# Cloning and characterization of the mouse homologue of the human dendritic cell maturation marker CD208/DC-LAMP

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DC-LAMP, a member of the lysosomal-associated membrane protein (LAMP) family, is specifically expressed by human dendritic cells (DC) upon activation and therefore serves as marker of human DC maturation. DC-LAMP is detected first in activated human DC within MHC class II molecules-containing compartments just before the translocation of MHC class II-peptide complexes to the cell surface, suggesting a possible involvement in this process. The present study describes the cloning and characterization of mouse DC-LAMP, whose predicted protein sequence is over 50% identical to the human counterpart. The mouse DC-LAMP gene spans over 25 kb and shares syntenic chromosomal localization (16B2-B4 and 3q26) and conserved organization with the human DC-LAMP gene. Analysis of mouse DC-LAMP mRNA and protein revealed the expression in lung peripheral cells, but also its unexpected absence from mouse lymphoid organs and from mouse DC activated either *in vitro* or *in vivo*. In conclusion, mouse DC-LAMP is not a marker of mature mouse DC and this observation raises new questions regarding the role of human DC-LAMP in human DC.

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**Key words:** Mouse / Dendritic cells / Cellular activation

## 1 Introduction

The unique capacity of dendritic cells (DC) to prime naive T cells relies on a coordinated combination of specialized functions. Indeed, DC switch from an immature "antigen-capturing" stage to a mature "antigen-presenting" one with optimal ability to activate specific T cells [1]. Several mature DC markers directly underlie those functional changes. Expression of CCR7 enables maturing DC to migrate, in response to CCL19/21, from peripheral tissues to secondary lymphoid organs [2]. Up-regulation of CD80 and CD86 costimulatory molecules at the cell surface concurs with the increased ability of mature DC to present antigen to T cells and to activate them [3, 4]. In contrast, no definitive function has yet been reported for CD83 and DC-lysosomal-associated membrane protein (DC-LAMP), two of the most specific markers for mature human DC [5, 6], although recent reports suggest that CD83 might participate in the triggering of T cells [7].

DC-LAMP is a member of the LAMP family that has been used as the most discriminative marker of human mature DC [5, 8]. The LAMP family includes a group of heavily glycosylated proteins that all contain a conserved intracytoplasmic tyrosine-based lysosome-targeting motif ($YX_{\phi}$, where $X_{\phi}$ represents a bulky hydrophobic residue) [9]. This targeting signal is recognized by the adaptor complexes AP1, AP2 and AP3, which convey the LAMP from the trans-Golgi network to the lysosomal membrane [10].

Although cloned more than a decade ago [11], the functions of the ubiquitously expressed LAMP-1 and LAMP-2 remain largely unknown [12]. Protection by their glycosylated luminal part of lysosomal membranes from hydrolysis has been hypothesized [13, 14]. LAMP-1 cell surface expression has been linked to metastatic properties of cancer cell lines [15] and Th1 costimulation by macro-

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**Abbreviations: LAMP:** Lysosomal-associated membrane protein **MIIC:** MHC class II molecules-containing compartment **FISH:** Fluorescence *in situ* hybridization **ODN:** Oligodeoxynucleotide

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phages [16]. LAMP-1-deficient mice display normal lysosomal morphology and functions [17], but the structurally related LAMP-2 glycoprotein is up-regulated, suggesting a functional overlap between both LAMP. Indeed, LAMP-1 and LAMP-2 double-knock-out mice are not viable, whereas both single-deficient mice are fertile and viable [18]. CD68, the closest relative to human DC-LAMP (hDC-LAMP) [5, 19] is described as a scavenger receptor responsible for oxidized low-density lipoprotein uptake by monocytes and macrophages [20, 21]. Macrosialin, the mouse homolog of human CD68 [22], was also shown to reach the cell surface of elicited macrophages and to rapidly internalize, consistent with a receptor function.

The latest identified member of the LAMP family, DC-LAMP, is transiently expressed upon human DC activation at the limiting membrane of the MHC class II molecules intracellular storage compartments (MHC class II molecules-containing compartments, MIIC) [5, 23, 24] involved in MHC class II peptide loading and transfer to cell surface [25]. With time, MHC class II molecules are translocated to the plasma membrane, while DC-LAMP concentrates in perinuclear lysosomes [5]. Although a role in sorting MIIC membrane-associated molecules has been proposed, no definitive function has been ascribed to DC-LAMP yet.

To get further insights into the function of the molecule, and with the hope to define a novel marker of mature mouse DC, we have cloned the mouse DC-LAMP (mDC-LAMP) homologue that shows high conservation of both genomic organization and protein sequence. However, the expression pattern of mDC-LAMP is unexpectedly different from that reported in human: mDC-LAMP was detected only in the lung, but never in mouse DC, no matter the source of cells (*in vitro* derived, *ex vivo* purified or *in vivo*) or the type of activation. Therefore, the mouse appears not to be the most relevant animal to elucidate DC-LAMP function in DC. Moreover, the hypothesis regarding the putative functions of DC-LAMP in human DC should be revisited in light of those results.

