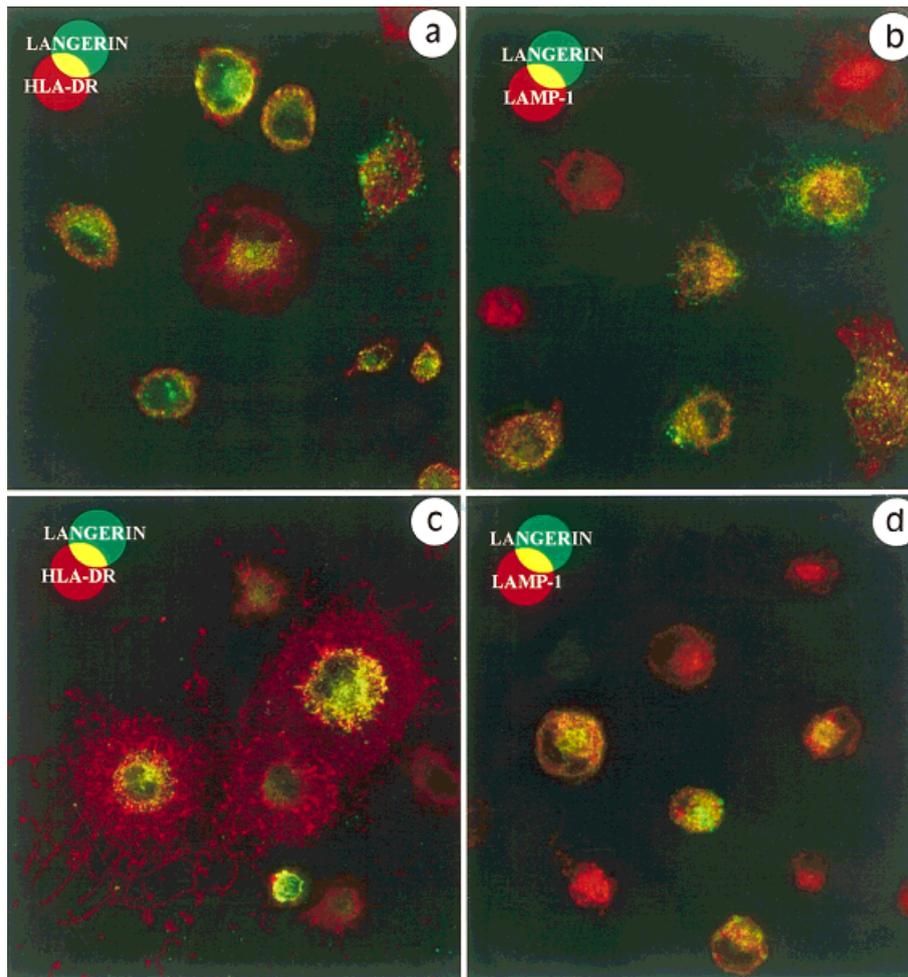


Figure 7. Langerin does not colocalize with HLA-DR or LAMP-1. CD34-derived DC were supplemented with 1 ng/ml TGF- β from day 7 to day 9 of culture. Double-color confocal laser scanning microscopy was performed at day 9 (a, b) and after two subsequent days of CD40 ligation (c, d). No colocalization is detected between Langerin and HLA-DR (a, c) or LAMP-1 (b, d), irrespective to CD40L engagement.

cursors routed from bone marrow towards skin and epithelia, Langerin is likely not acquired at a pre-LC maturation stage. This is consistent with relatively late (day 8–12) appearance of Langerin on CD34-derived DC.

Several lines of evidence indicate that Langerin is down-regulated beyond the LC maturation stage. First, loss of reactivity with mAb DCGM4 was observed after culture of epidermal explants, a condition that induces LC maturation [5], and in response to CD40L, a cell surface molecule of activated T cells that provides a strong signal for DC activation and maturation [8]. Second, the antigen was neither detected in blood, which contains a subset of mature DC [13], nor significantly in areas of secondary lymphoid tissue where mature DC present antigen. A residual intracellular expression of Langerin was

observed, however, following CD40 ligation *in vitro*. This apparent discrepancy with *in vivo* distribution of Langerin may reflect that CD40L is not the major maturation signal that LC receive *in situ*. Finally, mAb DCGM4 did not react with DC derived from peripheral blood monocytes in GM-CSF and IL-4 [7]. As such cells also represent immature DC, this result indicates that Langerin expression is not a general feature of an early differentiation state of DC, but is restricted to cells of the LC pathway.

