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Identification and expression of mouse Langerin (CD207) in dendritic cells

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Abstract

We have cloned the mouse homologue of human Langerin (h-Langerin), a type II transmembrane protein with a single external C-type lectin domain. Mouse Langerin (m-Langerin) displays 65 and 74% homologies in total amino acid and lectin domains with those of h-Langerin. The cognate mouse and rat genes were assigned to chromosome 6D1–D2 and chromosome 4q33 distal–q34.1 proximal respectively, syntenic to the h-Langerin gene on chromosome 2p13. With RT-PCR, m-Langerin transcripts were as expected detected in MHC class II$^+$, but not MHC class II$^-$, cells from epidermis and the expression level was reduced by culture. However, m-Langerin transcripts were also expressed in spleen, lymph nodes (LN), thymus, liver, lung and even heart, but not gut-associated lymphoid tissues. In single-cell lymphoid suspensions, m-Langerin transcripts were mainly detected in the CD11c$^+$ dendritic cells (DC), especially the CD11b^{low}/CD8^{high} fraction of spleen and LN. DC generated from bone marrow precursors by granulocyte macrophage colony stimulating factor (GM-CSF) expressed m-Langerin, but this was shut down during maturation with CD40 ligand or lipopolysaccharide. DC derived from blood monocytes by GM-CSF + IL-4 lacked m-Langerin unless the cultures were supplemented with transforming growth factor (TGF)-β1. Unexpectedly, significant amounts of m-Langerin transcripts were detected in skin and LN of TGF-β1-deficient mice, although in much lower amounts than littermate controls. Recombinant m-Langerin could form multimers and bind to mannan–agarose. These findings indicate that Langerin expression is regulated at several levels: by TGF-β1, DC subsets, DC maturation and the tissue environment.

Introduction

Dendritic cells (DC) are potent antigen-presenting cells that develop from bone marrow (BM) progenitors (1,2). DC are localized as sentinels in many organs, where they endocytose foreign and self-antigens, and then migrate to secondary lymphoid organs to present antigenic peptides in the context of MHC molecules to specific T cells. A subset of DC, designated Langerhans cells (LC), is located in the epidermis of skin. An important marker to define LC is the racket-shaped cytoplasmic Birbeck granules, which can play a role in endocytosis (3). This organelle is recognized with electron microscopy, although anti-Lag ('Langerhans-associated granule') antibody has been established for human LC (4). Recently, a new C-type lectin called Langerin (CD207) has been cloned using the human LC-specific antibody DCGM4.

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This lectin binds both anti-Lag and DCGM4 mAb but via different epitopes (5,6).

LC also express E-cadherin, presumably for establishing homologous contacts with E-cadherin on keratinocytes (7). Both E-cadherin and Langerin expression depend on transforming growth factor (TGF)-β1, using cultured monocytes and $CD34^+$ progenitors, and even immature $CD11c^+$ DC in peripheral blood (8–10). TGF-β1-deficient mice lack LC but not other DC (11). A low level of E-cadherin expression is detected in a small fraction of lymph nodes (LN) DC, where its reduced expression is ascribed to the maturation of LC during migration from epidermis to draining node, since LC also significantly lose E-cadherin expression upon culture (7). Like E-cadherin, Langerin is also reduced upon maturation of LC (5). Langerin is a useful marker to study LC development and to identify subsets of DC, especially skin-derived LC (12). Therefore, we have independently cloned mouse Langerin (m-Langerin) cDNA. Here, we describe its chromosomal mapping, tissue distribution and down-regulation with DC maturation. Unexpectedly, m-Langerin transcripts are expressed in a variety of organs. In spleen and lymph nodes, expression appears to be restricted to the $CD11b^{low}$ or $CD8^{high}$ DC subset, though m-Langerin is not found in Peyer's patches. In addition, we show that recombinant m-Langerin binds to mannan–agarose and can form multimers.

Methods

Mice

Female BALB/c and male DBA/2 mice were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). These mice were bred under specific pathogen-free condition and (BALB/c × DBA/2)F_1 ($CD2F_1$) mice were used at 8–12 weeks old. TGF-β1-deficient mice (13) were treated with 4 μg/g of rapamycin (Sigma, St Louis, MO) 3 times a week from day 10 after birth until use. All experiments were conducted according to institutional guidelines.

Cells and cultures

For all cell cultures, RPMI 1640 containing 10% FCS, 50 μM β-mercaptoethanol and antibiotics was used, unless otherwise indicated. BM-DC were prepared as described (14). To obtain fully mature late-stage BM-DC, aggregates of growing DC were collected at day 6, transferred into new plates at a cell density of $<5 \times 10^5$ cells/ml and re-cultured in the presence of baculovirus-expressed soluble CD40 ligand (sCD40L, ×100 dilution) or lipopolysaccharide (LPS, 1 μg/ml; Sigma) for another 2 days. BM-DC of early, intermediate and late stage were sorted using a FACStar Plus (Becton Dickinson, Mountain View, CA) based on the expression levels of CD86 and MHC class II. Monocyte-derived mouse DC were prepared as described (15). Briefly, white blood cells were cultured for 2 h at 37°C in 24-well plates at 2.5×10^6 cells/well. After extensive washing, adherent cells were cultured with 20 ng/ml recombinant granulocyte macrophage colony stimulating factor (rGM-CSF) and 400 U/ml rIL-4 in the presence or absence of 10 ng/ml TGF-β1. Then, cells were harvested on days 2, 4, 6 and 7. rGM-CSF was a kind gift from Kirin Brewery (Takasaki, Gunma, Japan), while the other cytokines were obtained from R & D Systems (Minneapolis, MN).

Epidermal cell suspensions (fresh) were prepared from ear halves as described (16,17) and MHC class II^+ LC were positively enriched using TIB-120 mAb (M5/114.15.2; ATCC, Rockville, MD) followed by immunomagnetic beads (sheep anti-rat IgG Dynabeads; Dynal, Oslo, Norway). LN and spleen DC were prepared as previously described (18). DC were further fractionated into $CD11c^+CD8^{high}$ ($CD11c^+CD11b^{low}$) and $CD11c^+CD8^{low/-}$ ($CD11c^+CD11b^{high}$) by sorting with either FACStar Plus or Epics Altra flow cytometers (Coulter, Hialeah, FL).