## 2 Results

### 2.1 Mouse DC-LAMP is highly homologous to its human counterpart

Northern blot analysis of poly(A)$^+$ RNA from different mouse tissues with an hDC-LAMP probe showed a band of approximately 3 kb in the lung, but not in the heart, brain, spleen, liver, skeletal muscle, kidney or testis (Fig. 1A). Detection of a single band suggested the existence of only one gene with no transcriptional isoforms. Hybridizing a mouse lung cDNA library with an hDC-LAMP probe led to the isolation of a 3272-bp clone. This cDNA starts with a candidate 20-bp 5' untranslated sequence (nucleotides 1–20), followed by a methionine codon located in a consensus Kozak context (Fig. 1B) that opens a 1236-bp open reading frame (nucleotides 21–1256), a 3' 2016-bp untranslated sequence (nucleotides 1257–3272) and a polyadenylation site (AATAAA) at position 3232–3237 followed by a poly(A) tail. The cDNA sequence (Fig. 1B) predicts a type I integral membrane protein of 411 amino acids that reveals a strong homology with hDC-LAMP (Fig. 2A). As for hDC-LAMP [5], two predominantly hydrophobic regions indicate a putative signal peptide (amino acids 1–21) and a transmembrane segment (amino acids 377–401). The luminal domain of 355 amino acids shares the characteristic features of LAMP family members [11, 26]: it has a bipartite structure, divided by a serine/proline-rich region. The membrane proximal domain contains four conserved cysteines, and two potential N-linked glycosylation sites. In contrast to the human sequence, the membrane distal domain does not contain any predicted N-linked glycosylation site. The sequence also indicates that there are several putative O-glycosylation sites (stretches of proline, serine and threonine). The cytoplasmic tail of ten residues contains a conserved tyrosine-based lysosomal targeting motif [27]. Sequence alignment confirmed that mDC-LAMP is a new LAMP family member (Fig. 2B) being most homologous to hDC-LAMP (51% overall identity), in particular in the membrane proximal domain (67.5% identity). It also shows 16%, 17% and 16% identities with mCD68, mLAMP-1 and mLAMP-2, respectively.

### 2.2 The genomic organizations of mouse and human DC-LAMP genes are highly conserved

A mouse genomic cosmid library was hybridized with a 1.9-kb PstI mDC-LAMP cDNA probe. One positive clone of ~40 kb was subcloned and sequenced. To complete the 5' portion of the gene, a second genomic clone was sequenced using the transposition reaction. Sequences of subcloned fragments were used to determine exon sequence, intron/exon borders, and intron sizes (Table 1). The deduced genomic structure was compared with hDC-LAMP and CD68 (clones NT_022676 and NT_010687, respectively) [28] (Fig. 3A and data not shown). Similarly to hDC-LAMP, the mDC-LAMP gene spans over more than 25 kb, and is composed of six exons, varying in length from 69 to 2141 bp, and five introns. This same organization is found for mouse and human CD68 ([29] and data not shown). Using fluorescence *in situ* hybridization (FISH), the mDC-LAMP gene was mapped to chromosome 16 in the B2-B4 region

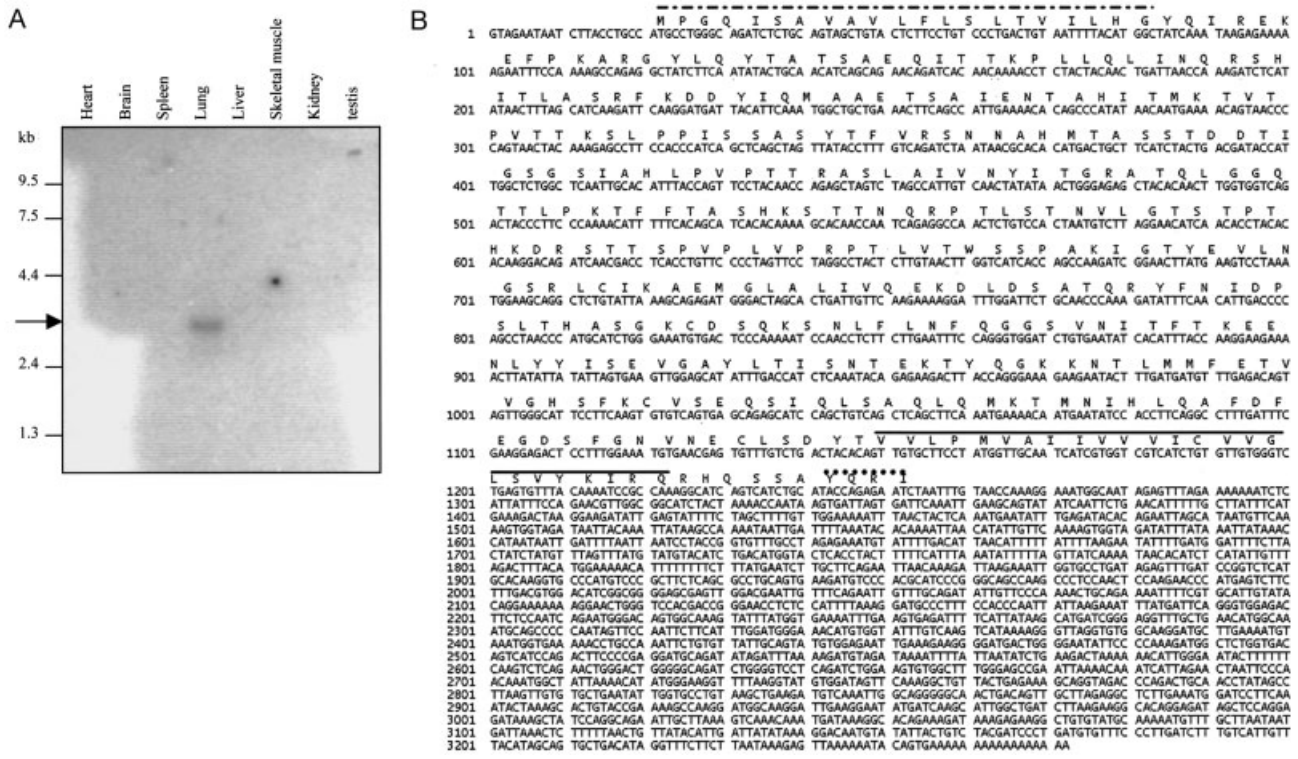


*Fig. 1.* Cloning of mDC-LAMP cDNA. (A) Commercially available mouse tissue Northern blot membrane hybridized with $^{32}$P-labeled hDC-LAMP coding sequence probe. Among the eight tissues analyzed, only the lung expresses detectable amounts of DC-LAMP mRNA which appears as a single 3.2-kb band (arrow). (B) Nucleotide and predicted amino acid sequence of mDC-LAMP cDNA (EMBL accession number: AJ510130). Dashed line (nucleotides 21–83) represents the predicted signal peptide, while the full over-lined region highlights the predicted transmembrane domain (nucleotides 1149–1223). C-terminal tyrosine-based lysosomal targeting motif (nucleotides 1242–1253) is dashed over-lined. Translation was performed using the Vector NTI program (InforMax, Bethesda, MD).

