DC-SIGN, but not sDC-SIGN, can modulate IL-2 production from PMA- and anti-CD3-stimulated primary human CD4 T cells

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Abstract

Dendritic cell (DC)-specific intercellular cell adhesion molecule-3 (ICAM-3)-grabbing non-integrin (DC-SIGN) is expressed on the surface of DCs and specialized macrophages and can support T cell proliferation. Antibody-mediated co-ligation of CD3 and ICAM-3, the ligand for both DC-SIGN and leukocyte function-associated antigen-1, leads to T cell activation. Therefore, we tested to see whether DC-SIGN or a splice variant of dendritic cell-specific intercellular cell adhesion molecule-3-grabbing non-integrin (sDC-SIGN) can co-stimulate primary human T cells. The sDC-SIGN lacking the transmembrane domain encoded by exon 3 localizes to the cytoplasm of cells and is not secreted. Both B7 and DC-SIGN co-stimulated phorbol myristate acetate-stimulated CD41 cells as compared with controls. However, unlike B7, both DC-SIGN and sDC-SIGN failed to co-stimulate CD4+ T cells treated with sub-optimal amounts of anti-CD3 (2 μg ml-1) as defined by a lack of CD69 and CD25 up-regulation, cell division and cytokine secretion. Instead, DC-SIGN, and not sDC-SIGN, induced a small but consistent down-regulation of IL-2 production by these CD41 T cells. In contrast, DC-SIGN in the presence of 30 μg ml-1 of anti-CD3 modestly up-regulated cytokine production as compared with control. These results suggest that DC-SIGN can differentially modulate T cell stimulation.

Introduction

Antigen-independent formation of T cell and dendritic cell (DC) clusters (1) is mediated by the initial interactions between adhesion and co-stimulatory molecules. These interactions occur between molecules such as CD2–leukocyte function-associated antigen-3 (LFA-3), LFA-1–intercellular cell adhesion molecule 1 and 3 (ICAM-1 and -3) and CD28–CD80/86. However, more recent studies have suggested that it is the ICAM-3–dendritic cell-specific intercellular cell adhesion molecule-3-grabbing non-integrin (DC-SIGN) interaction that is critical for facilitating TCR–peptide–MHC interactions that lead to immune synapse formation (2, 3). Other adhesion and co-stimulatory molecules are also highly expressed on most resting cells but feature lower affinities (TCR–peptide–MHC), reduced size (CD2, TCR, CD28) or require prior activation to function efficiently (LFA-1). These properties render them less important for the initial intercellular adhesion between T cells and antigen-presenting cells (APCs).

ICAM-3 belongs to the Ig superfamily and is structurally similar to ICAM-1 and ICAM-2 but is highly expressed on all resting T cells and thus can facilitate initial interaction between a T cell and an APC. The ICAM-3 is re-distributed to the interface of T cells and DCs regardless of whether the interaction is antigen independent or dependent (2). Furthermore, ICAM-3-, and not LFA-1- or ICAM-1-, blocking antibodies can inhibit antigen-independent T cell–APC conjugate formation, while both LFA-1- and ICAM-3-blocking antibodies can prevent antigen-dependent conjugate formation. These and other studies showed that ICAM-3 could play an important role in the initial T cell–APC interaction. Anti-ICAM-3 antibody treatment of Jurkat T cells can elevate the levels of intracellular calcium and cause tyrosine phosphorylation (4) and T lymphoblast homotypic aggregation in an LFA-1–ICAM-1-dependent manner (5). Further, under conditions of sub-optimal TCR stimulation, anti-ICAM-3 antibody can up-regulate
CD69 and CD25 expression (6, 7) and cause increased cell proliferation and IL-2 production (8). These studies indicate that ICAM-3 can provide critical signaling required for T cell stimulation. However, these studies used stimulating or blocking anti-ICAM-3 antibodies, instead of natural ligands.

In vivo, DC-SIGN is expressed at significant levels on subsets of APCs that include immature (CD83–) DCs, mature DCs, specialized macrophages in the lungs and placenta, and a subset of DC precursors in the blood. The DC-SIGN is a type II membrane protein that can bind HIV gp120 protein (9) and sequester HIV (10) and other pathogens until they are presented to T cells. Further, DC-SIGN has been shown to bind ICAM-2, which is highly expressed on endothelial cells and thus may mediate DC trafficking (11).

