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*J Immunol* 2007; 179:6052-6063; doi: 10.4049/jimmunol.179.9.6052
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The Novel Endocytic and Phagocytic C-Type Lectin Receptor DCL-1/CD302 on Macrophages Is Colocalized with F-Actin, Suggesting a Role in Cell Adhesion and Migration

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C-type lectin receptors play important roles in mononuclear phagocytes, which link innate and adaptive immunity. In this study we describe characterization of the novel type I transmembrane C-type lectin DCL-1/CD302 at the molecular and cellular levels. DCL-1 protein was highly conserved among the human, mouse, and rat orthologs. The human DCL-1 (hDCl-1) gene, composed of six exons, was located in a cluster of type I transmembrane C-type lectin genes on chromosomal band 2q24. Multiple tissue expression array, RT-PCR, and FACS analysis using new anti-hDCL-1 mAbs established that DCL-1 expression in leukocytes was restricted to monocytes, macrophages, granulocytes, and dendritic cells, although DCL-1 mRNA was present in many tissues. Stable hDCL-1 Chinese hamster ovary cell transfectants endocytosed FITC-conjugated anti-hDCL-1 mAb rapidly (t_{1/2} = 20 min) and phagocytosed anti-hDCL-1 mAb-coated microbeads, indicating that DCL-1 may act as an Ag uptake receptor. However, anti-DCL-1 mAb-coated microbead binding and subsequent phagocytic uptake by macrophages was ~8-fold less efficient than that of anti-macrophage mannose receptor (MMR/CD206) or anti-DEC-205/CD205 mAb-coated microbeads. Confocal studies showed that DCL-1 colocalized with F-actin in filopodia, lamellipodia, and podosomes in macrophages and that this was unaffected by cytochalasin D, whereas the MMR/CD206 and DEC-205/CD205 did not colocalize with F-actin. Furthermore, when transiently expressed in COS-1 cells, DCL-1-EGFP colocalized with F-actin at the cellular cortex and microvilli. These data suggest that hDCL-1 is an unconventional lectin receptor that plays roles not only in endocytosis/phagocytosis but also in cell adhesion and migration and thus may become a target for therapeutic manipulation. The Journal of Immunology, 2007, 179: 6052–6063.

Mononuclear phagocytes, such as monocytes (Mo)3/Macrophages (Mph) and dendritic cells (DC) act as APC and link the innate and adaptive immune responses. Mo/Mph are recruited to sites of inflammation where they phagocytose pathogenic microbes and damaged tissue components and mediate local effector functions. Resident and recruited DC also phagocytose pathogens but, after migrating into draining lymph nodes, present processed Ags to T and B lymphocytes to elicit Ag-specific adaptive immune responses (1, 2). The different functions of these APC are mediated, at least in part, by C-type lectin receptors.

C-type lectin receptors are a superfamily of cell surface proteins whose biology is complex but highly relevant to several human diseases (3–7). Both Mph and DC are equipped with an array of C-type lectin receptors that act as pattern recognition receptors. These include the classical (Ca^{2+}-dependent sugar binding proteins, e.g., macrophage mannose receptor (MMR/CD206, MMR hereafter), DC-specific ICAM-3 grabbing nonintegrin (DC-SIGN)/CD209, and macrophage C-type galactose/N-acetyl galactosamine-specific lectin (MGL)/CD301; Refs. 8–12) but also the nonclassical C-type lectin receptors (e.g., dectin-1; Refs. 13 and 14) that retain structural homology with the classical C-type lectins but have evolved to bind sugar and nonsugar ligands without classical Ca^{2+} and sugar binding motifs.

Many C-type lectin family members have more than one functional capacity, but these in general relate to innate and adaptive immune cell activity. These include: 1) specific binding to carbohydrate-pathogen-associated molecular patterns expressed on pathogenic microbes (e.g., MMR, MGL/CD301, DC-SIGN/CD209, and dectin-1); 2) triggering phagocytosis of pathogens (e.g., MMR, MGL/CD301, DC-SIGN/CD209, and dectin-1; Refs. 10 and 14–17); 3) directing transport of phagocytosed pathogens to appropriate intracellular compartments (e.g., human DEC-205/CD205, denoted hDEC-205 hereafter; Ref. 18); 4) signaling to initiate and regulate innate/adaptive immune responses (e.g., DC immuno activating receptor (DCAR), DC immunoreceptor (DCIR), dectin-1, and blood DC Ag-2 (BDCA-2/CD303; Refs. 19–25); and 5) specific interactions with self ligands to mediate cellular functions such as adhesion (e.g., DC-SIGN/CD209; Refs. 11 and 26).
While cloning human DEC-205, we identified a cDNA encoding a novel type I transmembrane C-type lectin receptor, termed DCL-1/CD302 (derived from the underlined letters of DEC-205-associated C-type lectin-1 and denoted hereafter as DCL-1 or human DCL-1 [hDCL-1]), as a genetic fusion partner of hDEC-205 in the Hodgkin’s lymphoma cell lines (i.e., L428, HDLM2, and KM-H2; Refs. 27 and 28). The hDCL-1 gene was localized directly ~5 kb downstream of the hDEC-205 gene, and these cell lines produced a 9.5-kb hDEC-205/hDCL-1 fusion mRNA by cotranscription and intergenic splicing and translated it into a hDEC-205/hDCL-1 fusion protein in situ (28). We also identified a hDCL-1 4.2-kb mRNA in human myeloid-like cell lines (e.g., HEL, HL60, U937, and Mono Mac 6) but not in T and B lymphocyte cell lines (28), suggesting that the hDCL-1 protein was expressed independently. The predicted cytoplasmic sequence of DCL-1 implied that it might have endocytic activity. This prompted us to investigate DCL-1 biochemically and functionally to clarify its potential as a target for therapeutic manipulation. The data suggest that hDCL-1 is an unconventional C-type lectin that may play roles not only in endocytosis/phagocytosis but also in APC adhesion and migration.