Peritoneal exudate macrophages were obtained from mice that had been i.p. injected with 2 ml of 4% thioglycollate (Difco, Detroit, MI) 4 days previously. Macrophages were prepared as adherent cells by incubating for 2 h. T cells were purified as $CD3^+$ cells using FITC-conjugated anti-mouse CD3ε (PharMingen, San Diego, CA) and anti-FITC microbeads (Miltenyi Biotech) from nylon wool non-adherent spleen and LN cells depleted of MHC class II $(TIB\text{-}120)^+$, $F4/80\ (HB198)^+$ and $B220\ (6B2)^+$ cells by sheep anti-rat Ig Dynabeads M-450 (Dynal). Naive B cells were positively enriched as $B220^+$ cells from $CD43^-$ spleen cells.

cDNA cloning of m-Langerin

Total RNA was extracted from the mouse epidermal cells using TRIzol Reagent (Gibco/BRL, Rockville, MD) and used for making a template with Superscript II (Gibco/BRL) in accordance with the manufacturer's protocols. The cDNA was then used in RT-PCR with Z-Taq DNA polymerase (Takara Shuzo, Kusatsu, Shiga, Japan). Several primers were designed based on h-Langerin cDNA (accession no. AJ242859). In these primers, a pair, 5′-GCTTGGAGAATATGAGCAAGTTGC-3′ and 5′-GCACTTTGGACCTTGTTGAATGGC-3′, amplified a 304-bp fragment under the conditions of 95°C for 2 min, 30 repeats of a cycle at 98°C for 1 s, 55°C for 5 s and 72°C for 1 min, and a final extension at 70°C for 7 min. This fragment was cloned into pCRII vector (Invitrogen, Carlsbad, CA). For 3′-rapid amplification of cDNA end (RACE), a template was prepared from the RNA with a primer 5′-TACGCCAAGCT-CGAAATTAACCCTCACTAAAGGG$(T)_{16}$-3′. The first PCR was done using a primer pair, 5′-TACGCCAAGCTCGAAATT-AACCCTC-3′ and 5′-CTCGGGGAACTTCTATTACTTTTC-3′, under the conditions of 95°C for 2 min, 30 repeats of a cycle at 98°C for 1 s and 68°C for 1 min, and a final extension at 70°C for 7 min. The second PCR was done using a primer pair, 5′-TCGAAATTAACCCTCACTAAAGGG-3′ and 5′-ACGCACC-CCAAAGACCTGGTACAG-3′, under the same conditions as above.

For 5′-RACE, the SMART PCR cDNA library construction kit (Clontech, Palo Alto, CA) was used for making a template. With this template, the first PCR was done using the primer pair, 5′-TACGGCTGCGAGAAGACGACAGAA-3′ and 5′- CTGTGT-GGAATTCCATCTGCTGCC-3′, under the same conditions. The second PCR was done using the primer pair, 5′-GGCTGCGAGAAGACGACAGAAGGG-3′ and 5′-GCCTT-GTAGAGAAACTTTTGTTCC-3′, also under the same conditions. Entire cDNA was finally amplified again with PfuTurbo DNA polymerase (Stratagene, La Jolla, CA) for sequencing.

RT-PCR

Total RNA was extracted using TRIzol Reagent (Gibco/BRL) and used for making templates with Sensiscript (Qiagen, Valencia, CA) or SuperScript II (Gibco/BRL) in accordance with the manufacturer's protocols. Primers for m-Langerin (5′-ACGCACCCCAAAGACCTGGTACAG-3′, 5′-AGACACCC-TGATATTGGCACAGTG-3′), β-actin (5′-CAGGAGATGG-CCACTGCCGCA-3′, 5′-CTCCTTCTGCATCCTGTCAGCA-3′), MIP-3α (5′-CGTCTGCTCTTCCTTGCTTTG-3′, 5′-TTGACTCT-TAGGCTGAGGAGG-3′), CCR6 (5′-GGTACATTGCCATCG-TCCAG-3′, 5′-CCAAAGAACAGCTCCAGTCC-3′), TGF-β1 (5′-TTTCGATTCAGCGCTCACTGCTCTTGTGAC-3′, 5′-ATG-TTGGACAACTGCTCCACCTTGGGCTTGC-3′) and E-cadher-in (5′-TGCTCAGCCAGGATCCTGAG-3′, 5′-GGTCTGGATC-CAAGATGGTGC-3′) were used under the conditions of 95°C for 2 min, 30 repeats of a cycle at 98°C for 1 s, 64°C for 5 s and 72°C for 10 s, and a final extension at 70°C for 7 min.

Northern blot analysis

Poly(A)$^+$ RNA was purified from s.c. LN by FastTrack 2.0 (Invitrogen) in accordance with the manufacturer's protocols. The RNA (2 μg) was separated on 1.2% formaldehyde–agarose gel electrophoresis and transferred to Hybond N+ membrane (Amersham Pharmacia Biotech, Piscataway, NJ). A cDNA fragment (1 kbp), which included the entire coding sequence, was labeled by [^{32}P]dCTP using the Prime-It II random primer labeling kit (Stratagene) and hybridized with the filter in Rapid-hyb hybridization solution (Amersham Pharmacia Biotech) at 68°C for 1 h. The filter was washed twice in 2 × SSC/0.1% SDS at 68°C for 15 min and then twice in 0.2 × SSC/0.1% SDS at 68°C for 15 min.

Chromosome preparation and fluorescence in situ hybridization (FISH)

The direct R-banding FISH method was used for chromosomal assignment of the Langerin gene to mouse and rat chromosome. Preparation of R-banded chromosomes and FISH were performed as described previously (19). As a probe, 1.4-kbp cDNA fragment was amplified using a primer pair, 5′-ATGCCAGAGGCAGAGATGAAGGA-3′ and 5′-TACCAT-GAGGTGTTCTAATAC-3′ under conditions of 95°C for 2 min, 30 repeats of a cycle at 98°C for 1 s, 62°C for 5 s and 72°C for 1 min, and a final extension at 70°C for 7 min. The amplified fragment was purified with agarose gel electrophoresis.

Expression of N-terminal-tagged m-Langerin protein and binding assay with mannan–agarose

A coding sequence of m-Langerin was amplified with KlenTaq DNA polymerase (Sigma) and cloned into pcDNA4/HisMax TOPO TA (Invitrogen) according to the manufacturer's protocols. Then human 293-based cell line Phoenix (a kind gift from G. Nolan) was transfected using Lipofectamine 2000 (Gibco/BRL). After 48 h, cells (5×10^6) were harvested and lysed in 1 ml of lysis buffer 1 (50 mM Tris–HCl (pH 7.6), 150 mM NaCl, 25 mM $CaCl_2$, 0.5% Triton X-100, 1 mM PMSF and 20 μg/ml aprotinin) for 30 min on ice. The lysate was collected after centrifugation at 12,000 *g* for 20 min and diluted 10-fold with binding buffer (lysis buffer 1 without Triton X-100). Then 1 ml of the lysate was incubated with 20 μl of mannan–agarose beads (Sigma). After 12 h incubation at 4°C, the agarose beads were washed by 1 ml of the binding buffer that included 0.05% Triton X-100 5 times with or without 25 mM EDTA. Bound proteins to the agarose beads were separated on 12% SDS–PAGE under denaturing conditions and transferred to Immobilon-P membranes (Millipore, Bedford, MA). The membrane was probed with a horseradish peroxidase-labeled anti-Xpress antibody (Invitrogen) and visualized by LumiGLO chemiluminescent substrate (Cell Signaling Technology, Beverly, MA) according to the manufacturer's protocols.