(Fig. 3B), which is syntenic to the localization of hDC-LAMP (chromosome 3q26-q27). Altogether, these results establish that the mDC-LAMP gene identified corresponds to the orthologue of hDC-LAMP.

### 2.3 Mouse DC-LAMP mRNA is almost exclusively detected in the lung

mDC-LAMP distribution was next analyzed by reverse transcription (RT)-PCR on mRNA extracted from various tissues. Specific primers were designed to amplify the 1.3-kb full-length mDC-LAMP open reading frame, as confirmed by sequencing (data not shown). Samples were first normalized according to β2 microglobulin PCR results. After 25 PCR cycles, a strong mDC-LAMP signal was detected in the lung, but not in the small intestine, colon, spleen, skin, thymus, kidney, lymph nodes, stomach or liver (Fig. 4). A specific but faint PCR product was detected in lymph nodes and, even weaker, in the spleen only after 40 cycles of PCR. DC-LAMP lung expression has been previously reported [5, 30] and will be discussed in detail elsewhere (manuscript in preparation). However, the extremely weak expression in mouse lymph nodes was unexpected and in contrast to humans where lymphoid organs are major sources of DC-LAMP mRNA. Similarly, the lack of signal in mouse thymus and appendix contrasted with human Northern blot data [5].

Altogether these results demonstrate that mDC-LAMP mRNA is mainly detected in the lung, with no expression observed in other organs except a very faint signal in lymph nodes. These observations differ strikingly from hDC-LAMP, which is most abundant in mature DC and, consequently, in lymphoid organs [5].

### 2.4 Mouse DC activated *in vitro* do not express DC-LAMP

To look for mDC-LAMP protein expression, monoclonal antibodies (mAb) were raised against the recombinant molecule. One clone (1006F7) was used for biochemistry, flow cytometry and immunohistological studies. mAb 1006F7 detected a strong and specific intracytoplasmic signal in mDC-LAMP-transfected COP5 cells by flow

