

A novel cluster of lectin-like receptor genes expressed in monocytic, dendritic and endothelial cells maps close to the NK receptor genes in the human NK gene complex

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The NK gene complex is a region on human chromosome 12 containing several families of lectin-like genes including the CD94 and NKG2 NK receptor genes. We report here that the region telomeric of *CD94* contains in addition to the *LOX-1* gene the novel human *DECTIN-1* and the *CLEC-1* and *CLEC-2* genes within about 100 kb. Sequence similarities and chromosomal arrangement suggest that these genes form a separate subfamily of lectin-like genes within the NK gene complex. *DECTIN-1* is selectively expressed in dendritic cells and to a lower extent in monocytes and macrophages. mRNA forms with and without a stalk exon are observed. During functional maturation of dendritic cells the level of DECTIN-1 mRNA is down-regulated several-fold. *CLEC-1* is found to be not only expressed in dendritic cells, but also in endothelial cells and in the latter aspect resembles the *LOX-1* gene. Whereas recombinant full-length DECTIN-1 and LOX-1 are transported to the cell surface, CLEC-1 proteins accumulate in perinuclear compartments. We propose that this family of lectin-like genes encodes receptors with important immune and/or scavenger functions in monocytic, dendritic and endothelial cells.

Key words: DECTIN-1 / CLEC-1 / LOX-1 / NK gene complex / C-type lectin-like receptor

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1 Introduction

Receptors with type II transmembrane orientation and a C-type lectin-like domain (CTLD) in their extracellular part have been found to be frequently expressed on hematopoietic cells and several have been shown to play important roles in the immune response [1]. Beside proteins such as CD69 [2], which are widely expressed in cells of the hematopoietic lineage, some are more restricted in cell type expression such as the lectin-like NK cell receptors [3, 4]. The lectin-like *NKR-P1* and *Ly49* genes, initially detected in rodent NK cells [5], and the *CD94* and *NKG2* genes, described first from human NK cells [6, 7], occur on syntenic regions of mouse chro-

mosome 6 and human chromosome 12 [8–10]. These regions have therefore been termed NK gene complex (NKC).

It is now well established that the lectin-like NK receptors possess important roles for monitoring proper MHC class I expression according to the missing-self hypothesis [11]. In human NK cells the CD94 chain forms heterodimeric receptors with different NKG2 isoforms, which can bind the non-classical MHC class Ib molecule HLA-E [12, 13] and thus monitor biosynthesis of MHC class I molecules [3], which can be downmodulated by many viruses. CD94/NKG2A functions as an inhibitory receptor, the CD94/NKG2C receptor can activate NK cells [14, 15]. The activating NKG2D receptor, only distantly related to the other NKG2 molecules, forms homodimers and recognizes the class I-like molecule MICA, which is up-regulated by stress, e.g. viral infection, and on some tumor cells [16].

We have previously investigated the genomic structure and transcription of the human NK receptor genes [10,

[122055]

The first two authors contributed equally to this work.

Abbreviations **NKC:** NK gene complex **ITAM:** Immunoreceptor tyrosine-based activation motif **CTLD:** C-type lectin-like domain **LDL:** Low-density lipoprotein **KIR:** Killer immunoglobulin-like receptor **DC:** Dendritic cells **MoDC:** Monocyte-derived DC **HPDC:** Hematopoietic progenitor-derived DC **HUVEC:** Human umbilical vein endothelial cells

17, 18]. During these studies we have found the *LOX-1* gene [19] just 200 kb telomeric of *CD94* [10]. *LOX-1* encodes a CTLD receptor, which binds oxidized low-density lipoproteins (LDL) and fulfills scavenger functions in endothelial cells and macrophages [20]. In an attempt to find additional CTLD receptor genes we have extended these studies to cover the flanking areas. Whereas we have been unable to detect any further human equivalents of the murine *Ly49* genes [8], besides the *LY49L* pseudogene [10], we describe here a cluster of related lectin-like genes telomeric of *CD94*. This cluster contains, in addition to the *LOX-1*, the novel human *DECTIN-1* and the *CLEC-1* and *CLEC-2* genes [21].

2 Results

2.1 The human *DECTIN-1*, *CLEC-1*, and *CLEC-2* genes are closely linked to *LOX-1*

We have tested several lectin-like genes, which we identified in the GenBank expressed sequence tagged database (dbEST), for their presence in a PAC contig covering the centromeric part of the human NKC [10]. In addition to the recently described *CLEC-1* and *CLEC-2* cDNA [21], one novel gene sequence corresponding to EST clone AI741964 tested positive using specific oligonucleotides in PCR (Fig. 1). As described below, the gene represented by AI741964 encodes the human homologue of the recently described murine Dectin-1 [22]. All three genes occur telomeric of the previously mapped *LOX-1* gene [10] within about 100 kb. The order is *LOX-1* followed by *DECTIN-1*, *CLEC-1* and *CLEC-2*. Based on partial sequences available from the human genome project and our mapping data all four genes are

of the same transcriptional orientation. This is similar to the *NG2* gene cluster, which is localized about 200 kb centromeric of *LOX-1* [10]. Based on the available partial genomic sequences and additional sequencing we established the exon-intron structures. All three genes consist of exons encoding cytoplasmic, transmembrane, stalk and three CTLD exons (Fig. 2). At the exon 3/intron 3 boundary *DECTIN-1* has a GC instead of the conserved GT dinucleotide (Table 1), a feature occasionally occurring in the lectin-like genes. The *DECTIN-1* transcripts show splicing variation resulting in forms with and without a stalk exon (see below, Fig. 6).

