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# Ligation of dendritic cell-associated lectin-1 induces partial maturation of human monocyte derived dendritic cells

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## Abstract

Dendritic cell-associated lectin-1 (DCAL-1), also known as C-type lectin-like-1 (CLECL1), is a novel C-type lectin-like molecule expressed by antigen presenting cells including dendritic cells (DCs). Here we report that incubation of immature DCs (iDCs) with an anti-DCAL-1 monoclonal antibody (mAb) induced downstream signaling, including phosphorylation of c-Jun N-terminal kinase (JNK) and p44/42 MAP kinase. Furthermore, ligation of DCAL-1 expressed by iDCs specifically enhanced HLA-DR expression, whereas the expression of other co-stimulatory molecules remained unchanged and minimal cytokine secretion was detected. DCs that express high levels of major histocompatibility complex (MHC) class II in the absence of high levels of other co-stimulatory molecules and inflammatory cytokine secretion may play an important role in the maintenance of immune tolerance. Therefore, our data suggests an important role for DCAL-1 in the regulation of the immune response.

### Keywords

Innate immune response; MHC class II; Co-stimulatory molecules; MAPKinase

## 1. Introduction

Dendritic cells (DCs), the sentinels of the immune system, sample their environment via a wide variety of pathogen recognition receptors (PRRs) [1]. PRRs include various different families of molecules such as Toll-like receptors (TLR), NOD-like receptors, RIG-I-like receptors [2, 3], as well as C-type lectin receptors [4,5]. TLR agonists, in particular, potently activate innate immune responses [3]. However, it is becoming increasingly clear that the initiation of innate immune responses is fine tuned by integrating signals derived from the recognition of multiple pathogen components by various receptors [6].

DCs express many C-type lectin and C-type lectin-like molecules, including dendritic cell-associated lectin-1 (DCAL-1), DCAL-2, dendritic cell immunoreceptor (DCIR), Dectin-1, DC-SIGN (CD209), BDCA-2 (CD303), and Langerin (CD207) [4]. Evidence that the ligation of C-type lectins expressed by DCs may modulate DC functionality is beginning to emerge [7]. For example, the interaction of mannose-capped lipoarabinomannan (ManLAM) derived from


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*Mycobacterium bovis* with DC-SIGN prevents mycobacteria- or lipopolysaccharide (LPS)-induced DC maturation [8]. Furthermore, Dectin-1 activated DCs promoted the differentiation of Th17 cells *in vitro* [9]. Targeting DCIR on human plasmacytoid DCs resulted in enhanced antigen presentation but inhibited IFN-$\alpha$ production [10]. Recently, the CLR CLEC5A (MDL-1) was shown to bind to dengue virus (DV) and to mediate proinflammatory responses to DV rather than viral uptake [11]. Thus C-type lectins may regulate the proinflammatory responses of immature DCs (iDCs) in response to a diverse array of pathogens.

We have previously described DCAL-1, a novel DC-associated, C-type lectin-like molecule [12]. *DCAL-1* is located on human chromosome 12p13.31 just 3′ to *CD69*. DCAL-1 expression is restricted to human hematopoietic cells, with highest levels expressed by DCs and B cells. A population of $CD4^+CD45RA^+$ T cells can bind to a recombinant His-tagged DCAL-1 fusion protein, suggesting that a subset of T cells may express a DCAL-1 ligand. Co-incubation with a soluble DCAL-1-His-tagged fusion protein enhanced the proliferation of $CD4^+$ T cells in response to CD3 ligation and significantly increased levels of interleukin (IL)-4 secretion. Therefore, DCAL-1 may act as a T cell co-stimulatory molecule, which skews $CD4^+$ T cells toward a Th2 response by enhancing T-cell secretion of IL-4.

In this study, we characterized the signaling pathways activated after DCAL-1 ligation by a specific mAb, and examined the downstream effect this had on the phenotype and function of human iDCs.

