Dendritic-cell–associated C-type lectin 2 (DCAL-2) alters dendritic-cell maturation and cytokine production

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Dendritic-cell (DC)–associated C-type lectin receptors (CLRs) take up antigens to present to T cells and regulate DC functions. DCAL-2 is a CLR with a cytosolic immunoreceptor tyrosine-based inhibitory motif (ITIM), which is restricted to immature DCs (iDCs), monocytes, and CD1a+ DCs. Cross-linking DCAL-2 on iDCs induced protein tyrosine phosphorylation and MAPK activation as well as receptor internalization. To test if DCAL-2 is involved in DC maturation and cytokine expression, we stimulated iDCs with anti–DCAL-2 mAb with or without LPS, zymosan, or CD40L. While anti–DCAL-2 did not induce iDCs to mature, it did up-regulate CCR7 expression and IL-6 and IL-10 production. DCAL-2 signals augmented DC maturation induced by LPS or zymosan, increasing both CCR7 and DC-LAMP expression. Of interest, DCAL-2 ligation had the opposite effects on TLR versus CD40L signaling: anti–DCAL-2 suppressed TLR-induced IL-12 expression, but significantly enhanced CD40L-induced IL-12 production. DCAL-2 ligation also suppressed the ability of TLR-matured DCs to induce IFN-γ–secreting Th1 cells but augmented the capacity of CD40L-matured DCs to polarize naïve T cells into Th1 cells. Thus, DCAL-2 may program DCs differently depending on whether DCs are signaled via TLRs or by T cells. DCAL-2 may be a potential immunotherapeutic target for modulating autoimmune diseases or for developing vaccines.

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only binds to β-1,3- and β-1,6-linked glucans on yeast, but also can bind to an unknown endogenous ligand on T cells and promote TCR-dependent T-cell proliferation.\textsuperscript{22,36}

The ITAM-like motifs within dectin-1 and DC-SIGN are involved in transducing signals to promote macrophage or DC activation and can integrate with TLR signaling.\textsuperscript{37,38} The integration of CLR signals with TLR signals can lead to different outcomes. Cross-linking Dectin-1 with TLR-2/6 by zymosan can enhance TNF-α and IL-12 expression, leading to strong Th1 response.\textsuperscript{37} On the other hand, simultaneous ligand binding of DC-SIGN and TLR-4 can inhibit TLR-mediated IL-12 production and DC maturation.\textsuperscript{39} Thus, studies suggest CLRs may either synergize or antagonize TLR signals depending on the particular combination of receptors. While the balance between TLR and CLR stimulation may play a significant role in initiating DC functions, the mechanisms by which TLRs and CLRs interact with each other are still unclear.

Previously, we defined a novel C-type lectin, DCAL-1, which is expressed on DCs. DCAL-1 can bind to T cells and serve as a costimulatory molecule to promote T-cell proliferation and Th2 differentiation.\textsuperscript{30} Here we describe another DC-associated type II CLR, dendritic-cell–associated C-type lectin-2 (DCAL-2). DCAL-2 is restricted to myeloid DCs and upon ligation internalizes, suggesting it can be used by DCs as an Ag capture receptor. Ligation of DCAL-2 with a mAb triggers the expression of chemokines and cytokines (MIP-3α, IL-6, IL-10). Of most importance, DCAL-2 can selectively interact with TLR signaling pathway or the T-cell signal CD40L to alter cytokine production by DCs and thereby regulate the quality of downstream T-cell activation. DCAL-2 thus may be a useful target for modulating immune responses toward protective immunity or tolerance induction.