2.2 Similarity and potential functional elements of human and murine *DECTIN-1* proteins

The sequence of AI741964 displayed high similarity to the murine Dectin-1 cDNA with the exception that a stalk exon was missing. Since the human *DECTIN-1* gene contained a potential stalk exon and we could show that this is found correctly spliced in a fraction of about 5 to 10% of the transcripts (Fig. 7), we have compared the corresponding predicted full-length sequences of the mouse and human proteins (Fig. 3). Both sequences show an identity of 59% (similarity 69%). Close to the N-terminus of the cytoplasmic domain an ITAM-like sequence is present in the human as well as the murine versions (Fig. 3). This sequence differs slightly from a classical ITAM motif as it is found in the CD3 γ chain or the DAP12 molecule [23]. The first tyrosine (residue 3) in the motif is 4 amino acid before the leucine (residue 7), whereas this distance is 3 amino acid in the classical $Yx_2Lx_{6-8}Yx_2L$ motif [24].

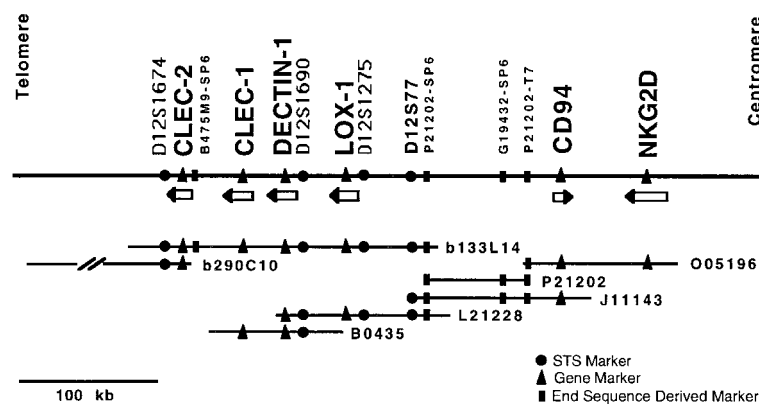


Fig. 1. A novel gene cluster telomeric of D12S77. Physical map of the 400 kb region telomeric of the CD94 and NKG2 receptor genes in the human NKC. The cluster consisting of the *LOX-1*, *DECTIN-1*, *CLEC-1* and *CLEC-2* genes is shown. The PAC and BAC clones used and the genes, STS markers and PAC or BAC end sequences tested by PCR are indicated.

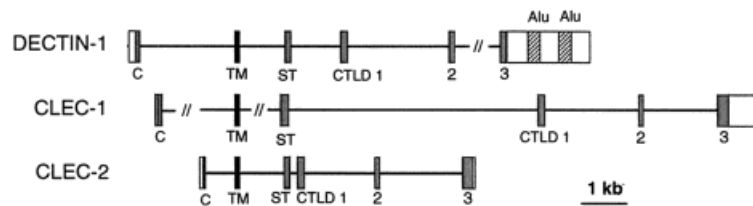


Fig. 2. Exon-intron structures of the DECTIN-1, CLEC-1 and CLEC-2 genes. The cytoplasmic (C), transmembrane (TM), stalk (ST) and three CTLD exons are displayed. Untranslated exon regions are shown by open boxes, translated regions by shaded or filled boxes. Two Alu sequences present in the DECTIN-1 3'-untranslated region are marked.

2.3 DECTIN-1, CLEC1, CLEC2 and LOX-1 form a separate subfamily within the NKC

To establish the relationship of *DECTIN-1*, *CLEC-1* and *CLEC-2* to *LOX-1* and the other genes of the NKC the amino acid sequences of the CTLD were aligned and a phylogenetic tree was constructed (Fig. 4). The obtained data display that DECTIN-1 forms together with LOX-1, CLEC-1 and CLEC-2 a subfamily of closer related lectin-like receptors of the NKC. The highest similarity of DECTIN-1 was seen with LOX-1 (44%), CLEC-1 and CLEC-2 are somewhat more distantly related (37%). The

subfamily of the NKG2 and the CD94 proteins as well as the AICL/LLT-1/CD69 group and the KLRP1, MAFA and NKR-P1 receptors are again more distant and displayed in separate branches.

2.4 DECTIN-1 is preferentially expressed in DC and CLEC-1 in DC and endothelial cells

Since *LOX-1*, *DECTIN-1* and *CLEC-1* are members of one subfamily, we investigated the expression of DECTIN-1 and CLEC-1 mRNA in primary human mono-

Table 1. Exon-intron boundaries of the DECTIN-1, CLEC-1 and CLEC-2 genes

| | exon | length | 5' splice donor | intron | length | 3' splice acceptor |
|-----------------|------|--------|-----------------|--------|--------|--------------------|
| DECTIN-1 | 1 | 103 | GAGAAAG gtatata | 1 | 2135 | tcctcag GATCGTG |
| | 2 | 99 | ACCATGG gtgagta | 2 | 1028 | tgtgaag CTATTTG |
| | 3 | 136 | ACCACAG gcaaggg | 3 | 1124 | ttccag GGGTTC |
| | 4 | 153 | TGAATTG gtaagtg | 4 | 1932 | attacag GGATTTA |
| | 5 | 119 | CTAACTT gtaagtg | 5 | nd | ttaacag ATTTACG |
| | 6 | 132 | TTCAATG-TAA | | | |
| CLEC-1 | 1 | 115 | CGCACAG gtaccct | 1 | 9585 | cctgcag AGCACAG |
| | 2 | 99 | CTTTTGT gtaagtc | 2 | >650 | gggttag TTTTCA |
| | 3 | 176 | GCTGGAG gtaagag | 3 | 5581 | ttccaag CACACAG |
| | 4 | 152 | AGACCTG gtaagat | 4 | 2092 | ttcacag GAATTTG |
| | 5 | 119 | CTGAACT gtaagcc | 5 | 1779 | tcttaag GTTCCAT |
| | 6 | 1013 | CAGGTAC-TGA | | | |
| CLEC-2 | 1 | 121 | GTCTCCG gtaagaa | 1 | 661 | cctacag TTGGCTC |
| | 2 | 99 | ATTTGGT gtaagtg | 2 | 1004 | aacctag CTGTCAT |
| | 3 | 119 | ACTTTCA gtaagta | 3 | 159 | tctccag AAGGTCA |
| | 4 | 156 | GAACATP gtggtaa | 4 | 1597 | acgatag GAGTACA |
| | 5 | 107 | AAAATAT gtaagtc | 5 | 1853 | ttcacag GTTTGAG |
| | 6 | 145 | ACTACCT-TAA | | | |

The sequences at the exon-intron boundaries and the sizes of the exons and introns are given. For exons 1 and 6 the sizes of the translated regions are calculated. nd: size not determined.