Materials and Methods

Purification of leukocytes and production of Mo-derived DC (MoDC) and Mφ

Blood was obtained from volunteer donors and “inflamed” palate tonsils were obtained at routine tonsillectomy with informed consent as approved by the Mater Hospital Human Research Ethical Committee. To isolate pure (>98% purity) T lymphocytes, B lymphocytes, and NK cells with minimal contamination, the cell suspensions were first layered on Histopaque 1077 (Sigma-Aldrich; Miltenyi Biotec) and then FACS using a FACSVantage device (BD Biosciences) as described previously (30). T lymphocytes were CD3+CD11c−HLA-DR+. B lymphocytes were CD19+CD20+CD3−CD11c−. NK cells were CD3−CD14−CD19−CD20+CD31+HLA-DR+. CD235a+CD16+CD56+. Mo were CD14+CD3+CD20+CD3−. B lymphocytes (BDC) subsets were CD3+CD14+CD19−CD20+CD34+CD11c−CD235a+ (lineage) or CD4−CD11c+ (malignant BDC). MoDC and Mφ were produced by culturing CD14+ Mo with GM-CSF and IL-4 in RPMI 1640 (Invitrogen Life Technologies) and 10% FCS (Invitrogen Life Technologies) (for MoDC; Ref. 31) or CD3− (10,000 U/ml) in RPMI 1640 (Invitrogen Life Technologies) and 10% AB serum (Australian Red Cross, Brisbane, Queensland, Australia) (for Mφ) for 5−7 days. As required, the cells were activated with Escherichia coli LPS (100 ng/ml; Sigma-Aldrich) for 1 day. CSF-1 was a gift from David Hume (University of Queensland, St. Lucia, Queensland, Australia).

hDCL-1 mRNA expression analysis

A commercial multiple tissue expression array (MTE Array; Clontech) was probed with [32P]dCTP-labeled, 1.6-kbp hDCL-1 cDNA produced by the primers MK062 (5'-GACCATGGAGCGGACATGATA-3') and MK063 (5'-GGCTCTACCATCTGGGTTTGT-3') on pB30-1 plasmid DNA (28) serving as a template and labeled by random priming (RediPrime II DNA labeling system; Amersham Bioscience). The final washing condition was 0.1× SSC with 5% SDS at 55°C. The blot was quantitated by scintillation counting using a1450 MicroBeta counter (Packard Instrument Co.) and the mAb was purified by protein G column chromatography. Similarly, one clone secreting high levels of 3XFLAG-hDCL-1-Ig was chosen by staining with FITC-conjugated sheep anti-human IgG F(ab')2 antibody. The hybridomas (MMRI-18, -19, -20, and -21) were adapted to a serum-free medium (Hybridoma-SFM; Invitrogen Life Technologies) and the mAb was purified by protein G column chromatography. As described later, the hybridomas (MMRI-18, -19, -20, and -21) were adapted to a serum-free medium (Hybridoma-SFM; Invitrogen Life Technologies) and the mAb was purified by protein G column chromatography. As required, the mAbs were conjugated with FITC (Sigma-Aldrich) or PE (PhycoLight R-PE conjugation kit; Prozyme).

To map hDCL-1 mAb epitopes, HB12 cells were preincubated with unconjugated hDCL-1 mAbs (10 µg/ml) on ice, washed, and stained with 10 µg/ml FITC-conjugated PE-MMRI-19 or FITC-MMRI-20 for flow cytometry analysis.

IP and WB analysis

Cells were surface biotinylated using sulfo-NHS-LC-biotin (Pierce), lysed in a lysis buffer (1% Triton X-100, 0.25% sodium deoxycholate, 0.15 M NaCl, 50 mM Tris-HCl (pH 7.4), and 5 mM EDTA) containing a mixture of protease inhibitors (Complete (Roche Applied Science) and 1 mM PMSF). The lysate was subjected to IP analysis using the DCL-1 mAb and probed with [32P]dCTP-labeled, 1.6-kbp hDCL-1 cDNA produced by the primers MK062 (5'-TTCAGTGACAGCAAGCCGACATGATA-3') and MK063 (5'-GGCTCTACCATCTGGGTTTGT-3') on pB30-1 plasmid DNA (28) serving as a template and labeled by random priming (RediPrime II DNA labeling system; Amersham Bioscience). The final washing condition was 0.1× SSC with 5% SDS at 55°C. The blot was quantitated by scintillation counting using a1450 MicroBeta counter (Packard Instrument Co.) and the mAb was purified by protein G column chromatography. Similarly, one clone secreting high levels of 3XFLAG-hDCL-1-Ig was chosen by staining with FITC-conjugated sheep anti-human IgG F(ab')2 antibody. The hybridomas (MMRI-18, -19, -20, and -21) were adapted to a serum-free medium (Hybridoma-SFM; Invitrogen Life Technologies) and the mAb was purified by protein G column chromatography. As required, the mAbs were conjugated with FITC (Sigma-Aldrich) or PE (PhycoLight R-PE conjugation kit; Prozyme).

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CHAPS and 1 mM PMSF and digested with 10 U of N-glycosidase F (Roche Applied Science) at 37°C overnight. The samples were concentrated with Microcon YM30 ultrafiltration units (Millipore) and subjected to SDS-PAGE.

For IP/WB analysis, 5–10 million cells were lysed with 1 ml of the lysis buffer. Two different concentrations of the cell lysate (final protein concentrations 400 and 133 μg/ml) were immunoprecipitated with the rabbit peptide Ab to the hDCL-1 cytoplasmic domain (CP) and protein A-Sepharose as described previously (28) and Western blotted with MMRI-20, IgG (GAM) F(ab')2 on ice and subjected to FACS using a FACSCalibur device (BD Bioscience).

DCL-1 endocytosis by HB12 cells
Near confluent HB12 cells cultured for 36–48 h in 24-well plates were incubated at 37°C in a CO2 incubator for the indicated time periods with FITC-MMRI-20 or 401.21 (10 μg/ml) diluted in Ham’s F12 medium with 10% FCS and 10 mM HEPES (pH 7.4). For the cells at t = 0 min the cells were stained on ice for 1 h with the FITC-conjugated mAbs. At the end of incubation, the cells were chilled on ice, washed once with cold culture medium, and harvested in cold MACS buffer. The cells were stained with biotinylated MMRI-21 (2.5 μg/ml) on ice followed by allophycocyanin-streptavidin (BD Bioscience) to detect cell surface hDCL-1, fixed with 4% paraformaldehyde (PFA) in PBS, and analyzed by FACS. Geometrical mean of fluorescence was determined using FCS Express version 3 software (De Novo Software) and relative hDCL-1 expression was calculated using the hDCL-1 cell surface expression at t = 0 min as 100%.