## 2. Subjects and methods

### 2.1. Reagents

Anti-DCAL-1 (Clone No. UW50) and anti-DC-SIGN (Clone No. UW60) were prepared as previously described [12]. An isotype control antibody (murine IgM, Sigma, St. Louis, MO) was included in all experiments. All batches of UW50 used in this study were prepared aseptically in our laboratory and tested negative for endotoxin contamination. Recombinant human IL-4 was purchased from Research Diagnostics Inc. (Concord, MA). Recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) was purchased from Amgen (Seattle, WA).

### 2.2. Cell culture

Human monocytes (>97-99% $CD14^+$) purified from leuka-pheresis products by $CD14^+$ immunomagnetic-positive selection were obtained from the Cellular Therapy Laboratory at the Fred Hutchinson Cancer Research Center (Seattle, WA). Monocytes were either directly used for experiments or frozen in RPMI 1640 (Gibco Invitrogen, Carlsbad, CA), 30% human AB serum (ICN Biomedicals, Aurora, OH), and 10% dimethyl sulfoxide (Sigma, St. Louis, MO) and stored in liquid nitrogen until use. Freezing had no effect on DC phenotypes or the performance in experiments. Monocyte-derived iDCs were generated as previously described [13]. Human dense tonsillar B cells were purified by density gradient centrifugation as described [14].

### 2.3. Western blotting

Immature DCs ($2 \times 10^6$ cells/sample) were incubated for varying times (5, 30, 60, 120 minutes) with anti-DCAL-1 (100 $\mu$g/ml), isotype control (mouse IgM) as a negative control, and anti-CD40 (G28-5) or PMA/Ionomycin (Sigma) as a positive control. Cell lysates were prepared, and protein levels determined using the BCA assay (Pierce, Rockford, IL). Proteins were separated on 10% sodium dodecyl sulfate-polyacryalimide gel electrophoresis gels, transferred to nitrocellulose membranes, and blotted with the following antibodies: Phospho-JNK, Phospho-ERK1/2, Phospho-p38, Phospho-AKT all from Cell Signaling (Beverly, MA), and

Phospho-Tyrosine (Clone No. 4G10, a generous gift from Michael Gold, University of British Columbia). Initial blotting was followed by anti-rabbit-HRP or anti-mouse-HRP conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were then developed using ECL (Amersham, Aylesbury, UK). Total p38 MAPK levels were analyzed as a loading control (Santa Cruz).

### 2.4. Analysis of DC maturation

Graded doses of a purified anti-DCAL-1 or dialyzed control mouse IgM (0, 1, 5, 10, 50, 100 $\mu$g/ml) were incubated with iDCs, for 6, 12, 24 or 48 hours. In some experiments the antibody was pre-bound to the plate in PBS for 24 hours at 4°C. After incubation the cells were washed and labeled with antibodies to the following: CD1a (Beckman Coulter, Fullerton, CA), CD40 (G28-5, prepared in our laboratory), and HLA-DR, CD83, CD80, CD86, CCR5, CCR7, and CXCR4 (all from BD BioSciences). Secretion of cytokines/chemokines was analyzed in supernatants by enzyme-linked immunoabsorbent assay using commercially available matched pairs of mAbs specific for IL-6, IL-10, and TNF-$\alpha$ (from BD BioSciences), IL-8, IL-12p40, CCL2, CCL17 and CCL22 (R&D Systems, Minneapolis, MN).

## 3. Results

### 3.1. Ligation of DCAL-1 on iDCs induces the tyrosine phosphorylation of downstream signaling molecules

To investigate whether crosslinking DCAL-1 activates downstream signaling in DCs, iDCs were stimulated with anti-DCAL-1 for varying times, cell lysates prepared, and levels of protein tyrosine phosphorylation examined by western blotting (Fig. 1a). DCAL-1 ligation significantly upregulated expression of both a 38 kDa band at 120 minutes; an identical band was also present in DCs treated with PMA/Ionomycin (P/I), indicating an association with cell activation. To determine the identity of the phosphorylated proteins we performed western blotting of the same samples with phospho-specific antibodies (Fig. 1b). There was a slight increase in the levels of phospho-AKT and phospho-p38 MAPK over time in cells treated with anti-DCAL-1 (Fig. 1b). There were significantly higher expression levels of phospho-p44/42 MAPK and phospho-JNK in iDCs stimulated with anti-DCAL-1 than in the cells stimulated with isotype control (Fig. 1b). Thus, DCAL-1 ligation induces activation of a protein tyrosine kinase (PTK) as detected by new protein tyrosine phosphorylation and activation of JNK.

