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Retagging Identifies Dendritic Cell-specific Intercellular Adhesion Molecule-3 (ICAM3)-grabbing Non-integrin (DC-SIGN) Protein as a Novel Receptor for a Major Allergen from House Dust Mite*

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Background: Allergen uptake by DCs is central to allergic sensitization.
Results: DC-SIGN recognizes major allergens from house dust mite and dog. However, silencing DC-SIGN leads to Th2 differentiation.
Conclusion: DC-SIGN is a newly identified receptor for Der p 1 and Can f 1 that appears to support Th1 cell differentiation.
Significance: Understanding of how allergic responses are selected and propagated is essential for developing novel therapies.

Dendritic cells (DCs) have been shown to play a key role in the initiation and maintenance of immune responses to microbial pathogens as well as to allergens, but the exact mechanisms of their involvement in allergic responses and Th2 cell differentiation have remained elusive. Using retagging, we identified DC-SIGN as a novel receptor involved in the initial recognition and uptake of the major house dust mite and dog allergens Der p 1 and Can f 1, respectively. To confirm this, we used gene silencing to specifically inhibit DC-SIGN expression by DCs followed by allergen uptake studies. Binding and uptake of Der p 1 and Can f 1 allergens was assessed by ELISA and flow cytometry. Intriguingly, our data showed that silencing DC-SIGN on DCs promotes a Th2 phenotype in DC/T cell co-cultures. These findings should lead to better understanding of the molecular basis of allergen-induced Th2 cell polarization and in doing so paves the way for the rational design of novel intervention strategies by targeting allergen receptors on innate immune cells or their carbohydrate counterstructures on allergens.

Dendritic cells (DCs)[4] act as sentinels of the immune system and serve as a bridge between innate and adaptive immunity. Internalization of antigens by DCs is an important step in the sequence of events that leads to the induction of the adaptive immune response (1). DCs can efficiently sample their microenvironment using a plethora of receptors such as C-type lectin receptors (CLRs), Toll-like receptors, or scavenger receptors (2). Immature DCs take up antigens in peripheral tissues, process them into peptides, and then migrate to lymph nodes where they acquire a fully mature status capable of stimulating naïve T cells (3, 4). Immature DCs are characterized by their superior capacity for antigen uptake which can be attributed to the numerous CLRs that are highly expressed on these cells. These CLRs include mannose receptor (MR, CD206), dendritic cell-specific intracellular adhesion molecule (ICAM)-3-grabbing non-integrin (DC-SIGN, CD209), and dendritic and epithelial cells, 205 kDa (DEC-205, CD205) (5–7). C-type lectins are calcium-dependent carbohydrate-binding glycoproteins with a wide range of biological functions characterized by the presence of at least one carbohydrate recognition domain that interacts with and recognizes carbohydrates via either mannose or galactose side chains (8–11).

DCs have been shown to play a key role in the initiation and maintenance of immune responses to microbial pathogens as well as to allergens, but the exact mechanisms of their involvement in allergic responses and Th2 cell differentiation have remained elusive (12, 13). Given the importance of antigen recognition and uptake by DCs on downstream events leading to T cell differentiation, there is considerable interest in identifying potential receptors for allergens on DCs. Within this context, we and others have shown that MR is partially involved in the uptake of Der p 1, the major allergen from house dust mite (14, 15). Blocking MR by mannan (14) or its down-regulation using siRNA (15) leads to approximately 60–70% reduction in Der p 1 uptake by human monocyte-derived DCs. The residual uptake after blocking MR does not seem to be due to macropinocytosis by DCs, and as such it is reasonable to suggest the presence of other putative allergen receptors on DCs. In this study, using a number of different approaches including confocal microscopy, receptor activity-directed affinity tagging (retagging) (16, 17) and gene silencing, we show that Der p 1 and Can f 1 uptake by human DCs is also mediated by DC-SIGN. Although down-regulation of MR inhibits Th2 differen-

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[4] The abbreviations used are: DC, dendritic cell; CLR, C-type lectin receptor; CT, control; DC-SIGN, dendritic cell-specific ICAM-3-grabbing non-integrin; ICAM, intercellular adhesion molecule; MR, mannose receptor; qRT-PCR: quantitative real time PCR; retagging, receptor activity-directed affinity tagging; sDC-SIGN, soluble DC-SIGN.

tiation (15, 18), intriguingly we have shown that knocking down DC-SIGN expression on human DCs leads to a bias toward Th2 cell differentiation in autologous DC-T cell co-cultures, suggesting an antagonistic relationship between the two main allergen receptors expressed on DC surface.

Early events at the interface of allergens and DCs play a key role in downstream events leading to allergic sensitization. Therefore, identifying receptors that are involved in the initial recognition and uptake of allergens by DCs would not only lead to better understanding of the molecular basis of allergen-induced Th2 cell polarization but also pave the way for the rational design of novel intervention strategies.