Materials and methods

Primary cell culture and cell lines

Human peripheral blood CD14\textsuperscript{+} monocytes were obtained as described\textsuperscript{40} and were routinely 97% to 99% pure as assessed by flow cytometry. These CD14\textsuperscript{+} cells were then differentiated into immature DCs (iDCs) by culturing monocytes in IL-4 and GM-CSF for 5 to 7 days as described.\textsuperscript{40} Immature DCs were defined as CD14\textsuperscript{+}, CD1α\textsuperscript{+}, CD86\textsuperscript{+}, and HLA-DR\textsuperscript{+}. To mature DCs, iDCs were stimulated with graded doses of E. coli LPS (Sigma-Aldrich, St Louis, MO), yeast zymosan (Sigma-Aldrich), and CD40L-transfected L cells in the presence or absence of anti–DCAL-2 mAb or IgM control for 24 hours. CD1α\textsuperscript{+}, BDCA-2\textsuperscript{+} pDCs, B cells, and natural killer (NK) cells were obtained from peripheral blood buffy coats (Oregon Redcross, Portland, OR) using anti-CD1a, anti–BDCA-2, anti-CD19, anti-CD56 magnetic beads (Miltenyi, Auburn, CA). Total CD3\textsuperscript{+} T cells were isolated by sheep red blood cell (SRBC) (Triple J Farms, Bellingham, WA) agglutination. Dense human tonsillar B cells were prepared as previously described.\textsuperscript{41,42} The purity of the cells was more than 97% by flow cytometric analysis. T cells were activated for 24 hours with a mAb to CD3 (64.1) at 1 μg/mL in solution. B cells were activated with 1 μg/mL anti-CD40(G28-5) and 10 μg/mL anti-IgM (Jackson Immunoresearch Laboratories, West Grove, PA) for 24 hours. The BJAB, Daudi, HL-60, Jurkat, MP-1, Nalm-6, and U937 cell lines were cultured in RPMI1640 supplemented with 10% FCS at 37°C and 5% CO\textsubscript{2}.

Reverse transcriptase–polymerase chain reaction (RT-PCR) expression analysis of hDCAL-2

To prepare RNA from the purified primary cells or cell lines, RNA was isolated as described by the manufacturer or directly isolated using Qiagen RNeasy kit (Qiagen, Chatsworth, CA). First-strand cDNA synthesis was performed using oligo dTs and avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI) in standard reverse-transcription reactions. Human DCAL2 expression was analyzed by PCR of the cDNA using the following specific primers: 5’-cattagctgttgaactactattc-3’ and 3’-agccagaggtattatatcacc-5’ and 30 cycles of 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 45 seconds. β-Actin primers were used as loading controls: forward, 5’-gctgctagaaggtttccggtcgt-3’ and reverse, 3’-cattgagaggttgtgttgccagatc-5’.

Flow cytometric analysis and intracellular staining

To investigate the expression of DCAL-2 on peripheral blood mononuclear cells (PBMCs) or cell lines, cells were single or double stained with anti–DCAL-2 mAb or mouse IgM (TEPC183; Sigma-Aldrich) as an isotype control, washed, and then stained with FITC-conjugated goat F(ab\textsuperscript{2}) anti-mouse (IgM + IgG, GAM Ig-FITC, Jackson Immunoresearch Laboratories) or PE-conjugated anti-mouse IgM (BD Pharmingen, San Diego, CA), then washed and incubated with PE- or FITC-conjugated anti-CD3, anti-CD16, or anti-CD14 mAb (BD Pharmingen). To stain DCAL-2 on blood DCs and pDCs, cells were purified from PBMCs using anti-CD1a and anti–BDCA-2 magnetic beads and then stained with anti–DCAL-2 mAb, followed by PE-conjugated heavy chain-specific anti-mouse IgM.

To study the effect of DCAL-2 signal on DC maturation, iDCs were stimulated with the indicated stimuli for 24 hours. At the indicated times, cells were double stained with the following mAbs: anti–human CCR7 (2H4) and anti-CD86 (IT2.2), anti-CD83 (HB15; BD Bioscience Pharmingen, San Diego, CA), or anti–DC-LAMP (Lampinitch Beckman Coulter, Somerset, NJ). For detecting CCR7 and CD86 expression, cells were first stained with anti–CCR7 followed by a second step with GAM Ig-FITC. After fixing, cells were then double stained with anti–CD86-PE. To detect DC-LAMP and CD83, cells were first fixed with 1% paraformaldehyde, then permeabilized with PBS containing 0.1% saponin and 1% FBS; cells were then stained with anti–human DC-LAMP followed by GAM Ig-FITC. Cells were fixed and double stained with anti–CD83-PE. The stained cells were analyzed using a FACScan analyzer and CellQuest software (BD Bioscience, San Diego, CA).