Incubation of DC at 37°C resulted in rapid endocytosis of mAb DCGM4. The endocytic function of Langerin agrees with its distribution pattern restricted to immature DC specialized in capture of antigen. The down-regulation of Langerin expression in response to CD40L

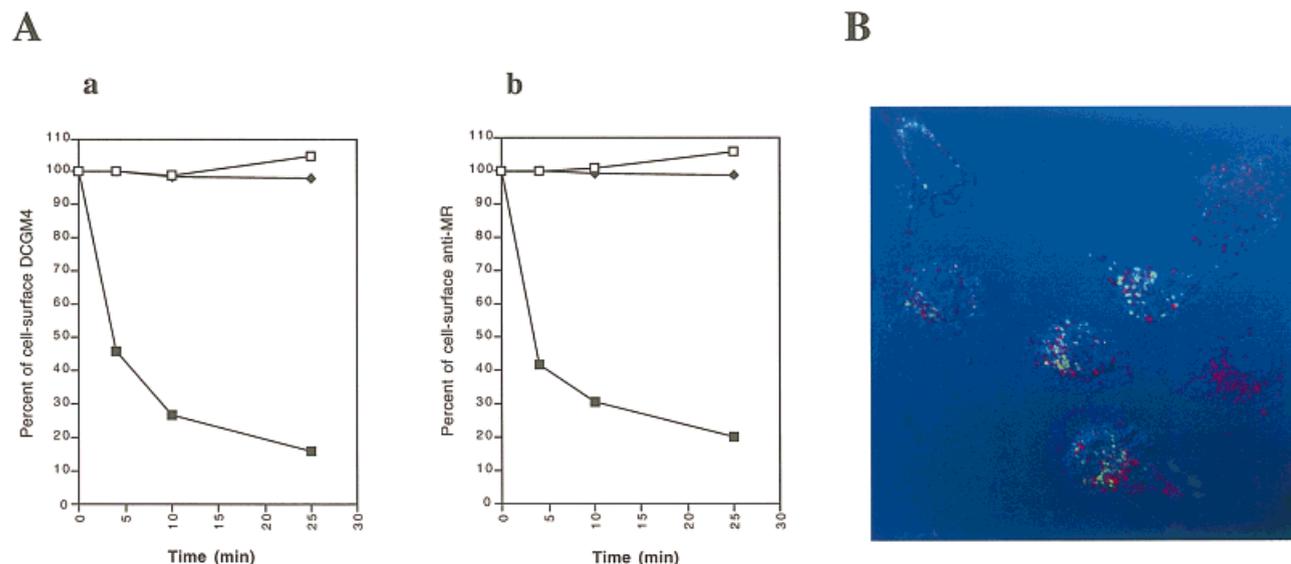


Figure 8. mAb DCGM4 is rapidly internalized at 37 °C but not detected in HLA-DR⁺ compartments. (A) CD34-derived DC supplemented with 1 ng/ml TGF- β were labeled at 4 °C with mAb DCGM4 or mAb DCGM1 directed against the MR, followed by biotin-labeled F(ab')₂ goat anti-mouse IgG. Cells were incubated at 37 °C for the time periods indicated, cooled to 4 °C and stained with streptavidin-PE. Internalization was measured as decrease in cell surface-bound antibody determined by FCM analysis. mAb DCGM4 is rapidly internalized at 37 °C (a), with kinetics similar to that of an anti-MR mAb (b) (■). In paraformaldehyde-fixed cells, no decrease of cell surface fluorescence was detected (◆). Results are expressed as the percentage decrease of MFI as compared to control samples kept at 4 °C (□). (B) Confocal microscopy analysis following internalization (20 min at 37 °C) of mAb DCGM4 (green), illustrating lack of colocalization with HLA-DR (red).