To determine whether DCAL-1 ligation could promote calcium flux, human dense tonsillar B cells or iDCs were loaded with indo-1 (Molecular Probes, Invitrogen, Carlsbad, CA) at 37°C for 30 minutes. Cells were stimulated with 1, 10, 100 $\mu$g/ml doses of anti-DCAL-1. Intracellular calcium flux was measured by flow cytometry on a BD LSR (BD BioSciences) over 30 minutes. Data were analyzed using FloJo software (Tree Star, Inc., San Carlos, CA). Calcium mobilization was not induced following anti-DCAL-1 treatment of either iDCs or B cells, whereas B cells treated with anti-IgM, as a positive control, showed a marked increase in calcium flux (data not shown).

### 3.2. Ligation of DCAL-1 upregulates HLA-DR expression but does not affect other DC maturation markers

Immature DCs were incubated with graded doses of anti-DCAL-1, equivalent concentrations of mouse IgM (isotype control) or LPS (1 $\mu$g/ml) as a positive control for 48 hours, and then the expression of a panel DC maturation markers (CD1a, CD83, CD80, CD86, HLA-DR, CCR7, and CCR5) was examined by flow cytometry. The dose-response experiments determined 10 $\mu$g/ml of anti-DCAL-1 as the optimum concentration. Treatment of iDCs with the isotype control had no effect on the expression of maturation markers. Stimulation with LPS significantly upregulated the expression of HLA-DR, CD86, and CCR7, and

downregulated CCR5, whereas anti-DCAL-1 stimulation, selectively upregulated the expression of HLA-DR in all donors tested ($n = 10$) (Figure 2, isotype control vs. anti-DCAL-1 treatment; $p < 0.01$ by Wilcoxon signed rank test).

Stimulation of iDCs with soluble anti-DCAL-1 from 0.1 to 100 $\mu$g/ml did not induce secretion of any of the cytokines or chemokines tested (including IL-6, IL-8, IL-10, IL-12p40, CCL2, TNF-$\alpha$, CCL17, or CCL22; data not shown). To determine whether increasing the strength of the DCAL-1 signal by clustering the receptors changed the downstream effect of anti-DCAL-1 stimulation, we incubated iDCs in plates coated with graded doses of anti-DCAL-1 for 6, 12, 24, and 48 hours. When iDCs were stimulated in this manner with anti-DCAL-1, they secrete significantly more IL-6 than iDCs treated with isotype control antibody (Table 1). However, levels of IL-6 detected were consistently much lower than when the same cells were stimulated with LPS (Table 1). Treating iDCs with anti-DCAL-1 before LPS activation did not alter LPS induction of co-stimulatory molecules or cytokine secretion (data not shown).