EXPERIMENTAL PROCEDURES

Cell Preparation—Immature DCs were generated from monocytes isolated from peripheral blood of nonatopic healthy donors (obtained with consent and after ethical committee approval) in the presence of interleukin (IL)-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (250 units/ml and 50 ng/ml, respectively) (R&D Systems) in RPMI 1640 medium (Sigma-Aldrich) supplemented with 100 units/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine (all from Sigma-Aldrich), and 10% low endotoxin FCS (Autogen Bioclear, Calne, UK) for 6 days as described before (19). In some experiments, we used NIH-3T3 fibroblast transfectants stably expressing DC-SIGN (3T3/DC-SIGN) (a kind gift from Dr. Vineet KewalRamani, National Cancer Institute, Bethesda, MD) (20) and mock fibroblast cells lacking DC-SIGN.

Confocal Microscopy—To promote the adherence of DCs, glass base dishes (IWAKI, Asahi Techno Glass Corp., Japan) with an internal core diameter of 1 cm were coated with 6 μg/ml (w/v) of poly-L-lysine (Sigma) overnight. To deplete the cell membrane from cholesterol, immature DCs were treated with 10 mM (2-hydroxypropyl)-β-cyclodextrin (Sigma) which is a more water-soluble/toxicologically benign alternative to α-, β-, and γ-cyclodextrin (21–24). 5×10^5/200 μl β-cyclodextrin-treated or untreated DCs were then seeded in each dish for 1 h at 37 °C, 5% CO_2. Dishes were then placed under a Zeiss confocal microscope (LSM501uv META combi, Welwyn Garden City, UK) (equipped with a hot plate and tissue culture chamber) and received 5 μg/ml Cy5-labeled Der p 1. For each condition, confocal images were taken after 20 min.

Retagging Technique—Der p 1-binding proteins were purified as described previously for the *Helicobacter pylori* SabA adhesin (16, 17) with some modifications. Briefly, Der p 1, conjugated to the Sulfo-SBED multifunctional cross-linker (Pierce, Thermo Scientific), was incubated with DCs or monocytes according to the manufacturer's recommendations. The photoreactive cross-linker group was activated by 2 min of UV irradiation, and the biotin-(re)tagged proteins were purified with streptavidin-coated magnetic beads.

Mass Spectrometry Analysis—Extracted biotin-tagged proteins were separated by SDS-PAGE, and bands were digested with sequencing-grade trypsin (Promega) as described previously (19) and analyzed using a Micromass ToF-Spec E (Micromass). Briefly, the nanoflow LC-tandem MS was done on a 7-Tesla linear trap quadrupole-Fourier transform mass spectrometer (Thermo Electron) equipped with a nanospray source modified in-house. The spectrometer was operated in data-dependent mode, automatically switching to tandem MS mode. MS spectra were acquired in the FTICR, whereas tandem MS spectra were acquired in the linear trap quadrupole trap. For each scan of FTICR, the three most intense, doubly or triply charged, ions were sequentially fragmented in the linear trap by collision-induced dissociation. All the tandem mass spectra were searched using MASCOT PMF data base search engine (Matrix Science, London, UK) or Aldente (expasy site) against the databases (Sprot 54.0) for the human proteins as appropriate. Search parameters included a peptide mass accuracy tolerance of 0.2Da and allowed for modifications such as alkylation of cysteine during the tryptic digest procedure and the possible formation of methionine sulfoxide (19).

Binding of Natural Allergens to Soluble DC-SIGN by ELISA—ELISA-based binding assays were used to determine soluble DC-SIGN (sDC-SIGN; R&D Systems) binding to different allergens. All washing steps were performed in lectin buffer consisting of 10 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$, 0.154 M NaCl, and 0.05% (w/v) Tween 20. Maxisorp plates (Nunc, Thermo Scientific) were coated overnight with different test allergens or mannan (5 μg/ml) and blocked with 1% BSA. Plates were sequentially incubated with 5 μg/ml of sDC-SIGN and 5 μg/ml DC-SIGN mAb (clone 120507; R&D Systems). Bound antibodies were detected by incubation with phosphatase-conjugated goat anti-mouse Ab diluted to 1/1000, and *p*-nitrophenyl phosphate solution (Sigma) was added to the plates at concentration of 1 mg/ml. Absorbance was measured at 405 nm (Expert Plus, SLS; Nottingham, UK).

Binding of Der p 1 to NIH-3T3 Cells Expressing DC-SIGN—Allergens were labeled with Cy3 or Cy5 (GE Healthcare) labeling kits according to the manufacturer's instructions and as described before (15). Binding assays were conducted in presence and absence of EDTA. 3T3/DC-SIGN fibroblasts were used to study binding of Der p 1 to DC-SIGN. Briefly, cells (2×10^5/100 μl) were incubated with Cy5 only or Cy5 Der p 1 at 37 °C for 45 min; cells were then washed and fixed in 0.5% formaldehyde. To investigate DC-SIGN specificity, NIH-3T3 fibroblasts that lack DC-SIGN expression (mock cells) were used, and binding was detected by flow cytometry on an Altra flow cytometer (Beckman Coulter).

Blocking Experiments—Immature DCs were resuspended in uptake medium (RPMI 1640 medium + 30% PBS with Ca^{2+} and Mg^{2+} +10% FBS) and preincubated with (200 μg/ml) mannan (Sigma), or different concentrations (10 and 20 μg/ml) of Lewis-x (PAA-Lewis-x; Lectinity, Moscow, Russia) for 30 min at 37 °C and subsequently incubated with 5 μg/ml Cy5 Der p 1 for another 30 min. In some experiments, Cy5 Der p 1 was preincubated with varying concentrations (5, 10, and 20 μg/ml) of sDC-SIGN prior to addition to cells. BSA was used as a control. Cells were then washed and fixed in 0.5% formaldehyde, and Der p 1 uptake was determined by flow cytometry. Results are expressed as median fluorescence intensity (MFI).