For confocal microscope analysis, HB12 cells cultured on round coverslips (13 mm in diameter) were incubated with FITC-MMRI-20 or 401.21 as described above. At the end of incubation the cells were chilled on ice, washed twice with cold culture medium, and stained with biotinylated MMRI-21 followed by streptavidin-Alexa Fluor 633 (Invitrogen Life Technologies) in the cold medium. After fixing with PFA, the cells were permeabilized with 0.1% Triton X-100 in HEPES-buffered saline (1 mM CaCl2, 1 mM MgCl2, 0.15 M NaCl, and 10 mM HEPES [pH 7.4]), stained with Alexa Fluor 546-phalloidin (Invitrogen Life Technologies) and 4',6-diamidino-2-phenylindole (DAPI), postfixed with 4% PFA in HEPES-buffered saline, mounted with Prolong Gold (Invitrogen Life Technologies), and observed under a laser-scanning confocal microscope (LSM) using a ×100 objective (LSM510 META; Carl Zeiss).
Phagocytosis of MMRI-20-coated microbeads by HB12 cells

Rat anti-mouse IgG1-microbeads (4.5 μm in diameter; Dynabeads, Invitrogen Life Technologies) were incubated with a saturating concentration of MMRI-20 or the isotype control mAb (10 μg of mAb per 100 μl of bead suspension), washed, and resuspended in CHO cell culture medium. HB12 cells cultured on round coverslips in 24-well plates were incubated on ice with the microbeads (4 × 10^5 beads/well) for 1 h to allow the cells to bind the beads. After washing extensively with the cold culture medium, the cells were incubated at 37°C for the indicated periods, washed with cold HEPES-buffered saline, fixed with PFA, and permeabilized as described above. The cells were stained with Alexa Fluor 488-GAM (Invitrogen Life Technologies), Alexa Fluor 546-phalloidin, and DAPI, postfixed, and subjected to LSM. To quantitate the microbeads bound on HB12 cells at t = 0 h, the cells incubated with the microbeads on ice (in triplicate) were harvested with cold MACS buffer, stained with Alexa Fluor 488-GAM, and subjected to FACS analysis.

Binding of anti-C-type lectin mAb-coated microbeads to Mph

The rat anti-mouse IgG1-conjugated microbeads were coated with anti-DEC-205 mAb (MMRI-7; Ref. 30) or anti-MMRI mAb (mAb 15-2; Ref. 36) as described above, washed, and resuspended in RPMI 1640, 1% BSA, and 10 mM HEPES (pH7.4) (Mph binding buffer). Mph cultured on coverslips in 24-well plates were incubated on the beads (4 × 10^5 beads/well) in the binding buffer for 1 h. The cells were washed extensively with the binding buffer, fixed, and permeabilized as described above. The cells were stained with Alexa Fluor 488-GAM, Alexa Fluor 546-phalloidin, and DAPI and subjected to LSM using a ×20 objective. Randomly selected field images (10–20 fields/sample) were taken and the numbers of beads associated with the cells (60–90 cells/field) were counted. Statistical significance was assessed by Student’s t test (two-tailed, unpaired) using GraphPad Prism software.

Cellular localization of C-type lectins in Mph

Mph cultured on coverslips were incubated with 0.5% DMSO (a solvent control) or 10 μM cytochalasin D (stock solution was 2 mM cytochalasin D in DMSO; Sigma-Aldrich) at 37°C for 30 min. The cells were washed twice with prewarmed Hanks-buffered saline (Invitrogen Life Technologies) at 37°C and fixed with 4% PFA plus 3% sucrose in PBS (8) or acetone at room temperature for 20 min. Both fixation methods yielded similar results. The cells were blocked and/or permeabilized with 10% BSA, 10% donkey serum, and 0.5% Triton X-100 in PBS for 30 min and stained with MMRI-20 mAb clone 15-2, MMRI-7, or 401.21 followed by Alexa Fluor 488-donkey anti-mouse IgG (Invitrogen Life Technologies) and counterstained with Alexa Fluor 546-phalloidin and DAPI. After fixation, the cells were mounted and observed with a LSM using a ×100 objective.

DNA sequencing and bioinformatics

The DNA sequences of the expression vectors were confirmed by sequencing (Australian Genome Research Facility, St Lucia, Queensland, Australia). DCL-1 orthologs were identified by searching the nonredundant and expressed sequence tag (EST) database from the National Center for Biotechnology Information (NCBI) using the basic local alignment search tool (BLAST) search engine with the entire human DCL-1 or its CP coding sequence (GenBank accession no. AY311407) as query. As required, the contigs were assembled to obtain a consensus sequence using a Sequencher 3.0 program (Gene Codes). The full or partial coding sequences were submitted to GenBank and assigned GenBank Third Party Annotation Database accession numbers for possum (Trichosurus vulpecula; BK0006290), dog (Canis lupus familiaris; BK0006291), bovine (Bos taurus; BK0006292), and pig (Sus scrofa; BK0006293). Multiple sequence alignment was performed using ClustalW available on the Australian National Genomic Information Service Bioinformatics service (ANGIS; www.angis.org.au). Potential serine/threonine/tyrosine phosphorylation sites and isoelectric point (pI) were predicted using the programs NetPhos 2.0 (37) and ProtParam, respectively, on the ExPASy Molecular Biology server (http://au.expasy.org; Ref. 38).

