

Identification and characterization of DC-SIGN-binding glycoproteins in allergenic foods

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Abstract

Background: DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin) is a C-type lectin receptor expressed on macrophages and dendritic cells. DC-SIGN has high affinity for fucosylated glycans in several plant glycoproteins and pathogens. DC-SIGN is thought to be crucial for the development of allergic sensitization. However, the precise role of DC-SIGN in food allergy pathogenesis is not yet understood.

Objective: We sought to characterize DC-SIGN-binding glycoproteins in a panel of allergenic and non-allergenic foods.

Methods: Fluorescent-labeled peanut and soy extracts were used to test protein binding to human monocyte-derived dendritic cells (DCs) by flow cytometry. DC-SIGN-blocking assays were performed by incubating DCs with food extracts followed by staining with anti-DC-SIGN antibody. Using a DC-SIGN-Fc chimera, food extracts were tested for binding by ELISA and autoradiography. IgE immunoblotting was performed with pooled sera from food-allergic subjects. DC activation and maturation were assessed by flow cytometry.

Results and Conclusions: We demonstrate that peanut agglutinin, a minor peanut allergen, is a novel ligand for DC-SIGN. Peanut agglutinin activates DCs to induce the expression of costimulatory molecules *in vitro*. We present a comprehensive report on the characterization of DC-SIGN-binding proteins in common allergenic foods such as peanut, soy, tree nuts, egg, and milk. Foods that rarely induce allergy, such as pine nuts, chickpea, and corn, showed no binding to DC-SIGN. Several DC-SIGN-binding proteins show reactivity in serum IgE immunoblots. We have also identified novel non-IgE-binding proteins that interact with DC-SIGN; these proteins may be important for regulating immune responses to these foods.

DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin) also known as CD209 is a C-type lectin receptor, which in humans is encoded by the *CD209* gene (1). DC-SIGN is present on both macrophages and dendritic cells. DC-SIGN binds various microorganisms by recognizing 'pathogen-associated molecular patterns (PAMPs)', which consist of high mannose-/fucose-containing glycoproteins on their envelopes. DC-SIGN can also initiate innate immunity by modulating toll-like receptors (2). DC-SIGN has been identified as a receptor for several viruses such as HIV and hepatitis C (1). On myeloid and preplasmacytoid DCs, DC-SIGN mediates dendritic cell rolling interactions with blood endothelium and the activation of CD4⁺ T cells, as well as the recognition of pathogenic haptens (3). DC-SIGN together with other C-type lectin receptors is involved in the recognition of tumors by dendritic cells and is a potential engineering target for dendritic cell-based cancer vaccines (4, 5).

Carbohydrate moieties in a variety of allergens were shown to bind to C-type lectin receptors such as DC-SIGN, mannose receptors, dectin on dendritic cells. DC-SIGN has a high affinity for fucosylated glycans in several plant glycoproteins and pathogens. This initial binding is thought to be crucial for the development of allergic sensitization (6, 7). The major peanut allergen Ara h 1 has been shown to bind DC-SIGN; Ara h 1

lium and the activation of CD4⁺ T cells, as well as the recognition of pathogenic haptens (3). DC-SIGN together with other C-type lectin receptors is involved in the recognition of tumors by dendritic cells and is a potential engineering target for dendritic cell-based cancer vaccines (4, 5).

alone was sufficient to activate DCs to prime naive T cells toward a Th2-biased phenotype (8). Several other common allergens such as BG60, Der p 2, Cyn d 1, Cor a 11 have been shown to bind DC-SIGN to induce Erk and Raf kinases (9, 10). In contrast, the mouse homologue SIGNR1 was shown to be required for oral tolerance to foods (11).

The precise role of DC-SIGN in food allergy pathogenesis is yet to be clarified. Hsu et al. (9) have used whole-food extracts and shown that some food proteins can also bind to DC-SIGN. However, the nature and identities of specific proteins in allergenic food are not fully elucidated. Because binding and uptake of glycosylated antigens mediated by C-type lectin receptors are crucial for immune response, we sought to characterize DC-SIGN-binding glycoproteins in a variety of foods. Using flow cytometry and recombinant DC-SIGN-Fc chimera labeled with I^{125} , we identified several DC-SIGN-binding proteins in a wide panel of allergenic foods and compared the MW of bands with that of published allergenic proteins from the respective foods. We then tested whether those DC-SIGN-binding glycoproteins are also targets for IgE binding (i.e. capable of inducing allergic reactions) using serum from respective food-allergic subjects. To our knowledge, this is the first comprehensive report on the characterization of DC-SIGN-binding proteins in major food allergens.

Materials and methods

DC-SIGN-Fc purification and radiolabeling

The DC-SIGN-human IgG-Fc construct was a kind gift from Dr. Wayne Shreffler (8); 293 cells were transfected with the DC-SIGN-Fc expression construct, followed by the isolation and purification of DC-SIGN-Fc proteins from cell culture supernatants by affinity chromatography (Arvys Proteins Inc., Stamford, CT, USA) and by radiolabelling with I^{125} (DiaMed Inc., Windham, ME, USA). Purified I^{125} -DC-SIGN-Fc protein was used as a primary probe in immunolabeling assay on protein extracts of food allergens.

DC-SIGN-ELISA

The wells of high-binding 96-well plates (Costar, Fisher Scientific, Pittsburgh, PA, USA) were coated in duplicate with indicated concentrations of peanut or soybean extracts for 18 h at 4°C, blocked with 1% bovine serum albumin (BSA) in TBST with $CaCl_2$ for 1 h at room temperature followed by incubation with purified recombinant DC-SIGN-Fc dissolved in blocking buffer for 1 h at room temperature. The plates were washed and incubated with horseradish peroxidase-conjugated anti-human IgG, followed by color development with TMB substrate for 10 min. The reaction was stopped by adding 50 μ l 2N sulfuric acid and the absorbance recorded at 450 nm in an ELISA reader.