RNA Interference—RNA interference (RNAi) was performed by transfecting DCs with small interfering RNA (siRNA) (50 nM), using the Lipofectamine RNAiMax (Invitrogen), to specifically knock down DC-SIGN. For DC-SIGN mediated gene silencing, the following oligonucleotides were employed:

(GGCAAUGGCUGGAACGACGACAAAU), and (AUUUGUCGUCGUUCCAGCCAUUGCC) (Invitrogen) and the nonsilencing control (CT) siRNA used was all star negative control (Qiagen). Gene knockdown was estimated quantitatively at message level by quantitative real time PCR (qRT-PCR) and at protein level by flow cytometry and Western blotting.

Real Time PCR—Real time PCRs were conducted using a MX3005P thermal cycler (Stratagene, Agilent Technologies), and PCR amplifications were performed with the SYBR Green method using the following primers: CD209 (forward, 5′-CCAAAGGAGGAGACAAGCAG-3′ and reverse, 5′-GGACGACAGCTTCAGTGTGA-3′) and GAPDH (forward, 5′-GAGTCAACGGATTTGGTCGT-3′ and reverse, 5′-GACAAGCTTCCCGTTCTCAG-3′). Each reaction was performed in a final volume of 25 μl comprising 5 μl of template (cDNA), 12.5 μl of SYBR Green master mix (Stratagene), 1 μl (200 nm) of each primer, and 0.38 μl of ROX dye. Amplifications were performed starting with an initial denaturation step of 95 °C/30 s, followed by 40 cycles of 95 °C/30 s,56 °C/60 s, and 72 °C/60 s.

Western Blotting—DC-SIGN knockdown was assessed by Western blotting as described elsewhere (25). Briefly, proteins were run on SDS-PAGE, transferred to nitrocellulose membranes (Amersham Biosciences, GE Healthcare), blocked with (PBS + 0.05% Tween 20 + 5% milk), and subsequently probed with rabbit polyclonal Ab to human CD209 (2 μg/ml; AbD Serotec, Kidlington, UK) or mouse anti-human mAb to β-actin (Sigma). Membranes were washed and reprobed with HRP-conjugated goat anti-rabbit IgG (Fc) or rabbit $F(ab')_2$ anti-mouse IgG:HRP (AbD Serotec). Bands were visualized with ECL reagent (Amersham Biosciences), and the intensity of each protein was normalized against β-actin as an internal loading control. In retagging experiments the blots were probed with streptavidin directly conjugated with HRP (1:5000 dilution) (Thermo Fischer Scientific).

Effect of DC-SIGN Knockdown on Der p 1 and Can f 1 Uptake—DCs were transfected at day 1, and the effect of DC-SIGN gene silencing on uptake was studied. Briefly, DCs that have previously been treated with CT or DC-SIGN siRNA were washed in PBS, resuspended in uptake medium, and incubated with 5 μg/ml of either Cy5 Der p 1 or Cy3 Can f 1 for 30 min. Cells were then washed and fixed in 0.5% formaldehyde, and the quantitative uptake of Cy5 Der p 1 and Cy3 Can f 1 was then estimated by flow cytometry.

Effect of DC-SIGN Down-regulation on T Cell Polarization—Naïve ($CD3^+/CD45RA^+$) T cells were isolated by immunomagnetic cell sorting (Miltenyi Biotec) according to the manufacturer's instructions. DCs that had previously been treated with either CT or CD209 siRNA were loaded or not for 6 h with 5 μg/ml natural Der p 1 (Indoor Biotechnologies, Wiltshire, UK). DCs were washed and cultured in 96-well U-bottom plates (Nunc) with $CD3^+CD45RA^+$ autologous T cells (ratio DC/T cell: 1/5) in RPMI 1640 medium plus 5% human AB serum (Sigma). On day 3, rh-IL-2 (20 units/ml; R&D Systems) was added to the cells, and feeding with fresh medium and IL-2 was performed every 3–4 days. After 16 days, T cells were restimulated for 8 h with autologous DCs (ratio DC/T cell: 1/1) loaded or not with Der p 1. For intracellular staining, brefeldin A (10 μg/ml; Sigma) was added for the last 6 h, and the production of IL-4 and interferon (IFN)-γ was detected on $CD4^+$ cells by intracellular staining (26) using anti-IL-4-PE (clone 4D9) and anti-IFN-γ-FITC (clone 45.15); both were purchased from Beckman Coulter.