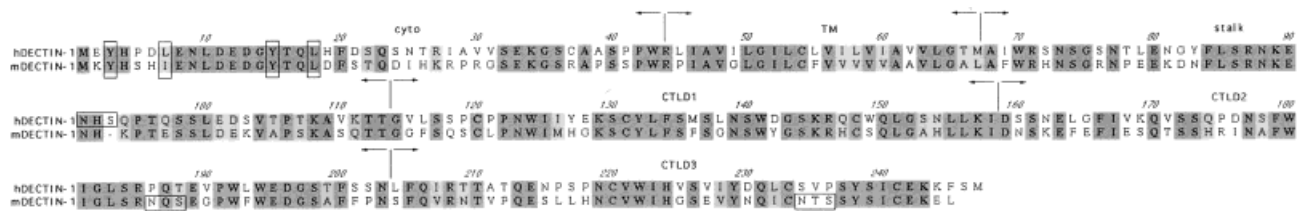


Fig. 3. Comparison of the amino acid sequences of human and mouse DECTIN-1. The predicted amino acid sequences of full-length transcripts for human and mouse (AF262985) DECTIN-1 were aligned. Identical and similar amino acids are shown by dark and light shaded areas, respectively. The boundaries corresponding to the cytoplasmic (cyto), transmembrane (TM), stalk and three CTLD domain exons are indicated. An ITAM-like motif in the aminoterminal part and potential N-glycosylation sites in the stalk and CTLD exons are boxed.

cytes, DC, macrophages, T and B lymphocytes as well as endothelial cells. The data obtained show that human DECTIN-1 mRNA is abundantly and selectively expressed in monocyte-derived DC (MoDC) and in DC derived from CD34⁺ hematopoietic progenitor cells (HPDC). A smaller amount of DECTIN-1 mRNA was also present in monocytes and macrophages, whereas T and B lymphocytes as well as endothelial cells, did not detectably express DECTIN-1 transcripts (Fig. 5A and B). No expression was visible in Northern blots including several additional cell lines, *i.e.* Jurkat, RPMI8866, NKL and NK92 (data not shown). The size of the RNA in DC was estimated to be 3.0 to 3.5 kb from the Northern blots, which corresponds to the expected transcript sizes assuming the use of the first or several consecutive polyadenylation signals detected in the gene in a region 1.6 to 1.9 kb downstream from the stop codon.

We further reinvestigated the expression of *CLEC-1* including endothelial cells in this analysis. These data confirm the previously established expression in DC. Some lower expression was detectable in the monocytic cell line U937. Interestingly, *CLEC-1* mRNA was found to be significantly expressed in HUVEC at a level comparable to primary DC (Fig. 5C).

2.5 DECTIN-1 expression is down-regulated during functional maturation of DC

We have further tested whether stimulation of DC by inflammatory mediators, CD40 ligand or zymosan A would lead to changes in expression levels for DECTIN-1 mRNA. As shown in Fig. 6A, treatment of MoDC with a combination of IL-1 β and TNF- α for 18 to 24 h resulted in a three- to fivefold reduction of DECTIN-1 mRNA. Alternatively, HPDC were activated by mAb specific for the CD40 receptor in the absence or presence of zymosan A

stimulating phagocytosis of the yeast particles (Fig. 6B). It appears that down-modulation of DECTIN-1 mRNA is specially pronounced in cultures treated with strong inducers of maturation such as TNF- α plus IL-1 β or CD40 ligation combined with phagocytosis stimulation.

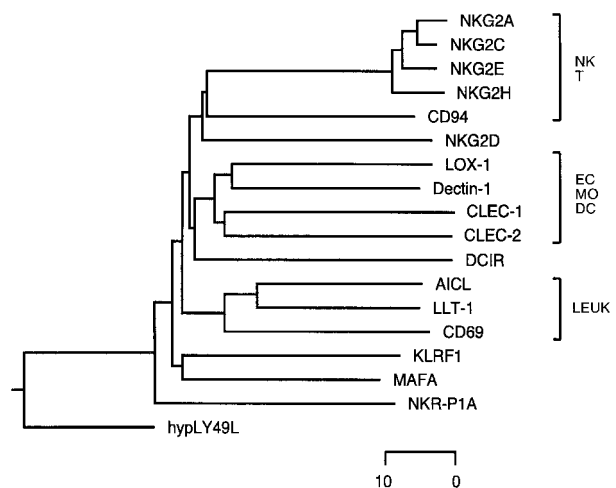


Fig. 4. Phylogenetic tree including the lectin-like genes of the human NKC. The sequences of the CTLD of the genes were aligned and a phylogenetic tree constructed. NKG2E and H are two alternatively spliced transcripts from the same gene [33]. For *LY49L* a hypothetical sequence containing all three CTLD exons spliced comparably to the other genes were chosen, although only aberrantly spliced transcripts have been detected for this gene [10, 34]. The genes consistently found to be closer related to each other are indicated by brackets on the right. Preferential expression of the sub-families in NK cells, T cells, endothelial cells (EC), monocytic cells (MO), dendritic cells (DC) or more broadly in leukocytes (LEUK) is indicated.