Fluorescent labeling, blocking assays, and flow cytometry

Crude protein extracts were prepared as previously described (12). Peanut or soy protein extracts were labeled with

DyLight 488 dye at a concentration of 1 μ g/ml as per manufacturer's instructions (Pierce Biotechnology, Rockford, IL, USA). About 1×10^5 DCs were reconstituted in 10% FCS/PBS containing 100 μ g/ml calcium chloride ($CaCl_2$) and magnesium chloride ($MgCl_2$) (Sigma Aldrich Inc., St. Louis, MO, USA). About 2.5 μ g DyLight 488-labeled proteins were incubated with DCs on ice for 30 min, washed, and acquired in a BD-LSR2 flow cytometer. Antibody-blocking assays were performed by incubating DCs with 100 μ g/ml crude protein extracts from indicated foods for 30 min, followed by additional 30-min incubation with 10 μ l PE-conjugated anti-DC-SIGN antibody (Clone DCN46; BD BioSciences, San Jose, CA, USA), washing, and flow cytometry.

Deglycosylation and immunoprecipitation

DC-SIGN-Fc deglycosylation was performed using PNGase F (New England Biolabs Inc., Ipswich, MA, USA) as per the manufacturers' recommendations. The extent of deglycosylation was confirmed by the shift in MW on the gel (data not shown). Native or deglycosylated DC-SIGN-Fc proteins were bound to Dynabeads® M-270 Epoxy beads (Life Technologies, Grand Island, NY, USA). Immunoprecipitation of peanut agglutinin (PNA; 20 μ g) was performed in the presence of 10 mM $CaCl_2$ or 2 mM EDTA as per the manufacturer's recommendations. The eluate was run on SDS-PAGE/Western blots and probed with either anti-PNA antibody (0.5 μ g/ml) or DC-SIGN-Fc (1 μ g/ml) followed by appropriate alkaline phosphatase-conjugated secondary reagent and chemiluminescence development.

Immunoblotting and protein identification

Crude protein extracts were separated on reducing SDS-PAGE gels and transferred to nitrocellulose membrane, blocked with BSA. Autoradiography was performed by probing with I^{125} -labeled DC-SIGN-Fc in the presence or absence of 10 mM $CaCl_2$. The developed films were scanned, and image was processed with Adobe Photoshop. The molecular weights of the bands were estimated using Quantity One software (Bio-Rad Laboratories Inc., Hercules, CA, USA) and compared to known allergens (Table 1).

IgE immunoblotting was performed with pooled sera from food-allergic subjects diluted 1 : 5 for hazelnut and walnut extracts and 1 : 30 for the rest of the extracts with or without $CaCl_2$. After washing, the blots were probed with I^{125} -labeled anti-human IgE. Protein identification from SimplyBlue-stained bands was performed at the Wistar Institute Proteomic Facility using nanocapillary reverse-phase HPLC interfaced directly with hybrid linear ion trap mass spectrometers (LC/MS/MS). Purified peanut agglutinin (Sigma-Aldrich Inc., St. Louis, MO, USA) was tested for DC-SIGN-binding by immunoblot with DC-SIGN-Fc antibody in the presence or absence of 10 mM $CaCl_2$. Alkaline phosphatase-conjugated goat anti-human IgG was used as a secondary antibody followed by chemiluminescence.

Table 1 Characterization of DC-SIGN-binding food proteins

Binding score	Approximate MW of bands	Known allergen close to MW	
Peanut			
+++	65/62	62	Ara h 1
++	45/42	45, 42	Ara h 1/h 3 fragments
++	38	?	Ara h 1 fragment
++	33	?	Ara h 1 fragment?
+/-	28	30	Peanut agglutinin?
		23	Ara h 3 fragment
+/-	15	14	Ara h 6
Soy			
++	90	?	
+++	65	63-67	Gly m Bd 60 I/Gly m 5
+++	49	47-55	7S globulin-Gly m 5 partial/Gly m Bd 28K
+	38	35-40	Glycinin G1 (11S globulin)
+++	33	30	Gly m Bd 30K, P34-Gly m1
+/-	17	17	Gly m 4
		14	Gly m 3
Walnut			
+++	62	?	Jug r 2
		56	Jug n 2
+	52	?	Jug r 2
+	49	?	
		44	Jug r 2
++	35	?	
+	28	?	
+/-	15	14	Jug r 1
+/-	10		
Hazelnut			
+++	75	?	
+++	55	?	
		48	Cor a 11
+/-	45	?	
		40	Cor a 9
+/-	30	?	
Milk			
+++	70	69	BSA
+	49	?	
		36	β -Lactoglobulin
+	25	27	α -Casein
		23	β -Casein
		14-15	α -Lactalbumin
Egg			
++	98	?	
		76-77	Gal d 3 (ovotransferrin)
++	62	65-70	Gal d 5 (a-livetin) (yolk)
+	52	?	
+	45	45	(Gal d 2)
++	35	35	Phosvitin (transferase?)
		28	Gal d 1 (ovomucoid)
+/-	14	14.3	Gal d 4 (egg lysozyme)

The intensity of bands was visually scored as +++ high; ++ medium; and – low. The text in italics is the molecular weights of known allergens. '?' indicates that the identity of the bands is not known.