Geijtenbeek et al. showed that blood-derived monocytes use LFA-1 to bind ICAM-3 on a T cell but monocyte-derived DCs, which express both LFA-1 and DC-SIGN, bind ICAM-3 mostly through DC-SIGN (3). Further, they demonstrated that an anti-DC-SIGN-blocking antibody could decrease allo-stimulation of human T cells by monocyte-derived DCs. These studies suggested that both DC-SIGN and LFA-1 could facilitate interactions between T cells and monocyte-derived DCs. However, whether DC-SIGN can co-stimulate ICAM-3-bearing cells or only facilitate initial T cell and APC adhesion is not known. A clearer understanding of its function would provide further insights into the mechanisms involved in T cell stimulation.

In this study, we cloned and expressed DC-SIGN, a natural ligand for ICAM-3, and a splice variant of dendritic cell-specific intercellular cell adhesion molecule-grabbing non-integrin (sDC-SIGN) and tested for their ability to provide co-stimulatory signals to human T cells.

Methods

Media and antibodies

DAP-3 and 293T cells were grown in DMEM medium (Invitrogen, Carlsbad, CA, USA) supplemented with antymycotic/antibiotic, β-mercaptoethanol and 10% FCS (Mediatech, Herndon, VA, USA). CHO cells were grown in RPMI medium (Invitrogen) containing the above-mentioned supplements. Human PBMCs and T cells were cultured ex vivo in RPMI supplemented with 5% FCS. Stable cell lines transfected with various cDNAs were further supplemented with 250 μg ml⁻¹ of G418 (Sigma–Aldrich, St Louis, MO, USA). Polyclonal anti-DC-SIGN antiserum was produced by immunizing rabbits with a peptide containing C-terminal 20 AAs (AAVGELPE-KSKQKEIYQ) of the DC-SIGN coupled to keyhole limpet hemocyanin. Antibody response against the specific peptide was monitored using an ELISA. After three immunizations, the antibody titers reached ~1/200 000 and at that time the rabbits were bled and sacrificed. Antibodies against human antigens CD45RA (H100), CD50 (TU41), CD69 (FN50), CCR7 (2H4), CD25 (M-A251) and CD4 (RPA-T4) were purchased from BD Pharmingen (San Diego, CA, USA). Anti-human CD45RO-ECD was purchased from Beckman Coulter Immunotech (Fullerton, CA, USA). Anti-DC-SIGN (MAB1621) was obtained from R&D Systems (Minneapolis, MN, USA).

PBMC and CD4+ T cell isolation

PBMCs were isolated using lympholyte-H reagent (Cedarlane, Vancouver, Canada) following the manufacturer’s protocol. Briefly, 14 ml of peripheral blood from a healthy donor was mixed with 20 ml of PBS and was under layered with 9 ml of lympholyte-H. After centrifugation at 750 × g for 20 min at room temperature, the ‘buffy layer’ was harvested and washed with PBS. PBMCs were used directly for co-stimulation assays and monocyte differentiation or further processed to isolate CD4+ T cells.

CD4+ T cells were isolated by negative selection using CD4+ T Cell Isolation Kit (Miltenyi Biotec, Auburn, CA, USA), following manufacturer’s protocol. Non-CD4+ T cells that were bound to beads were subjected to an automacs separation using the ‘deplete’ program. Over 95% of the live enriched cells were CD4+.

Monocyte-derived DCs

Isolated PBMCs (5–10 × 10⁶ cells per well) were plated into six-well dishes in RPMI containing 5% FCS and incubated at 37°C for 2 h. Wells were gently washed three times with PBS to eliminate non-adherent cells and the remaining cells were cultured in RPMI supplemented with granulocyte macrophage colony-stimulating factor (GM-CSF; 800 U ml⁻¹; PeproTech, Rockyhill, NJ, USA) and IL-4 (400 U ml⁻¹; PeproTech). Cultures were replenished with fresh GM-CSF- and IL-4-supplemented medium every 3 days. To induce differentiation, monocyte-derived immature DCs were treated with LPS (10 μg ml⁻¹, L2654), human IFN-γ (1000 U ml⁻¹, L-2396) or human tumor necrosis factor α (10 μg ml⁻¹, T-0157) (Sigma–Aldrich) for 24 h.