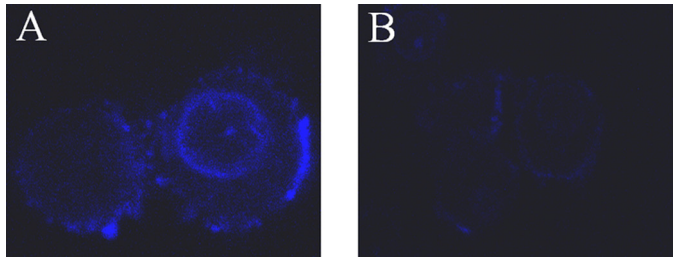

FIGURE 1. **Confocal microscopy showing the uptake of Cy5 Der p 1 by DCs before and after treatment with β-cyclodextrin.** The uptake of Cy5-labeled Der p 1 by DC before (*A*) and after (*B*) treatment with 10 mM (2-hydroxypropyl)-β-cyclodextrin for 1 h is shown. The loss of Der p 1 localization (hot spots) after depletion of cholesterol from cell membrane is indicative of the involvement of membrane rafts (*i.e.* a receptor-ligand interaction).

Statistical Analysis—Statistical analyses were performed by using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA). Student's *t* tests or the Mann-Whitney *U* test was used for pairwise comparisons analysis. One-way ANOVA with Bonferroni's post hoc test was used for multiple comparisons analysis. Significance was accepted when p values ≤ 0.05, where *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$. Data were expressed as mean ± S.E.

RESULTS

Glycoallergen Uptake by Human DCs Is a Receptor-mediated Event—Using cyclodextran (27), we depleted the DC membrane from cholesterol thereby disrupting all receptor-mediated endocytosis (28). This led to almost complete abrogation of Der p 1 uptake by DCs compared with control cultures which were not treated by cyclodextran (Fig. 1). We did not observe any changes in DC viability upon cyclodextran treatment (data not shown). This observation together with our previous data (15) showing that MR is only responsible for 60–70% of Der p 1 uptake by human DCs clearly suggest the presence of other putative receptors on DCs that are capable of allergen uptake.

DC-SIGN Is Novel Receptor for Major House Dust Mite Allergen Der p 1—To identify other receptors on human DCs that are involved in Der p 1 uptake, we used retagging, a powerful tool for identifying cell surface receptors (16, 17). Using this technique, we were able to identify DC-SIGN as a novel receptor for Der p 1 (Fig. 2). Subsequently, the functional importance and contribution of DC-SIGN to uptake of allergens were assessed using a number of complementary approaches. First, we investigated the binding of DC-SIGN to different allergens using ELISA. Der p 1 (OD values = 1.416 ± 0.078), Can f 1 (1.153 ± 0.043), and Ara h 1 (OD = 0.930 ± 0.003), which was previously identified as DC-SIGN ligands (29), were all found to bind to DC-SIGN, albeit with different strengths, whereas the cat allergen Fel d 1 did not show any binding (Fig. 3).

A more physiologically relevant approach to study the binding of Der p 1 to DC-SIGN involved NIH-3T3 fibroblast transfectants stably expressing DC-SIGN. This showed that Cy5-labeled Der p 1 binds to NIH-3T3 fibroblasts expressing DC-SIGN (Fig. 4) but not mock cells as detected by flow cytom-

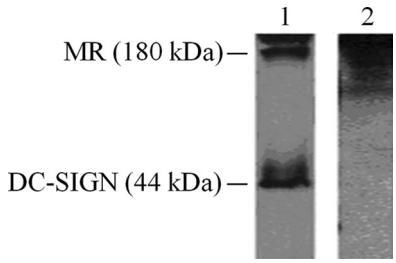

FIGURE 2. **Identification of DC-SIGN as a novel receptor for Der p 1 on DCs by retagging technique.** Der p 1, conjugated with Sulfo-SBED multifunctional cross-linker, was incubated with DCs or monocytes. After photoreactive cross-linking, the biotin-(re)tagged proteins were purified with streptavidin-coated magnetic beads. Extracted biotin-tagged proteins were separated by SDS-PAGE. Western blotting analysis revealed a band at 44 kDa in DC condition (*lane 1*) corresponding to DC-SIGN. No bands were obtained in the case of monocytes (*lane 2*). Mannose receptor (MR, 180 kDa), which has previously been identified as Der p 1 receptor on DCs, was also obtained in the retagging experiments.

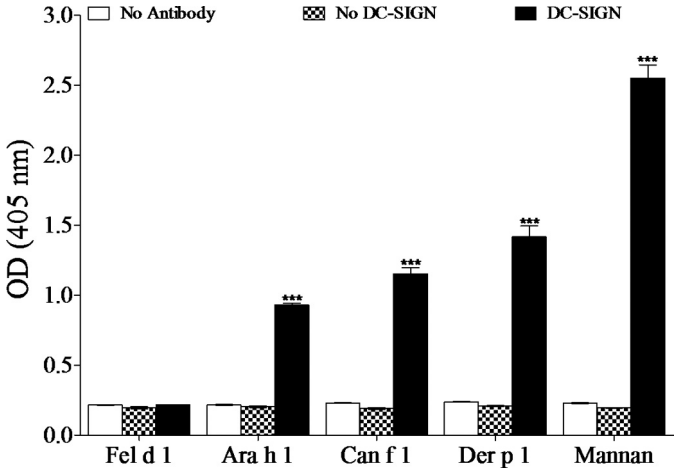

FIGURE 3. **Detection of natural allergen binding to sDC-SIGN by ELISA.** ELISA plates were coated overnight with Der p 1, Can f 1, Ara h 1, Fel d 1, or mannan. Binding to sDC-SIGN was investigated using DC-SIGN mAb (clone 120507). Der p 1, Can f 1, and Ara h 1 were found to bind sDC-SIGN with referral to mannan as a control, whereas no binding was detected with Fel d 1. Data presented are the average of four independent experiments ± S.E. (*error bars*). ***, $p \leq 0.001$.