DC activation

Human monocyte-derived dendritic cells (MDDCs) were generated from freshly isolated CD14⁺ monocytes as before (13, 14). MDDCs ($1 \times 10^5/200 \mu\text{l}$ medium) were incubated for 18 h at 37 °C with 50–200 μg PNA, 100 μg endotoxin-free peanut extract, or 10 ng lipopolysaccharide (LPS, positive control). The cells were washed and stained with a cocktail of fluorescent-conjugated mAb against CD80, CD86, CD83, HLA-DR (BD Biosciences, San Jose, CA, USA) and Live/Dead stain (Invitrogen) followed by flow cytometric acquisition and analysis (BD-LSR2 flow cytometer, BD Biosciences, San Jose, CA, USA and FlowJo software, Tree Star Inc., Ashland, OR, USA.).

Human subjects

De-identified serum samples used in this study were provided by the Food Allergy Resource Initiative, which is supported by funding from Food Allergy Research and Education and maintained at the Elliot and Roslyn Jaffe Food Allergy Institute, Icahn School of Medicine at Mount Sinai. Serum samples were obtained from food-allergic subjects who had a positive for oral food challenge or had a convincing history of food allergy with high IgE levels against culprit foods. Pooled serum samples were used in IgE immunoblots. The IgE levels of the subjects are given in Table 2.

Results

Peanut and soybean proteins interact with DC-SIGN

Soybeans share amino acid sequence similarity to several peanut allergens. However, the allergenicity of soy is generally weaker and the incidence of soy allergy is low compared to peanut allergy (15, 16). We sought to determine whether this difference is due to DC-SIGN interaction with soy allergens (or its absence). We first compared the binding of DC-SIGN to peanut and soybean extracts by ELISA. ELISA plates were coated with peanut or soybean protein extracts (10 $\mu\text{g}/\text{ml}$) and probed with various concentrations of DC-SIGN-Fc followed by anti-human IgG-HRP and TMB substrate (Fig. 1A). The presence of DC-SIGN-binding proteins in protein extracts is revealed by higher absorbance in ELISA. Both peanut and soybean extracts bound to DC-SIGN-Fc in a dose-dependent fashion. Surprisingly, the absorbance was even higher with soybean extract than with peanut, indicating the presence of DC-SIGN interacting proteins in soy.

We tested whether peanut and soy proteins interact with DC-SIGN in a more physiological setting. Human MDDCs were incubated with 2.5 $\mu\text{g}/100 \mu\text{l}$ of peanut or soy extracts conjugated with DyLight 488 fluorescent dye for 30 min on ice, washed, and analyzed by flow cytometry. Both peanut–DyLight 488 and soy–DyLight 488 conjugates showed binding to MDDCs (Fig. 1B). However, the MFI for soy proteins is higher than for peanut, which is consistent with the ELISA results (Fig. 1A). We then performed a DC-SIGN-blocking

Table 2 Allergen-specific IgE levels of food-allergic subjects

Food-allergic subject	Specific IgE kU/l
Peanut	
1	7.35
2	47.7
3	<0.35
4	8.85
5	1.59
Soy	
1	59.4
2	35.4
3	55.5
4	25.5
Walnut	
1	2.83
2	1.74
3	1.47
4	7.71
5	<0.35
6	1.36
7	2.62
Hazelnut	
1	16.2
2	4.87
3	33.4
4	2.88
Milk	
1	>100
2	>100
3	>100
4	>100
5	>100
6	>100
7	>100
Egg	
1	>100
2	>100
3	>100
4	60.1
5	80.4

assay in which human MDDCs were incubated with crude protein extracts from peanut or soy for 30 min on ice followed by staining with PE-conjugated anti-DC-SIGN antibody and flow cytometry. The antibody binding was compared to MDDCs alone. Incubation of MDDCs with either peanut or soy extracts reduced the MFI of antibody staining (Fig. 1C, D). Consistent with Fig. 1A, B, the binding of soy extract to MDDCs was higher than that to peanut as shown by a reduction of about 30–40% of anti-DC-SIGN binding with soy compared to 10–15% with peanut-incubated MDDCs (Fig. 1E). These results suggest that both peanut and soy proteins bind to DC-SIGN expressed in MDDCs. The increased DC-SIGN binding to soy extract might be either due to higher binding efficiency or a greater number of DC-SIGN-binding molecules present in soy, or both.

We further sought to characterize the molecular nature of DC-SIGN-binding proteins in both peanut and soy. Crude protein extracts from peanut and soy were run on SDS-PAGE/Western blots and probed with DC-SIGN-Fc chimera labeled with I^{125} in the presence or absence of $CaCl_2$ followed by autoradiography. At least six protein bands were identified in autoradiographs of peanut extract with DC-SIGN-Fc- I^{125} when probed in the presence of calcium ions (Ca^{2+} , calcium chloride) (Fig. 1F, lane 3). The MWs of specific DC-SIGN-binding proteins ranged from >100–14 kDa. Most prominent bands were seen at approximate MWs of 100, 65, 55, 38, 33, and 28 kDa (Table 1). In the absence of Ca^{2+} , a single prominent band at about 40 kDa was observed (Fig. 1F, lane 2), indicating that this protein binds to DC-SIGN in a nonspecific, Ca^{2+} -independent manner. To test whether the DC-SIGN-binding proteins correspond to known peanut allergens, we compared the autoradiography results to IgE immunoblots using pooled sera from peanut-allergic subjects. The IgE immunoblots showed prominent reactivity to Ara h 1, Ara h 2, and Ara h six bands. Notably, there were three bands with approximate MWs of 38, 33, and 28 kDa in peanut extracts bound to DC-SIGN, but were not identified in IgE immunoblots. It is unlikely that these DC-SIGN-binding proteins belong to Ara h three fragments as the MW of the bands differs from published Ara h three fragment sizes of 45, 42, and 23 kDa (17).