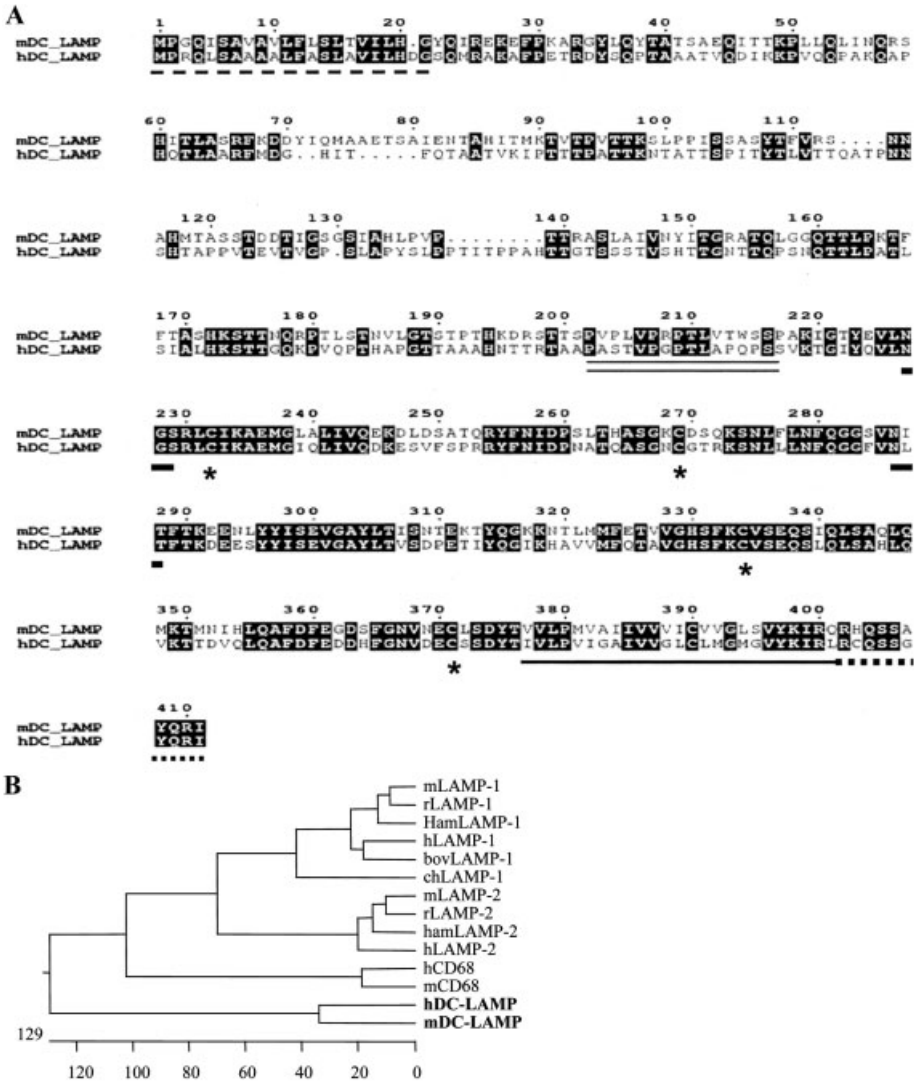


*Fig. 2.* Alignment of mDC-LAMP predicted sequence with related molecules. (A) Alignment of hDC-LAMP and mDC-LAMP amino acid sequences. The N-terminal signal peptide is indicated by broken underlining. The serine/proline-rich hinge region is doubly underlined; the transmembrane domain is singly underlined. The C-terminal intracytoplasmic part containing the lysosomal targeting signal is highlighted by a broken line. Potential N-linked glycosylation sites are bold underlined, and the conserved cysteines are indicated with a star. (B) Phylogenetic tree of the LAMP family members. Amino acid sequences of different species (h: human, m: mouse, ham: hamster, bov: bovine, ch: chicken, r: rat) were compared using the Megalin program with Clustal method and then displayed graphically in a phylogenetic tree. Units indicate the number of substitution events.

cytometric analysis (Fig. 5A) and immunoprecipitated a 45-kDa protein from transfectants lysates, corresponding to the predicted size of mDC-LAMP (Fig. 5B).

To directly address the issue of DC-LAMP expression by mouse DC, such cells were either purified *ex vivo* from spleen or derived *in vitro* from bone marrow precursor cultures. Both types of DC were activated *in vitro* for 24 h in the presence of LPS, activating CpG oligodeoxynucleotides (ODN), anti-CD40 mAb, or with a combination thereof. Flow cytometric analysis of the *ex vivo* purified $CD11c^+$ splenic DC showed an up-regulation of CD86 cell surface expression after 24 h LPS activation, confirming their maturation (Fig. 5C). Human monocyte-derived DC exhibited a strong up-regulation of hDC-LAMP under the same experimental conditions (data not shown). Similar maturation was observed with all other activatory signals tested, namely activating CpG ODN, anti-CD40 mAb, or a combination thereof (data not shown). However, no mDC-LAMP could be detected in these *in vitro* activated DC (Fig. 5C and data not shown). Similar results were obtained with bone marrow-derived

**Table 1.** Exon/intron boundary sequences of the mDC-LAMP gene, as determined by sequencing and computer analysis[a)]

| Exon | Intron-EXON-intron junctions | Exon (bp) | Intron (bp) |
|---|---|---|---|
| | | | |
| 1 | GTAGA--- ATG CCT GGG------- TCC CTG ACT G gtaagtgaca | 69 | |
| | M P G S L T | | |
| | | | 4833 |
| 2 | taatttgcag TA ATT TTA--------- AAG GAT TTG gtaagttggg | 695 | |
| | V I L K D L | | |
| | | | 946 |
| 3 | tttggtttag GAT TCT GCA -------- TTT ACC AAG gtaaggcatg | 128 | |
| | D S A F T K | | |
| | | | nd |
| 4 | tcttttacag GAA GAA AAC ------- TCA AAT ACA G gttagtaaaa | 57 | |
| | E E N S N T | | |
| | | | 2431 |
| 5 | ttgtcttcag AG AAG ACT---------- TTT GGA AAT G gtaagttaaa | 170 | |
| | E K T F G N | | |
| | | | 4575 |
| 6 | tctctcatag TG AAC GAG--------- CAG AGA ATC TAA--- TGAA | 2143 | |
| | V N E Q R I | | |
| | | | |

[a)] nd: Not exactly determined, bp: base pairs

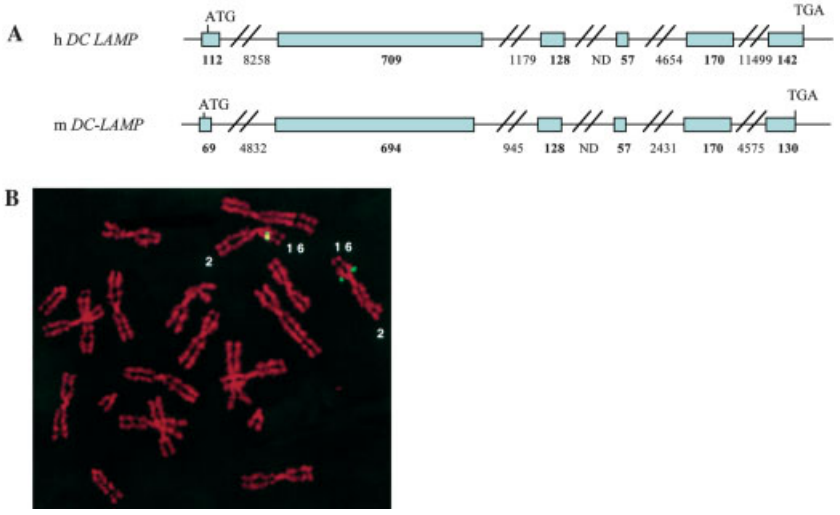


*Fig. 3.* Compared organization of hDC-LAMP and mDC-LAMP genes. (A) Structure of hDC-LAMP and mDC-LAMP genes. Exons are shown by the heavy line from the 5'- to the 3'-end of the gene; introns are shown by the thin line. Start (ATG) and stop (TGA) codons are shown in exons 1 and 6, respectively. The size (in nucleotides) is indicated below each exon and intron. hDC-LAMP genomic structure was deduced from computer analysis of the genomic clone NT_022676, which contains the entire hDC-LAMP gene. Mouse gene structure was obtained as described in Sect. 4. ND: not exactly determined. (B) FISH localization of mDC-LAMP gene. R-banded metaphase chromosomes appear red-stained with propidium iodide. The FITC fluorescent signals (green) are located to chromosome 16 in the B2-B4 region, which is syntenic to the localization of the hDC-LAMP gene (3q26.3-q27).