Analysis of multimer formation of m-Langerin molecules

The lysate of m-Langerin-transfected 293 cells was analyzed by 10% SDS–PAGE under native or denaturing conditions followed by immunoblotting as described above. For cross-linking experiment, bis-(sulfosuccinimidyl)suberate (BS^3; Pierce, Rockford, IL) was used in accordance with the manufacturer's protocol. Briefly, cell lysate was prepared using lysis buffer 2 (20 mM sodium phosphate buffer, pH 7.6, 150 mM NaCl, 0.5% Triton X-100, 1 mM PMSF and 20 μg/ml aprotinin). The lysate of 20 μl was diluted 10-fold with PBS (20 mM sodium phosphate buffer, pH 7.6 and 150 mM NaCl) and various concentrations of BS^3 added. After 2 h incubation at 24°C, the reactions were quenched with glycine (final 50 mM) for 1 h at 24°C and analyzed by 7.5% SDS–PAGE under reducing conditions followed by immunoblotting as described above.

Results

Identification of m-Langerin cDNA

Primer pairs constructed from the lectin part of h-Langerin cDNA amplified a 304-bp fragment from epidermal cell cDNA. As a result of 5′- and 3′-RACE, a 1.5-kb cDNA sequence (accession no. AY026050) was obtained. The cDNA contained a short 5′-non-coding region, a 996-bp open reading frame, a 497-bp entire 3′-non-coding region with a polyadenylation signal (AATAAA) at position 1453 bp, followed by a poly(A) tail (data not shown). The open reading frame encoded a 331-amino-acid polypeptide that showed 65% identity in overall domains and 74% identify in the lectin domain with those of h-Langerin (Fig. 1A). Like h-Langerin, the putative m-Langerin was a type II transmembrane protein, with a single C-type lectin carbohydrate recognition domain (CRD) containing a EPN-motif to bind mannose. m-Langerin also contained two potential *N*-glycosylation sites in the neck domain and a proline-rich motif as a potential signal transduction site in the intracellular domain (20). Furthermore, heptad repeats (see Fig. 5B), possibly forming an α-helical coiled-coil stalk (21) for dimerization/multimerization, were found in the neck domain.

Comparison of the amino acid sequence of the CRD domain of m-Langerin revealed significant homology with several C-type lectins having a single CRD in the type II membrane protein. Among them, m-Langerin displayed the highest homology with mouse Kupffer cell receptor at 46.6%. Degree of homology of m-Langerin and other lectins ranged from 40.2% with m-Mincle to 24.6% with m-Dectin 1 (Fig. 1B).

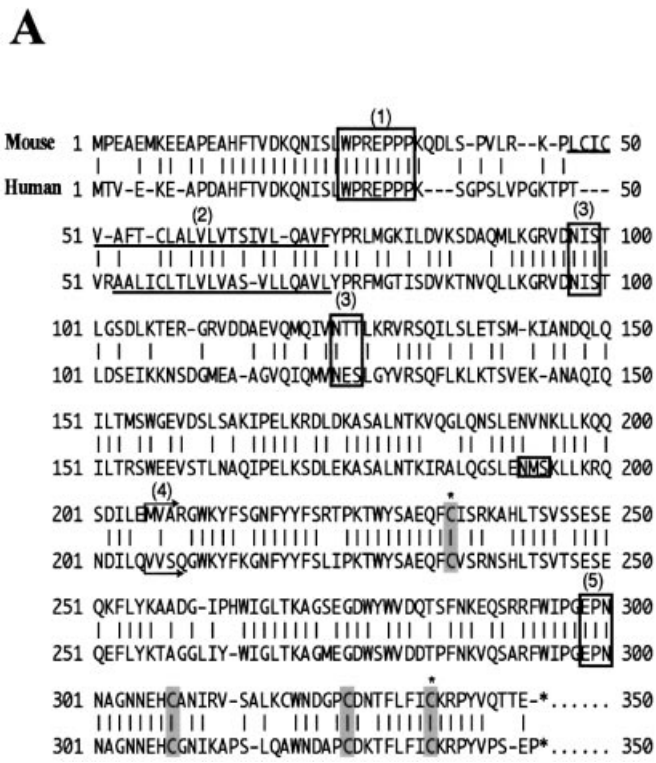
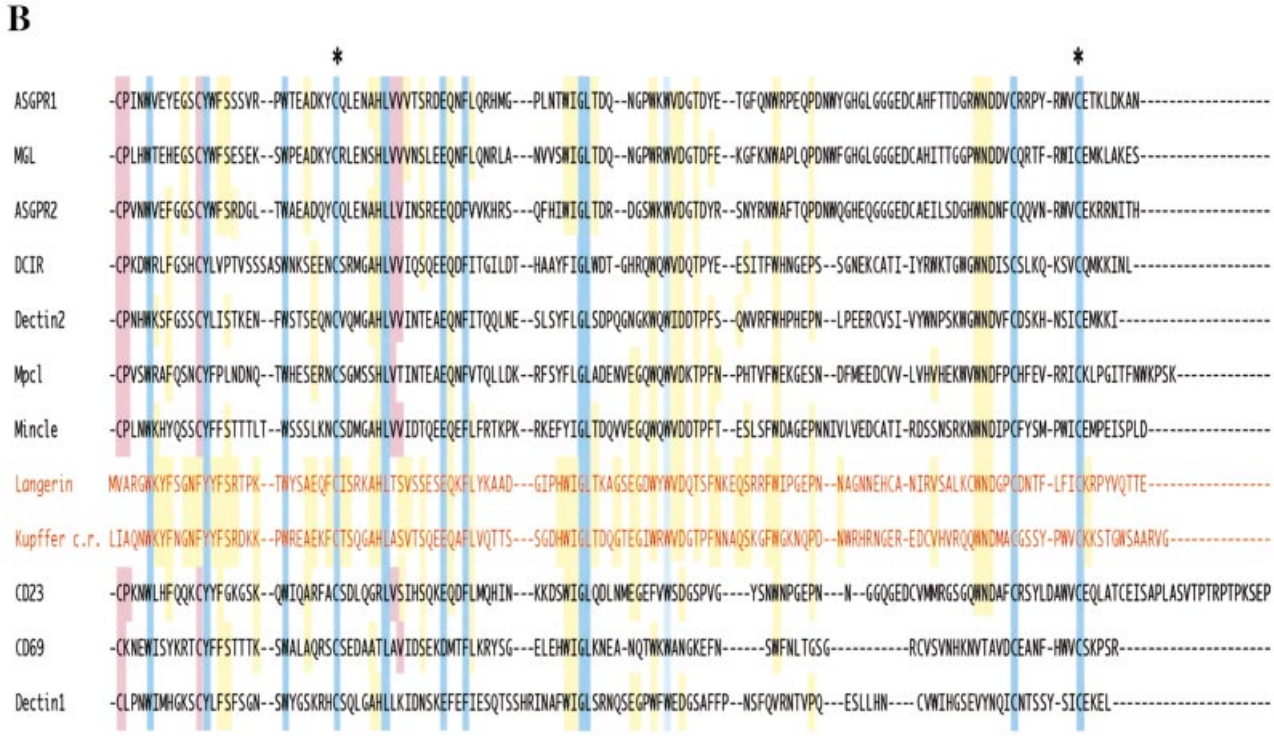