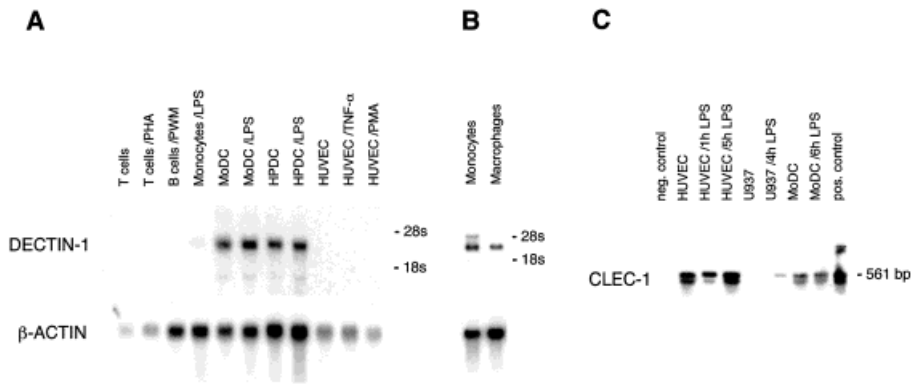


Fig. 5. DECTIN-1 and CLEC-1 mRNA expression in monocytes, DC and endothelial cells. A, B. Northern blots of DECTIN-1 mRNA. Total RNA of T and B lymphocytes, monocytes, MoDC, HPDC and HUVEC (A) or monocytes and macrophages (B) was subjected to Northern blotting. T and B lymphocytes, monocytes and DC were treated with PHA, PWM or LPS, respectively, for 6 h as indicated. HUVEC were incubated with TNF- α or PMA for 5 h. Hybridization of the same filter with a β -actin probe is shown as control. C. RT-PCR of CLEC-1 mRNA. Reverse transcription of CLEC-1 cDNA was primed on total RNA followed by PCR amplifying a 651-bp DNA fragment. The amplified DNA was Southern blotted and the filters hybridized with a CLEC-1 probe. RNA from uninduced HUVEC, U937 cells and MoDC as well as cultures treated with LPS were used. The negative and positive control show PCR reactions without template and with CLEC-1 cDNA, respectively.

Since MDC chemokine (CCL22) is known to be strongly induced in DC upon their maturation, we hybridized the same blot with a MDC probe to confirm activation of MoDC or HPDC. As seen in Fig. 6, a strong induction of MDC parallels downregulation of DECTIN-1 transcripts.

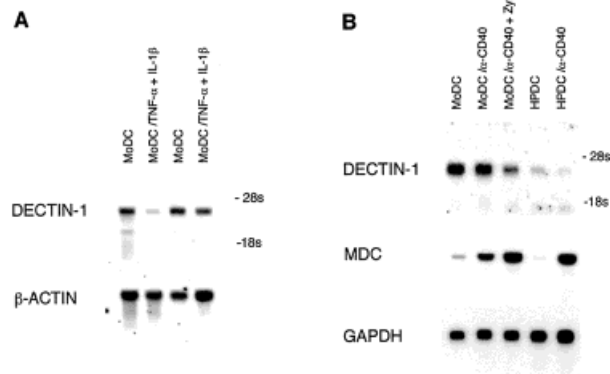


Fig. 6. Down-regulation of DECTIN-1 mRNA during maturation of DC. A. Northern blot of total RNA isolated from immature MoDC and cultures stimulated for 18 h with a combination of TNF- α and IL-1 β . Hybridization with a DECTIN-1 cDNA and a β -actin probe as control is shown. B. Northern blot with RNA isolated from immature MoDC and HPDC, MoDC and HPDC stimulated with anti-CD40 antibodies or MoDC treated with a combination of anti-CD40 antibodies and zymosan A for 24 h. The blot was consecutively hybridized with probes for DECTIN-1, MDC and GAPDH.

2.6 DECTIN-1 transcripts are variably spliced

Since AI741964 lacked the stalk exon we have investigated the presence of the stalk exon in DECTIN-1 transcripts. Northern blot analysis was unable to distinguish transcripts which were predicted to differ by only 141 bases. Therefore reverse transcription PCR was performed using a primer pair to amplify a fragment between the second and fourth exons. Two products of 505 bp and 367 bp were obtained corresponding in size to the predicted fragments with and without the stalk exon, respectively (Fig. 7). We estimate that approximately 5 to 10 % of the products contained the stalk exon. This upper band was excised from the gel, sub-cloned and two of the obtained clones sequences. Both clones displayed correctly spliced stalk exons as predicted from the genomic sequence (data not shown). We have further analyzed the PCR fragments obtained with primers which amplify fragments of the mRNA from the 5'-untranslated region to the stalk exon and from the stalk exon to the sixth exon, respectively (see Fig. 7 B). In both cases the major obtained products corresponded to the predicted sizes.

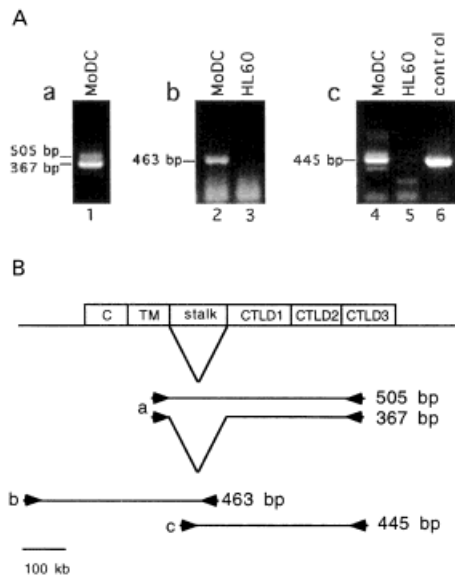


Fig. 7. A fraction of human DECTIN-1 mRNA contains the stalk exon sequences. **A.** RT-PCR of DECTIN-1 mRNA. RNA from MoDC was reverse transcribed using a DECTIN-1 primer and PCR performed on the cDNA using different primer pairs as indicated in **B.** (a) Displays the amplification of transcripts with and without the stalk exon, (b) amplification of the 5'-part and (c) of the 3'-part of the stalk containing transcript. Lane 6 displays the product obtained from the full-length DECTIN-1 cDNA. **B.** Schematic drawing of the oligonucleotides used for RT-PCR. The first primer pair (a) was designed to test the presence of the stalk exon fragment resulting in predicted 505-bp and 367-bp PCR products with or without the stalk exon fragment, respectively. The second primer pair (b) tested correct splicing of the 5' part, the third primer pair (c) correct splicing of the 3' part of the stalk exon-containing transcripts.