Results

The DCL-1 proteins and genes are conserved

DCL-1 is the simplest type I transmembrane C-type lectin discovered to date; it contains a SP, one C-type lectin-like domain (CTLD), and one short spacer followed by one transmembrane domain and one CP. Our BLAST search identified human DCL-1 orthologs in mice, rats, and other mammalian (e.g., monkeys, bovines, pigs, and dogs) and marsupial species (e.g., possums) but not in other vertebrates (e.g., birds, reptiles, and fish) and nonvertebrates (e.g., insects and nematodes). Furthermore, there was no paralog detected within several mammalian genomes examined, suggesting that DCL-1 is a mammalian/marsupial-specific protein with no redundancy (data not shown). The amino acid comparison between human, mouse (GenBank Accession No. AK004267), and rat DCL-1 (GenBank Accession No. BC089829) indicated that DCL-1 was highly conserved (Fig. 1A). The overall amino acid identity and similarity between hDCL-1 and the mouse or rat ortholog was 76 and 81%, respectively. Mouse DCL-1 was nearly identical to rat DCL-1 (92% identity and 94% similarity). In addition to six highly conserved cysteines for a typical C-type lectin motif (3), DCL-1 CTLD were rich in acidic amino acids (D and E), which comprised 21.5, 19.4, and 18.6% of CTLD amino acids for human, mouse, and rat DCL-1, respectively. The predicted pI values were 4.14, 4.24, and 4.21, respectively. One potential N-glycosylation site (NX(S/T)) was also conserved. In the CP, putative serine/threonine/tyrosine phosphorylation sites, an acidic amino acid cluster (EE(N/D)E), a potential lysosome targeting signal (Ref. 18), a hydrophobic amino acid cluster (LVV, a potential

FIGURE 2. Characterization of hDCL-1 protein expressed in CHO-hDCL-1 transfectants. A, Surface hDCL-1 protein expressed on HB12 cells was detected with anti-FLAG mAb M2 by flow cytometry (bold line). The gray fill indicates an isotype control staining. B, Characterization of hDCL-1 protein expressed by HB12 cells. Cell lysate from HB12 cells was immunoprecipitated with anti-hDCL-1 cytoplasmic domain (aDCL-1 CP) or preimmune rabbit Ab (Preimmune) and protein A beads and fractionated by SDS-PAGE under reducing (+DTT) or nonreducing conditions (−DTT), followed by WB with anti-FLAG (aFLAG) mAb M2 and HRP-conjugated goat anti-mouse IgG. The signals were detected by ECL. Asterisks indicate nonspecific bands. C, Effect of N-glycosidase F (N-glyaseF) digestion on HB12-derived hDCL-1 protein. The DCL-1 protein immunoprecipitated from HB12 was digested with N-glycosidase F and subjected to WB analysis as described above. The positions of molecular mass standards are shown on the right.
and a putative tyrosine-based internalization motif (FST/PAP(Q/L)SPY; Ref. 5) were all conserved between the species.

To determine the full genomic structure of hDCL-1, we performed a BLAST search of the NCBI genomic sequence database and determined the intron-exon boundaries using the "GT-AG" splice site consensus by comparing the genomic sequence with these cDNA sequences (40, 41). We found the hDCL-1 gene consisted of six exons spanning 29 kbp (Fig. 1, B and C). The SP was encoded by exon 1, CTLD by exons 2–4, the spacer by exon 5, and the transmembrane domain and CP by exon 6. Similarly, both the mouse and rat DCL-1 genes consisted of six exons with conserved exon-intron junctions spanning over 33 kbp. In mice, we detected an alternatively spliced DCL-1 mRNA lacking exon 5 (GenBank Accession No. AK004267), but no such deletion was found in human or rat DCL-1 by extensive BLAST search. The hDCL-1 gene was mapped to the chromosome band 2q24 containing the type I transmembrane C-type lectin cluster, which includes the phospholipase A2 receptor (PLA2R), DEC-205, and DCL-1. This gene cluster was also conserved in mice (2C1.2) and rats (3q21). These data suggested that hDCL-1 protein structure and function was highly conserved during evolution.

Recombinant hDCL-1 protein characterization

To assess the nature of hDCL-1 at protein levels, we established HB12 cells by stably transfecting CHO-K1 cells with the pSec3XFLAG-hDCL-1 expression vector. After labeling with the anti-FLAG mAb M2, flow cytometry analysis confirmed significant 3XFLAG-hDCL-1 cell surface expression (Fig. 2A). Immunoprecipitation with a rabbit anti-hDCL-1 CP peptide Ab (28) and Western blotting with an anti-FLAG mAb identified 3XFLAG-hDCL-1 as a broad band with modal sizes of 32 kDa in nonreduced and 35 kDa in reduced conditions (Fig. 2B), confirming the presence of the intermolecular disulfide bonds expected in a C-type lectin domain (3). N-glycosidase F treatment focused the 3XFLAG-hDCL-1 into a more defined single band of 30 kDa.

FIGURE 4. Production and characterization of mAbs against hDCL-1 protein. A, HB12 cells were stained with a series of anti-hDCL-1 mAb (MMRI-18, 19, 20 and 21) and subjected to flow cytometry analysis. The gray fills indicate an isotype control staining. B, IP of hDCL-1 protein from PBMC using the anti-DCL-1 mAb. Cell surface biotinylated PBMC lysate was immunoprecipitated with the anti-hDCL-1 mAb or an isotype control IgG1 (Ctr IgG1) and subjected to Western blotting under nonreduced conditions. The positions of molecular mass standards are shown on the left. Arrows indicate the specific hDCL-1 protein bands. C, Inhibition of PE-conjugated MMRI-19 and FITC-conjugated MMRI-20 binding to HB12 by unconjugated anti-hDCL-1 mAb. HB12 cells were preincubated with unconjugated anti-DCL-1 mAb (10 μg/ml), stained with PE-conjugated MMRI-19 (left panel) and FITC-conjugated MMRI-20 (right panel), and their binding was detected by flow cytometry. An isotype control IgG1 (Ctr IgG1) was used as negative control.
when reduced (Fig. 2C), indicating that the DCL-1 CTLD N-glycosylation motif was glycosylated in CHO cells. The 3XFLAG-hDCL-1 was also glycosylated in transfected COS-1 and HEK293 cells (data not shown).

hDCL-1 mRNA is predominantly expressed by phagocytes and DC

Multiple tissue expression array analysis revealed that hDCL-1 mRNA was present in several different issues but at variable levels (Fig. 3A). Adult liver, lung, PBMC, and spleen expressed hDCL-1 mRNA at relatively high levels, whereas neuronal tissues (e.g., brain and spinal cord), skeletal muscle, and ovary had low levels. In the limited fetal tissues examined, lung, liver, spleen, and kidney all had relatively high levels of hDCL-1 mRNA.