DC-SIGN-Fc autoradiography with soy extracts identified several proteins ranging from MW 98 to 6 kDa (Fig. 1G, lane 3 & Table 1). Six prominent bands were seen at approximate MWs of 90, 65, 49, 38, 33, and 17 kDa. Interestingly, most of these DC-SIGN-binding proteins in soybeans showed reactivity to IgE when probed with serum from soy-allergic subjects except two bands at approximate MWs of 10 and 3 kDa. Among these bands, five corresponded to well-known soy allergens (18), namely Gly m 1 [Gly m Bd 30K (33 kDa)], Gly m Bd 60 l (65 kDa), Gly m 5 [7S globulin (49 kDa)], Gly m 6 [glycinin G1 (38 kDa)], and Gly m 4 (17 kDa). These data suggest that several proteins in peanut and soy bind to DC-SIGN (in addition to Ara h1 in peanut) and that many of these proteins correspond to well-known allergenic proteins.

Peanut agglutinin is a specific ligand for DC-SIGN

To better characterize DC-SIGN-binding, but non-IgE-inducing, proteins in peanut, we performed LC-MS/MS analysis on the individual protein bands (Fig. 1F). In addition to the presence of Ara h1 fragments in these bands, LC-MS/MS data revealed the presence of galactose-binding lectin, late embryogenesis-abundant protein group 4 and group 2 proteins (~25–30 kDa); Arachin 6 and late embryogenesis-abundant protein group 2 protein (~38 kDa). It is likely that the Arachin 6 binds to DC-SIGN in a nonspecific manner as this protein was detected in control autoradiograph probed in the absence of $CaCl_2$. Among these protein bands, galactin-binding lectin, also known as peanut agglutinin (PNA), was reported to be a minor allergen (19). As Ara h1 fragments