2.7 Following ectopic expression full-length DECTIN-1 and LOX-1 are found on the cell surface, whereas CLEC-1 accumulates intracellularly

We have evaluated the surface expression of FLAG-tagged recombinant DECTIN-1, CLEC-1 and LOX-1 in HUVEC following transfection with the respective expression constructs. To evaluate surface expression staining of non-permeabilized cells with an anti-FLAG antibody was compared to parallel samples of cells permeabilized with Triton X-100 before the staining. For DECTIN-1 we used constructs with and without the stalk exon. LOX-1 and full-length DECTIN-1 efficiently accumulated at the cell surface of HUVEC as shown by strong staining of non-permeabilized cells (Fig. 8A). In contrast, DECTIN-1 without stalk and the full-length CLEC-1 protein were not detectable, although signifi-

cant staining of permeabilized cells was obtained. A closer analysis of permeabilized cells at higher magnification revealed that cells transfected with LOX-1 and full-length DECTIN-1 expression constructs display strong staining of peri-nuclear cellular compartments as well as surface expression visible over the whole cell (Fig. 8B). Recombinant CLEC-1 can only be detected intracellularly in peri-nuclear compartments.

3 Discussion

This work has identified a novel subfamily of related CTLD receptor genes adjacent to the NK receptor genes in the NKC. This family contains the *LOX-1*, *CLEC-1* and *CLEC-2* genes as well as the novel human *DECTIN-1* gene. All four genes occur within 100 kb just 200 kb telomeric of *CD94* and all four genes display the same transcriptional orientation. In addition to this tandem arrangement, the sequence similarities of the CTLD support a common evolutionary history by consecutive gene duplications. Phylogenetic tree construction using the CTLDs of the human NKC genes have shown *LOX-1*, *DECTIN-1*, *CLEC-1* and *CLEC-2* as a subfamily parallel to a subfamily consisting of *CD94* and the *NKG2A* and *C* genes as well as subfamilies constituted by *CD69*, *AICL* and *LLT-1* or *KLRF-1* and *NKR-P1*. Whereas the *NKG2A* and *C* genes are over 90 % similar in their CTLD, the different members of the *DECTIN-1* group are more distantly related, comparable to the similarity of *CD94* to *NKG2A* and *C* (about 40 %). *NKG2D*, *DCIR* and *MAFA* could be variably associated in the tree depending on slight variations in the alignment.

Based on the DC-specific expression previously reported for the murine *Dectin-1* and the human *CLEC-1* [21, 22], we tested the expression of both genes in DC, monocytes and macrophages. In addition, we have included endothelial cells, since *LOX-1* was found to be related to both genes. *LOX-1* was originally identified as a gene encoding a receptor for oxidized LDL in endothelial cells and macrophages [19, 20]. Indeed, DC derived from either monocytes or hematopoietic progenitor cells were found to contain high levels of DECTIN-1 transcripts. Lower amounts were further seen in monocytes and macrophages, whereas no detectable levels were found in endothelial cells or several other cell types tested. A specificity of DECTIN-1 for the dendritic phenotype is indicated by the fact that differentiation of monocytes to DC up-regulates, whereas differentiation to macrophages rather reduces DECTIN-1 mRNA. CLEC-1 mRNA expression was detected in both endothelial cells and DC. However, CLEC-1 expression in