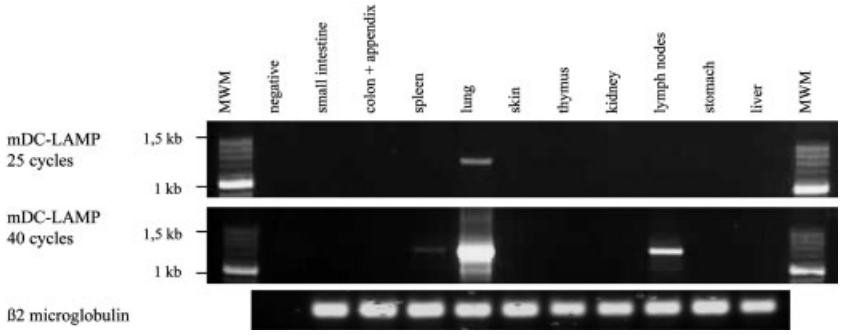


*Fig. 4.* mDC-LAMP mRNA expression analysis. RT-PCR was performed on total mRNA isolated from different adult mouse organs. A single 1.3-kb specific band was amplified in the lung, but not in thymus, spleen or lymph nodes after 25 PCR cycles. A faint specific band is detected in lymph nodes and spleen only after 40 PCR cycles. Comparison of β2 microglobulin RT-PCR products after 20 cycles confirmed the use of comparable amounts of starting material in all PCR reactions. MWM: Molecular Weight Marker.

DC after activation, in both BALB/c and C57BL/6 mouse strains (data not shown). Furthermore, no DC-LAMP mRNA could be PCR-amplified from either of those mature DC populations (data not shown). These results demonstrate that neither DC-LAMP mRNA nor protein expression could be detected in *in vitro* activated mouse DC.

### 2.5 Mouse DC activated *in vivo* do not express DC-LAMP

Since a faint mRNA signal was amplified from naive mouse lymph nodes, CD11c$^+$ cells were purified from pooled peripheral lymph nodes, and a quadruple stain-

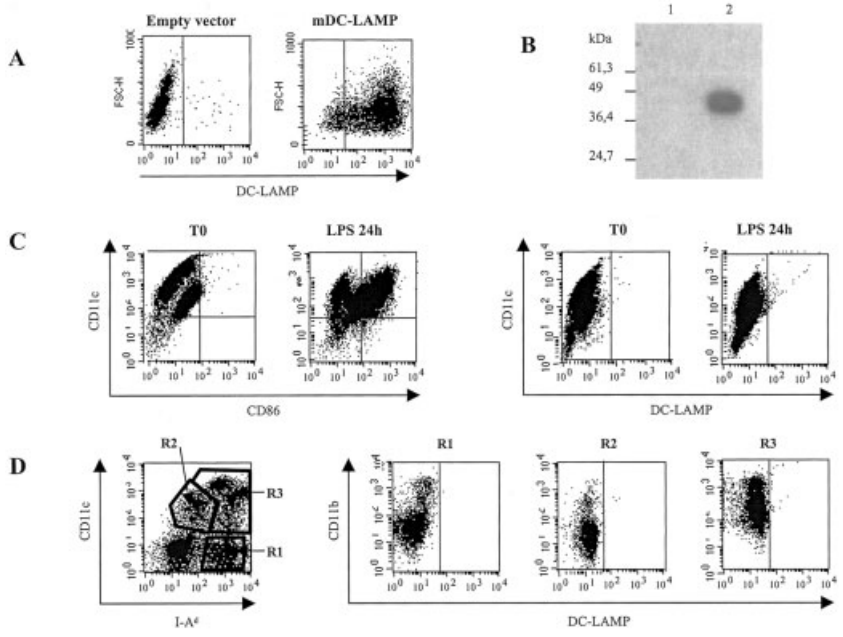


*Fig. 5.* mDC-LAMP expression analysis in DC. (A) Flow cytometric analysis of mDC-LAMP-transfected COP5 cells with rat anti-mDC-LAMP specific mAb (clone 1006F7). This mAb specifically recognizes mDC-LAMP, as shown by the absence of signal in empty vector-transfected cells. (B) Biochemical analysis of mDC-LAMP. mAb 1006F7 specifically immunoprecipitates a 45-kDa protein from mDC-LAMP-transfected COP5 cell lysates (lane 2). Western blot revelation on immunoprecipitates with isotype-matched negative control antibody from same lysates shows no signal (lane 1). (C) Flow cytometric analysis of *ex vivo* purified CD11c$^+$ splenic cells after 24 h LPS activation *in vitro*. CD86 is up-regulated (left panel), but DC-LAMP expression can not be detected with the 1006F7 mAb (right panel). (D) Flow cytometric analysis of *ex vivo* purified CD11c$^+$ cells from pooled peripheral lymph nodes. DC-LAMP is neither expressed by the CD11c$^{high}$/I-A$^{d\ high}$ DC population, nor by the other subpopulations.

ing with anti-CD11c, anti-I-$A^d$, anti-CD11b and anti-DC-LAMP mAb was performed on those cells and analyzed by flow cytometry (Fig. 5D). DC-LAMP expression could be detected neither in $CD11c^+$/I-$A^{d\ high}$ splenic DC [31] nor in any other cell population. The cellular source of the very low signal of DC-LAMP mRNA in mouse lymph nodes remains unclear, but the present data clearly demonstrate that it is not DC, or that the level of protein expression, if any, is too low to be detected with specific mAb. Given the extremely low level of mRNA expression and the absence of protein detection in lymph nodes, the cellular source of this signal was not investigated further.