Semiquantitative RT-PCR indicated that hDCL-1 mRNA was expressed in MoDC, myeloid and plasmacytoid BDC, Mo, Mph, and granulocytes but not in T lymphocytes, B lymphocytes, or NK cells (Fig. 3B). The mRNA levels in MoDC and Mph decreased considerably upon activation by LPS.

These data suggested that hDCL-1 expression was restricted to phagocytes, including APCs, and that its ubiquitous hDCL-1 mRNA expression in human tissues might be explained by the residential tissue phagocytes.

hDCL-1 mAb production and characterization

To characterize hDCL-1 protein in leukocytes, we generated hybridomas producing mAbs to hDCL-1 by immunizing mice with HB12 cells (primary immunization) and 3XFLAG-hDCL-1-Ig protein. We screened 3000 hybridomas from two independent fusions and established four individual DCL-1 mAb producing cloned hybridomas: MMRI-18, -19, and -20 from the first fusion and MMRI-21 from the second fusion (all mouse IgG1 isotype). These mAbs labeled HB12 cells (Fig. 4A), bound to the 3XFLAG-hDCL-1-Ig fusion protein in ELISA, immunoprecipitated the 3XFLAG-hDCL-1 in IP/WB analysis (data not shown), and also immunoprecipitated 24- and 30-kDa bands (in nonreduced conditions) from cell surface-biotinylated PBMC lysate (Fig. 4B).

Epitope mapping analysis indicated that MMRI-18, -19, and -20 bound to similar epitopes, distinct from that of MMRI-21, because the preincubation of HB12 cells with unconjugated MMRI-18, -19, or -20 inhibited the binding of directly conjugated PE-MMRI-19 and FITC-20 to the HB12 cells, whereas MMRI-21 preincubation had no effect (Fig. 4C). MMRI-20 preincubation completely blocked PE-MMRI-19 binding, whereas MMRI-18 or -19 preincubation only partially blocked PE-MMRI-20 staining, suggesting that MMRI-20 had the highest affinity among MMRI-18, 19, and 20.

hDCL-1 is expressed on phagocytes and DC

Using the new DCL-1 mAb, we investigated hDCL-1 expression on human leukocytes, including T lymphocytes (CD3⁺ CD11c⁻ HLA-DR⁻), B lymphocytes (CD20⁺ HLA-DR⁻ CD11c⁻), NK cells (CD56⁺ HLA-DR⁻), Mo (CD14⁺ HLA-DR⁺ CD19⁻), and the myeloid (Lin⁻ HLA-DR⁻ CD11c⁺) and plasmacytoid control staining, respectively. D, Cell lysate from FACS-purified leukocytes, monocyte-derived Mph, and MoDC (400 and 133 μg/ml; indicated by filled triangles) was immunoprecipitated with anti-hDCL-1 cytoplasmic domain (αDCL-1 CP) or preimmune rabbit Ab (Preimmune) and protein A beads and fractionated by SDS-PAGE under nonreducing conditions, followed by Western blotting with MMRI-20. HB12 cells were used as a positive control.
considerably upon LPS activation (Fig. 5). MoDC expressed low levels of hDCL-1, and the levels decreased at moderate levels (data not shown). Monocyte-derived Mph and lymphocytes, and NK cells. Granulocytes also expressed hDCL-1 at moderate levels (data not shown). MoDC expressed low levels of hDCL-1 on their surface. No hDCL-1 expression was detected on T lymphocytes, B cells, and Mo, whereas the 30-kDa band was more prominent in Mph and MoDC than the 24-kDa band. LPS activation consistently decreased both signal levels. The exact nature of these two hDCL-1 bands requires further clarification.

hDCL-1 colocalizes with F-actin in HB12 cells and is endocytosed upon hDCL-1 mAb binding

Cellular localization of hDCL-1 in HB12 cells and its relationship with F-actin was assessed by LSM (Fig. 6A). Both x-y and x-z optical sectioning of the staining indicated that the majority of hDCL-1 protein in HB12 cells colocalized with F-actin in filopodia and the cellular cortex at basal surfaces (x-y sectioning) and the cellular cortex (x-z sectioning).

Because hDCL-1 CP contained potential internalization motifs (tyrosine based and hydrophobic amino acid based), we investigated hDCL-1 internalization by HB12 cells (Fig. 6, B and C) using flow cytometry and laser scanning confocal microscopy. When incubated with FITC-MMRI-20 at 37°C, the cells took up the Ab for up to 60 min and then reached a plateau. The increase of FITC-MMRI-20 uptake was due to the continuous transport of intracellular hDCL-1 to the cell surface. This uptake was specific because the FITC-isotype control mAb was not taken up by the cells. In contrast, the remaining cell surface hDCL-1 detected with biotinylated MMRI-21, which bound to a distinct epitope from that of MMRI-20 (see Fig. 4C), decreased concomitantly, indicating that hDCL-1 was endocytosed when bound to MMRI-20. The half-life of cell surface hDCL-1 was ~20 min. The level of cell surface hDCL-1 was relatively unchanged when HB12 cells were incubated with the FITC-isotype control mAb. These flow cytometric data were confirmed by LSM, showing that hDCL-1 endocytosis was hDCL-1 mAb specific. Interestingly, endocytosed hDCL-1 at t = 30 min no longer colocalized with F-actin, whereas cell surface hDCL-1 colocalized with F-actin at t = 0 min (Fig. 6C).

These data indicated that: 1) cell surface hDCL-1 is endocytosed when bound by hDCL-1 mAb; 2) intracellular hDCL-1 (up to 50% of cell surface hDCL-1) was transported to the cell surface and internalized upon hDCL-1 mAb binding; and 3) once internalized, hDCL-1 did not colocalize with F-actin and was not recycled to the cell surface for up to 120 min at least.

hDCL-1 on HB12 cells behaves as a phagocytic receptor

One of the presumed functions of C-type lectins on phagocytes and DC is phagocytosis. Therefore, we investigated whether hDCL-1 behaved as a phagocytic receptor by using HB12 cells (Fig. 7A).