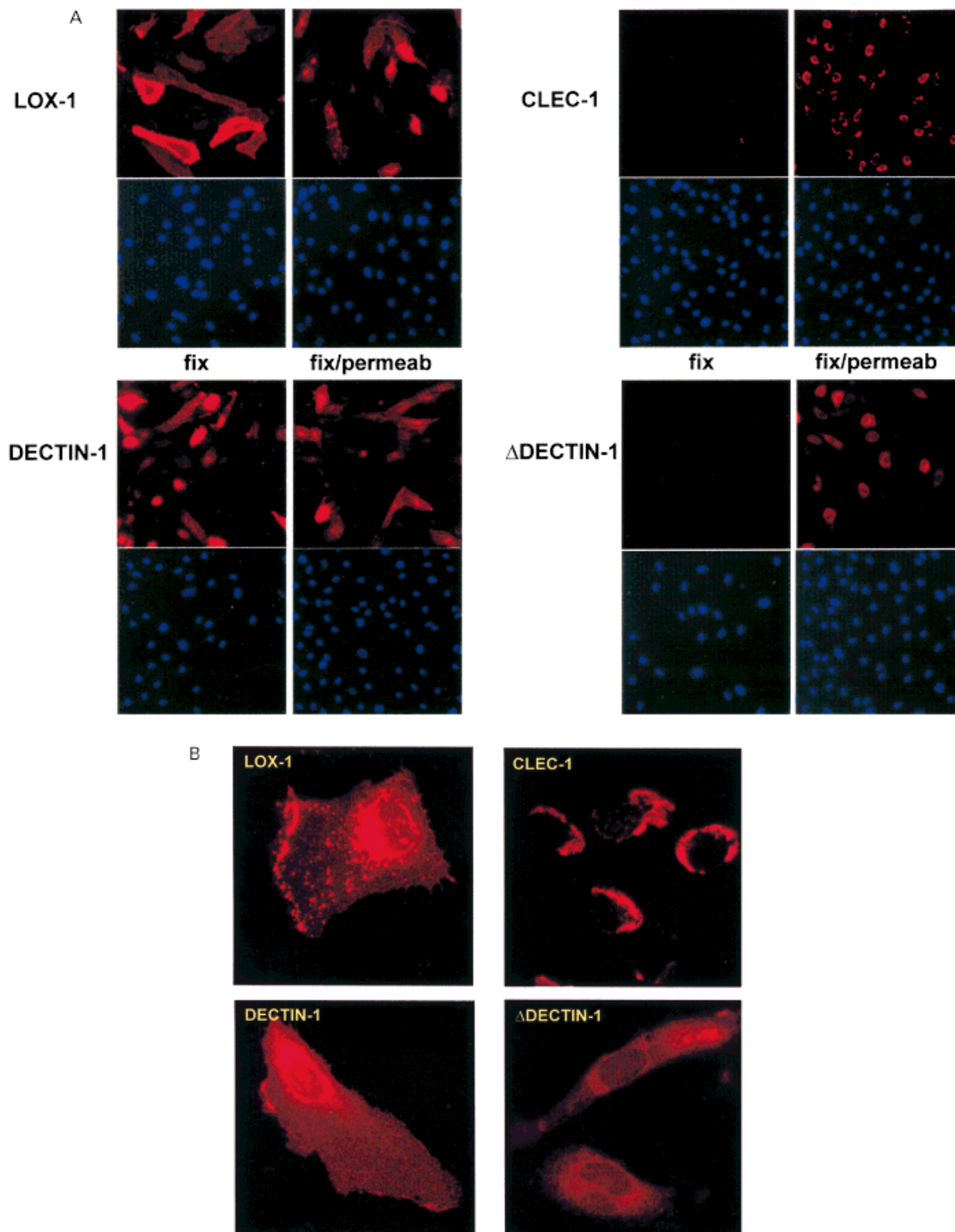


Fig. 8. Expression of recombinant LOX-1, DECTIN-1 and CLEC-1 in HUVEC. Expression plasmids for C-terminally FLAG-tagged LOX-1, DECTIN-1 and CLEC-1 were transfected into HUVEC. Expression was analyzed following staining with anti-FLAG antibodies and secondary Texas Red-labeled antibodies. **A.** Comparison of the staining of non-permeabilized and permeabilized cells. In the left lanes formaldehyde-fixed non-permeabilized cells (fix) and in the right lanes permeabilized cells (fix/permeab) are displayed. The upper rows show staining with the anti-FLAG antibody, the lower rows staining of the DNA with Hoechst 333258 to visualize total cell number. The DECTIN-1 construct without the stalk exon is indicated by Δ DECTIN-1. **B.** Comparison of the staining of permeabilized cells for all four expression constructs at higher magnification.

MoDC or HPDC appeared to be considerably lower when compared to DECTIN-1. Thus, the current data suggest that the members of this subfamily display distinct, but partially overlapping expression specificities: CLEC-1 shares DC expression with DECTIN-1, endothelial cell expression with LOX-1 and all three can be further found in monocytes.

Following antigen uptake immature DC are known to home into lymphoid organs and this process is associated with profound phenotypic and functional changes known as DC maturation [25]. Molecules involved in DC function are likely to be modulated during this final maturation. Therefore, we have evaluated changes of DECTIN-1 mRNA expression after activation of MoDC or HPDC. We have consistently observed DECTIN-1 mRNA to be severalfold downregulated upon stimulation with strong inducers including cross-linked anti-CD40 mAb and zymosan A, whereas the gene for MDC (CCL22) was strongly up-regulated. This provides evidence for an important function of DECTIN-1 in immature DC.

The finding of CTLD genes for receptors on monocytic cells and DC, which are related to NK cell receptor genes in structure, chromosomal linkage and evolution, is reminiscent of the leukocyte receptor complex on chromosome 19 [26]. In the leukocyte receptor complex the KIR gene family of MHC class I receptors of NK cells is flanked by the related ILT genes, which are expressed by monocytic cells and other leukocytes. Some of them have been proposed to be important for DC function [27]. This suggests the structural and functional coevolution of these receptors on NK, monocytic and dendritic cells. The coevolution of DECTIN-1 and CLEC-1 with the CTLD NK receptors could indicate ligands related to non-classical MHC class I molecules as it is was found for NKG2D or the CD94/NKG2 receptors. Alternatively, since DECTIN-1 and CLEC-1 form a group with LOX-1, it could be that their predominant ligand and function might be in lipoprotein metabolism [19], removal of apoptotic cells [28] or antigen capture. It seems that all the receptors of the DECTIN-1 and the NKG2 group lack most of the characteristic residues involved in Ca^{++} binding and therefore non-carbohydrate ligands are likely [1].

The human DECTIN-1 gene was found to be expressed as a major mRNA transcript lacking the stalk exon and a minor form representing about 10 % of the mRNA that contained this exon. This splicing variation is likely due to the presence of a GC instead of a GT dinucleotide at the 5'-end of intron 3. A similar change has been observed at the 5'-end of intron 4 in the CD94 gene, although in this case splicing is occurring properly [29]. Splicing variation involving the stalk exon is a common feature of the CTLD receptors, e.g. NKG2B is a stalkless

transcript of the NKG2A gene. In this case, previously reported data suggest that the stalk region is partly dispensable for expression and function [12]. As previously suggested [21], in the case of the stalk-containing CLEC-1 it appears likely that a second chain, not present in sufficient amount in the transfected cells, will be necessary for surface expression. A similar situation has been reported for the NKG2A and NKG2C chains, which need heterodimerization with the CD94 chain to be stably displayed on the cell surface [30]. Alternatively, a specific function of the protein inside the cells is possible, as recombinant CLEC-1 accumulated in perinuclear compartments similar to rough endoplasmic reticulum and Golgi-like structures.

Both, DECTIN-1 and CLEC-1 proteins contain tyrosine residues in their cytoplasmic domains. The tyrosines of DECTIN-1 are within an ITAM-like motif, which only slightly differs from the consensus ITAM as found in the CD3 chains or the DAP12 molecule [24]. This putative ITAM motif could function in activation of DC following binding to a ligand. In preliminary experiments using ectopic expression of tagged DECTIN-1 we were unable to detect tyrosine phosphorylation (data not shown). It is however possible that binding of unknown specific ligands would be required for significant phosphorylation. CLEC-1 has a single tyrosine within a sequence that does not conform to any known motif, but it could as well be part of a novel motif involved in activation/inhibition or, alternatively, in subcellular distribution.