Internalization assay

One million iDCs were incubated with anti–DCAL-2 or control mouse IgM for 30 minutes at 4°C. After washing twice with cold PBS, cells were resuspended and cultured with warm media (37°C). At the indicated time points, cells were washed and then fixed with 4% paraformaldehyde. The level of cell surface-bound anti–DCAL-2 remaining on cells was detected using GAM Ig-FITC. After fixing, cells were then double stained with anti–CD86-PE. To detect DC-LAMP and CD83, cells were first fixed with 1% paraformaldehyde, then permeabilized with PBS containing 0.1% saponin and 1% FBS; cells were then stained with anti–human DC-LAMP followed by GAM Ig-FITC. Cells were fixed and double stained with anti–CD83-PE. The stained cells were analyzed using a FACScan analyzer and CellQuest software (BD Bioscience, San Diego, CA).

Western blotting

iDCs were incubated with anti–DCAL-2 mAb for 30 minutes at 4°C. After washing twice with cold PBS, cells were resuspended and cultured with warm media (37°C) and lysed at the indicated time points by lysis buffer.\textsuperscript{43} Total protein concentrations were measured using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of protein were mixed with the Laemmli protein sample buffer and boiled, then separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellular membranes. After blocking with 5% fatfree milk, the blots were probed with antibodies against phosphotyrosine (4G10), anti–phospho-p38 MAPK, anti–phospho-ERK, and anti–phospho-JNK (Cell Signaling, Beverly, CA). The blots were washed and incubated with horseradish peroxidase (HRP)–labeled secondary Abs. Blots were then visualized by an enhanced chemiluminescence (ECL) detection reagent. The blots were stripped and reprobed with anti–total p38 MAPK (Santa Cruz Biotechnology, Santa Cruz, CA) as a loading control.

Cytokine analysis

iDCs were stimulated with LPS, zymosan, or CD40L-transfected L cells in the presence or absence of anti–DCAL-2 or IgM isotype control for 24 or 48 hours. The supernatants were collected, and then cytokines and chemokines were analyzed by enzyme-linked immunosorbent assay (ELISA) (IL-6, IL-10, IL-12 p40, IL-12 p70, and MIP-3β; R&D Systems, Minneapolis, MN).

T-cell proliferation and differentiation

Immature DCs were stimulated with graded doses of the indicated stimuli in the presence or absence of anti–DCAL-2 mAb or IgM control (10 μg/10\textsuperscript{6} cells) for
24 hours and then washed. Naive CD4⁺/CD45RA⁻ T cells were purified by negative selection using anti-CD8 and anti-CD45RO magnetic beads and generally were 95% to 98% of purity. For T-cell proliferation assays, naive CD4⁺/CD45RA⁻ T cells were cultured with the pretreated DCs for 5 days and then analyzed by flow cytometry. To monitor T-cell differentiation, naive CD4⁺/CD45RA⁻ T cells were cultured with the pretreated DCs. After 6 days, cells were harvested and stimulated with PMA and ionomycin for 6 hours with the addition of Brefeldin A (Sigma-Aldrich) for the last 5 hours of culture. Cells were fixed and permeabilized with PBS containing 0.1% saponin and 1% FBS. Cytokine production profiles were determined by intracellular cytokine staining using anti–IFN-γ (B27) and anti–IL-4 (MP4-25D2) (BD Bioscience Pharmingen) and analyzed by flow cytometric analysis.

Results

Identifying the type II C–type lectin, DCAL-2

Human DCAL2 was identified by creating position-specific scoring matrices (PSSMs) based on the CRD of DC-SIGN and DCIR then blasted against human EST databases. The full-length hDCAL2 contained a putative open-reading frame (ORF) of 798 bp (GenBank accession no AY426759), encoding a 265–amino acid (aa) polypeptide with predicted size of approximately 31 kDa. Comparisons between hDCAL-2 protein sequence and BLAST alignments revealed features typical of a type II transmembrane C–type lectin with a cytoplasmic tail that contains a tyrosine at amino acid position 7 centered in the sequence VTYA DL. This is identical to the consensus sequence IVXXVL of an immunoreceptor tyrosine-based inhibitory motif (ITIM). hDCAL-2 protein also has a putative hydrophobic transmembrane region (residues 44–60) and an extracellular region containing a single carbohydrate recognition domain (CRD) with 6 cysteines to form 3 disulphide bonds. While it has no clear calcium-binding motif within the CRD, it does have 6 potential N-linked glycosylation sites, at amino acid positions 78, 86, 96, 103, 156, and 217 (Figure 1A).