DC-SIGN and sDC-SIGN cloning and expression in CHO cells

cDNAs encoding DC-SIGN and sDC-SIGN were obtained by reverse transcription–PCR using RNA isolated from monocyte-derived immature DCs as a template and F1-AGAGTGGGGGTGACATGAGTG and B1-GAAGTTCTGCTAGAGTGGG primers (3). Amplified products were cloned, sequenced and sub-cloned into the pIREs-2 eGFP vector (Clontech, Palo Alto, CA, USA), which was used to establish permanently transfected cells. Briefly, CHO, DAP-3 or 293 T cells were seeded (2 × 10⁵ cells per well in a six-well plate) the day before transfection. Two micrograms of DNA was mixed with 8 μl of Superfect reagent (Qiagen, Valencia, CA, USA) at room temperature for 5 min in 150 μl of serum-free medium and added to cells in a total volume of 1 ml. After 2 h of incubation, cells were washed twice with PBS and allowed to grow for 2 days. Permanently transfected cells were established by culturing cells in the presence of G418 (1 mg ml⁻¹) for at least 1 week. Cells expressing high levels of protein were obtained by sorting on the basis of green fluorescent protein (GFP) expression.

Co-stimulation assays

Human PBMCs or CD4+ T cells were mixed with irradiated CHO cells (8000 rads of gamma irradiation) at a ratio of 3.5: 1 in 96-well flat-bottom plates and grown in 5% RPMI at 37°C
and 5% CO₂. Two separate anti-CD3 antibodies were used for T cell stimulation. For the first set of experiments, flat-bottom 96-well plates were coated with 2 µg ml⁻¹ (sub-optimal) or 30 µg ml⁻¹ (large dose) of anti-CD3 antibody (UCHT1, Pharmingen), incubated overnight at 4°C and gently washed once with PBS before cell mixtures were added. For the second set of experiments, soluble stimulating anti-CD3 antibody (10 ng ml⁻¹ of HIT3a, Pharmingen) or phorbol myristate acetate (PMA) (4 ng ml⁻¹) was added to the cell mixtures. Cells and culture supernatants harvested at 24 h were used to determine the levels of expression of CD69 and CD25 and cytokines. Cells harvested on day 3 or 4 were used to determine the cell division.

**Cell division**

Cells were washed twice with PBS and then incubated in the dark (10⁷ cells ml⁻¹) with 0.250 µM of 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE; Molecular Probes, Eugene, OR, USA) in PBS at 37°C for 10 min. To quench the reaction, cells were diluted with PBS containing 10% FCS and incubated for an additional 20 min. These cells were washed with PBS containing 2% FCS and used in the co-stimulation assays. Cell division was determined by the extent of CFSE dilution detected using a flow cytometer (FACSCalibur; BD, San Jose, CA, USA) followed by data analysis using Win MDI 2.8 software (http://facs.scripps.edu/software.html). Live lymphocyte-gated cells were assessed for forward scatter (FSC) and green (FL1) fluorescence properties. Quadrants were set up using live CFSE-loaded untreated T cells as negative controls for cell size (FSC) and cell division (FL1 channel for CFSE).

**Cell staining**

Lymphocytes were re-suspended separately from triplicate wells gently so as to harvest only the non-adherent lymphocytes. CHO cells inadvertently carried over were gated out by size exclusion. Lymphocytes were incubated for 10 min on ice with Fc-block reagent (Pharmingen) in PBS + 2% FCS. All subsequent staining was carried out on ice for 30 min in the dark using 50 µl per reaction of antibodies (5 µg ml⁻¹) in PBS + 2% FCS. The CD4+ lymphocytes were stained with FITC-CD69 (Pharmingen) or CD25 (Pharmingen) and then with 4',6-diamidino-2-phenylindole (DAPI) (Sigma–Aldrich), Cy-chrome CD45RA and CD45RO-ECD. CCR7 staining was performed using a mouse anti-CCR7 antibody (Pharmingen) followed by the addition of biotinylated rat anti-mouse IgM (R6-60.2, Pharmingen) and streptavidin–PE (Pharmingen). Cells were washed once with 200 µl of PBS + 2% FCS. DAPI-negative cells were analyzed by FACS to determine the expression levels of various markers. The results were analyzed using Win MDI software (http://facs.scripps.edu/software.html).