## 4. Discussion

In this study we determined the effect of cross-linking DCAL-1 expressed by iDCs on the induction of downstream signaling and the phenotype of the cells. Incubation of iDCs with a DCAL-1 specific mAb led to the tyrosine phosphorylation of a protein approximately 38 kDa in size (Fig. 1a), indicating that anti-DCAL-1 treatment activates a PTK, despite the fact that DCAL-1 contains no defined signaling motifs in its cytoplasmic tail. Blotting these samples with antibodies to other phospho-proteins revealed that ligation of DCAL-1 expressed by immature DCs results in phosphorylation of both p44/p42 MAPK and JNK (Fig. 1b). However, upon repeating these experiments with dense human tonsillar B cells (also express DCAL-1), no phosphorylated JNK or p44/42 MAPK could be detected. This may indicate that B cells lack the adaptors or other downstream components of the signaling pathway that integrate DCAL-1 signaling in DCs. Immune signaling pathways are differentially regulated in DCs and B cells; for example, the set of p38 MAPK-dependent genes in DCs (IL-12p40 and CIAP-2) is different from B cells (IL-10 and IL-1$\beta$) [15]. MAPK signaling pathways (p38 MAPkinase, ERK1/2, and JNK) differentially regulate all aspects of phenotypic maturation, cytokine production, and functional maturation of DCs. Thus, distinct maturation of DCs may be induced by modulating the balance of the phosphorylation of these molecules [16]. The ligation of DCAL-1 with a specific mAb did not lead to a classical mature DC phenotype ($CD80^{hi}CD86^{hi}CCR7^{+}CD83^{+}$) as seen when iDCs are exposed to LPS (Figure 2). However, anti-DCAL-1 treatment of iDCS was as effective as LPS in inducing upregulation of HLA-DR expression (Fig. 2b). Anti-DCAL-1 treatment of iDCs induced the secretion of low levels of IL-6 (Table 1) but none of the other cytokines tested.

Similarly, ligation of DC-SIGN (a c-type lectin also expressed by DC) with a specific mAb resulted in ERK and PI3 kinase phosphorylation and enhanced IL-10 secretion but had no effect on the expression of DC maturation markers [17]. In addition, ligation of the Ig superfamily member TREM-2 promoted partial DC maturation, specifically the upregulation of the chemokine receptor CCR7 [18]. Together with the data we present here, these study findings highlight the fact that there are alternate programs of DC differentiation that result in DCs with different phenotypes and cytokine secretion patterns.

We found significantly increased levels of IL-6 in the supernatant of anti-DCAL-1 stimulated DCs compared with control DCs (Table 1). IL-6 is a pleiotropic cytokine that has a crucial role to play in the regulation of the immune response. For example, IL-6 is required in the development of autoimmune disease in several experimental models including collagen-induced arthritis [19] or experimental autoimmune encephalomyelitis [20], possibly because of a role in the differentiation of Th17 cells [21]. Although IL-6 does not induce conventional

maturation of DCs, it does alter the pH of the early endosomes, altering antigen processing and thus enabling IL-6 treated DCs to activate a number of T cell clones against determinants that were previously cryptic due to poor display [22]. However, IL-6 has also been reported to have an immunosuppressive role, with DCs matured by LPS in the presence of IL-6 expressing less CCR7, producing lower levels of tumor necrosis factor-$\alpha$ and CXCL10 and being less effective in allogenic MLR assays. The inhibitory effect of IL-6 resulted in part from the induction of autocrine IL-10 [23]. Further studies are required to elucidate the role of IL-6 produced as a result of DCAL-1 ligation in the regulation of the immune response.

The current dogma regarding DC maturation states that iDCs with the capacity to endocytose antigen remain in the periphery in a surveillance role; once a pathogen is encountered, a program of maturation occurs resulting in the upregulation of co-stimulatory molecules such as CD80 and the chemokine receptor CCR7, allowing the cells to traffic to the draining lymph node, where they secrete pro-inflammatory cytokines and interact with naïve T cells, and thus initiating an immune response [1]. However it is now evident that a range of DCs with differing phenotypes and cytokine secretion patterns exist that have important roles in maintaining tolerance as well as initiating immunity [24]. Anti-DCAL-1 treatment of iDCs had no consistent effect on their ability to drive lymphocyte proliferation in mixed lymphocyte reaction (MLR) assays (data not shown). Further investigation into the signals that drive the differentiation of DCs into cells capable of inducing immunity or maintaining tolerance is required. A recent study revealed that DCAL-1 (CLECL1) expression in the liver strongly correlated with the single nucleotide polymorphism (SNP) at chromosome 12p13 that increases susceptibility to developing type 1 diabetes [25]. Whether or not levels of DCAL-1 expressed by liver DCs or other immune cell subsets influences the development of autoimmune diseases will be the subject of future studies.