Analysis of the genomic sequence revealed that DCAL2 is located at human chromosome 12p13 in a region near a cluster of C-type lectins and that the gene has 6 exons. Mouse Dcal-2 was also identified from partial ESTs and the full-length sequence obtained by 5' and 3' rapid amplification of complementary DNA ends (RACE) PCR (Figure S1A; see the Supplemental Figures link at the top of the online article, at the Blood website); mDcal-2 had a similar genomic structure to hDCAL2 and its nucleotide sequence is 72% homologous to hDCAL2 (Figure S1B). Human and mouse DCAL-2 have been previously described as myeloid inhibitory C-type lectin (MICL)⁴⁴ and C-type lectin molecule-1 (CLL-1),⁴⁵ respectively. Compared with the primary CRD protein sequence of other C-type lectins, hDCAL-2 has the greatest identity to Dectin-1 (31%), CLEC-1 (30%), and CLEC-2 (30%), with lower homology with DCIR (24%), CD69 (21%), and DCAL-1 (11%) (Figure 1B). The phylogenetic tree of these similar C-type lectins
indicates hDCAL2 forms a cluster with dectin-1, CLEC1, and CLEC2 (Figure 1C).

**DCAL-2 expression is restricted to monocytes, macrophages, and DCs**

The expression of DCAL-2 in human tissues was determined by Northern blotting, which detected a single band at the predicted size of 1.6 kb (Figure S2A). DCAL-2 mRNA is expressed in PBMCs and at lower levels in the lung, but was not detectable in placenta, liver, spleen, small intestine, kidney, colon, skeletal muscle, and brain, indicating a selective expression of hDCAL-2 in hematopoietic tissues. RT-PCR analyses of primary hematopoietic cells showed that hDCAL-2 mRNA is restricted to CD14+ monocytes, CD1a+ blood DCs, monocyte-derived iDCs, and macrophages (Figure 2A). DCAL-2 mRNA was not detected by RT-PCR in NK cells, plasmacytoid DCs (pDCs), CD3+ T cells, activated CD3+ T cells stimulated with anti-CD3, peripheral blood B cells, dense tonsillar B cells, or tonsillar B cells stimulated with anti-IgM and anti-CD40. RT-PCR analyses of cell lines confirmed this restricted expression pattern. A low level of mRNA expression of hDCAL-2 was detected in myeloid cell lines (HL60 and U937) and at very low levels in a pre–B-cell line (Nalm6) but not in mature B-cell lines (Daudi, REH, and Bjab) (Figure S2B). DCAL-2 was also not detected in nonhematopoietic cells, including a follicular dendritic-cell line (FDC-1) (Figure S2B), HeLa cells, or in T-cell lines (CEM, Jurkat, and Molt-4) and primary gingival epithelial cells (data not shown).

DCAL-2 protein expression on the cell surface was analyzed using an anti–DCAL-2 mAb we developed (IgM isotype, UW70). Similar to RT-PCR analyses, DCAL-2 protein was expressed on CD14+ monocytes, CD1a+ blood DCs, and iDCs, but not on blood pDCs, CD3+ T cells, CD16+ NK cells, or B cells (Figure 2B-C). Only the myeloid cell lines HL60 and U937 showed weak surface expression of hDCAL-2, while HeLa cells or B- or T-cell lines were negative (Figure 2C). These results are consistent with and extend the previous reports that DCAL-2 mRNA is restricted to myeloid cells. Neither DCAL-2 RNA nor protein expression changed on DCs compared with iDCs after they were matured with either LPS, zymosan, or CD40L (data not shown).