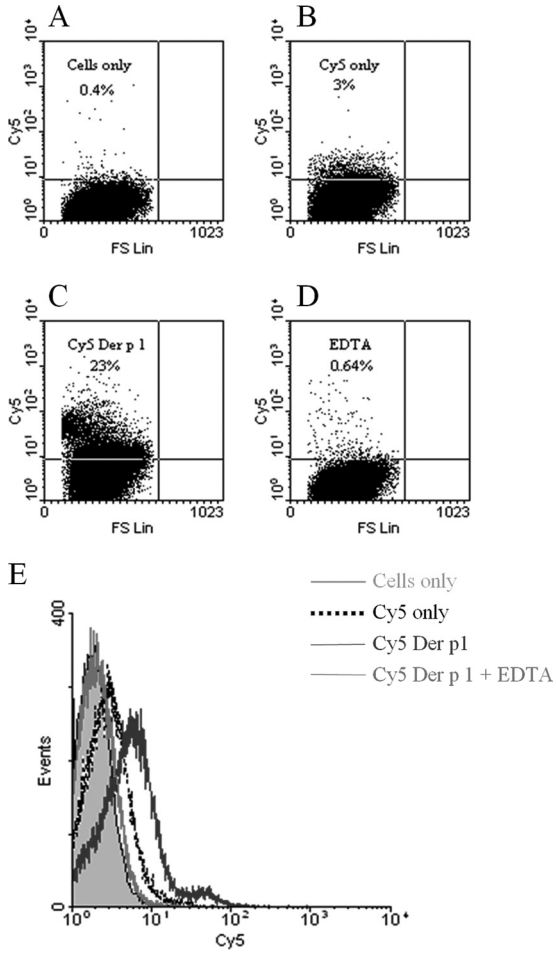

FIGURE 4. **Binding of Der p 1 to NIH-3T3 cells expressing DC-SIGN.** Cells (2×10^5/100 μl) were incubated with Cy5 Der p 1 or Cy5 alone, and binding was determined by flow cytometry. Results obtained demonstrate that Der p 1 binds to NIH-3T3 fibroblasts expressing DC-SIGN. *A–D*, dot plots corresponding to cells only (*A*), cells incubated with Cy5 only (*B*), cells incubated with Cy5 Der p 1 (*C*), cells treated with EDTA (*D*). *E*, histogram demonstrating Der p 1 binding to NIH-3T3 cells expressing DC-SIGN.

etry. Incubation with EDTA completely abrogated the binding, indicating that binding is Ca^{2+}-dependent.

Furthermore, based on the carbohydrate specificity of Lewis-x for DC-SIGN (30, 31), we sought to block DC-SIGN on DCs using Lewis-x antigen to investigate further the impact of DC-SIGN on Der p 1 uptake by DCs. This resulted in a reduction of Der p 1 uptake by DCs. Results obtained (Fig. 5*A*) demonstrate that Der p 1 uptake was inhibited by Lewis-x in a dose-dependent manner with blocking reaching a plateau at 20 μg/ml ($p < 0.05$, $n = 3$). Mannan was used as a control and inhibited Der p 1 uptake by 60% which was in line with previous observations (14, 15).

Preincubation of Der p 1 with Soluble DC-SIGN Reduces Der p 1 Uptake by DCs in Dose-dependent Manner—We also sought to study the interaction between Der p 1 and DC-SIGN by flow cytometry through preincubation of Der p 1 with different concentration of sDC-SIGN. This resulted in a dose-dependent and statistically significant decrease in Der p 1 uptake ($p < 0.05$, $n = 3$) further confirming the role of DC-SIGN in recognition of Der p 1 (Fig. 5*B*). Similar concentrations of BSA did not lead to any changes in Der p 1 uptake (data not shown).

DC-SIGN Down-regulation Reduces Der p 1 and Can f 1 Uptake by DCs—To evaluate further the role of DC-SIGN in the uptake of Der p 1 and Can f 1 by DCs, we investigated the impact of DC-SIGN down-regulation following siRNA experiments. Here, we describe an efficient siRNA strategy for the systematic knockdown of DC-SIGN on human cells, as a simple and highly reproducible method. The quantitative measurement of gene silencing at the mRNA and protein levels was achieved via qRT-PCR (Fig. 6*A*), flow cytometry (Fig. 6, *B* and *C*), and Western blotting (Fig. 6*D*), obtaining percentage inhibition of 70 – 80%. In line with our previous studies (15, 32), DC viability after siRNA treatment as well as the expression and localization of MR was not affected by silencing DC-SIGN expression (data not shown). Allergen uptake data obtained following specific DC-SIGN gene silencing demonstrated a significant reduction in Der p 1 (32%; $p < 0.01$, $n = 9$) and Can f 1 (33%; ($p < 0.01$, $n = 4$) uptake by DCs (Fig. 7). No effect was observed in the uptake of the negative control, FITC-SO_4-3-galactose, a specific ligand that is taken up by MR, confirming