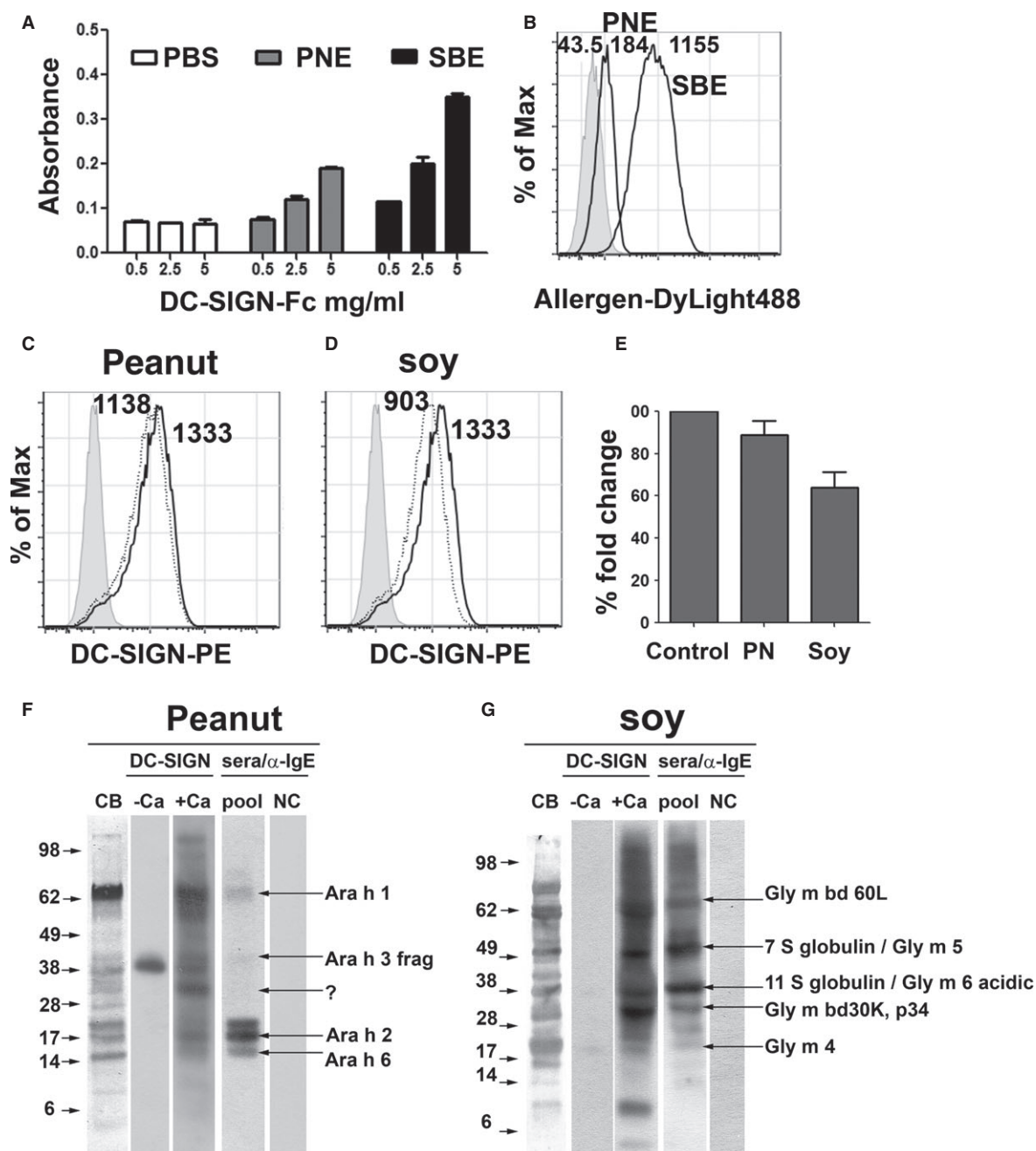


Figure 1 Molecular characterization of DC-SIGN-binding proteins in peanut and soy. ELISA plates were coated in duplicate with peanut or soybean protein extracts (10 μ g/ml), washed, and probed with various concentrations of DC-SIGN-Fc followed by anti-human IgG-HRP and TMB substrate (A). MDDCs were incubated with 2.5 μ g/100 μ l of peanut or soy extracts conjugated with DyLight 488 fluorescent dye for 30 min on ice, washed, and analyzed by flow cytometry (B). Human MDDCs from three healthy volunteers were incubated with 100 μ g/ml of crude protein extracts from peanut (C) or soy (D) for 30 min on ice followed by staining with PE-conjugated anti-DC-SIGN antibody and flow cytometry. A representative

histogram is shown. The % fold change in mean fluorescent intensity is shown as bar graph (E). Crude protein extracts from peanut (F) or soy (G) were run on SDS-PAGE/Western blot. The transferred proteins were stained with Coomassie Blue stain (lane: CB) or probed with ^{125} I-DC-SIGN-Fc chimera in the presence or absence of CaCl_2 followed by autoradiography (lanes: -Ca and +Ca, respectively). IgE immunoblots were performed using pooled serum from peanut (F)- or soy (G)-allergic subjects (lane: pool) or nonspecific serum from healthy volunteers (lane: NC). The molecular weight of the bands was estimated using standards run along with the extracts.

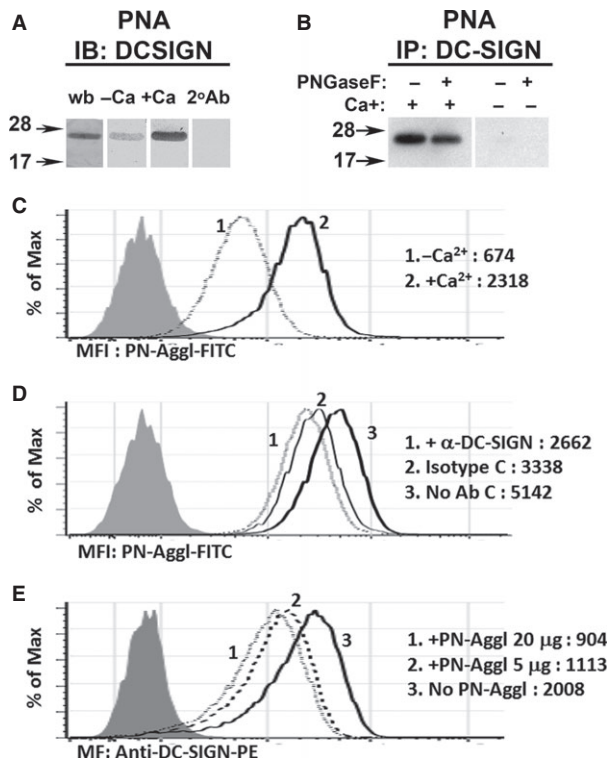


Figure 2 Peanut agglutinin is a novel ligand for DC-SIGN. Purified PNA was run on SDS-PAGE/Western blot (A). The transferred proteins were stained with Ponceau stain (lane: wb) or probed with DC-SIGN-Fc in the presence or absence of CaCl_2 (lanes: -Ca and +Ca, respectively) followed by immunoblot with anti-human alkaline phosphatase and chemiluminescent substrate. PNA was immunoprecipitated with native or PNGase F-deglycosylated DC-SIGN in the presence of CaCl_2 (Ca+) or EDTA (Ca-). The eluate was run on SDS-PAGE/Western blot and probed with anti-PNA antibody (B). Human MDDCs from a healthy volunteer are stained with PNA-FITC in the presence or absence of CaCl_2 (C) or in the presence of anti-DC-SIGN-blocking antibody or isotype control (D) followed by flow cytometry. MDDCs were stained with DC-SIGN-PE in the presence of purified PNA along with no PNA control followed by flow cytometry (E).

were also present in bands of ~25–30 kDa and identified as hits in LC-MS/MS, we further investigated whether PNA is indeed a ligand for DC-SIGN or just presents as a nonspecific band in our DC-SIGN autoradiograms. To this end, we performed immunoblots with pure PNA and probed with DC-SIGN-Fc antibody followed by detection with alkaline phosphatase-conjugated secondary antibody (Fig. 2A). A prominent single band of PNA at ~30 kDa was observed on Western blots, and this band bound to DC-SIGN in Ca^{2+} -dependent manner.

To further confirm the specificity of PNA binding to DC-SIGN, we performed immunoprecipitations with native and DC-SIGN-Fc protein deglycosylated with PNGase F (Fig. 2B). PNGase F treatment cleaves the GlcNAc-Asn linkage of N-linked oligosaccharides in glycoproteins. DC-SIGN-Fc deglycosylated under nondenaturing conditions resulted in

low MW DC-SIGN-Fc bands in SDS gels, confirming deglycosylation of native glycoprotein (data not shown). Native or deglycosylated DC-SIGN-Fc proteins were covalently bound to Dynabeads and immunoprecipitated with PNA in the presence of CaCl_2 or EDTA. The eluate was probed with anti-PNA antibody in immunoblots. Both native and deglycosylated DC-SIGN-Fc were able to bind and precipitate PNA in the presence of CaCl_2 . This binding was not seen when immunoprecipitation was performed in calcium-free conditions (in the presence of EDTA) (Fig. 2B). These results confirm that DC-SIGN–PNA interaction is calcium dependent and not specific to GlcNAc-containing sugar moieties in DC-SIGN-Fc. In addition, these data also indicate that the glycosylated moieties in the 'Fc' portion of the DC-SIGN-Fc chimera do not bind to PNA (because the Fc portion contains GlcNAc structures cleaved by PNGases and also due to the fact that the Fc part was immobilized to paramagnetic beads).