**Ligation of hDCAL-2 induces signal transduction in iDCs**

Marshall et al demonstrated that human MICL/DCAL-2 transfected into the mouse Raw cell line associates with phosphatases such as SHP-1 or SHP-2 upon pervanadate stimulation. To investigate if DCAL-2 ligation signals human DCs, we used our anti–DCAL-2 mAb to cross-link hDCAL-2 on iDCs. Protein tyrosine phosphorylation (PTP) at different time points was analyzed by Western blotting using antiphosphotyrosine mAb (Figure 3A). Within 5 minutes after DCAL-2 ligation, several changes in PTP patterns were evident: PTP of a 100-kDa band gradually diminished by 60 minutes, while PTP was induced on major proteins about 90-, 70-, 60-, and 45-kDa in size. We further examined if ligand binding to DCAL-2 could induce activation of MAP family kinases including p38 MAPK, ERK, and JNK. Anti–DCAL-2 induced phosphorylation of both p38 MAPK and ERK (Figure 3B-C) but not JNK phosphorylation (data not shown). These data demonstrate that DCAL-2 expressed on DCs possesses a functional signaling motif and that ligand binding of DCAL-2 may signal DCs.

**DCAL-2 internalizes after ligand binding**

Several C-type lectins internalize after mAb and/or ligand binding including DC-SIGN, dectin-1, and BDCA-2. The cytoplasmic tail of DCAL-2 does not appear to have any of the known internalization consensus motifs. To investigate if DCAL-2 can internalize, iDCs were coated with anti–DCAL-2 mAb at 4°C, washed, and then warmed up to 37°C for the indicated times to allow for receptor internalization. Cells were fixed and the amount of anti–DCAL-2 mAb remaining on cell surface was detected by GAM-Ig-FITC and flow cytometry. A gradual internalization of DCAL-2 was evident after mAb binding, demonstrating that after cross-linking DCAL-2 can be internalized by iDCs (Figure 3D). To exclude the possibility that the disappearance of bound anti–DCAL-2 was due to shedding or cleavage, we also analyzed total anti–DCAL-2 by intracellular staining; similar levels of total anti–DCAL-2 were detected in all samples (data not shown).
DCAL-2 signaling up-regulates CCR7 and cytokine expression but does not induce DC maturation

Since DCAL-2 cross-linking could signal iDCs by promoting tyrosine phosphorylation, and p38 MAPK and ERK activations, we next examined if ligation of DCAL-2 induces DC maturation or cytokine expression. After we stimulated iDCs with anti–DCAL-2 or IgM isotype control for 24 hours, we measured the expression of DC maturation markers such as CD83, CD86, CCR7, and DC-LAMP (Figure 4A-B, left panels). Only 4% to 5% of iDCs expressed CD83 and CD86 and only 2% to 3% expressed DC-LAMP after culturing in medium only or control IgM (i.e., they maintained their immature state). Stimulation of iDCs with anti–DCAL-2 did not induce up-regulation of CD83, CD86, or MHC II (data not shown) and only slightly increased DC-LAMP expression. Surprisingly, DCAL-2 signaling significantly up-regulated CCR7 expression even though these DCs did not express the well-defined maturation markers such as CD83 and CD86.

Since activation of p38 MAPK and ERK is involved in regulating cytokine expression in DCs, we also examined the cytokine/chemokine expression profile from DCAL-2–stimulated iDCs.43 We analyzed supernatants from DCs stimulated with anti–DCAL-2 or isotype control for 24 hours using ELISA. Anti–DCAL-2 stimulation alone induced modest production of IL-6, IL-10, MIP-3β, and TNF-α; low levels of IL-12p40; and barely detectable levels of IL-12p70 (Figure 5A). These results suggest that a DCAL-2 signal induces iDCs to express CCR7 and modest levels of some cytokines and chemokines while maintaining DCs at a relatively immature stage.