In summary, the results presented here show that ligation of DCAL-1 expressed by iDCs by a specific mAb induces the phosphorylation of p44/42 and JNK MAPK and the up-regulation of MHC class II expression, resulting in a partially mature DC phenotype that may have an important immunoregulatory role.

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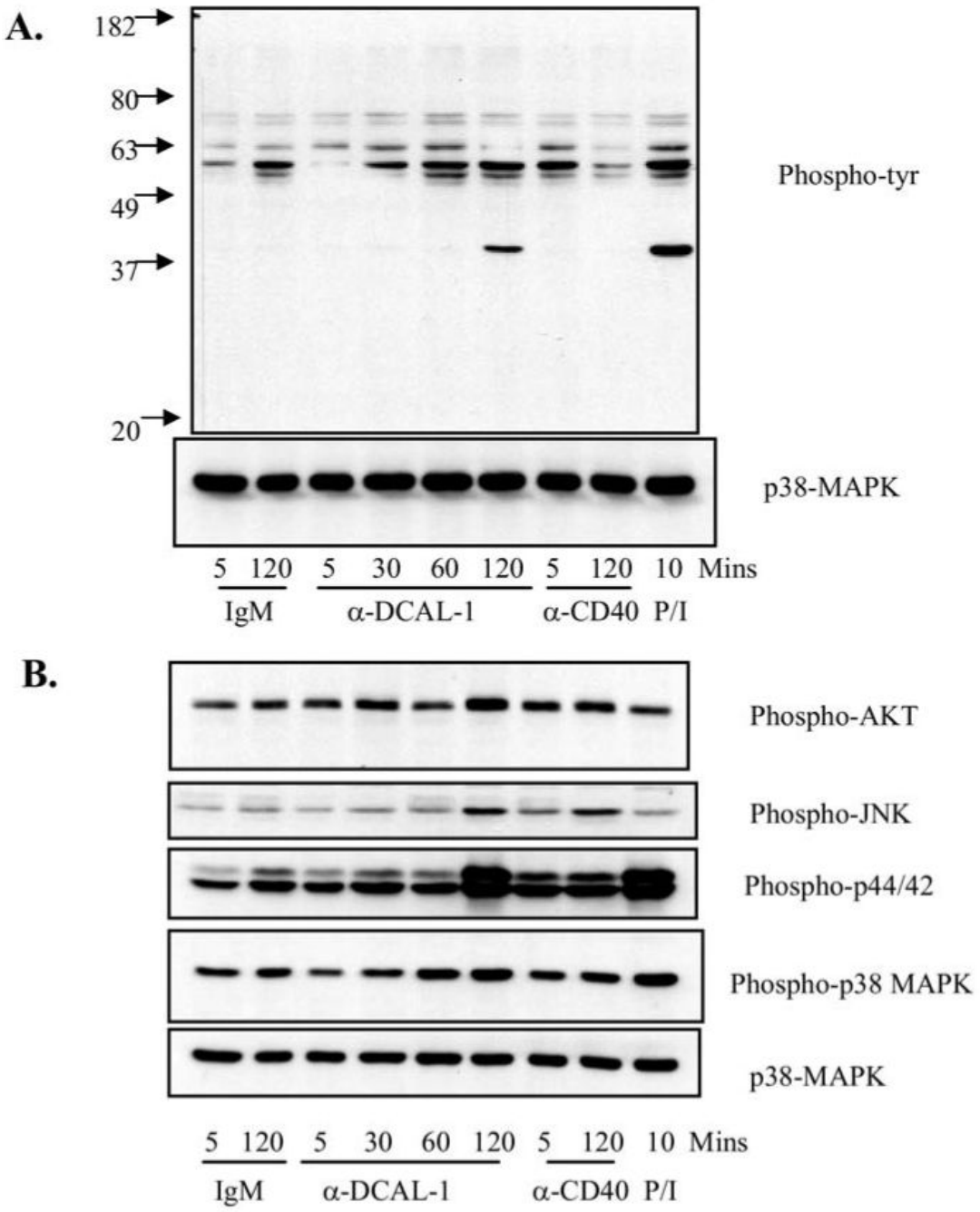