Thus, it will be of interest to determine the ligands and functions of CLEC-1 and DECTIN-1 receptors in monocytic, dendritic and endothelial cells and to see whether they would possess MHC-like ligands and be involved in the modulation of cellular activity or whether they would rather have scavenger functions for apoptotic cells and pathogens which could be connected to efficient antigen presentation.

4 Materials and methods

4.1 Database search and sequence alignments

The GenBank expressed sequence database (dbEST) was searched for selection of new lectin-like genes by using the CTLD domain sequences of NKG2A, NKG2D, CD94, LY49L and LOX-1. Two clones, AI741964 and AI1050791 were selected, sequenced using the chain termination method and shown to contain cDNAs of the novel *DECTIN-1* and the *CLEC-1* genes. The amino acid sequence of the DECTIN-1 protein was aligned with the homologous murine Dectin-1 (AF262985) using MacVector 6.5 software (Oxford Molecular, Inc.). Multiple alignment of the CTLD domains of the

lectin-like genes of the NKC was performed using the ClustalW method of the MacVector 6.5 software. Accession numbers for the molecules used: NKG2A (X54867), NKG2C (X54869), NKG2E (L14542), NKG2H (AF078550), NKG2D (X54870), LOX-1 (AF035776), CLEC-1 (AF200949), CLEC-2 (AF124841), DCIR (AJ133532), AICL (X96719), LLT-1 (AF133299), CD69 (L07555), KLRF1 (AF175206), MAFA (AF081675), and NKR-P1A (U11276).

Phylogenetic trees were constructed by the MegAlign program of the DNASTAR software version 3.0 according to the nearest neighbor method.

4.2 Contig and gene mapping

A PAC contig of the centromeric part of the NKC has been previously described by us [10, 17]. The *DECTIN-1* gene was detected by corresponding primers and PCR analysis in the most telomeric PAC of the contig, L21228. An additional PAC clone, B0435, was selected as described previously [10] and contained the *DECTIN-1* as well as the *CLEC-1* genes. To verify the order of the clones and genes in the region gene specific primers and primers for STS markers and PAC and BAC ends were used on clones of the PAC [10] and BAC [31] contigs. The oligonucleotides used are: LOX-1: forward: 5'-TTCATCCAGCAAGCAATTC-3', reverse: 5'-AAGTGGGCATCAAGGAG-3'; *DECTIN-1*: forward: 5'-AACAAAGTGTCTTCCCAACCTG-3', reverse: 5'-AGAGAATGTTGATCCATCCTCC-3'; *CLEC-1*: forward: 5'-TTCTACTCTTATTGGACAGGGC-3', reverse: 5'-AGTTCAGAAGTGAAGGGGTTCC-3'; *CLEC-2*: forward: 5'-TGAGTTTTTGG-AAGATGGAA-3', reverse 5'-AGCCCTTATCTGTGTTATC-3'.

Primers for the STS markers and BAC ends were from the Human Genome Database (<http://sequence.aecom.yu.edu/chr12/>), primers for the PAC ends and the CD94 and NKG2D genes have been described previously [10, 17].

4.3 Isolation of cells and cell culture

Cord blood (CB) samples were collected at the Lainzer Hospital, Vienna. Mononuclear cells (MNC) were isolated by Lymphoprep® (Nycomed Pharma AS, Oslo, Norway) density gradient centrifugation. CD34⁺ cells were isolated from CB-MNC using the MACS CD34⁺ cell isolation kit from Miltenyi (Miltenyi Biotech, Bergisch Gladbach, Germany). Purified CD34⁺ progenitor cells were seeded at a cell density of 5×10^4 cells/ml and cultured in RPMI1640 medium (Life Technologies Ltd., Paisley, Scotland) supplemented with 10 % FCS, 2 mM glutamine and 50 µg/ml each of streptomycin and penicillin. The medium was further supplemented with SCF (50 U/ml) and Flt3-L (25 U/ml) purchased from R&D Systems Inc., Minneapolis, MN. On day 6, cell cultures were split and fed with culture medium containing SCF (25 U/ml), Flt3-L (12.5 U/ml), GM-CSF (300 U/ml, Novartis, Basel, Switzerland), TNF- α (50 U/ml; R&D) and TGF- β (5 ng/ml; R&D).

Human monocytes obtained by countercurrent elutriation were seeded at 8×10^5 cells/ml in CM supplemented with GM-CSF (300 U/ml) and rIL-4 (200 U/ml). Cultures were fed on day 3 exchanging half of the medium for fresh CM supplemented with GM-CSF and IL-4 to the concentrations indicated above.

The MNC fraction of buffy coats was used to isolate primary monocytes via the monocyte isolation kit, Miltenyi Biotech. > 95 % pure monocytes were seeded at 2×10^6 cell/ml in CM in the presence of 200 U/ml of M-CSF (R&D). After 6 days of culture, the phenotype of mature macrophages (CD13⁺, CD14⁺, CD16⁺, CD1a⁻, CD40⁻) was verified by flow cytometry.

Human T cells, B cells and monocytes were obtained by countercurrent elutriation. T and B cells were cultured in CM and stimulated for 24 h by PHA (10 µg/ml, Pharmacia Biotech, Uppsala, Sweden) or PWM (10 µg/ml, Sigma, St. Louis, MO) and rhIL-4 (100 U/ml, Novartis Pharma, Basel Switzerland) respectively. Monocytes, MoDC or HPDC were stimulated with 100 ng/ml LPS (Sigma) or in the presence of 4 µg/ml of anti-CD40 mAb (mAb clone 626.2) further cross-linked by 2 µg/ml of F(ab')₂-fragments of goat-anti mouse IgG (Pierce Chemical Corp, Rockford, IL). In some cases, zymosan A (25 µg/ml, Sigma) was used in conjunction with anti-CD40 mAb cross-linked by F(ab')₂ fragments of goat anti-mouse IgG. Alternatively, final maturation of MoDC was induced by incubation in the presence of inflammatory cytokines such as TNF- α (200 U/ml) and IL-1 β (100 U/ml) for 18 h to 24 h.