FACS analysis using FITC-MMRI-20 revealed that moderate levels of hDCL-1 were present on both Mo and myeloid BDC (Fig. 5A). Plasmacytoid BDC expressed low levels of hDCL-1 on their surface. No hDCL-1 expression was detected on T lymphocytes, B lymphocytes, and NK cells. Granulocytes also expressed hDCL-1 at moderate levels (data not shown). Monocyte-derived Mph and MoDC expressed low levels of hDCL-1, and the levels decreased considerably upon LPS activation (Fig. 5, B and C). These data supported the hDCL-1 mRNA analysis (see Fig. 2) and showed that hDCL-1 expression was restricted to the phagocytic, monocyte, Mph, granulocyte, and DC leukocyte populations.

We used semiquantitative IP/WB to analyze DCL-1 expression on Mo, Mph, and MoDC (Fig. 5D; Ref. 30). We identified two bands in Mo, Mph, and MoDC of 24 and 30 kDa in nonreducing conditions. The ratios of these two bands differed, depending on the cell type: the 24-kDa band was more abundant than the 30-kDa band in Mo, whereas the 30-kDa band was more prominent in Mph and MoDC than the 24-kDa band. LPS activation consistently decreased both signal levels. The exact nature of these two hDCL-1 bands requires further clarification.

Figure 6. hDCL-1 colocalizes with F-actin and is internalized upon hDCL-1 mAb binding. A, Colocalization of hDCL-1 with F-actin in HB12 cells. B, hDCL-1 internalization by HB12 cells by flow cytometry. The cells were incubated with FITC-MMRI-20 at 37°C for various time periods. At the time points, cells were chilled, harvested in cold MACS buffer, and stained with biotinylated MMRI-21, which recognized a distinct epitope on hDCL-1 from that of MMRI-20 (see Fig. 4C), decreased concomitantly, indicating that hDCL-1 was endocytosed when bound to MMRI-20. The half-life of cell surface hDCL-1 was ~20 min. The level of cell surface hDCL-1 was relatively unchanged when HB12 cells were incubated with the FITC-isotype control mAb. These flow cytometric data were confirmed by LSM, showing that hDCL-1 endocytosis was hDCL-1 mAb specific. Interestingly, endocytosed hDCL-1 at t = 30 min no longer colocalized with F-actin, whereas cell surface hDCL-1 colocalized with F-actin at t = 0 min (Fig. 6C). These data indicated that: 1) cell surface hDCL-1 is endocytosed when bound by hDCL-1 mAb; 2) intracellular hDCL-1 (up to 50% of cell surface hDCL-1) was transported to the cell surface and internalized upon hDCL-1 mAb binding; and 3) once internalized, hDCL-1 did not colocalize with F-actin and was not recycled to the cell surface for up to 120 min at least.

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As expected, the majority of HB12 cells (>85%) bound the MMRI-20-coated microbeads (4.5 μm in diameter), whereas little binding was observed with the isotype control mAb-coated beads. When the cells were incubated at 37°C, HB12 cells phagocytosed the MMRI-20-coated beads (Fig. 7B): At t = 0 h we observed colocalization of F-actin at the contacts between the beads and the cells. In 2–4 h, HB12 cells began to phagocyte the majority of the microbeads surrounded by phagocytic cups. In some cases, the microbeads were fully phagocytosed. Occasionally we observed Alexa Fluor 488 dye released in the cytoplasm, suggesting that some beads were transported to phagolysosomes for proteolytic degradation. These data indicated that hDCL-1 behaves as a phagocytic receptor.

Anti-C-type lectin-coated beads binding to Mph

Mph are prototypical phagocytes and express an array of C-type lectin receptors, including MMR and DEC-205, which may play a role in phagocytosis. We assessed the surface expression of these C-type lectin receptors on macrophages by using a quantitative indirect immunofluorescence analysis (34, 35) (Fig. 8A). The analysis revealed that, although the expression levels varied among donors, MMR was the most abundant C-type lectin receptor, whereas the levels of DCL-1 and DEC-205 were approximately one-half and one-fifth that of the MMR, respectively. We coated rat anti-mouse IgG1-conjugated beads with mAbs specific to MMR (mAb 15-2; Ref. 36), hDEC-205 (MMRI-7; Ref. 30), and DCL-1 (MMRI-20) and investigated their binding and phagocytosis by LSM (Fig. 8, B and C). Nonspecific and complement receptor-mediated binding and phagocytosis by the Mph were minimized by incubating the mAb-coated microbeads on ice in the

![Image](https://via.placeholder.com/150)

**FIGURE 7.** HB12 cells bind and phagocytose MMRI-20-coated microbeads specifically. A, Rat anti-mouse IgG-conjugated microbeads (4.5 μm in diameter) were coated with MMRI-20 or an isotype control IgG1 (Ctr IgG1) and incubated with the clone HB12 in on ice. After washing to remove unbound microbeads, the cells were harvested with cold MACS buffer, stained with Alexa Fluor 488-GAM, and the binding of the microbeads was analyzed by flow cytometry. B, The clone HB12 cells cultured on coverslips were incubated with the mAb-coated microbeads on ice as described above. After washing, the cells were replenished with the tissue culture medium and incubated for various time periods. At the time points the cells were chilled on ice and fixed with PFA. After permeabilization, the cells were stained with Alexa Fluor 488-GAM (green), Alexa Fluor 546-phalloidin (red), and DAPI (blue) and analyzed by LSM with x-y-z sectioning using a x100 objective. Large panels, x-y sectioning at centers of nuclei; horizontal strips, x-z sectioning; vertical strips, y-z sectioning. White arrowheads indicate phagocytic cup or phagosomes. White arrows indicate mouse IgG dissociated from the microbeads. Bar, 10 μm.