**Fig. 1.**

Ligation of dendritic cell-associated lectin-1 (DCAL-1) induced the phosphorylation of downstream signaling molecules in dendritic cells (DCs). Immature DCs were incubated with an isotype matched control (IgM), anti-DCAL-1 (UW50), anti-CD40 (G28-5) or PMA/Ionomycin for the time points indicated, cell lysates were prepared, and the levels of phosphorylated proteins were determined by western blotting. (A) Stimulation of immature DCs with anti-DCAL-1 induces tyrosine phosphorylation. (B) Levels of specific phospho-proteins were analyzed. Total p-38 MAPK levels were used as a control for equal protein loading. The results shown are representative of two experiments performed with iDCs obtained from different donors.

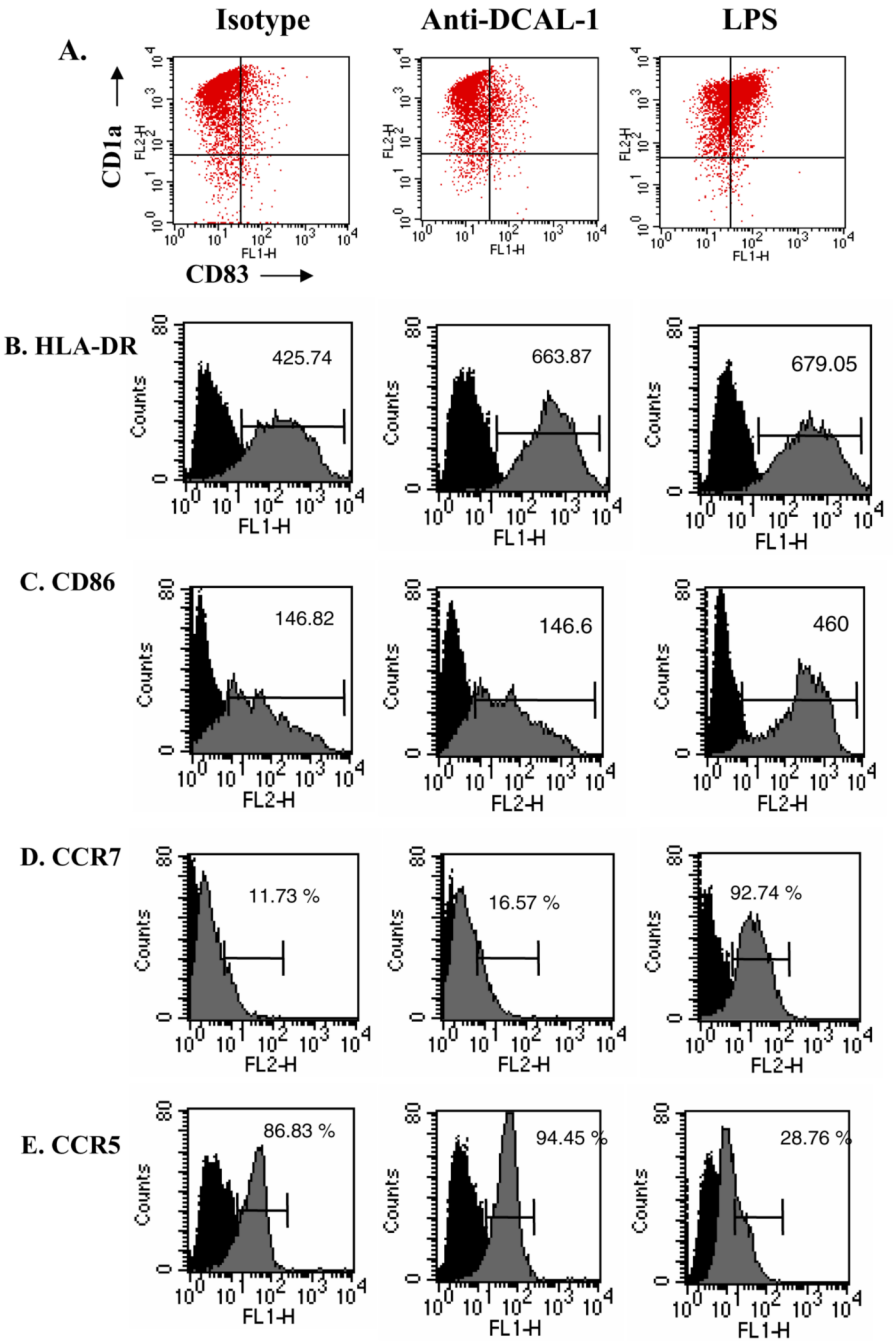


**Fig. 2.**

Anti-dendritic cell-associated lectin-1 (anti-DCAL-1) treatment of immature dendritic cells (DCs) specifically upregulates HLA-DR. Immature DCs were treated with either 10 $\mu$g/ml of soluble Anti-DCAL-1, isotype control (mouse IgM) or 1 $\mu$g/ml of *E. coli* lipopolysaccharide as a positive control for 48 hours and the expression of DC maturation markers analyzed by flow cytometry. (A) Dot plots show the expression of CD1a versus CD83. (B) HLA-DR expression; the grey histograms represent the expression of HLA-DR following the different treatments and the black histogram indicates the staining of an isotype control antibody. Numbers represent the M.F.I. Isotype control versus anti-DCAL-1 treatment; $p < 0.01$ by Wilcoxon signed rank test. (C) CD86 (Numbers represent the M.F.I.), (D) CCR7 (Numbers