**ELISA for cytokines**

Supernatants, harvested from co-stimulation assays performed in triplicate, were frozen at −80°C until assayed. A volume of 50 µl of supernatant was used to assay for IL-2, IL-4 and IFN-γ as outlined in the manufacturer’s instructions (IL-4 and IFN-γ: eBioscience, San Diego, CA, USA; IL-2: OptEIA human IL-2 kit, Pharmingen). ACH8122 and biotinylated AHC7129 (Biosource, Camarillo, CA, USA) antibodies were used to capture and detect IL-12, respectively. MAXI-SORP ELISA plates (439 454; Nunc, Rochester, NY, USA) were coated with ACH8122 antibody in (50 µl per well) carbonate buffer (0.1 M Na₂CO₃/NaHCO₃, pH = 9.0) overnight at 4°C. Next day plates were washed three times with PBS + 0.05% Tween-20 and then blocked for 1 h with 100 µl PBS + 10% FCS at room temperature. Plates were washed extensively between the following steps. Fifty microliters per well of culture supernatant was added in triplicate and incubated for 2 h, biotinylated AHC7129 antibody was added and incubated for 1 h and streptavidin–HRP was added and incubated for 30 min; the reaction was developed using 50 µl of tetramethylbenzidine (TMB) substrate (BD) for 15 min and stopped with 25 µl of 1 M HCl. Plates were read at 450 nm using a Bio-Rad 550 plate reader (Bio-Rad, Hercules, CA, USA) and then analyzed using Excel software (Microsoft, Redmond, WA, USA).

**DC-SIGN ELISA**

DAP-3 and 293T cells stably expressing DC-SIGN or sDC-SIGN were grown to confluence in six-well dishes containing 3 ml of medium. Culture supernatants were tested in triplicate for the presence of soluble DC-SIGN using a sandwich ELISA. A polyclonal rabbit antiserum against the C-terminal 20 AAs of DC-SIGN was used as the capture antibody. For detection, monoclonal anti-DC-SIGN antibody (MAB1621, R&D Systems) was used at 1 µg ml⁻¹ in PBS + 10% FCS followed by the addition of HRP-labeled goat anti-mouse IgG2a polyclonal antibody (Caltag, Burlingame, CA, USA) and TMB substrate as outlined above. DAP-3 sDC-SIGN cell lysate was used as a positive control.

**Western blot**

293T, sDC-SIGN-293T and DC-SIGN-293T cells (0.8 × 10⁶) were washed and re-suspended in 30 µl of 1 × boiling protein sample buffer supplemented with β-mercaptoethanol. Samples were boiled for 5 min and subjected to SDS-PAGE on a 10% gel (Bio-Rad) using a commercial electrophoresis buffer (161-0732, Bio-Rad). Proteins were then transferred to a nitrocellulose membrane (162-0115, Bio-Rad) using a mini-gel transfer apparatus (Bio-Rad) overnight at 4°C at 30 V in transfer buffer (3 g Tris l⁻¹, 14.4 g glycine l⁻¹ and 20% methanol). The nitrocellulose membrane was blocked with 2% skim milk in PBS (milk/PBS) for 2 h at room temperature. Rabbit anti-DC-SIGN antibody was added (20 µg ml⁻¹ in 1% milk/PBS) and incubated for 2 h at room temperature with gentle rocking. The membrane was washed three times with PBS + 0.05% Tween-20 for 5 min each. The secondary HRP-labeled anti-rabbit (Caltag) antibody was added at 1/500 in 1% milk/PBS and washed as before. Western blotting detection system (RPN2132; Amersham Bioscience, Piscataway, NJ, USA) was used following the manufacturer’s instructions.

**Results**

sDC-SIGN is not secreted and resides in the cytoplasm of cells

We characterized sDC-SIGN which was identified on PCR amplification of DC-SIGN from human cells. Although previously
identified (12), it had not been characterized. This isoform lacks the putative transmembrane region encoded by exon 3 of DC-SIGN (Fig. 1A–C) and relative to DC-SIGN it was expressed at very low levels in the IL-4- and GM-CSF-treated PBMCs of three different individuals (Fig. 1B). Both DC-SIGN and sDC-SIGN were stably expressed, and comparison of sDC-SIGN and DC-SIGN protein expression in 293T cells (Fig. 1D–F), CHO (Fig. 3) and DAP-3 cells (not shown) demonstrated that sDC-SIGN is not expressed on the cell surface. This was not due to a lack of protein expression since sDC-SIGN could be readily detected on a western blot prepared using lysates of 293T cells (Fig. 1E). Further, anti-DC-SIGN antibodies were able to stain permeabilized 293T cells expressing sDC-SIGN (Fig. 1F).