Immunohistochemistry studies on various tissues were next performed to characterize DC-LAMP expression *in vivo* (Fig. 6). In agreement with mRNA expression data, a strong expression of the DC-LAMP protein was detected in the lung, in particular within a population of peripheral lung cells that we characterized as type II pneumocytes (Fig. 6B; manuscript in preparation). However, mDC-LAMP could not be detected in any other tissue analyzed, including peripheral lymph nodes (Fig. 6D), Peyer's patches, mesenteric lymph nodes, thymus and spleen, although all these organs contained $CD11c^+$/$CD40^+$ mature DC (Fig. 6C and data not shown).

Considering the lack of DC-LAMP expression in mouse DC under steady-state conditions, a combination of activating CpG ODN and anti-CD40 mAb was injected to mice, and spleens were collected 24 and 48 h later. This protocol triggers a strong and prolonged maturation of splenic DC, as illustrated by CD11c/CD40 double staining (Fig. 6E). However, no DC-LAMP could be detected in those spleens at any time after injection (Fig. 6F and data not shown), confirming that *in vivo* activated mouse DC do not express detectable amounts of DC-LAMP. Therefore, mature mouse DC do not express DC-LAMP under any of the conditions studied, which is strikingly different from the data obtained in humans [5], where this protein is used as a specific marker of DC maturation.

## 3 Discussion

We have cloned the mouse homologue of the hDC-LAMP gene and found a striking difference of distribution

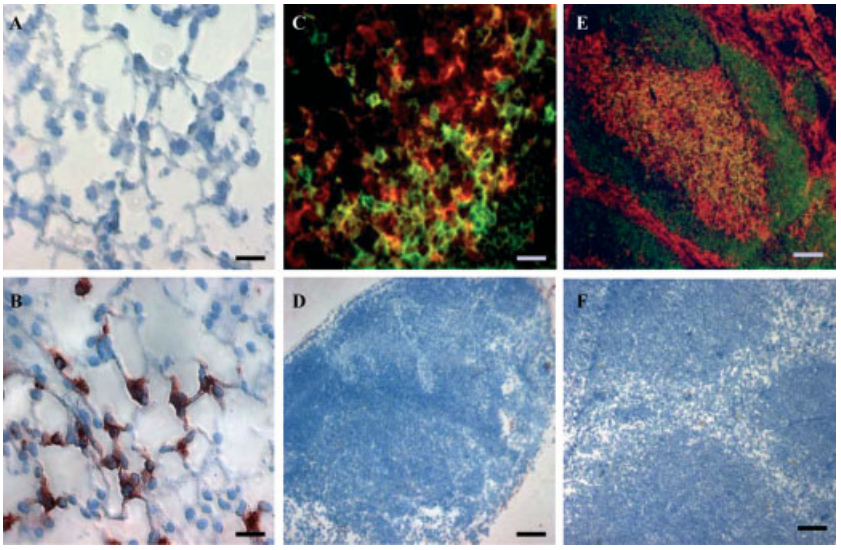


*Fig. 6.* mDC-LAMP expression in DC *in vivo*. (A, B) Validation on mouse lung frozen sections of the 1006F7 mAb for immunohistochemistry studies. Sections were stained with biotin-coupled 1006F7 mAb or isotype-matched negative control antibody, and binding was detected using HRP-coupled streptavidin and AEC substrate. mAb 1006F7 specifically stains a subpopulation of alveolar epithelial cells (B), while no staining was observed with the isotype-matched control antibody (A). (C, D) Peripheral lymph nodes contain mature DC, but no DC-LAMP-expressing cells. Peripheral lymph node frozen sections were double-stained with anti-CD11c (red) and anti-CD40 (green) mAb (C); double-positive yellow cells indicate the presence of activated DC, but no DC-LAMP expression could be detected using the 1006F7 mAb (D). (E, F) Presence of mature DC but lack of DC-LAMP expression in activated mouse DC in spleen from stimulated mice. Spleen cryosections from mice injected intraperitoneally 24 h before with rat anti-mouse CD40 mAb and activating CpG ODN were double-stained with anti-CD11c (red) and anti-CD40 (green) mAb (E). Despite the presence of activated DC (yellow), no DC-LAMP-expressing DC could be detected (F). Bars: (A, B, C) 25 μm; (D, E, F) 250 μm. (A, B, D, F) Counter coloration with Harry's hematoxylin.

between the two species. Indeed, while hDC-LAMP is considered as a reliable marker of DC maturation, we could not detect any expression of this protein in mouse DC, under any of the conditions studied. In contrast, and similarly to human, mouse type II pneumocytes were found to strongly express DC-LAMP (manuscript in preparation).

The absence of DC-LAMP in mouse mature DC raises two questions. The first issue concerns the claim that the gene we have identified is the orthologue of hDC-LAMP. The mouse gene reported here shares with its human counterpart syntenic chromosomal localization and organization that is also similar to mouse and human CD68 genes ([29] and data not shown). Furthermore, mDC-LAMP and hDC-LAMP predicted proteins share high sequence homology, with 67.5% identity between the two membrane proximal domains and a well-conserved and functional cytoplasmic tyrosine-based motif that targets both proteins to lysosomal membrane in transfected cells (data not shown). Collectively, these data clearly establish that the new gene described here corresponds to the mouse orthologue of hDC-LAMP.

The second issue deals with the sensitivity and the specificity of the methods used to look for DC-LAMP expression by mouse DC. Both the mRNA and protein detection (by Northern blot and PCR, and with specific mAb, respectively) were internally controlled by the positive signals observed in the lung. To avoid overlooking some subsets of cells, fully mature mouse DC obtained from several sources (BALB/c, 129sv and C57BL/6 strains, DC generated *in vitro*, DC purified *ex vivo*, DC analyzed in lymphoid organs *in situ*) after different modes of activation (LPS, CpG ODN, anti-CD40 mAb) were analyzed. However, neither the protein nor the mRNA could be detected in any mouse mature DC analyzed.