indicate the percentage of cells expressing CCR7), (E) CCR5 (Numbers indicate the % of cells expressing CCR5). This experiment was performed on different donors ($n$ = 10) with similar results and one of these experiments is shown.

**Table 1**

Anti-DCAL-1-dependent induction of cytokine secretion (pg/ml) by monocyte-derived dendritic cells

| IL | Isotype control (1 μg/ml) | Isotype control (10 μg/ml) | Isotype control (100 μg/ml) | Anti-DCAL-1 (1 μg/ml) | Anti-DCAL-1 (10 μg/ml) | Anti-DCAL-1 (100 μg/ml) | LPS (1 μg/ml) |
|---|---|---|---|---|---|---|---|
| IL-6 | 119 (± 19) | 285 (± 29) | 264 (± 21) | 368* (± 14) | 421* (± 16) | 507* (± 65) | 5517 (± 3319) |
| IL-8 | 1345 (± 674) | 1557 (± 323) | 1129 (± 176) | 1536 (± 492) | 2070 (± 554) | 1906 (± 524) | 8218 (± 3528) |
| IL-10 | 37 (± 8) | 37 (± 7) | 40 (± 6) | 43 (± 8) | 44 (± 11) | 43 (± 12) | 2910 (± 1024) |
| IL-12 (p40) | 485 (± 297) | 548 (± 170) | 465 (± 167) | 354 (± 289) | 442 (± 409) | 423 (± 430) | 1454 (± 594) |

DCAL-1 = dendritic cell-associated lectin-1; IL = interleukin; LPS = lipopolysaccharide.

Monocyte-derived dendritic cells were incubated with graded doses of plate-bound anti-DCAL-1, an isotype control antibody or lipopolysaccharide (1 μg/ml) for 24 hours; supernatants were harvested and cytokine levels were determined by enzyme-linked immunoabsorbent assay. Results shown as picograms per milliliter (pg/ml).

* $p \leq 0.05$ compared with cells incubated with isotype control using the Wilcoxon signed rank test.