Fig. 1. Analysis of the m-Langerin gene. Alignments of predicted amino acid sequences of m-Langerin and h-Langerin. Box 1: proline-rich putative signal transduction site; underline 2: transmembrane region; box 3: potential *N*-glycosylation site; arrow 4: lectin domain start site; box with asterisk: two cysteine forming disulfide bridge; box 5: EPN motif suggesting mannose-type specificity. (B) Alignment of CRD domain of mouse C-type lectins using CLUSTALW. Amino acids of m-Langerin and Kupffer cell receptor (Kupffer c. r.) are shown in red. Blue: common amino acids in all lectins; violet: common amino acids except for m-Langerin and Kupffer cell receptor; yellow: common amino acids in m-Langerin, Kupffer cell receptor and other lectins. Accession numbers for Kupffer cell receptor, asialoglycoprotein receptor (ASGPR1), asialoglycoprotein receptor 2 (ASGPR2), macrophage galactose/*N*-acetylgalactosamine-specific C-type lectin (MGL), FcεRII (CD23), dendritic cell immunoreceptor (DCIR), dendritic cell-associated C-type lectin-1 (Dectin1), dectin-2 α isoform (Dectin2), CD69 antigen (CD69), macrophage C-type lectin (Mpcl) and macrophage-inducible C-type lectin (Mincle) are D88577, U08372, NM_007493, AF132744, M99371, AJ133533, AF262985, AF240357, L23638, NM_010819 and AB024717.



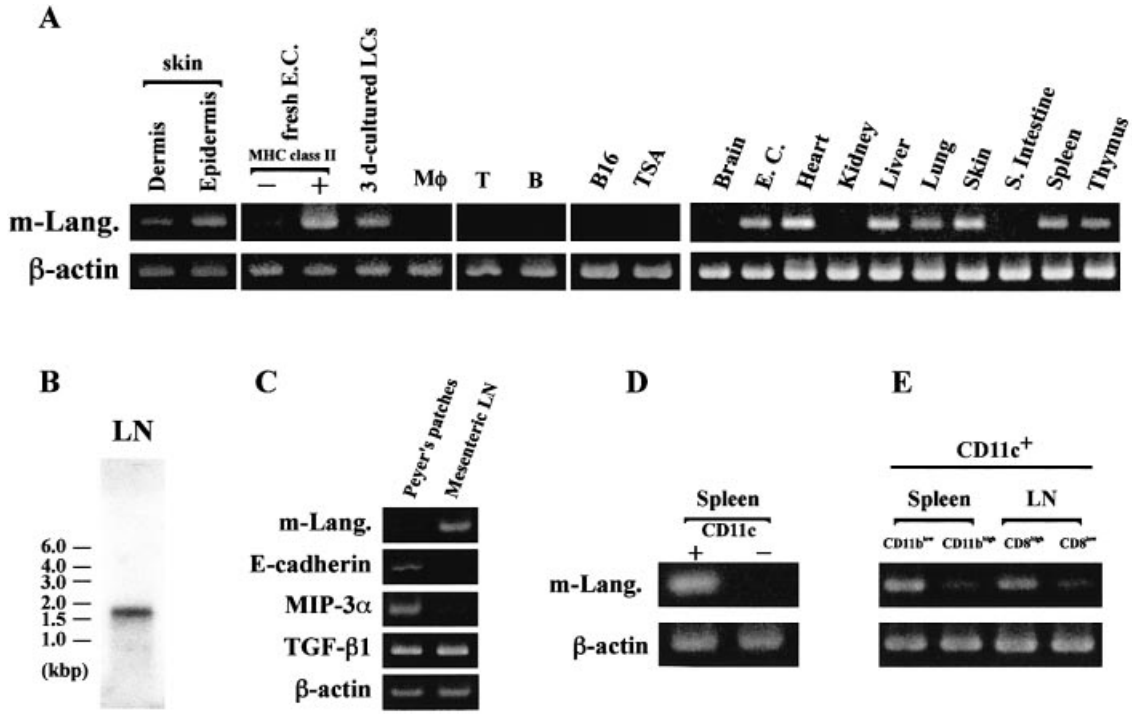


Fig. 2. Expression of m-Langerin transcripts. (A) Expression pattern of m-Langerin in dermis and epidermis, epidermal MHC class II$^+$ cells (LC), macrophages (Mϕ), T cells, B cells and other cell lines, and several organs. EC: epidermal cells prepared with trypsinization; S. Intestine: small intestine. (B) Northern blot analysis of m-Langerin transcripts in s.c. LN. (C) Differential expression of m-Langerin transcripts between Peyer's patches and mesenteric LN. (D) Exclusive expression of m-Langerin in CD11c$^+$ cells of spleen. For preparation of CD11c$^-$ cells, spleen cells were depleted with anti-CD11c microbeads twice. (E) m-Langerin expression in DC subsets. CD11c$^+$ cells were positively selected by anti-CD11c microbeads from low-density cells negative for Thy-1, B220 and F4/80. CD11c$^+$ fractions were further fractionated into CD11b^{low} and CD11b^{high} (spleen) or CD8^{high} and CD8^{low} (LN) using flow cytometry. For RT-PCR of T, B cells (A) and sorted cells (E), Sensiscript was used with 50 ng of total RNA. For others, SuperSccript II was used with 5 μg of total RNA to synthesize a template cDNA.

Expression and distribution of m-Langerin transcripts

h-Langerin has been shown to encode a molecule associated with the Birbeck granules of epidermal LC (5). Thus, we first examined the expression of putative m-Langerin transcripts in mouse epidermal MHC class II$^+$ cells by comparing with MHC class II$^-$ cells and other cell types. Tissue distribution was also studied. As shown in Fig. 2(A), m-Langerin transcripts were largely detected in epidermis, although relatively low but significant levels of expression were also seen in dermis. m-Langerin-expressing cells in epidermal cell suspension were MHC class II$^+$ LC. Other cell types including macrophages, T and B cells, and tumor cell lines, such as B16 melanoma and TSA mammary adenocarcinoma, were all detected as negative. The level of m-Langerin expression was substantially diminished during cell maturation in 3-day cultured LC. In contrast to h-Langerin which is expressed in lung, skin and tonsil, but not other non-lymphoid tissues (5,6), m-Langerin transcripts were detected not only in skin and lung, but also heart, liver and various lymphoid tissue, such as spleen, thymus and LN. Furthermore, the small intestine expressed no m-Langerin transcripts, whereas mesenteric LN, which drains gut-associated mucosal tissue, did express m-Langerin. With Northern blot analysis, ~1.8-kb m-Langerin transcripts were detected in s.c. LN (Fig. 2B), and a similar size transcript was seen in spleen and thymus (data not shown). Interestingly, there were no m-Langerin transcripts in Peyer's patches, although expression of E-cadherin, MIP-3α and TGF-β1 was detected (Fig. 2C).

To further define the cell types expressing m-Langerin, we first examined CD11c$^+$ DC and CD11c$^-$ non-DC from spleen, since spleen should not contain any skin-derived DC, except in the *aly* mouse that lacks LN and Peyer's patches (22). The results shown in Fig. 2(D) demonstrated that CD11c$^+$ spleen DC expressed m-Langerin. Then, we fractionated CD11c$^+$ DC from spleen and LN into CD11b^{low} CD8α^{high} cells and CD11b^{high} CD8α$^{-/low}$ cells and found that m-Langerin transcripts were predominantly detected in the former population (Fig. 2E).