HUVEC were prepared and cultured in 199 medium supplemented with 20 % SCS (HyClone, Logan, Utah), 1 U/ml heparin and 50 µg/ml endothelial cell growth supplement (ECGS, Technoclone, Vienna) [32]. HUVEC were induced with 10 U/ml TNF- α or 0.1 pM PMA. 293 cells were grown in MEM α medium with 10 % NCS (GIBCO/BRL, Rockville, MD). U937 cells were grown in RPMI 1640 medium supplemented with 10 % FCS (GIBCO/BRL, Rockville, MD) and were induced with 300 ng/ml LPS.

4.4 Northern blots and RT-PCR

Total cellular RNA was isolated following cell lysis in TRIzol reagent (GIBCO/BRL, Rockville, MD), chloroform extraction and precipitating the RNA by isopropanol. Twenty micrograms of total RNA were fractionated on a 1.3 % agarose – 6.4 % formaldehyde gel, transferred to a nylon membrane (Amersham, Little Chalfont, GB) and covalently linked by ultraviolet irradiation.

Membranes were hybridized with radioactively labeled probes and washed as previously described [32]. For *DECTIN-1* a 927 bp HindII-HindIII fragment of the *DECTIN-1* cDNA was used as probe. MDC mRNA was detected using the oligonucleotide 5'-CCTTATCCCTGAAGGTTAGCAA-

CACCACGCC-3' corresponding to nucleotides 209–239 of the cDNA. The probes were labeled by random priming using a Random Primer Kit (Stratagene Cloning Systems, La Jolla, CA) or by a 5'-end kinasing reaction, respectively. Bound radioactivity was visualized by exposure on phosphor screen cassettes of a PhosphorImager (Molecular Dynamics/Amersham).

RT-PCR was performed according to following protocol. Primers (40 µg) specific for CLEC-1 (5'-TTTTCTGGTCTTTAATCTCT-3') or DECTIN-1 mRNA (5'-GATTCCATGCTAGGGATCTAC-3') were annealed with 3 µg of total RNA in 10 µl containing 50 mM Tris-HCL (pH 8.3), 75 mM KCl and 3 mM MgCl₂ by heating to 85 °C followed by cooling to 42 °C. First-strand synthesis was performed in 20 µl containing 50 mM Tris-HCL (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 0.5 mM dNTP, 20 mM DTT and 100 U Superscript Reverse Transcriptase (GIBCO/BRL, Rockville, MD). The cDNA contained in 1 µl of the reaction mix was employed as template in a PCR reaction. The oligonucleotides used to amplify cDNA are for CLEC-1: 5'-TCTGTCGTGAGCTGTATAAC-3' and 5'-AAACCCAAGCTCAGAAATGC-3'. For DECTIN-1: 2nd-4th EXON: forward: 5'-GGGAATCCTATGCTTGGTAAT-3', reverse: 5'-TGGAGATGGGTTTTCTTGGG-3'; 5'-UT-STALK: forward: 5'-CTCCCGTAAGTACCTAGCCCA-3', reverse: 5'-GTGGTTTTGACAGCTTTGGT-3'; STALK-3'-END: forward: 5'-GAGATCCAATTCAGGAAGCA-3', reverse: 5'-TGGAGATGGGTTTTCTTGGG-3'. The PCR products were size fractionated on a 1 % agarose gel, Southern blotted and hybridized with CLEC-1 and DECTIN-1 probes.

4.5 Expression constructs and cell transfections

Expression constructs for LOX-1, DECTIN-1 and CLEC-1 were obtained in a modified pcDNA3.1/HisA vector (Invitrogen, Groningen, Netherlands) providing a cytomegalovirus enhancer/promoter and a sequence encoding a Flag peptide. In the pcDNA3.1/N-Flag vector the Flag-encoding sequence was inserted between the HindIII and BamHI sites of the multiple cloning sites, in the pcDNA3.1/C-Flag modified vector between the XhoI and ApaI sites. The two vectors can be used to provide an N-terminal or C-terminal Flag epitope to protein. The coding regions of all three cDNA were amplified by PCR using oligonucleotides providing EcoRI/NotI sites for insertion into the pcDNA3.1/N-Flag vector or EcoRI/XhoI sites for the pcDNA3.1/C-Flag vector. All constructs were verified by sequencing of the inserted DNA fragments.

HUVEC were seeded 24 h prior to transfection in six-well plates. Transient transfections of HUVEC cells were carried out using Lipofectamine™ reagent (GIBCO/BRL, Rockville, MD) as previously described [32].

4.6 Immunofluorescence studies

Immunofluorescence assays were performed as previously described [32]. Following transfection cells were washed with PBS, fixed for 10 min at room temperature with 3.7 % formaldehyde, 2 % sucrose in PBS and permeabilized for 5 min with 0.5 % Triton X-100 in PBS. Primary antibodies (anti-Flag M2 monoclonal antibody, Sigma, St. Louis, MO) were incubated with the cells for 1 h at room temperature, cells washed with PBS, incubated with Texas Red-labeled donkey anti-mouse IgG (Accurate Chemical, Westbury, NY) for 1 h at room temperature and washed with PBS. Cell nuclei were visualized by incubation with a fluorescent groove-binding probe for DNA (Hoechst 333258, Sigma Chemicals, St. Louis, MO). Results were analyzed in a Nikon Diaphot TMD fluorescence microscope (Nikon Ltd.).

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Nomenclature: According to the Human Gene Nomenclature Database (www.gene.ucl.ac.uk/nomenclature) part of the genes described have obtained the following symbols: *KLRA1* (LY49L), *KLRB1* (NKR-P1A), *KLRC1* (NKG2A), *KLRC2* (NKG2C), *KLRC3* (NKG2E), *KLRC4* (NKG2F), *KLRD1* (CD94), *KLRG1* (MAFA-L), *OLR-1* (LOX-1).

Human DECTIN-1 cDNA sequences have been submitted to the EMBL and GenBank databases under the accession numbers AJ312372 and AJ312373.