or TNF- α is consistent with diminishing endocytic capacity of DC during late stages of maturation [3, 18]. Remarkably, internalization via Langerin proved to be as rapid as that mediated via the MR. The MR is internalized through coated pits and recycled through early endosomes [3, 19], allowing delivery of exogenous antigen into specialized compartments for processing and loading onto MHC class II molecules for presentation to T cells. We neither observed colocalization of intracellular Langerin with HLA-DR nor with LAMP-1 molecules indicative of late endosomes and MIIC lysosomes [20, 21]. In addition, internalization of mAb DCGM4 did not result in routing of the antibody into HLA-DR⁺ compartments. These data strongly suggest that the function of Langerin is not related to antigen delivery into the MHC class II pathway. Consequently, it will be of particular interest to examine whether Langerin could be involved in routing extracellular antigen into the MHC class I pathway for induction of cytotoxic T cell responses. This may be relevant as DC are highly efficient cells for the uptake of apoptotic cell bodies and subsequent routing of internalized material for presentation on MHC class I molecules [22].

In conclusion, the present report describes mAb DCGM4 which recognizes Langerin, a novel protein selectively expressed by LC. In particular, cytomembrane reactivity

allows to use mAb DCGM4 for cell sorting and functional studies. The highly restricted distribution and efficient endocytic capacity of Langerin suggests a particular role of this molecule in routing cell surface-bound material in LC.

4 Materials and methods

4.1 Generation of mAb DCGM4

BALB/c mice (Iffa Credo, Les Oncins, France) were immunized with CD34-derived DC (10^6 cells) and mAb were obtained as described [23]. Supernatants were screened for reactivity with CD34-derived DC and three unrelated cell types, namely peripheral blood polynuclear cells, T lymphocytes activated with PHA, and the myeloid cell line KG1 (ATCC; Rockville, MD). Selected hybridomas were cloned by limiting dilution and ascites were produced in BALB/c mice. mAb DCGM4 was purified by anion-exchange chromatography on DEAE A50 (Pharmacia Biotech, Uppsala, Sweden). Ig isotype was determined by ELISA using a mouse hybridoma subtyping kit (Boehringer Mannheim, Mannheim, Germany).

4.2 Media and reagents

The medium used was RPMI 1640 supplemented with 10 % heat-inactivated FBS (Flow Laboratories, Irvine, GB), 2 mM L-glutamine, and 80 µg/ml gentamicin (referred to as complete medium). Recombinant human (rh) GM-CSF (specific activity: 2×10^6 U/mg; Schering-Plough Research Institute, Kenilworth, NJ) was used at 100 ng/ml (200 U/ml). rhTNF- α (specific activity: 2×10^7 U/mg; Genzyme, Boston, MA) was used at 2.5 ng/ml (50 U/ml). rhstem cell factor (specific activity: 4×10^5 U/mg; R&D, Abington, GB) was used at 25 ng/ml and rhIL-4 (specific activity: 10^7 U/mg; Schering-Plough Research Institute) was used at 5 ng/ml. rhTGF- β 1 (R&D) was used at 1 ng/ml.

4.3 Cells and tissues

DC were generated from cord blood CD34⁺ HPC as described [9]. In some experiments, TNF- α was replaced by TGF- β at day 7, or DC were activated with murine Ltk fibroblastic L cells transfected with human CD40L [24].

Human epidermal cell suspensions were obtained as described [6].

4.4 Cytofluorimetric analysis

For single staining, cells were labeled using the mAb anti-E-cadherin (SHE 79.7; Takara, Shiga, Japan), anti-MHC class II (HLA-DR) (Becton Dickinson, Mountain View, CA), anti-S100 (SH-B1, Sigma) and anti-Lag [16], revealed by FITC-conjugated goat anti-mouse Ig (Dako, Glostrup, Denmark). For double staining, cells were labeled with mAb DCGM4, revealed by PE conjugated goat anti-mouse Ig (Dako), and with FITC-conjugated anti-CD1a (Ortho, Raritan, NJ). Fluorescence was determined with a FACScan flow cytometer (Becton Dickinson). For intracytoplasmic phenotyping, cells were stained in PBS, 0.3 % saponin (Sigma) and 5 % BSA, using the same procedure.