DCAL-2 interacts with TLR signaling pathways

After encountering pathogens, iDCs can be activated and undergo a maturation process via ligation of PRRs such as TLRs. At this stage, the function of DCs switches from Ag capture toward Ag presentation and T-cell programming.14,46,47 TLRs and CLRs can interact and modulate DC functions (e.g., by altering cytokine expression).38,48 Cross-linking of dectin-1 and TLR2/6 by the yeast cell component, zymosan, enhances IL-12 and TNF-α production.37 Also, DC-SIGN binding interferes with TLR4-induced IL-12 production.32 Thus, we examined if DCAL-2 signaling could alter TLR-induced DC maturation and/or cytokine production: iDCs were stimulated with either LPS or yeast zymosan in the presence or absence of anti–DCAL-2 mAb for 24 hours, and then analyzed for changes in DC maturation markers. Stimulation of iDCs with either LPS or zymosan, as expected, induced DC maturation and increased levels of CD83, CD86, and DC-LAMP and slightly increased CCR7 expression (Figure 4A-B, upper and bottom panels). DCAL-2 signaling did not alter either LPS- or zymosan-induced DC maturation as measured by expression of CD83 and CD86 (Figure 4A-B, middle panel). However, anti–DCAL-2 did significantly increase LPS- and zymosan-induced...
DCAL-2 did not alter CD40L-induced increases in either CD83 or CD86, but did augment CCR7 and DC-LAMP expression and also enhanced CD83 and CD86 expression somewhat when combining with a low dose of CD40L (Figure 6A). Anti–DCAL-2 also increased levels of IL-12p40, IL-12 p70, IL-10, IL-6, and TNF-α when combined with graded doses of CD40L (Figure 6B and Figure S4). Taken together these data suggest that DCAL-2 can synergize with CD40 signaling to promote inflammatory cytokine expression and possibly Th1 responses.

**Discussion**

DCAL-2 is the latest addition of a growing family of type II CLRs, including DC-SIGN, dectin-1, and BDCA-2, which modulate DC functions upon ligand binding. This group of C-type lectins possess several special features that make them attractive targets for immunotherapy or antigen-delivering vehicles for vaccine development: (1) they are expressed mainly on APCs such as macrophages and dendritic cells; (2) they can serve as endocytotic receptors; and (3) they modulate immune responses when combined with other immune stimuli such as TLRs. Of interest, they do not share similar cytoplasmic signal motifs for signaling or internalization; DC-SIGN and dectin-1 possess ITAM-like motifs, BDCA-2 contains a relatively short intracellular domain and lacks any known motif for signal transduction, and DCAL-2 contains an ITIM. However, the diversities of signaling motifs used by these CLRs may contribute to their interaction with other PRRs such as TLRs.

**DCAL-2 signaling modulates the capacity of DCs to induce T-cell polarization**

Our results suggested that DCAL-2 signaling could down-regulate TLR-induced IL-12p40/p70 but augment CD40L-induced IL-12 production. To examine if DCAL-2 ligation during DC maturation can affect the ability of mature DCs to activate resting T cells, we initially tested if DCAL-2 signaling would affect DC-dependent allogeneic T-cell proliferation. First, iDCs were stimulated with LPS, zymosan, or CD40L with or without anti–DCAL-2 for 24 hours, then they were mixed with allogeneic CD4+CD45RA+ T cells labeled with CFSE. After 4 days, T-cell proliferation was measured by flow cytometry. Anti–DCAL-2 treatment slightly decreased the ability of zymosan-matured DCs to induce naive T-cell proliferation, but had no effect on DCs matured by either LPS or CD40L (Figure 7A). Similar results were also observed when T cells were pulsed with [3H] thymidine after a 5-day stimulation (data not shown).

We next investigated if the reduced level of IL-12 expression in LPS/DCAL-2– and zymosan/DCAL-2–treated DCs would affect T-cell differentiation. When naive T cells were cocultured with LPS- or zymosan-matured DCs, around 33% ± 4% and 34% ± 2% of differentiated T cells produced IFN-γ (Figure 7B). However, when LPS/DCAL-2– or zymosan/DCAL-2–matured DCs were used to differentiate naive T cells, significantly fewer T cells produced IFN-γ after restimulation (23% ± 2% and 20% ± 3%, respectively [P < .01] vs LPS or zymosan DCs; n = 4 for each condition). By contrast, the CD40L/DCAL-2–matured DCs had the opposite effect: more IFN-γ–producing T cells (41% ± 4% [P < .02] vs CD40L DCs; n = 4 for each condition) were detected when CD40L/DCAL-2–matured DCs were cultured with T cells compared with CD40L-matured DCs (30% ± 4%). These data suggest that the regulation of IL-12 expression caused by the presence of DCAL-2 signaling during DC maturation could influence downstream T-cell differentiation.