To investigate DC-SIGN–PNA interaction in a physiological setting, we then performed PNA binding assays with human MDDCs. FITC-conjugated PNA specifically bound to MDDCs in the presence of Ca^{2+} (Fig. 2C). This binding was inhibited in the presence of competing anti-DC-SIGN-blocking antibody (clone 120507) in the presence of Ca^{2+} (Fig. 2D). The efficiency of anti-DC-SIGN-blocking antibody is modest as direct competitive binding of this mAb with another PE-conjugated anti-DC-SIGN mAb (clone DCN46) resulted in binding inhibition of about 35% (data not shown) and is consistent with the data shown in Fig. 2D. We further confirmed the binding activity of PNA by incubating MDDCs with purified PNA followed by staining with PE-conjugated anti-DC-SIGN antibody in the presence of Ca^{2+} (Fig. 2E). The decrease in MFI of DC-SIGN staining indicates that PNA binding to DC-SIGN is specific and dose dependent. These results demonstrate that the minor peanut allergen, PNA, is a novel ligand for DC-SIGN.

PNA-induced DC maturation and expression of costimulatory molecules

We investigated the functional role of PNA by assessing DC activation and maturation *in vitro*. Human MDDCs were incubated with 100 μg PNA for 18 h at 37 °C. PNA induced the expression of DC activation markers CD80, CD86, HLA-DR and maturation marker CD83 in a dose-dependent fashion (Fig. 3). The level of PNA-induced expression of these markers was equivalent to that seen with whole peanut extract. PNA induced a modest, but detectable increase in the expression of CD83 compared to peanut or LPS control (Fig. 3A, B). Moreover, we also observed that PNA induced a dose-dependent increase in the MFI of CD80 and CD83, but not HLA-DR staining. These data demonstrated the inherent DC activation potential of PNA.

DC-SIGN binding to food protein extracts

Identification of several DC-SIGN-binding proteins in peanut and soy that showed reactivity to serum IgE led us to exam-

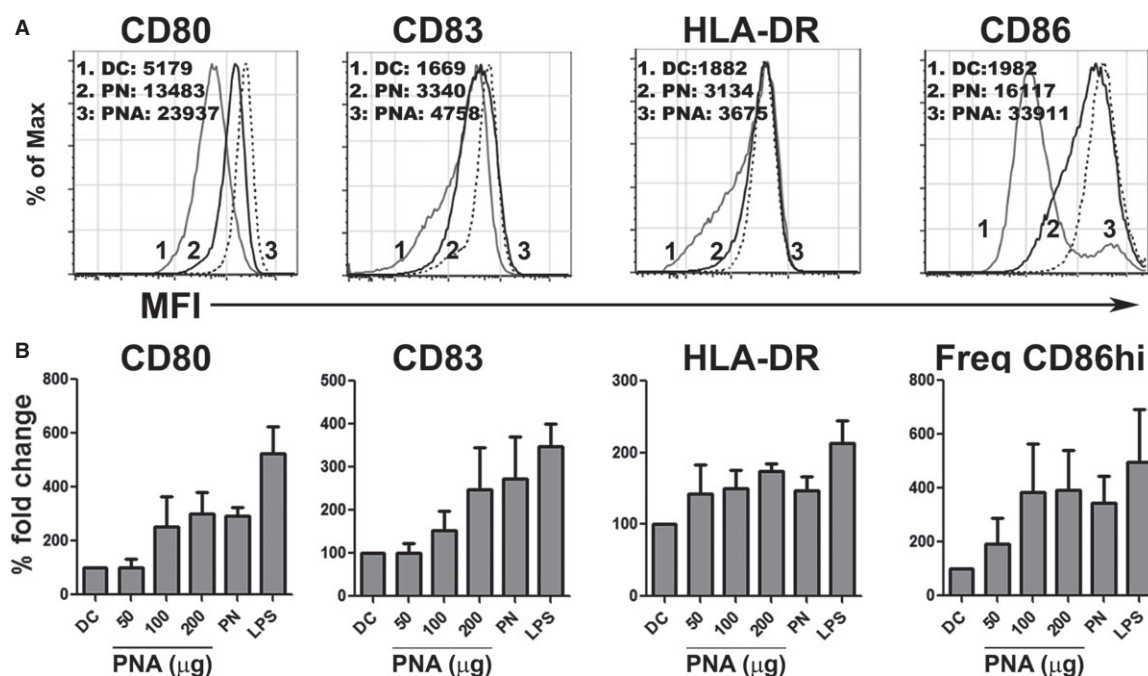


Figure 3 Peanut agglutinin induces DC activation and maturation. Human MDDCs were activated with PNA (100 μ g), peanut extract (100 μ g), or LPS (10 ng) for 18 h at 37°C and stained with CD80, CD83, CD86, and HLA-DR (A). The percentage fold increase com-

pared to medium along controls (100%) in MFI of CD80, CD83, and HLA-DR and the frequency of CD86hi DCs at PNA concentrations of 50, 100, or 200 μ g were plotted as bar graphs (B). Data from four individual experiments are shown.