Since sDC-SIGN could be found in the cytoplasm of stably expressing cells, we tested whether it was secreted into the medium. Supernatants from cultures of cells stably expressing sDC-SIGN and from both immature and stimulated monocyte-derived DCs were tested for the presence of sDC-SIGN using a newly developed DC-SIGN ELISA. No sDC-SIGN was found in the supernatant of confluent DAP-3 (Fig. 2A) or 293T cells (not shown) or CHO cells (not shown) after 3 days of culture. However, the protein was readily detected in the diluted lysates of sDC-SIGN-expressing DAP-3 cells (Fig. 2A). Similarly, sDC-SIGN was also not detected in the culture supernatants of immature and stimulated DCs (Fig. 2B). This was not due to a problem in the ability of DCs to secrete soluble factors since LPS-treated DCs secreted IL-12 (Fig. 2C). Taken together, these data suggest that sDC-SIGN is a soluble but non-secreted form of DC-SIGN localized to the cytoplasm of cells whose function remains unknown. This allowed us to use sDC-SIGN as a non-membrane-expressed control for DC-SIGN in co-stimulation assays.

Fig. 1. sDC-SIGN, an isoform of DC-SIGN, is not expressed on the cell surface. (A) Schematic representation of sDC-SIGN compared with DC-SIGN. Both molecules are identical; however, sDC-SIGN is missing exon 3, the putative transmembrane domain of the protein. (B) Using the same DC-SIGN primers, reverse transcription–PCR was performed from GM-CSF + IL-4-treated PBMC-RNA from three different individuals (lanes 1, 2 and 3) and PCR from plasmids containing sDC-SIGN (lane 4) and DC-SIGN (lane 5). Top arrow points to DC-SIGN, while bottom arrow points to sDC-SIGN. (C) Sequence difference between sDC-SIGN and DC-SIGN is highlighted in gray (i.e. exon 3). Putative splicing signals are indicated by arrows. (D) DC-SIGN and sDC-SIGN were cloned into an MCS-IRES-GFP vector where transcripts expressing (s)DC-SIGN also expressed GFP. 293T cells transfected with DC-SIGN and sDC-SIGN express GFP (upper histograms). Staining for cell surface DC-SIGN demonstrates that sDC-SIGN is not expressed on the cell surface (bottom histograms) of transfected 293T cells gated on GFP expression. However, sDC-SIGN is expressed inside the cell as revealed by western blot (E) and by staining against DC-SIGN in permeabilized sDC-SIGN-transfected 293T cells (F). Normal 293T cells were used as control in the permeabilized 293T cell experiment (light histogram in F).
DC-SIGN co-stimulates PMA and higher concentrations of anti-CD3—but not sub-optimal concentrations of anti-CD3—induced IL-2 production by human CD4+ T cells

T cell activation requires a primary signal through the TCR and co-stimulation from accessory molecules found on the surface of APCs. The most important of the co-signaling molecules is the B7 family members CD80 and CD86 that constitutively expressed CD28 on T cells. Accessory molecule, ICAM-3, expressed on all resting CD45RA+RO (naive) and CD45RO+RA (memory) CD4+ T cells (our unpublished observations) (13, 14) can also provide co-stimulation but to a lesser degree than CD28. Anti-CD28 (10 μg ml–1) treatment of anti-CD3 (2 μg ml–1)-stimulated CD4+ T cells increased proliferation by at least 5-fold, while anti-ICAM-3 (10 μg ml–1 of HP2/19 antibody) treatment increased T cell proliferation by 2-fold (our unpublished observations) (5) as compared with T cells treated only with this limiting amount (2 μg ml–1) of stimulating anti-CD3 antibody. However, unlike stimulating anti-ICAM-3 antibody HP2/19, other anti-ICAM-3 antibodies (2), such as 186-2G9, cannot co-stimulate T cell stimulation when used in the presence of the same anti-CD3 antibody (not shown). This suggested that simply binding to ICAM-3 on T cells is not sufficient and may require specific interaction with ICAM-3, to provide co-stimulation.