**DCAL-2 synergizes with CD40 signaling pathway to enhance cytokine expression**

CD40 ligation of iDCs is a potent stimulus for maturing DCs. Since DCAL-2 ligation modulated TLR-induced DC functions, we next examined whether DCAL-2 signaling could affect a T-cell–mediated DC signal via CD40. We stimulated iDCs with either CD40L-transfected L cells or CD40L:Fc fusion protein for 24 hours, and then analyzed DC maturation and cytokine expression.

CCR7 expression and also slightly enhanced DC-LAMP expression in zymosan-treated cells. These results suggest that DCAL-2 signals may interact with different TLR signaling pathways during DC maturation. The combination of DCAL-2 signaling with certain TLRs such as TLR2 and TLR4 may augment the DC maturation process including DC migration and Ag processing.

We next examined if combining a DCAL-2 signal with a TLR signal could modulate cytokine and chemokine expression. LPS alone induced DCs to produce significant amounts of IL-12p40 and moderate levels of IL-12p70, while zymosan stimulation induced high amounts of IL-12p40 but low levels of IL-12p70 (Figure 5B). Anti–DCAL-2 significantly suppressed both LPS- and zymosan-induced IL-12p40 and IL-12p70 expression (Figure 5B). DCAL-2 signaling also suppressed LPS-induced TNF-α expression but not zymosan-induced TNF-α expression (Figure S3A), but had no effect on either LPS- or zymosan-induced IL-6 and IL-10 expression by DCs (Figure S3B). These data show that DCAL-2 signals can antagonize TLR signals and modulate TLR-induced cytokine/chemokine expression selectively.
The functions of CLRs on DCs have been revealed by intensive studies on DC-SIGN and dectin-1. CLRs can serve as adhesion molecules, antigen recognition receptors, or signaling molecules, or even regulate T-cell activation. Since DCAL-2 is internalized after it is cross-linked, it may function as an Ag uptake receptor and promote antigen presentation. However, the endogenous ligand(s) for DCAL-2 remains to be identified. The possible ligands of C-type lectins on DCs include glycosylated self-proteins. Accordingly, it has been suggested that DCs may use CLRs to bind self-antigens to induce self-tolerance, as shown by the induction of tolerance when Ag was delivered via DEC-205/CD205. Tolerogenic DCs display a semimature phenotype (high MHC II, but low levels of costimulatory molecules such as CD80 and CD86) but require CCR7 to migrate to draining LNs in order to induce peripheral tolerance. Of interest, when we cross-linked DCAL-2 with mAb alone, those DCs displayed a partially mature phenotype (low CD83 and CD86, and no DC-LAMP expression), but up-regulated CCR7 suggests that they may have similar functions as the steady-state DCs (eg, tolerogenic DCs). Anti–DCAL-2–stimulated DCs also expressed significant amounts of IL-6/IL-10 that could suppress Th1 responses or even induce the generation of regulatory T cells. These data suggest signaling via DCAL-2 could be involved in generation of immune tolerance to certain Ag's. Thus, it will be important to determine if DCAL-2 can recognize any soluble self-proteins leading to induce immune tolerance.

C-type lectins contain various signaling motifs in their cytoplasmic regions and evidence suggests these motifs are involved in DC functions. CLRs including BDCA-2, DCAL-1 (E.J. Ryan, D.M., E.A.C.; unpublished data, November 2005), or dectin-1 induce calcium mobilization or tyrosine phosphorylation after Ab or ligand binding. More recently, Rogers et al demonstrated that ligand (zymosan) binding to dectin-1 can induce tyrosine phosphorylation on its ITAMs and recruit the Syk tyrosine kinase; this signaling pathway is critical for IL-2 and IL-10 production by murine DCs, providing direct evidence that signaling via C-type lectins shapes immune responses. Ligand binding to DCAL-2 can induce a series of signaling events including PTP and MAPK activation (p38 MAPK and ERK), suggesting that, similar to other CLRs, DCAL-2 is involved in programming DC activities. The presence of an ITIM in its cytoplasmic tail suggests DCAL-2 may serve as an inhibitory receptor. Phosphorylation of ITIMs allows recruitment of SH2 domain–containing protein tyrosine phosphatases, such as SHP-1, SHP-2, and SHIP. These phosphatases then dephosphorylate various protein tyrosine kinases, adaptor molecules, or enzymes to balance or suppress the activating signaling. Since our mAb did not immunoprecipitate or Western blot DCAL-2, we could not determine the proteins that associate with DCAL-2 in DCs. However, Marshall et al showed that the ITIM on MICL/DCAL-2 is able to recruit SHP-1 and SHP-2 in a pervanadate-treated cell line. It is possible that DCAL-2 could use these phosphatases to modulate DC signaling, especially since SHP-2 may be involved in activating ERK and cytokine expression.