Despite their high homology, mDC-LAMP and hDC-LAMP sequences display differences that may correlate with functional divergences. The lack of expression of mDC-LAMP by mature DC is likely linked to a difference observed between mDC-LAMP and hDC-LAMP promoters: the human promoter contains a conserved NF-κB-binding site that is not present in the mouse sequence (B. de Saint-Vis, personal communication). Since NF-κB is implicated during DC activation [32, 33], the activation-driven expression of DC-LAMP in human DC may rely, at least in part, on that site. Interestingly, differences between mouse and human CD68 promoter organization have also been shown. Indeed, despite extensive homology at the sequence level, the genetic elements responsible for macrophage-specific gene expression are differently organized in both species [34].

Moreover, while hDC-LAMP contains seven potential N-glycosylation sites, only two can be identified in the mouse molecule. hDC-LAMP was observed as a 70–90-kDa protein on SDS-PAGE [5], which is considerably higher than the 44-kDa predicted mass of the polypeptide core. This difference was most likely due to an extensive glycosylation of the protein, as previously described for all other LAMP family members in both human and mouse [19, 22, 35, 36]. In contrast, the molecular mass of the mDC-LAMP protein immunoprecipitated from transfectants approximately corresponds to the mass predicted by bioinformatics (Fig. 5B), confirming limited glycosylation, if any. Since the glycosylation pattern of LAMP-1 or CD68 has been related to specific functions such as metastatic potential and T cell costimulation [15, 16, 37], the low glycosylation of the mouse protein may modify its function when compared to hDC-LAMP.

The absence of DC-LAMP in mouse mature DC deprives us of a good model for understanding the role of the molecule in human DC and leads us to reconsider the hypothesis regarding its functions. Based on the local and temporal coincidence of DC-LAMP appearance on the limiting membrane of MIIC and MHC class II molecule translocation to the cell surface, a role for DC-LAMP in the reorganization and transfer of MIIC to the plasma membrane in human activated DC had been proposed [5, 23]. Whatever function(s) DC-LAMP might actually exert in human mature DC, those should be carried out by other molecule(s) in mice.

In conclusion, DC-LAMP is absent from mouse DC, but expressed in human mature DC and in type II pneumocytes of both species. This pattern of expression suggests to rather use the official nomenclature CD208 to designate this molecule (7$^{th}$ Workshop and Conference on Human Leukocyte Differentiation Antigens, Harrogate, June 2000). CD208 will not become a reliable marker of mature DC in mice, and mice will not represent an easy model to unravel the function(s) of CD208 in human DC, but will hopefully be useful to understand its role in the lung.

## 4 Materials and methods

### 4.1 Cloning of mouse DC-LAMP cDNA and generation of monoclonal antibodies

Northern blot was performed on Mouse Tissues Northern Blot membrane (Clontech, Palo Alto, CA) hybridized with the coding sequence of hDC-LAMP cDNA labeled by random priming with [$^{32}$P]-dCTP as described elsewhere [38]. Mouse lung poly(A)$^+$ RNA (2 μg; Clontech) was used to make a

cDNA library in pSport (Superscript Plasmid System Kit, GIBCO BRL), which was screened with a probe generated from hDC-LAMP. A 1296-kb cDNA (mDC-LAMP) containing a poly(A) tail but lacking the 5' end was isolated from this library. The full-length sequence was amplified from the lung library using the RACE Marathon™ kit (Clontech) and two oligonucleotides, 5'-TTCCCTGGTAAGTCTTCTCTGTATTTGA and 5'-ACAGCTGGATGCTCTGCTCACTGACACA, with the recommended cycling program 1. PCR products were cloned in the pCRII plasmid (Invitrogen, San Diego, CA).

Sequencing was performed on both strands by the dideoxynucleotide method using a Taq Dye Deoxy Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and an automated sequencer (Applied Biosystems). The full-length mDC-LAMP sequence was subcloned in the eukaryotic expression vector pMET7 (Schering Plough, Kenilworth, NJ). COP5 cells were transiently transfected using standard electroporation with pMET7-mDC-LAMP plasmid and used to immunize Lou rats to produce mAb as described previously for mice [38]. Antibodies were screened by immunocytochemistry and cytofluorometry on mDC-LAMP-transfected COP5 cells.

### 4.2 Genomic organization of the mDC-LAMP gene

The mDC-LAMP cDNA obtained as described above was used as a probe to hybridize a 129/Ola mouse spleen genomic cosmid 121 library (Resource Center/Primary Database, Max Planck Institute for Molecular Genetics, Heidelberg, Germany) as described previously [38]. Three cosmid subclones were first identified and entirely sequenced. Furthermore, direct sequencing from the cosmid DNA was also used to sequence areas difficult to clone using the transposition reaction, according to instruction guidelines (Primer Island Transposition kit, Perkin Elmer Cetus, Norwalk, CT). This enabled us to obtain the entire genomic organization of mDC-LAMP. The organization of the hDC-LAMP gene was obtained by BLAST nucleotide analysis using the GenBank high throughput genomic database, and the predicted proteins were aligned using the Megalign program (LASERGENE Navigator, DNASTAR, Madison, WI).

### 4.3 Fluorescence *in situ* hybridization

FISH was performed using standard methods [38]. Briefly, the mDC-LAMP cDNA was biotinylated by nick translation with biotin-16-dUTP, as recommended by the manufacturer (Roche, Meylan, France), and the hybridized probe was detected by means of fluorescein isothiocyanate (FITC)-conjugated avidin (Vector Laboratories Inc., Burlingame, CA).