![Image](https://via.placeholder.com/150)

**FIGURE 8.** Human monocyte-derived Mph preferably bind anti-MMR and anti-DEC-205 mAb-coated microbeads, but not anti-hDCL-1-coated microbeads. A, Cell surface expression of hDCL-1, MMR, and DEC-205 on Mph detected with a quantitative indirect immunofluorescence analysis. Mph differentiated from CD14+ Mo with CSF-1 were stained with a supersaturating concentration (20 μg/ml) of MMRI-20 (anti-hDCL-1), mAb 15-2 (anti-MMR), or MMRI-7 (anti-DEC-205) and FITC-GAM and analyzed by FACS. Gray fills indicate an isotype control IgG1 staining. The numbers in brackets indicate the number of specific Ab binding sites in the Mph preparation determined by the assay. B, Rat anti-mouse IgG-conjugated microbeads (4.5 μm in diameter) were coated with MMRI-20, mAb 15-2, MMRI-7, or the isotype control IgG1 (Ctr IgG1). Monocyte-derived Mph cultured on coverslips were incubated with the mAb-coated microbeads on ice, washed to remove unbound microbeads, and fixed with PFA. After permeabilization, the cells were stained with Alexa Fluor 488-GAM (green), Alexa Fluor 546-phalloidin (red), and DAPI (blue) and analyzed by LSM using a x20 objective. The insets correspond to magnified views of boxed areas. C, Quantitation of mAb-coated microbeads binding to Mph. The number of mAb-coated microbeads and the number of cells were counted from randomly selected confocal microscopic fields (10 fields for anti-hDCL-1, anti-MMR/CD206, and anti-DEC-205 and 20 fields for the isotype control IgG1) and expressed as number of microbeads/cell (mean ± SD).
absence of serum (14). Anti-MMR and anti-hDEC-205 mAb-coated microbeads bound to Mph effectively, and more than two microbeads were found to be associated with Mph (2.45/1100 ± 0.49 and 2.17/1100 ± 1.04 beads/cell, respectively, mean ± SD, n = 10). In contrast, only a small number of MMRI-20-coated microbeads bound to Mph (0.31/1100 ± 0.12 beads/cell, n = 10). Little or no staining (0.09/1100 ± 0.10 beads/cell, n = 20) of the isotype control mAb-coated beads was seen. The binding of C-type lectin mAb-coated beads to Mph compared with isotype control mAb microbeads was statistically significant (p < 0.0001 by Student's t test). The comparative p values for MMRI-20- vs anti-MMR mAb-coated beads and MMRI-20- vs anti-hDEC-205 mAb-coated beads were most significant at 9 × 10⁻⁸ and 3 × 10⁻⁴, respectively, but there was no significant difference between the binding of anti-MMR mAb- and anti-DEC-205 mAb-coated beads to Mph (p = 0.46). After incubation at 37°C, the C-type lectin mAb-coated beads were rapidly phagocytosed completely (data not shown). These data indicated that Mph could use hDCL-1 for particle binding and phagocytosis; however, DCL-1 was less efficient than MMR or hDEC-205.

hDCL-1 colocalizes with F-actin in Mph

It was possible that the relatively inefficient binding and subsequent phagocytosis of hDCL-1 mAb-coated microbeads was due to a distinct and/or alternative hDCL-1 cellular localization from that of MMR or DEC-205. We therefore investigated the localization of hDCL-1 and MMR in relation to F-actin in fixed and permeabilized Mph using LSM (Fig. 9). Staining with MMRI-20 and...
Alexa Fluor 546-phalloidin showed that hDCL-1 colocalized with F-actin structures at the near basal surface such as filopodia and lamellipodia (not shown) in the periphery (Fig. 9A). We also found hDCL-1 staining in relatively large dot-like structures (1–2 μm) or podosomes associated with F-actin in some Mph. In contrast, MMR and DEC-205 were dispersed and there was no apparent colocalization of MMR or DEC-205 with F-actin (Fig. 9A). The hDCL-1 colocalization with F-actin became more apparent when Mph were treated with cytochalasin D to disrupt F-actin extension (Fig. 9B). The treatment resulted in the formation of F-actin clumps at the periphery of the Mph and the marked proportion of hDCL-1 colocalized with the clumps, whereas there was no colocalization of MMR or DEC-205 with clumped F-actin.

To further investigate whether the colocalization of hDCL-1 to F-actin is intrinsic to hDCL-1 protein, we constructed an expression vector for hDCL-1-EGFP fusion protein (pEGFP-hDCL-1) and transiently transfected it into COS-1 cells (Fig. 9C). The hDCL-1-EGFP colocalized with F-actin at the cellular cortex and microvilli of the apical cell surface of the transfecants, whereas control EGFP expression was restricted within the cytoplasm and nuclei. These data indicate that the DCL-1 colocalizes intrinsically with F-actin involved in cell adhesion and migration, suggesting that hDCL-1 may play an additional or alternative role in Mph adhesion and migration.

Discussion

We have described a novel type I C-type lectin receptor, hDCL-1, whose expression is restricted to phagocytes including APCs. The hDCL-1 gene was located in a cluster of type I transmembrane C-type lectins on human chromosome band 2q24, which includes PLAR and DEC-205. The latter is the genetic fusion partner of hDCL-1, involved in producing the DEC-205/DCL-1 fusion protein identified in Hodgkin’s Reed-Sternberg cell lines (28). The DCL-1 CTLD contains six cysteine, a typical feature of a C-type lectin motif, to form an intramolecular disulfide bond-mediated C-type lectin fold (3). Unlike the Ca2+-dependent carbohydrate recognition domains (CRD) found in prototypical C-type lectin receptors such as DC-SIGN/CD209, MMR/CD206, and MGL/CD301, the DCL-1 CTLD is devoid of the known amino acid residues essential for Ca2+-dependent sugar binding, e.g., the EPN motif (for mannose binding), the QPD motif (for galactose binding), and the WND motif (for Ca2+ binding) (Fig. 10), suggesting that DCL-1 does not have classic sugar binding capacity. In preliminary studies using agarose beads conjugated with mannan, mannan, N-acetyl glucosamine, and N-acetyl galactosamine in the presence of Ca2+, we saw no sugar binding to the hDCL-1-Ig.

However, as exemplified by dectin-1, a nonclassical C-type lectin receptor for β-glucan in a Ca2+-independent manner (14), it is possible that DCL-1 has some alternative carbohydrate binding capacity. It is also possible that DCL-1 binds to protein ligands in the same manner that DC-SIGN/CD209 binds to ICAM-2/3 (11, 26) and that PLA2R and Endo180 bind to collagens (42, 43). Because the DCL-1 extracellular domain is rich in acidic amino acids and its predicted pl is ~4, we speculate that DCL-1 ligands are likely to have a high pl and be rich in basic amino acids.