Since DC-SIGN is a physiological ligand for ICAM-3, a classical co-stimulation assay was used to test for the ability of DC-SIGN and sDC-SIGN to provide co-stimulation. DC-SIGN and sDC-SIGN were sub-cloned into the pIRES-GFP2 vector and sorted on the basis of equivalent levels of GFP expression. DC-SIGN expression levels in stably transfected CHO cells used in the co-stimulation assays are shown in Fig. 3. Human PBMCs were stimulated with a sub-optimal dose of plate-bound UCHT1 anti-human CD3 (2 μg ml–1) antibodies in the presence of CHO cells expressing either DC-SIGN or sDC-SIGN or B7 molecules, CD80 and CD86 (shown in Fig. 3). As a test for T cell stimulation we assayed the supernatants for the production of IL-2. Both DC-SIGN and sDC-SIGN could not co-stimulate IL-2 production, while B7 molecules were able to co-stimulate a 5-fold increase in IL-2 production relative to PBMCs stimulated with anti-CD3 alone (not shown). Interestingly, DC-SIGN, but not sDC-SIGN, consistently inhibited anti-CD3-induced IL-2 production by PBMCs, relative to controls (not shown). In order to better characterize the T cell response in this system, CD4+ T cells were isolated via negative selection to maintain them in resting state and used in subsequent assays.

We performed the co-stimulation assay using PMA (Fig. 4A), which activates tyrosine kinases and the high-affinity form of LFA-1. We confirmed that CD80 and CD86 could co-stimulate cytokine production from PMA-treated T cells. To a lesser extent, but consistently, DC-SIGN but not sDC-SIGN could up-regulate IL-2 production as compared with controls. The enhancement of IL-2 production by DC-SIGN in PMA-stimulated T cells represented >40% of the levels of IL-2 production by T cells when they were treated with PMA and ionomycin.

We then tested supernatants of anti-CD3 (2 μg ml–1)-stimulated CD4+ T cells for IL-2, IL-4 and IFN-γ 24 h after stimulation. Only CHO cells expressing CD80 and CD86 significantly up-regulated cytokine production (minimum of 5-fold increase over GFP controls). Consistently, there was a modest but significant (P = 0.0017) inhibition of IL-2 in the presence of DC-SIGN as compared with control (100 μg ml–1 compared with 400 μg ml–1, respectively, Fig. 4B). This assay was also repeated with a different stimulatory anti-CD3 antibody (HIT3a anti-human CD3 antibody) with similar results (not shown). The level of IL-4 was very low (<10 pg ml–1) at the limit of detection and therefore is not shown.
Since DC-SIGN could provide limited co-stimulation to PMA-stimulated T cells, we wanted to determine whether a similar enhancement could be seen when T cells were stimulated with a non-limiting concentration of anti-CD3 (30 μg ml⁻¹) antibody. Relative to the control, there was a moderate but significant increase in the production of IL-2 and IFN-γ when T cells were stimulated in the presence of DC-SIGN (Fig. 4C).

We further explored the effects of DC-SIGN on weakly stimulated T cells. Previously, it was shown that anti-ICAM-3 treatment of anti-CD3-stimulated T cells could induce
expression of activation markers CD69 and CD25. Therefore, we tested for the ability of DC-SIGN or sDC-SIGN to up-regulate these activation markers on T cells stimulated with sub-optimal anti-CD3 or PMA (Fig. 5). CD80- and CD86-expressing CHO cells induced higher levels of CD69 and CD25 expression on anti-CD3-stimulated T cells when compared with control CHO cells (CD69: 59 and 44%, respectively, compared with 23%; CD25: 17% each compared with 8% for the control). A modest suppression of CD69 up-regulation was seen in the presence of CHO cells expressing DC-SIGN relative to control CHO cells or cells expressing sDC-SIGN (9% compared with 23 and 22%, respectively).

The levels of CD69 were higher on all T cells treated with PMA irrespective of co-stimulation. The levels of CD25, however, were up-regulated on T cells co-stimulated with CD80 and CD86 relative to controls (CD25: 77 and 75%, respectively, compared with 45%). When cells were further sorted into CD45RA+RO/CD25 (naive) and CD45R+RA/CD25 (memory), we found that CD80 and CD86 could co-stimulate both populations (not shown).