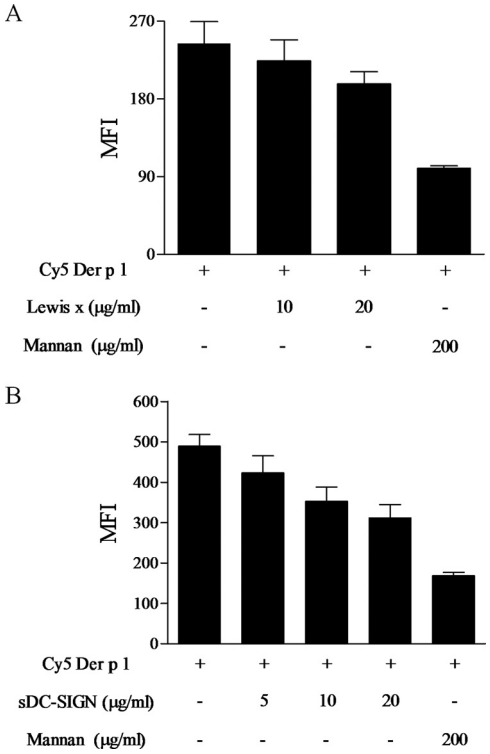

FIGURE 5. **Lewis-x and sDC-SIGN reduce Der p 1 uptake by DCs.** DCs were incubated with Cy5 Der p 1 previously preincubated or not with increasing concentrations of Lewis-x (10 and 20 μg/ml) (*A*) or sDC-SIGN (5, 10, and 20 μg/ml) (*B*). Mannan (200 μg/ml) was used as a control. Quantitative Cy5 Der p 1 uptake by DCs was estimated by flow cytometry. Dose-dependent inhibition of Der p 1 uptake was obtained. Result shown represents three independent experiments expressed as average median fluorescence intensity (*MFI*) ± S.E. (*error bars*).

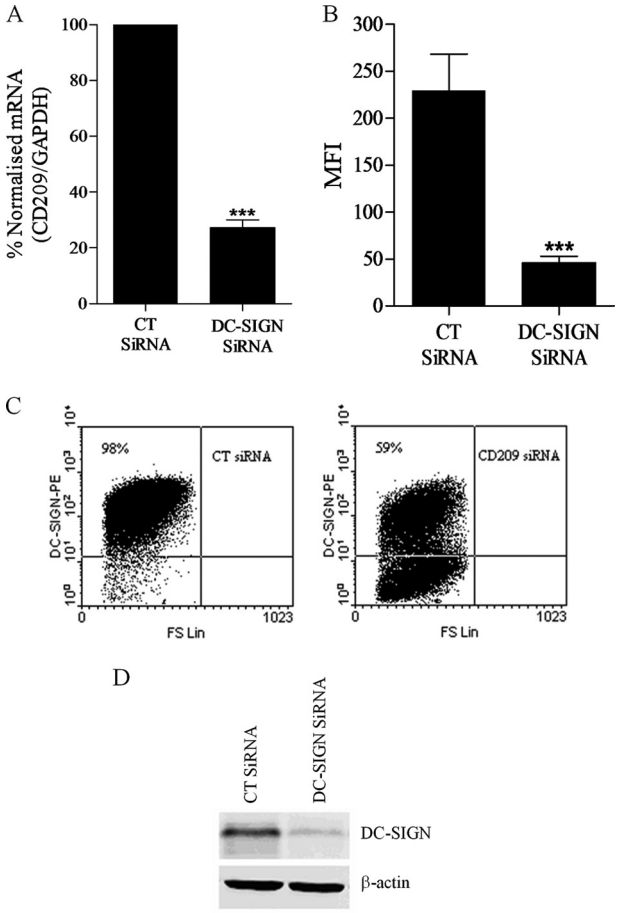

FIGURE 6. **Estimation of DC-SIGN gene silencing by various techniques.** *A*, DCs were transfected at day 1, and down-regulation of DC-SIGN expression was quantitatively analyzed at message level by qRT-PCR. Transcription of mRNA was inhibited by 73% ($n = 6$) after normalization against GAPDH. *B*, flow cytometric analysis of DC-SIGN silencing showed that siRNA treatment of DCs inhibits DC-SIGN surface expression by 80% compared with cells treated with CT siRNA. *C*, dot plot corresponds to cells treated with CT or DC-SIGN siRNA. *D*, assessment of DC-SIGN knockdown by Western blotting shows that DC-SIGN expression was inhibited by 82% with referral to β-actin as a reference internal control. ***, $p \leq 0.001$. *Error bars* indicate S.E.

that the reduction in uptake obtained following siRNA treatment was DC-SIGN-mediated.