ine DC-SIGN binding in other representative food allergens. We performed DC-SIGN-blocking assays, DC-SIGN-Fc autoradiography, and IgE immunoblotting with protein extracts of tree nuts (walnut and hazelnut) (Fig. 4), cow's milk, and chicken egg (Fig. 5) and less-allergenic foods such as pine nuts, chickpea, and corn (Fig. 6) (Table 1). The DC-SIGN-antibody-blocking assay in MDDCs showed that in the presence of either walnut or hazelnut extracts, the binding of anti-DC-SIGN antibody is decreased by about 25% and 20%, respectively (Fig. 4A–C). DC-SIGN autoradiography with walnut and hazelnut extracts showed multiple bands, but only a few of them react to serum IgE from walnut- and hazelnut-allergic subjects, respectively (Fig. 4D, E). We found that only Jug r 4 from walnut and Cor a 11 from hazelnut reacted to both DC-SIGN and IgE. The identity of DC-SIGN-binding, but non-IgE-reactive, proteins in walnut and hazelnuts is unknown. Likewise, we identified multiple DC-SIGN-binding proteins in cashew, Brazil nut, pecan, and pistachio (data not shown). However, IgE reactivity to these bands is not known.

Similar to tree nuts, milk and egg extracts prevented the binding of anti-DC-SIGN antibody by about 25% (Fig. 5A–C). In DC-SIGN autoradiography with cow's milk, bovine serum albumin and β -casein were bound to both DC-SIGN and IgE, while β -lactoglobulin and α -lactalbumin bound only to IgE, but not DC-SIGN (Fig. 5D). All known egg allergens such as Gal d 1, –2, –3, –4, and –5 were found reactive to both DC-SIGN and serum IgE from egg-allergic subjects.

Two unknown proteins of approximately 98 and 108 kD showed reactivity similar to both DC-SIGN and IgE immunoblots. An unknown 50-kDa band was seen in DC-SIGN immunoblots, but not in IgE immunoblot (Fig. 5E). Interestingly, DC-SIGN immunoblots from foods that generally do not induce allergic responses in humans, such as pine nuts, chickpea, and corn, showed no bands with the exception of a single 62-kDa band in corn (Fig. 6).

Discussion

We have identified several DC-SIGN-binding glycoproteins in a panel of allergenic food extracts. Traditional immunoblotting techniques to characterize DC-SIGN-binding proteins have proved tedious due to high background and nonspecific signals from secondary antibodies labeled with horseradish peroxidase or alkaline phosphatase (data not shown). To circumvent this issue, we have utilized a highly sensitive radioactively tagged DC-SIGN-Fc as a primary probe for autoradiography. The binding of DC-SIGN in the immunoblots is also specific and Ca^{2+} dependent, as we could not detect any bands in autoradiograms when the blots are processed with Ca^{2+} -free buffers.

We demonstrated the presence of many other DC-SIGN-binding proteins in peanut in addition to Ara h 1, which was previously shown to bind DC-SIGN (8). By employing DC-SIGN autoradiograms, mass spectrometry, DC-binding assays, and DC-blocking assays, we have systematically iden-

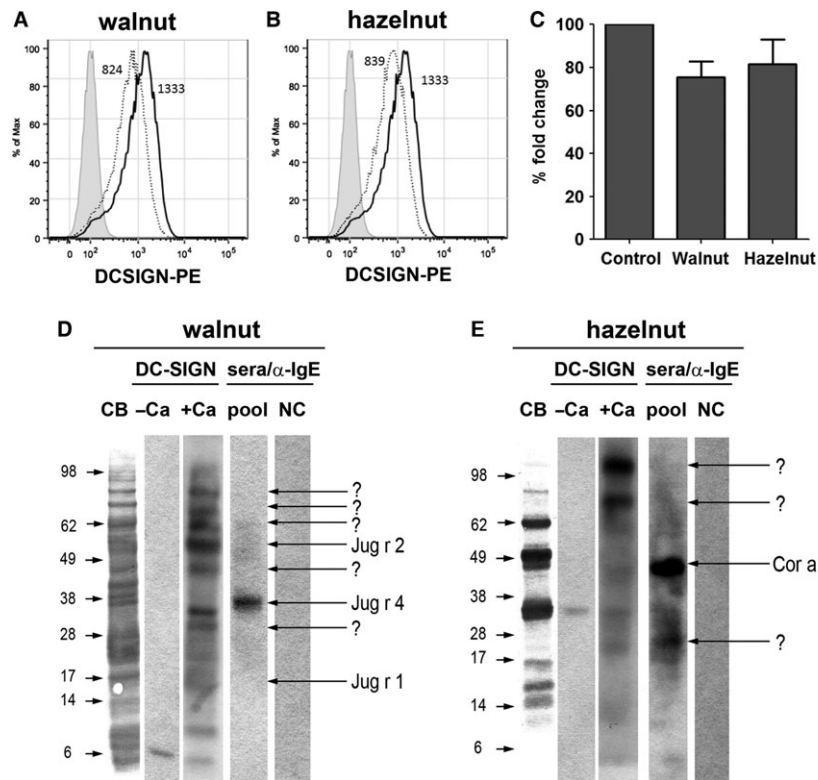


Figure 4 Molecular characterization of DC-SIGN-binding proteins in tree nuts. Human MDDCs from three healthy volunteers were incubated with 100 $\mu\text{g}/\text{ml}$ of crude protein extracts from walnut (A) or hazelnut (B) for 30 min on ice followed by staining with PE-conjugated anti-DC-SIGN antibody and flow cytometry. A representative histogram is shown. The % fold change in mean fluorescent intensity is shown as bar graph (C). Crude protein extracts from walnut (D) or hazelnut (E) were run on SDS-PAGE/Western blot. The trans-

ferred proteins were stained with Coomassie Blue stain (lane: CB) or probed with ^{125}I -DC-SIGN-Fc chimera in the presence or absence of CaCl_2 followed by autoradiography (lanes: -Ca and +Ca, respectively). IgE immunoblots were performed using pooled serum from peanut (A)- or soy (B)-allergic subjects (lane: pool) or nonspecific serum from healthy volunteers (lane: NC). The molecular weight of the bands was estimated using standards run along with the extracts.