Chromosomal mapping and genomic structure of the Langerin gene

The above results on the tissue distribution of m-Langerin transcripts were somewhat different from that of h-Langerin. Therefore, we attempted to define its localization on mouse chromosomes by the direct R-banding FISH method. As shown in Fig. 3(A), m-Langerin was mapped to the D1–D2 band of mouse chromosome 6 and rat Langerin gene to the q33 distal–q34.1 proximal band of chromosome 4. These regions are syntenic with human chromosome 2p13, where h-Langerin has been mapped (6,23). Therefore, the cloned

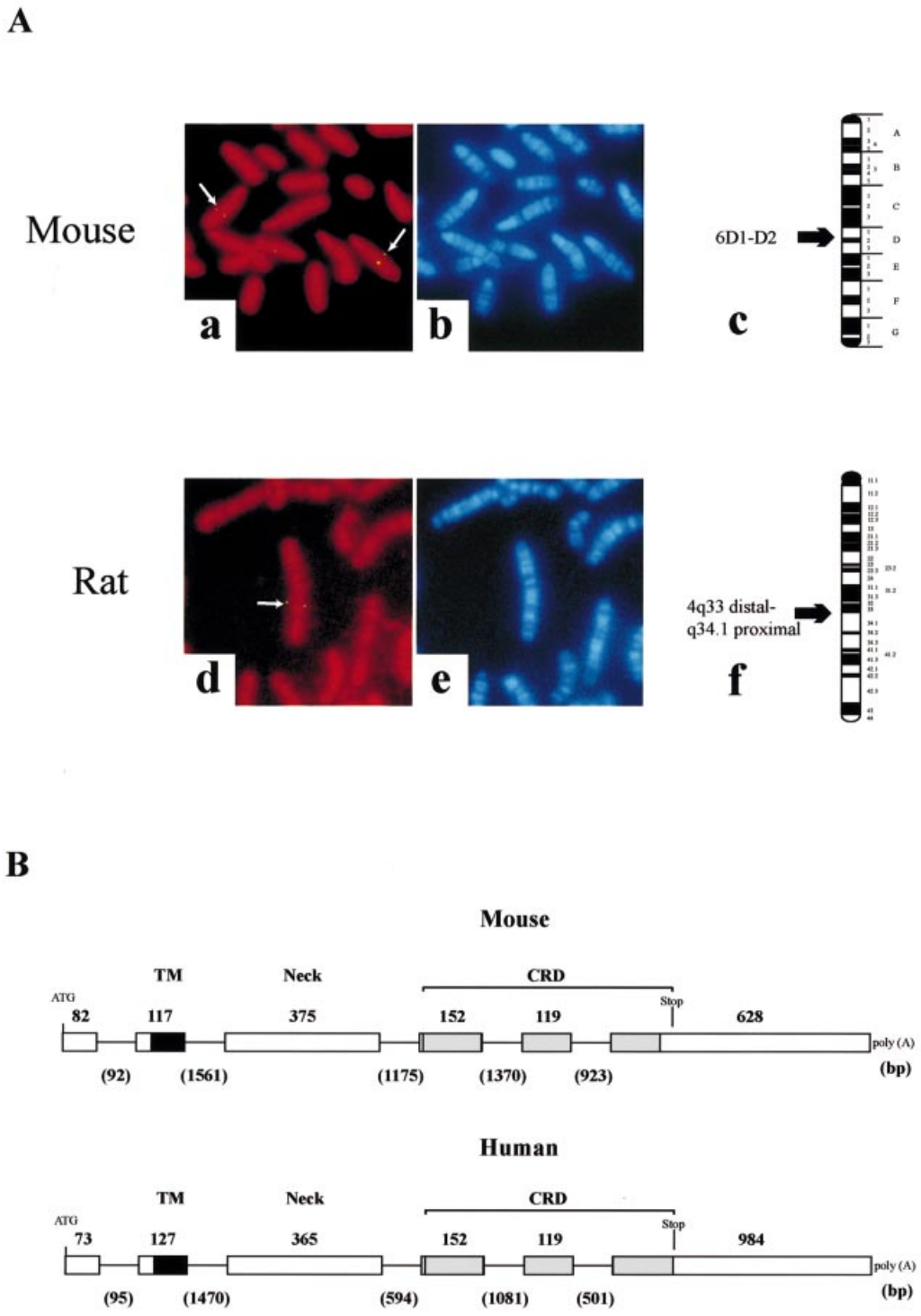


Fig. 3. Chromosomal mapping and genomic organization of the Langerin gene. (A) The Langerin gene was mapped by FISH method in mouse (a–c) and rat (d– f). Arrows indicate the hybridization signals (c and f). The Langerin gene was localized to mouse chromosome 6 D1–D2 and rat chromosome 4q33 distal–q34.1 proximal. R- and G-banded patterns are demonstrated in (a and d) and (b and e) respectively. (B) Exon–intron structures were described from mouse genomic draft sequence AC090647 and human BAC clone sequence AC007359. Intron and exon sizes are shown in base pairs with and without parentheses respectively. TM: transmembrane domain.

mouse gene is a mouse counterpart of h-Langerin. In terms of genomic organization, the m-Langerin gene consists of six exons including a large exon for the neck domain and three exons for the CRD (Fig. 3B). This structure was highly comparable to the h-Langerin gene, deduced from a complete genome sequence of BAC clone RP11-50401 (accession no.