DC-SIGN Down-regulation Favors Th2 Cell Polarization in DC-T Cell Co-cultures—DCs that have previously been treated with either CT or DC-SIGN siRNA were loaded or not for 6 h with 5 μg/ml natural Der p 1 and cultured with $CD3^+$/$CD45RA^+$ autologous T cells for up to 16 days. After restimulation with autologous DCs, the percentages of IL-4 and IFN-γ producing T cells were determined by intracellular staining. Given that the cells were isolated from peripheral blood of a nonatopic donor, not surprisingly both in the presence and absence of Der p 1 stimulation of naïve T cells by DC-SIGN-expressing DCs (CT-DCs) there was a Th1 bias as evidenced by high levels of IFN-γ production (Fig. 8). This is in line with previous observations showing that in healthy donors, the presence of natural Der p 1 dramatically enhanced Th1 polarization (33, 34). By contrast, our data show that silencing of DC-SIGN expression dampened the Th1 polarization in the unloaded as well as in the Der p 1 loaded condition mainly through a significant increase in IL-4 secretion as well as slight decrease in IFN-γ production. Interestingly, with the DC-SIGN-deficient cells, loading with Der p 1 resulted in a further reduction of the Th1 skewing.

DISCUSSION

We have previously shown that the uptake of a number of glycoallergens such as Der p 1 is mediated through MR; however, MR seems to be responsible for only approximately 60% of this uptake (15). Previous work (14) suggested that the residual Der p 1 uptake does not seem to be mediated through macropinocytosis, which is in line with our own observations (data not shown). Almost all endocytic pathways are sensitive to cholesterol perturbation (28), and clathrin-dependent (35) and -independent pathways (36) are both inhibited by the removal of cholesterol. In this study, using cyclodextran (27), we depleted the DC membrane from cholesterol thereby disrupting all receptor-mediated endocytosis (28). This led to almost complete abrogation of Der p 1 uptake by DCs compared with control cultures which were not treated by cyclodextran. These data clearly suggest the presence of other putative receptors on DCs that are capable of allergen uptake.

To identify such receptors we used retagging, which is mainly based on labeling the protein of interest with a multifunctional cross-linker composed of a biotin tag disulfide bonded to a photoreactive group. Thus, allowing the labeled allergen to interact with the cells and then subjecting the cells to UV irradiation will enable the photoreactive group to form a covalent bond with

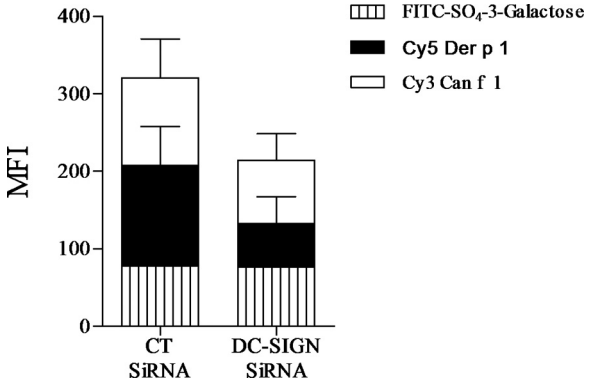

FIGURE 7. **Der p 1 and Can f 1 uptake by CT or DC-SIGN-deficient DCs.** DCs were transfected at day 1 with CT siRNA or DC-SIGN siRNA, and the impact of DC-SIGN knockdown on uptake was assessed at day 6. Cells that were treated with DC-SIGN siRNA showed a 32% reduction in Der p 1 uptake ($n = 9$) and 33% reduction in Can f 1 uptake ($n = 4$) compared with cells treated with CT siRNA. No effect on sulfated sugar was noticed after DC-SIGN gene silencing. Data are presented as average median fluorescence intensity (*MFI*) ± S.E. (*error bars*).

structures in the immediate vicinity. The bound receptor could then be extracted and identified by mass spectroscopy (16, 17). Using this technique, we identified DC-SIGN as a novel receptor for the major house dust mite and dog allergens, Der p 1 and Can f 1, respectively.

Having identified DC-SIGN as a potential receptor for Der p 1 on DCs, we set out to investigate its role in Der p 1 uptake by DCs. The functional importance and contribution of DC-SIGN to Der p 1 uptake by human DCs were assessed using different, but complementary approaches. First, we investigated the binding of DC-SIGN to different allergens using ELISA. Der p 1, Can f 1, and Ara h 1 were all found to bind to DC-SIGN in contrast to the cat allergen Fel d 1 which showed no binding. Another, perhaps more physiologically relevant, approach is to study the binding of Der p 1 to DC-SIGN using 3T3/DC-SIGN cells (20, 37). This showed that Cy5 Der p 1 binds to 3T3 fibroblasts expressing DC-SIGN, but not to mock cells as detected by flow cytometry. Incubation with EDTA completely abrogated the binding, indicating that binding is Ca^{2+}-dependent.

The above binding data prompted us to investigate whether DC-SIGN is involved in allergen uptake by human DCs. Based on the carbohydrate specificity of Lewis-x for DC-SIGN (30, 31), we therefore sought to target DC-SIGN on DCs using a natural ligand (Lewis-x antigen) to investigate further the impact of DC-SIGN engagement on Der p 1 uptake by DCs. Results obtained demonstrated dose-dependent and significant inhibition of Der p 1 uptake by Lewis-x with nearly 20% inhibition with highest concentration (20 mg/ml) of Lewis-x.