DCs can be activated and undergo maturation by one of 2 major pathways: (1) T-cell–independent pathways induced after recognition of PAMPs via PRRs such as TLRs or (2) T-cell–dependent pathways promoted via CD40 ligation by CD40L+ T cells. These 2 pathways can also coexist in the same APC. Indeed, TLR signals can induce CD40 up-regulation in macrophages and microglia, and regulate CD40-induced cytokine production. Although CLRs and TLRs can communicate with each other and this cross-talk may be important to fine tune the balance between immune activation or suppression, the nature of this cross-talk...
remains unclear. Simultaneous engagement of dectin-1 and TLR2/6 by zymosan enhances Th1 responses,37,64 but the combination of DC-SIGN and TLR4 leads to suppression of Th1 responses.32 Some pathogens such as mycobacteria may use CLRs as an escape mechanism to avoid host immune responses.38,39 When we mature iDCs with LPS or zymosan in the presence of anti–DCAL-2 mAb, DCAL-2 ligation altered TLR signaling, modulated the cytokine expression profile, and suppressed Th1 differentiation just as DC-SIGN ligation did.32 Surprisingly, anti–DCAL-2 synergized with CD40 signaling to promote Th1 responses by enhancing IL-12 production. Although CD40 signals are required to induce DC-mediated protective immune responses when targeting Ag to DCs via C-type lectins (eg, DEC-205),55,67 it was not known if CLRs such as TLRs65,68 could interact with the CD40 pathway. Our results show DCAL-2 ligation synergizes with CD40 signaling to modify downstream T-cell responses. Thus, the function of DCAL-2 on DCs may depend upon the receptors with which it interacts and its ability to recruit SHP-1/2 and other adaptor proteins. A number of TLR pathway signaling elements are tyrosine phosphorylated69 and thus are potential targets for DCAL-2–recruited phosphatases. The TLR and CD40 pathways share TRAF-6 in common,70 and use receptor-specific adaptor proteins.69,71,72 It is also possible that DCAL-2 signaling may interact with other CLRs and thus other CLR signals may overlap or alter the effects of DCAL-2. DCs express multiple lectins that may recognize various combinations of carbohydrate structures on the same pathogen.

The ability of directing immune responses has made DCs an attractive immunotherapeutic target.1,7-35 Recent studies suggest that antibodies against C-type lectins such as MMR, DEC-205/CD205, or DC-SIGN/CD209 can be used as antigen-delivering vehicles to induce immune tolerance or a desired immune response.35,52,55,67,76-78 Injection of anti–DEC-205 mAb coupled with ovalbumin alone induces regulatory T cells and leads to suppression of OVA-specific T-cell proliferation.39 However, simultaneous injection of anti–DEC-205 OVA conjugates with an agonistic anti-CD40 Ab significantly enhanced host immune responses against an OVA-expressed tumor and infection by an OVA-expressing vaccinia virus.67,78 Similarly, anti–DC-SIGN mediated targeting of antigen-induced, antigen-specific T-cell proliferation15,78 even without the presence of other DC maturation stimuli. These studies demonstrated the important roles of CLRs in regulating immune responses. A better understanding of how CLR signals interact with other DC stimuli will be important to develop optimal approaches to direct DC programming.

In conclusion, our data suggest DCAL-2 serves as a functional receptor on DCs and cross-linking of DCAL-2 modulates the ability of DCs to direct downstream T-cell polarization. The abilities of DCAL-2 to internalize and collaborate with TLR or CD40 signaling and regulate immune responses make it as a potential immunotherapeutic target for antibody-mediated antigen delivery.

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References

17. Figdor CG, van Kooyk Y, Adema GJ. C-type lectin
receptors on dendritic cells and Langerhans cells.


Dendritic-cell-associated C-type lectin 2 (DCAL-2) alters dendritic-cell maturation and cytokine production

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