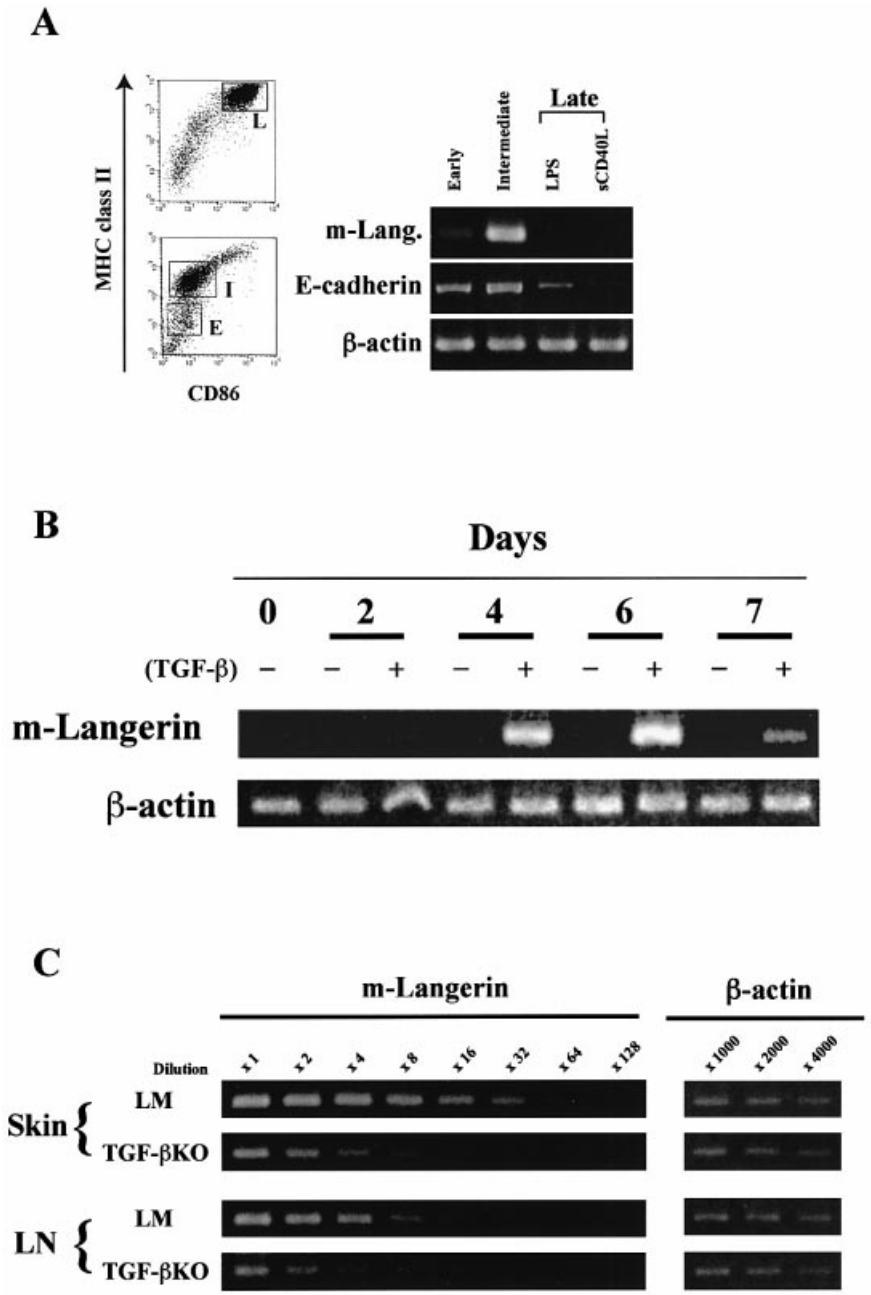


Fig. 4. m-Langerin expression in developing BM-DC and effect of TGF-β1. (A) m-Langerin expression during BM-DC maturation. The early (designated 'E') and intermediate (designated 'I') stages of BM-DC were sorted from day 6 BM cultures (left bottom panel). The mature (late) stage of BM-DC (designated 'L') was sorted from day 8 culture after stimulation with 1 μg/ml LPS (left top panel) or 1/100-fold dilution of sCD40L for the last 2 days. Expression of m-Langerin in each developmental stage was investigated by RT-PCR (right panel). (B) Adherent cells from mouse blood express m-Langerin in the presence of TGF-β1. m-Langerin transcripts were analyzed by RT-PCR. For this experiment, Sensiscript was used with 50 ng of total RNA to synthesize a template cDNA. (C) Semiquantitative analysis of m-Langerin transcripts in skin and LN from normal and TGF-β1-deficient mice. Skin and superficial (inguinal, brachial, axillary and cervical) LN were obtained from TGF-β1-deficient and littermate control mice at the age of 3–4 weeks. SuperScript II was used with 5 μg of total RNA to synthesize a template cDNA.

AC007359), except for the length of introns and exon 6. Splice donor and acceptor sites of each intron were typical, except for the donor sites of both mouse and human intron 1, where the dinucleotide sequences of the 5′-end of mouse and human intron 1 were GC and CA respectively (data not shown).

m-Langerin expression in BM-DC and DC derived from blood adherent cells

The generation of CD1a$^+$ DC from CD34$^+$ precursor cells is dependent on endogenous TGF-β1 and these cells express Langerin as well as E-cadherin (6). Therefore, we tested if BM-