The hDCL-1 extracellular domain contains one N-glycosylation signal near the center of the CTLD (Fig. 1A). The isolation of 3XFLAG-hDCL-1 from HB12 cells and N-glycosidase F digestion suggested that it was heterogeneously N-glycosylated at this site (~5 kDa carbohydrate moiety), and the reduced N-deglycosylated 3XFLAG-hDCL-1 protein was 30 kDa (Fig. 2C). The authentic hDCL-1 protein isolated from Mo, MoDC, and Mph consisted of two relatively sharp bands of 24 and 30 kDa in nonreducing conditions. The sharp nature of the 30-kDa band suggested that the DCL-1 had undergone little or no N-glycosylation, but how the 24-kDa DCL-1 band was produced is unclear. One possibility was that the 24-kDa band represented the transcribed product of the alternatively spliced DCL-1 mRNA (lacking exon 5 encoding the Ca2+-dependent carbohydrate recognition domain) found in mouse DCL-1, but our RT-PCR analysis failed to detect the truncated DCL-1 mRNA in human (data not shown). Perhaps the 24-kDa DCL-1 protein was produced by limited protease degradation.

The DCL-1 CP contains several putative functional motifs, including internalization signals (Tyr and hydrophobic amino acid based), late endosome targeting (acidic amino acid cluster), and serine/threonine/tyrosine phosphorylation, similar to that in DEC-205 CP (18, 27). Experiments using HB12 cells demonstrated that hDCL-1 was colocalized with F-actin in filopodia and cellular cortex and behaved as an endocytic (Fig. 6) and phagocytic receptor (Fig. 7). HB12 cells endocytozed cell surface 3XFLAG-hDCL-1 rapidly upon binding to FITC-MMRI-20 (t1/2 = 20 min). Manke et al. (18) showed that chimeric CD16 molecules containing MMR or DEC-205 CP expressed in mouse L cells could be endocytozed upon the binding of aggregated IgG and recycled back to the cell surface, suggesting that the CP of these C-type lectin receptors encode recycling signals. However, unlike the MMR and DEC-205 CP chimeric proteins, endocytozed hDCL-1 was not recycled to the cell surface, suggesting that DCL-1 CP differed in this function from the MMR or DEC-205 CP. The phagocytozed microbeads coated with anti-hDCL-1 appeared to reach phagolysosomes because the bead-bound Ab was released into cytospasm, suggesting that the acidic amino acid cluster in the...
Further data supporting the view that DCL-1 has novel functional properties were obtained in our studies comparing it to MMR and DEC-205. DCL-1 on Mph was less efficient than MMR or DEC-205 in binding anti-lectin mAb-coated microbeads and inducing phagocytosis (Fig. 8). Although significant numbers of microbeads could bind via DCL-1 to Mph, this was ~8-fold less efficient than MMR- and DEC-205-mediated binding. The quantitative indirect immunofluorescence analysis indicated that Mph expressed these C-type lectins at different levels (1–5-fold; MMR > DCL-1 > DEC-205) and, hence, the relatively poor bead binding of DCL-1 in contrast to that of DEC-205 did not correlate to the levels on the cell surface. We reasoned, therefore, that the differences might be, at least in part, a consequence of the relative accessibility (cellular localization) of the different C-type lectins. Our confocal microscope analysis revealed that MMR and DEC-205 had a dispersed distribution and there was no apparent colocalization with F-actin in Mph. In contrast, DCL-1 colocalized with F-actin, notably in filopodia, lamellipodia, and podosomes, the F-actin structures that play important roles in cellular adhesion and migration (Fig. 9). Furthermore, cytochalasin D treatment retained their colocalization. The fact that DCL-1 expressed in CHO cells (Fig. 6) and hDCL-1-EGFP fusion protein expressed in COS-1 cells (Fig. 9) colocalized with F-actin suggests that the DCL-1 CP contains an intrinsic signal for interacting with F-actin similar to that of other cell/matrix adhesion receptors such as the cadherins, ICAM, selectins, CD44, and integrins. The interaction between DCL-1 CP and F-actin is most likely to be indirect because all of the known cell/matrix receptor interactions with F-actin require adaptor proteins such as catenins (for cadherins; Ref. 44), ERM proteins (for ICAM, selectins, and CD44; Ref. 45), and α-actinin/talin/filamin (for integrins; Ref. 46). An indirect interaction is also suggested by the failure of a communoprecipitation experiment using nonionic detergent extraction to communoprecipitate β-actin (data not shown).

Podosomes in Mph and DC are regulated through the dynamic reorganization of actin microfilaments and contain many structural and signaling proteins commonly found in focal adhesions (47–49). These include prototypical cell adhesion receptors, integrins, and signaling proteins commonly found in focal adhesions (47–49). These include prototypical cell adhesion receptors, integrins, paxillin, talin, vinculin, Arp2/3 complex, and Wiskott-Aldrich syndrome protein (WASP). WASP is a hematopoietic cell-specific protein that participates in the actin organization of actin polymerization primarily through activation of Arp2/3 complex (48). WASP deficiency results in defective migration of neutrophils, Mo, Mph, and DC due to the failure of podosome formation and recruiting of cell/matrix receptors, primarily β₂ integrins (50–52). Our data suggest that DCL-1 may be another cell/matrix adhesion receptor integrated in cell adhesion complexes and that DCL-1 dysfunction may affect APC adhesion and migration, causing suppression of APC function.

In summary, it is likely that hDCL-1 is a potential multifunctional C-type lectin receptor that plays roles not only in endocytosis and phagocytosis but also in APC adhesion and migration and thus might become a target for therapeutic manipulation.

Acknowledgments

We thank Sonia Hancock, Georgina Crosbie, Nicola Gear, Hiroaki Mi­chiei, and Robert Wadeley for technical assistance and Georgina Clark for critical reading of this manuscript. We also thank David Hume, Mat Sweet, and Kate Stacy for providing CSF-1 and advice.