Next, we tested the abilities of DC-SIGN and sDC-SIGN to co-stimulate plate-bound anti-CD3- or PMA-induced division of T cells. CD4+ T cells were loaded with CFSE dye that dilutes on cell division. T cells were analyzed by flow cytometry 3 days post-stimulation for levels of CFSE and forward scatter, which is a crude measure of cell size (Fig. 6). In the anti-CD3 stimulation assay, both CD80 and CD86 could induce blast formation (77 and 61% of T cells compared with 11% in the presence of GFP control) and cell proliferation (53 and 20%, respectively, compared with 1% for GFP control) within 3 days. However, GFP vector control, DC-SIGN and sDC-SIGN could not induce any cell division. By day 3, PMA-treated T cells underwent significant cell division in the presence of CD80 (44%) and CD86 (30%) relative to 5% in the presence of CHO-GFP. Cell division in the presence of DC-SIGN and sDC-SIGN control was 10 and 4%, respectively. Non-dividing CD4+ T cells co-stimulated with GFP, sDC-SIGN, DC-SIGN, CD80 and CD86, nonetheless showed blast formation in 55, 54, 67, 54 and 54% of cells, respectively.

**Modulation of IL-2 production by DC-SIGN**

Prior experiments showed that DC-SIGN could not co-stimulate CD4+ T cells treated with sub-optimal amounts of

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**Fig. 5.** DC-SIGN, but not sDC-SIGN, partly inhibits the up-regulation of early activation marker CD69. Human CD4+ T cells were stimulated with PMA or sub-optimal doses of anti-CD3 and tested 24 h later for the up-regulation of CD69, CD25 (IL-2R) and expression of chemokine receptor CCR7. First row represents untreated CD4+ T cells. T cells were treated with either anti-CD3 or PMA in the presence of CHO-GFP (second row), CHO-sDC-SIGN (third row), CHO-DC-SIGN (fourth row), CHO-CD80 (fifth row) and CHO-CD86 (sixth row). Depicted in the dot plots are the percentages of cells that show CD69 or CD25 up-regulation as compared with the controls shown in the first row.
anti-CD3. However, we found that treatment of T cells with anti-CD28 and anti-CD3 in the presence of DC-SIGN caused lower IL-2 production (not shown). Therefore, we tested the effects of DC-SIGN on CD80-mediated co-stimulation, which can occur in trans, i.e. the ligands for TCR and CD28 can be present on two different cells.

Different ratios of CHO-DC-SIGN to CHO-CD80 (10 : 1 and 1 : 1) were used to co-stimulate anti-CD3-treated T cells. We tested for IL-2 secretion and activation marker up-regulation and compared them with T cells co-activated with the same ratios of CHO-vector and CHO-CD80 cells. When CHO-DC-SIGN : CHO-CD80 or CHO-GFP : CHO-CD80 cells were used at a ratio of 10 : 1, they induced 461 and 1049 pg ml⁻¹ of IL-2 and 550 and 1100 pg ml⁻¹ of IFN-γ, respectively. The effects of DC-SIGN were somewhat subdued when they were used at a 1 : 1 ratio (Fig. 7).

Discussion

In this study we expressed both sDC-SIGN and DC-SIGN and tested for their ability to provide co-stimulation to T cells stimulated with anti-CD3 and PMA. DC-SIGN increased IL-2 secretion when either 30 μg ml⁻¹ anti-CD3 or PMA was used to stimulate T cells. Interestingly, unlike CD80 and CD86, the DC-SIGN failed to provide co-stimulation when a sub-optimal amount of anti-CD3 (2 μg ml⁻¹) was used to treat CD4+ T cells as measured by cell division, cytokine secretion and up-regulation of CD69 and CD25. Moreover, DC-SIGN modestly suppressed anti-CD3-induced IL-2 production (Fig. 4) and CD69 expression (Fig. 5).

Unlike stimulating anti-ICAM-3 HP2/19 antibody, DC-SIGN was unable to co-stimulate human CD4+ T cells treated with the same anti-CD3 antibody at 2 μg ml⁻¹ (not shown and Figs 4–7). It is interesting to note that both the HP2/19- anti-ICAM-3-stimulating antibody and LFA-1 bind the first Ig-like domain of ICAM-3 (15), while DC-SIGN binds the second Ig-like domain (16). In our co-stimulation assay system, DC-SIGN decreased the efficiency of anti-CD3-induced T cell stimulation with respect to CD69 up-regulation, IL-2 production (Figs 4–6) and CD80-mediated T cell co-stimulation (Fig. 7). In contrast, DC-SIGN enhanced IL-2 production by T cells stimulated with PMA, which can substitute for TCR signaling and activate protein tyrosine kinases and induce the high-affinity form of LFA-1. Moreover, the observation that DC-SIGN can enhance IL-2 secretion in the presence of 30 μg ml⁻¹ of anti-CD3 suggested that a stronger stimulus (perhaps through increased tyrosine kinase activation) might be required to elicit the positive effects of DC-SIGN. These results revealed a complex role for DC-SIGN in the initial co-stimulation required for IL-2 production by T cells.