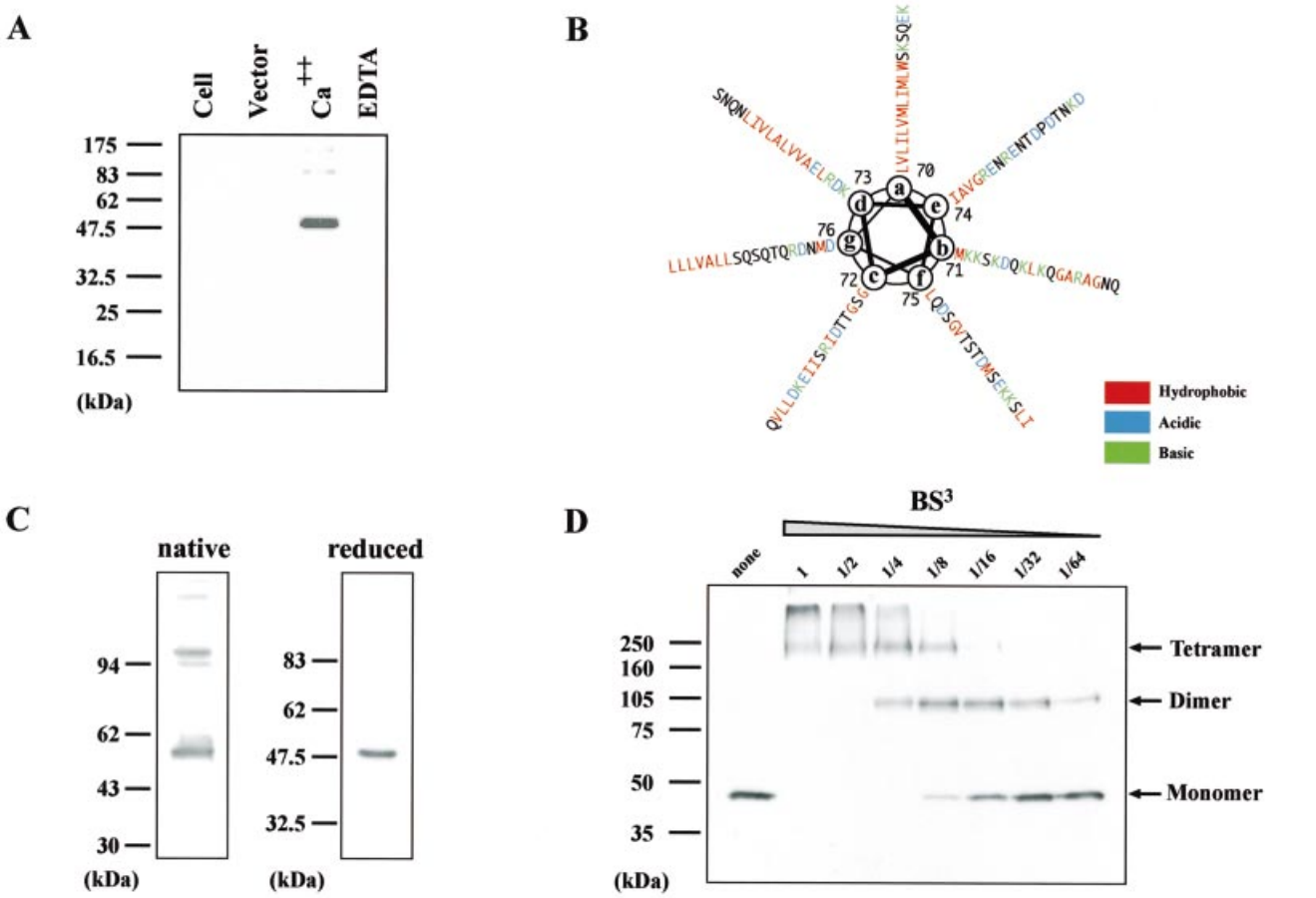


Fig. 5. Binding to mannan–agarose and possible formation of multimers of m-Langerin. (A) Cell lysates of parental cells (Cell), control plasmid-transfected cells (Vector) and m-Langerin-transfected cells were incubated with mannan–agarose beads. After washing with the binding buffer, bound fractions were analyzed by immunoblotting. EDTA: addition of 25 mM EDTA during the washing step. (B) Helical wheel diagram of the neck domain. (C) Cell lysates of m-Langerin-transfected cells were separated on native or denatured SDS–PAGE and detected by immunoblotting. (D) Chemical cross-linking experiment. The lysate of m-Langerin-transfected cells was subjected treatment with 2-fold serial dilutions of BS^3 from 30 μM (designated '1').

DC from mice expressed Langerin. To do this, the DC were sorted into early, intermediate and late stages of maturation based on the expression levels of MHC class II and CD86. Substantial amounts of E-cadherin and low levels of m-Langerin transcripts were detected in early-stage BM-DC (Fig. 4A). In the intermediate stage, m-Langerin transcripts were abundant, while maturation with LPS or sCD40L induced a dramatic decrease, as in human LC (Fig. 4A). This was also the case for E-cadherin, but some E-cadherin expression was still detected after LPS treatment.

These results suggest that endogenous TGF-β1 may be produced during culture and induce the observed m-Langerin expression. Therefore, we next examined the effect of exogenous TGF-β1 on the development of monocyte-derived DC, using adherent cells in the blood. First, we confirmed that the adherent fraction of blood cells expressed no detectable m-Langerin transcripts by RT-PCR (data not shown). Then adherent cells were cultured with GM-CSF and rIL-4 in the presence or absence of 10 ng/ml TGF-β1 for 7 days. Regardless of the presence or absence of TGF-β1, DC-like cells developed without significant proliferation (data not shown). In contrast to the case of BM-DC, m-Langerin transcripts were undetectable throughout the culture period in the absence of TGF-β1 (Fig. 4B). m-Langerin transcripts were not detected in day 2 cultures even in the presence of TGF-β1, but gradually increased thereafter and reached a maximum at day 6. By day 7, however, m-Langerin was reduced in association with spontaneous maturation of the DC.

TGF-β1 is not indispensable for the expression of m-Langerin transcripts

To examine the possibility that TGF-β1 plays an essential role to induce m-Langerin transcripts, we studied TGF-β1-deficient mice that are known to lack LC but not other DC populations (24). We stained epidermal sheets with anti-MHC class II mAb to confirm that there were no typical LC (data not shown). As shown in Fig. 4(C), both skin and LN cells in TGF-β1-deficient mice expressed small but significant amounts of m-Langerin transcripts, although the levels were less than 1/16 of littermates.

Binding to mannan–agarose and possible multimer formation of m-Langerin

Based on the primary structure of m-Langerin and the down-regulation of h-Langerin from the cell surface by mannan treatment (6), we analyzed the binding capacity of m-Langerin to mannan. To do this, N-terminal-tagged recombinant m-Langerin was expressed in 293 cells and the cell lysates were incubated with mannan–agarose. As shown in Fig. 5(A), the recombinant m-Langerin bound mannan–agarose and this binding was completely inhibited by EDTA, demonstrating m-Langerin is capable of capturing mannan.

As mentioned above, m-Langerin may form multimers due to heptad repeats of hydrophobic residues in the neck domain (Fig. 5B), since some of the tagged-m-Langerin migrated more slowly (~100 and >175 kDa) on native SDS–PAGE than m-Langerin (~47 kDa) on the denatured SDS–PAGE (Fig. 5C). In order to examine this possibility, we cross-linked molecules using BS^3. The results shown in Fig. 5(D) revealed that m-Langerin possibly formed dimers and multimers, as has been reported for other lectins, like asialoglycoprotein receptor (25), CD23 (26), pulmonary surfactant protein D (27), DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) and DC-SIGN receptor (28).

Discussion

It has been reported that h-Langerin is an LC-restricted type II lectin that plays a role as an endocytic receptor (3). Here we have identified m-Langerin through the use of primers derived from the functional lectin domain of h-Langerin. The deduced amino acid sequence of m-Langerin showed a high degree of homology with that of h-Langerin (Fig. 1A). Nevertheless, the expression patterns between m- and h-Langerin are different (5,6) (Fig. 2A). Therefore, we identified the Langerin genes on mouse and rat chromosomes in a syntenic position to h-Langerin (Fig. 3A). The genomic structures of m- and h-Langerin are closely related (Fig. 3B). These results demonstrate that our gene is a real counterpart of h-Langerin. Interestingly, the 5′-ends of mouse and human intron 1 are not 'GT'. These unusual splice donor sites suggest a potential regulation of Langerin mRNA splicing.

A comparison of m-Langerin with several type II lectins in amino acid sequence indicates that m-Langerin has a high degree of homology with Kupffer cell receptor (Fig. 1B). Based on the database analysis, m-Langerin and Kupffer cell receptor genes are located in a single genomic clone (accession no. AC090647) within ~200 kb proximity. However, sugar-binding specificities of these lectins are different: Langerin can bind mannose, whereas Kupffer cell receptor binds fucose and galactose. The close genomic linkage of these two endocytic lectins may indicate that these genes are generated by gene duplication.

In contrast to h-Langerin, m-Langerin was detected broadly in mouse tissues. Langerin expression in various lymphoid tissues also was of interest (Fig. 2). LC migrate from epidermis to draining s.c. LN (12). In addition, LC have been reported to express CD8α in LN, following injection into CD8α-deficient mice (29). Henri *et al.* have also reported similar observations demonstrating Langerin expression on $CD8\alpha^{low/moderate}$ DC subset in LN using specific mAb (12). Taking consideration of these studies and the demonstration that cultured LC still express some m-Langerin transcripts, $CD8\alpha^+$ LN DC with m-Langerin transcripts are possibly derived from LC. On the other hand, BM-DC activated by LPS or sCD40L abolished m-Langerin expression (Fig. 4A). Thus, strong activation signals seem to be required to shut off the synthesis of m-Langerin.