### 4.4 mRNA extraction, RT-PCR and sequencing

Total RNA was extracted from various tissues or *in vitro* cultured cells, reverse-transcribed and PCR-amplified as described [5]. PCR cycles were as follows: 1 min denaturation at 94°C, 1 min annealing at 55°C, 2 min elongation at 72°C. Forward primer was ATGCCTGGGCAGATCTCTGCAAT (nucleotides 1–23) and reverse primer was GGTATGCAGATGACTGCCTT (nucleotides 1245–1223). Positions are given based on the mDC-LAMP cDNA open reading frame. PCR amplification products were cloned into PCRII TOPO plasmid (Invitrogen) and sequenced with vector-specific primers on a ABI 373A sequencer (Applied Biosystems) using the dye terminator technology.

### 4.5 Biochemical studies

Standard calcium phosphate protocol was used to transfect COP5 mouse fibroblasts with c-myc-tagged mDC-LAMP cDNA. Transfected cells were lysed in 1% Nonidet-P40-containing buffer, and immunoprecipitation was performed on cells lysates with either rat anti-mDC-LAMP mAb or isotype-matched negative control antibody. Immunoprecipitates were analyzed by Western blotting with horseradish peroxidase (HRP)-coupled anti-myc mAb (Roche) as described in detail elsewhere [38].

### 4.6 Mice and cell culture

Six-week-old specific pathogen-free BALB/c, 129sv and C57BL/6 female mice were obtained from Charles River (Iffa Credo, L'Arbresle, France). All mice experiments were done following protocols approved by the institutional animal committee. Bone marrow-derived DC were generated as described in detail elsewhere [39] and cultured in 24-well plates at $10^5$ cells/ml for 6 days in complete culture medium [RPMI 1640 (Life Technologies, Paisley Park, GB), 5% fetal calf serum (Life Technologies), 2 mM L-glutamine (Life Technologies), 160 μg/ml gentalline (Schering Plough)] supplemented with GM-CSF (Schering Plough) at 10 ng/ml and TNF-α (R&D Systems, Abingdon, GB) at 100 U/ml.

Spleen and lymph node single-cell suspensions were obtained from manually dilacerated organs after 30 min digest with 1 mg/ml collagenase (Sigma-Aldrich, St. Louis, MO), crushing through a 0.22-μm cell strainer (BD Labware Falcon, Franklin Lakes, NJ) and final incubation in $NH_4Cl$ solution (Stem Cell Technologies, Vancouver, Canada). $CD11c^+$ cells were purified from total isolated cells by positive selection with $CD11c^+$ Microbeads and Minimacs (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Splenic $CD11c^+$ cells were cultured at $5\times10^5$ cells/ml in complete medium supplemented with mouse GM-CSF in 24-well plates.

*In vitro* activation was performed for 24 or 48 h with either LPS (10 ng/ml; Sigma-Aldrich), phosphorothioate-modified activating CpG ODN 1668 (TCCATGACGTTCCTGATGCT, referred to as CpG ODN; 10 μg/ml; MWG, Munich, Germany), anti-CD40 mAb (FKG45.5; 20 μg/kg; kind gift of Dr. Rolink, Immunology Department, Basel University, Switzerland) or with a combination of those stimuli. For mRNA extraction, $5\times10^6$ cells were kept aside. For DC activation *in vivo*, C57BL/6 mice were injected intraperitoneally with a combination of 100 μg anti-CD40 mAb (FKG45.5) and 50 μg of CpG ODN diluted in PBS, or with PBS alone as control.

### 4.7 Immunohistological studies

BALB/c mice organs were collected and snap-frozen in Cryojung tissue freezing medium (Leica GmbH, Nussloch, Germany). Tissue sections (7 μm thick) were fixed in acetone. For immunohistochemistry, endogenous peroxidase and biotin were blocked with 0.3% $H_2O_2$ (Sigma-Aldrich) and Avidin/Biotin Blocking kit (Vector Laboratories Inc.), respectively. After saturation with PBS/2% BSA/10% goat normal serum (Dako, Glostrup, Denmark), staining with biotin-coupled rat anti-mDC-LAMP mAb was performed. Binding was detected with streptavidin-HRP (Vector Laboratories Inc.) and AEC kit (Vector Laboratories Inc.) according to the manufacturer's instructions. Counterstaining was performed with Harry's hematoxylin (Vector Laboratories Inc.). Double immunohistological fluorescence was performed using a mix of hamster anti-mouse CD11c (MAC-11C5, Endogen, Cambridge, MA) and rat anti-mouse CD40 (3/23, BD PharMingen, San Diego, CA) mAb and detected with mixed goat anti-hamster IgG and goat anti-rat IgG $F(ab')_2$ coupled to Alexa 594 or Alexa 488, respectively (Molecular Probes).

### 4.8 Cytofluorometric analysis

Extracellular/intracellular stainings on DC were performed using standard techniques. Briefly, lymph node purified $CD11c^+$ DC were incubated with hamster anti-mouse CD11c (HL3, BD PharMingen), rat anti-mouse CD11b (M1/70, BD PharMingen) and mouse anti-mouse $I\text{-}A^d$ (AMS 32.1, BD PharMingen), respectively, coupled to APC, Cy5 and R-PE. Cells were then permeabilized (Fix and Perm kit, BD PharMingen), and incubated with rat anti-mDC-LAMP mAb coupled to Alexa 488. Fluorescence was analyzed with a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA). *In vitro* activated DC were stained extracellularly with R-PE-coupled hamster anti-mouse CD11c (HL3, BD PharMingen) and FITC-coupled rat anti-mouse CD86 (GL1, BD PharMingen). DC-LAMP staining was performed intracellularly.

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