Low-affinity interactions between accessory molecules allow T cells to probe the surface of APCs. Actin-binding proteins ezrin and moesin interact with ICAM-3, helping to localize it to the uropod of migrating lymphocytes (17, 18). Subsequently, ICAM-3 quickly re-distributes to the point of contact in an antigen-independent manner (2). This then facilitates further interactions between the T cell and APC allowing the formation of an immunological synapse. This results in re-distribution and co-localization of ICAM-3 with ICAM-1 and LFA-1 in the outer edges of the immunological synapse.
In vivo, both CD83+ and CD83− APCs and specialized macrophages express DC-SIGN. Monocytes differentiated into immature DCs, which induce tolerance, express higher levels of DC-SIGN as compared with matured DCs (3). Based on our observation that DC-SIGN can lower the levels of IL-2 production by T cells on sub-optimal stimulation by anti-CD3, we speculate that DC-SIGN may help maintain tolerance by inhibiting IL-2 production by weakly stimulated T cells (23, 24). How DC-SIGN brings about these differential effects under sub-optimal versus optimal T cell signaling is not fully understood but might involve negative signaling through ICAM-3 (25), interference with LFA-1 binding to ICAM-3 (26) or freeing up LFA-1 from ICAM-3 to allow homotypic ICAM-1–LFA-1 interaction (5). Further studies are required to delineate its mechanism of action both in vitro and in vivo.

**Abbreviations**

- APC: antigen-presenting cell
- CFSE: 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester
- DAPI: 4′,6-diamidino-2-phenylindole
- DC: dendritic cell
- DC-SIGN: dendritic cell-specific intercellular cell adhesion molecule-3-grabbing non-integrin
- FSC: forward scatter
- GM-CSF: granulocyte macrophage colony-stimulating factor
- ICAM: intercellular cell adhesion molecule
- LFA: leukocyte function-associated antigen
- PMA: phorbol myristate acetate
- sDC-SIGN: a splice variant of dendritic cell-specific intercellular cell adhesion molecule-3-grabbing non-integrin
- TMB: tetramethylbenzidine

**References**


**Fig. 7.** DC-SIGN can interfere with IL-2 and IFN-γ secretion from T cells stimulated by anti-CD3 and CHO-CD80. Ratios of CHO-DC-SIGN or CHO-GFP to CHO-CD80 cells of 1 : 1 or 10 : 1 were added to T cells stimulated by anti-CD3 and CHO-CD80. Ratios of CHO-DC-SIGN or GFP to CHO-CD80 cells of 1 : 1 or 10 : 1 were added to T cells stimulated by anti-CD3 and CHO-CD80. Ratios of CHO-DC-SIGN or GFP to CHO-CD80 cells of 1 : 1 or 10 : 1 were added to

**synapse.** TCR-mediated events within the synapse further strengthen the T cell–APC interaction by inducing the high-affinity form of LFA-1 (19). Previous studies using in vitro co-stimulation assays demonstrated that ICAM-1 signaling induced by LFA-1 could enhance T cell stimulation (20, 21) and restore co-stimulation in CD28 knockout mice (22). Further, cells lacking both pathways were unable to respond to anti-CD3-induced T cell signaling and suggested that in the absence of B7-CD80 signaling, LFA-1–ICAM-1 is the predominant co-stimulatory pathway.

Based on our current and others’ findings (19–22), we speculate that the DC-SIGN has two roles in stimulating a T cell. There is an initial and transient binding that occurs between APCs and T cells through DC-SIGN and ICAM-3, which allows for the engagement of TCR and the MHc–peptide complex. This initial TCR stimulation increases LFA-1 avidity for ICAM-1 (5) and decreases LFA-1–ICAM-3 interaction. Since DC-SIGN can interact with ICAM-3, and not ICAM-1, it can induce signaling through ICAM-3 and enhance IL-2 production by T cells that receive a strong TCR signal.