4.5 Immunohistology

Acetone-fixed cryocut tissue sections were incubated with mAb for 60 min, and subsequently with biotinylated sheep anti-mouse Ig (The Binding Site, Birmingham, GB) for 30 min. Following incubation with streptavidin coupled to alkaline phosphatase (Biosource, Camarillo, CA), enzyme activity was developed using Fast Red substrate (Dako). Double staining with mouse IgG1 antibody DCGM4 and IgG2b anti-CD1a (Immunotech) was revealed by sheep anti-mouse IgG1 (The Binding Site) followed by mouse anti-alkaline phosphatase-alkaline phosphatase complexes (APAAP technique; Dako), and biotinylated sheep anti-mouse IgG2b (The Binding Site) followed by ExtrAvidin-peroxidase (Sigma). Binding of goat anti-slgD-biotin and

mAb DCGM4-biotin (for double staining with anti-Lag) was directly revealed by ExtrAvidin-peroxidase. Alkaline phosphatase and peroxidase activity were revealed using Fast Blue substrate (Sigma) and 3-amino-ethylcarbazole (Sigma), respectively.

4.6 Internalization assay

CD34-derived DC supplemented with TGF- β were generated as detailed above and internalization was performed as described [25], using mAb DCGM4 or mAb DCGM1 (an anti-MR antibody raised in our laboratory). The measure of internalization is given by the percentage decrease of cell surface median fluorescence intensity (MFI) as compared to control samples kept at 4 °C. The percentage decrease of MFI observed in fixed cells was taken as measure of the off-rate of the antibody at 37 °C.

4.7 Electron microscopy

Langerhans cell-enriched epidermal cell suspensions were incubated with control mouse IgG1 (Sigma) or mAb DCGM4 for 1 h at 4 °C, followed by goat anti-mouse IgG conjugated with colloidal 5-nm gold particles (GAM-5nm) (Amersham, Arlington Heights, IL), and processed for electron microscopy as described [6]. Ultrathin sections were examined on a JEOL 1200 EX electron microscope (CMEABG, Université de Lyon, Lyon, France).

4.8 Confocal microscopy

Intracellular immunofluorescence staining was performed as previously described [20]. Cells on polylysine-coated coverslips were fixed with 4 % paraformaldehyde and incubated with anti-LAMP-1 (Pharmingen, San Diego, CA) or anti-HLA-DR (Becton Dickinson) followed by donkey anti-mouse Ig coupled to Texas Red (Vector Laboratories, Burlingame, CA), and finally incubated with mAb DCGM4 coupled to FITC. Coverslips were mounted onto glass slides with fluoromount (Southern Biotechnology Associates Inc., Birmingham, AL). Confocal microscopy was performed using a Confocal Laser Scanning Microscope TCS 4D (Leica Laser-technik GmbH, Heidelberg, Germany) interfaced with an argon/krypton ion laser and with fluorescence filters and detectors allowing to simultaneously record FITC and Texas Red markers [20].

4.9 Biochemical analysis

Proteins were extracted from CD34-derived DC supplemented with TGF- β by addition of 50 mM Tris-HCl pH 8 buffer with 150 mM NaCl, 5 mM EDTA, 1 % Triton X-100 and protease inhibitor (cOmplete Mini, Boehringer Mannheim) to

a frozen pellet of $100\ \mu\text{l}/10^7$ cells. Supernatants were then incubated with mAb DCGM4 covalently linked to Dynabeads M-450 sheep anti-mouse IgG magnetic beads (Dyna, Oslo, Norway). Beads were washed with extraction buffer by using a Dynal magnetic particle concentrator and boiled in the presence of $50\ \mu\text{l}$ SDS-PAGE sample buffer or resuspended in $100\ \mu\text{l}$ 0.5 M glycine, 0.15 M NaCl, pH 2.3, for 4 min. Then, supernatant was neutralized with $3.5\ \mu\text{l}$ of saturated Tris solution. SDS-PAGE analysis was performed with a PhastSystem in a 10–15 % gradient gel (Pharmacia Biotech), and gels were stained with Coomassie R250. Two-dimensional analysis was performed on a Multiphor II flat bed system with Immobililine DryStrip pH 3–10 and Excelgel SDS 8–18 % for the second dimension (Pharmacia Biotech), and gels were silver stained.

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