DC-SIGN has also been shown to mediate the uptake of various allergens such as the major peanut allergen (Ara h 1) (29), as well as the Bermuda grass pollen allergen (BG-60) and the major group 2 allergen from house dust mite (Der p 2) (38). As such, this and other reports clearly underscore the potential of CLRs (in particular MR and DC-SIGN), which are highly expressed on the surface of antigen-presenting cells, to serve as common receptors for recognition of a wide range of glycosylated allergens. Intriguingly, we have also shown for the first time that down-regulation of DC-SIGN expression through

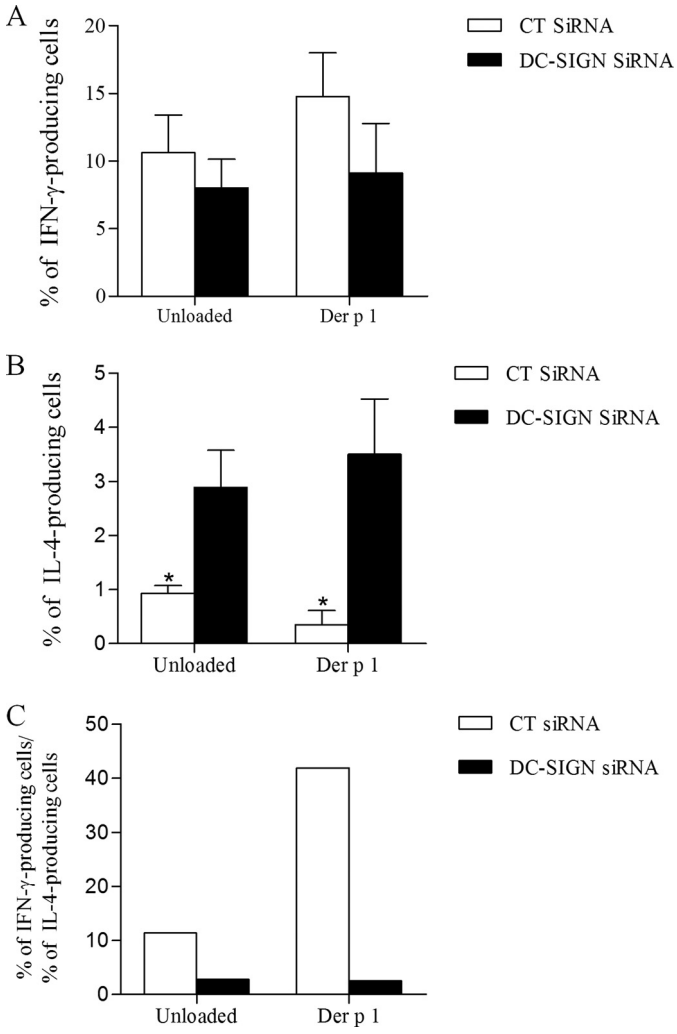

FIGURE 8. **T cell polarization after DC-SIGN knockdown.** *A* and *B*, DC-SIGN knockdown on DCs skews Th1/Th2 toward Th2. DCs (CT and DC-SIGN-deficient) from four different donors were preloaded or not with Der p 1 prior to establishing autologous DC-T cell co-cultures with naïve ($CD3^+/CD45RA^+$) T cells. On day 16, T cells were restimulated for 8 h with Der p 1-loaded or -unloaded DCs (not treated with siRNA), and IFN-γ (*A*) and IL-4 (*B*) production was analyzed by intracellular staining ($n = 4$). *C*, as an indicator of polarization, the ratio of IFN-γ to IL-4 based on the data obtained in *A* and *B* was calculated. *, $p \leq 0.05$.

siRNA leads to a bias toward Th2 polarization in autologous DC-T cell co-cultures. This is in contrast with our earlier work showing that down-regulation of MR, a major Der p 1 receptor expressed on DCs, leads to an opposite effect, *i.e.* bias toward Th1 polarization (15, 18). This could be partly explained by data showing that differentiation of naive T cells toward Th2 occurs upon co-stimulation through ICAM-1 and ICAM-2 instead of ICAM-3, which is thought to be the main DC-SIGN counterstructure on T cells (39). Within this context, it is interesting to note that we have shown previously (37) that Der p 1, a cysteine protease, in its enzymatically active form, can cleave DC-SIGN (its "Th1-promoting" receptor) but not MR (its "Th2-promoting" receptor), and this will further amplify its alleregenicity. Although the exact mechanism of polarized Th2 cell differentiation in the absence of DC-SIGN is yet to be determined, these data clearly indicate that glycoallergen uptake by DCs and events leading to downstream Th2 polarization are complex

and involve at least two receptors, MR and DC-SIGN, whose engagement by allergen leads to distinct and possibly antagonistic signaling events. Therefore, it is reasonable to assume that the overall fate of T cells in response to allergen exposure in different individuals could at least be partly determined by differential level of MR and DC-SIGN expression on DC subsets. In this connection, it is interesting to note that MR expression has been shown to be higher in atopic individuals (14).

Early events at the interface of DCs and allergens are clearly of great importance in determining T cell polarization. In this study, we have identified a novel receptor for two major allergens from house dust mite and dog, Der p 1 and Can f 1, respectively. This new insight could lead to better understanding of the molecular basis of allergen-induced Th2 cell polarization, and this should hopefully pave the way for the rational design of novel intervention strategies.

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