In Peyer's patches, m-Langerin transcripts were not detected even though there was significant expression of E-cadherin (Fig. 2C). This was also the case in small intestine (Fig. 2A). Furthermore, no m-Langerin transcripts was detected in $CD11c^+$ cells from Peyer's patches, even though there was LC-associated chemokine receptor, CCR6, transcripts in these $CD11c^+$ cells (data not shown). Nevertheless, it has been documented that $CCR6^+CD11b^+$ DC are localized in Peyer's patches, especially in the subepithelial dome region where MIP-3α/CCL20 is produced to attract LC (30). MIP-3α/CCL20 is known to be required for attraction of LC (31). In addition, CCR6 expression is shown to be restricted to LC or direct precursor of LC in DC populations, and to be induced by TGF-β1 and IL-10 (32,33). It is known that TGF-β1 and IL-10 are abundant in gut-associated mucosal tissue. Thus, it might be speculated that DC in mucosal tissue are not identical to epidermal LC.

Even though Peyer's patches and small intestine are negative for m-Langerin, mesenteric LN that drain these tissue do express Langerin transcripts. In earlier electron microscopic studies, LC and cells containing Birbeck granule-like structures were shown to be increased in number only after challenge with 2,4-dinitro-1-chlorobenzene (34). Taking into account this observation, Langerin-expressing cells in mesenteric LN may not be migratory DC from mucosal tissue. This possibility may also be supported by the results that a major population expressing Langerin was $CD11b^{low}/CD8\alpha^+$, but not $CD11b^{high}/CD8\alpha^-$, DC in mesenteric LN, spleen and liver (Fig. 2 and data not shown). Therefore, it is possible that Langerin-expressing DC in these organs originate from separate precursors other than authentic LC. Alternatively, Langerin-expressing cells in s.c. LN might consist of LC- and non-LC-derived DC.

Langerin transcripts expression in thymus should be also taken into consideration. DC from thymus have $CD11b^{low}/CD8\alpha^+$ cells as a major population and $CD11b^{high}/CD8\alpha^-$ cells as a minor population (18,35). It has been reported that typical Birbeck granules are present in medulla of rat and human thymus (36–39). Thymic DC are known to express another C-type lectin called DEC-205/CD205 that is a potent endocytic receptor for antigen-presenting function (40). The presence of these lectins raises the interesting possibility that T cell selection is influenced by antigen presentation via endocytic lectin receptors on DC for self-antigens.

LC development is known to depend on TGF-β1, since TGF-β1-deficient mice lack LC, but not other DC (11,24). As expected, BM-DC at the intermediate stage of maturation expressed m-Langerin transcripts, as well as E-cadherin, possibly due to endogenous TGF-β1 production, which acts on developing progenitor cells in a paracrine and/or autocrine manner (Fig. 4A) (8). Differentiation of LC from blood monocytes also allowed m-Langerin expression in the presence of exogenous TGF-β1, along with GM-CSF and IL-4, as in

the case of human LC (9,10,41). Unexpectedly, Langerin transcripts persisted in skin and lymph nodes of TGF-β1-deficient mice, although at markedly reduced levels (Fig. 4C). In addition, BM-DC of TGF-β1-deficient mice also expressed Langerin transcripts without exogenous TGF-β1 (data not shown). These results seem to indicate that Langerin expression *per se* does not depend solely on TGF-β1. Larregina *et al.* have recently reported that $CD14^+$ immediate LC precursors in dermis of human skin express Langerin, but not E-cadherin (42). We also detected Langerin transcripts in dermis, although relatively low amounts compared with epidermis (Fig. 2A). Therefore, cells expressing Langerin transcripts in the skin of TGF-β1-deficient mice are likely to be immediate precursors of LC in dermis. On the other hand, $CD11c^+$ cells in Peyer's patches did not express Langerin transcripts even when likely exposed to abundant intestinal TGF-β1. Taken together, synthesis of Langerin transcripts may be regulated at additional levels to TGF-β1. Cytokine(s) including the other TGF-β family members involved in the induction and/or regulation of m-Langerin synthesis remain to be investigated in the future study.

The genomic regions encoding the CRD of m- and h-Langerin consist of three exons. This organization is well conserved in other type II lectins. However, their neck domains show broad diversity in terms of length and number of repeat units. This diversity may generate specific functions of each lectin. For instance, it is thought that a long neck domain of human DC-SIGN facilitates initial contact with ICAM-3 on naive T cells to increase antigen presentation efficiency (43). Another example is dimer/multimer formation of lectin molecules using a heptad repeat in the neck domain (25–28). These examples suggest the functional importance of the neck domain.

The coiled-coil neck domain with a 7-amino-acid heptad repeat, $(a\text{-}b\text{-}c\text{-}d\text{-}e\text{-}f\text{-}g)_n$, including leucine, isoleucine and other hydrophobic amino acids at the *a* and *d* positions, indicates a possibility to form multimers for m-Langerin (Fig. 5B). In fact, m-Langerin could form dimers/tetramers. The results shown in Fig. 5(D) also demonstrate that monomers first form dimers and then further dimerize to a tetramer, since trimer forms of the molecule were not detected by SDS–PAGE. Such tetrameric extracellular clusters may allow the CRD to augment the affinity for glycans on molecules targeted by m-Langerin.

Langerin is a molecule associated with the subcellular structure, called Birbeck granules, a form of endocytic compartment (3,5,44). Along with maturation, LC are known to lose antigen-presenting capacity (45) and this is accompanied by the disappearance of acidic organelles, including Birbeck granules (46). As mentioned above, activated BM-DC lose Langerin transcripts expression, but cultured LC keep expressing it to some extent, suggesting that Langerin-expressing DC in s.c. and mesenteric LN and spleen are not fully activated, and rather in an intermediate stage or maturing state.

Like h-Langerin, m-Langerin is predicted to bind mannose-containing oligosaccharides because of its EPN motif in the CRD domain. This was confirmed by the experiment showing capability of binding to mannan–agarose by recombinant m-Langerin molecules, probably in their tetrameric form (Fig. 5A and C). It has been reported that the α-C region of the macrophage scavenger receptor exhibits a pH-dependent conformational change and possibly controls ligand release (47). This pH dependency is ascribed to the structure of the acidic amino acids stretch at adjacent positions to the hydrophobic core of the heptad repeat. The helical wheel diagram of m-Langerin reveals two stretches containing acidic and basic amino acids at position *b* and *e* (Fig. 5B). Although we have no direct evidence to anticipate pH-dependent multimer formation/degradation of the m-Langerin molecule, these stretches might be involved in antigen processing and in shaping the formation of the Birbeck granules in LC.

Collectively, Langerin is a unique marker for DC subsets including LC. However, the molecular regulation of its transcripts and protein expression requires further elucidation.

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Abbreviations

BM	bone marrow
BM-DC	bone marrow-derived dendritic cells
BS^3	bis-(sulfosuccinimidyl)suberate
CRD	carbohydrate recognition domain
DC	dendritic cell
DC-SIGN	DC-specific intercellular adhesion molecule-3-grabbing non-integrin
FISH	fluorescence *in situ* hybridization
GM-CSF	granulocyte macrophage colony stimulating factor
Lag	Langerhans-associated granule
LC	Langerhans cell
LN	lymph node
LPS	lipopolysaccharide
RACE	rapid amplification of cDNA end
sCD40L	soluble CD40 ligand

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