tified and demonstrated that galactose-binding lectin is a novel ligand for DC-SIGN in peanut (Figs 1 and 2). This receptor–ligand interaction is independent of GlcNAc-containing high mannose, hybrid, or complex glycan residues in the DC-SIGN molecule, as shown in immunoprecipitation experiments (Fig. 2). PNA, also known as galactose-binding lectin in peanut with a MW ranging from 24 to 30 kDa, is considered a minor allergen (19). PNA is a heat-stable homotetrameric nonglycosylated lectin in peanut (20). PNA uptake occurs rapidly from the gastrointestinal tract after peanut ingestion and can be detected in the circulation in a biologically active form at serum concentrations of up to 5 $\mu\text{g}/\text{ml}$ (21, 22). In contrast, the abundant major peanut allergens Ara h 1 and Ara h 3 proteins are susceptible to enzymatic digestion in the gut (23). PNA has been shown to induce the proliferation of rectal epithelial cells in human subjects at physiologically achievable systemic concentrations (24).

Soybean is recognized as one of the eight major food allergens, although reactions are typically milder and the allergy is generally outgrown. Soybean allergens share sequence simi-

larity with corresponding peanut allergens (18, 25). However, the incidence of exclusive soybean allergy is very low (16, 26), and the allergic symptoms are usually minor and often observed in subjects allergic to multiple foods and birch pollen (27). Why soy is less allergenic than peanut is a subject of interest and has been previously discussed (15, 16). We speculated that soy proteins may also show reduced/altered interaction with C-type lectin receptors such as DC-SIGN that are essential for priming a Th2 response. Surprisingly, soybean proteins show high binding to DC-SIGN in ELISA and antibody-blocking assays (Fig. 1) when used at concentrations similar to peanut, suggesting that there are multiple DC-SIGN proteins in soy, which was confirmed by DC-SIGN autoradiography (Fig. 2). Soybeans contain high quantities of immunomodulatory isoflavones and are the largest source of these phytochemicals in the human diet (28). The presence of isoflavones in soy extracts (which is predominantly protein bound) is likely responsible for the low severity in soy allergy, based on our previous results that these isoflavones are able to suppress allergic immune responses to peanut (13) and mucosal antigens (14) through their action

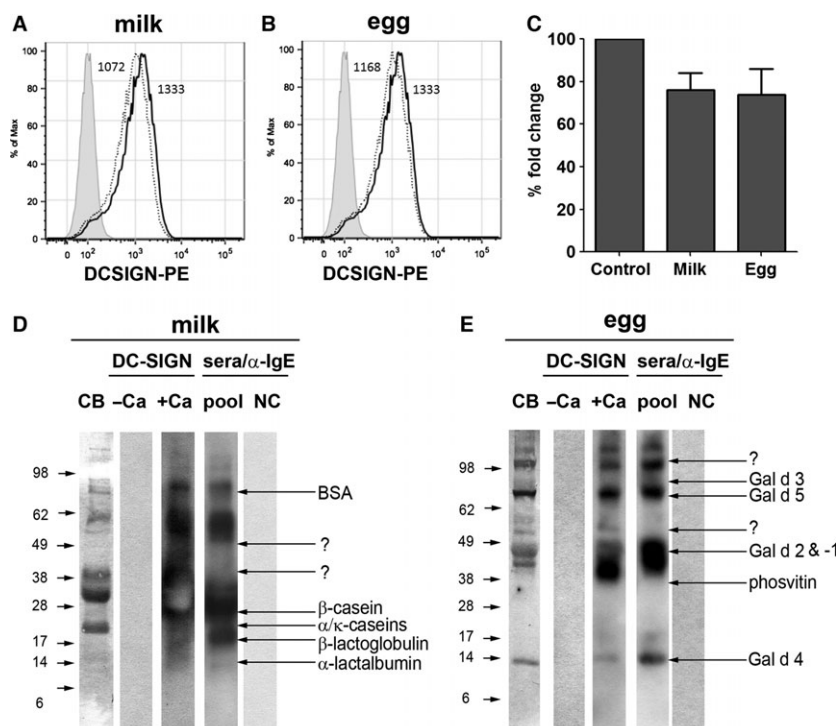


Figure 5 Molecular characterization of DC-SIGN-binding proteins in cow's milk and chicken egg. Human MDDCs from three healthy volunteers were incubated with 100 μ g/ml of crude protein extracts from cow's milk (A) or chicken egg (B) for 30 min on ice followed by staining with PE-conjugated anti-DC-SIGN antibody and flow cytometry. A representative histogram is shown. The % fold change in mean fluorescent intensity is shown as bar graph (C). Crude protein extracts from cow's milk (D) or chicken egg (E) were

run on SDS-PAGE/Western blot. The transferred proteins were stained with Coomassie Blue stain (lane: CB) or probed with ^{125} I-DC-SIGN-Fc chimera in the presence or absence of CaCl_2 followed by autoradiography (lanes: -Ca and +Ca, respectively). IgE immunoblots were performed using pooled serum from peanut (A)- or soy (B)-allergic subjects (lane: pool) or nonspecific serum from healthy volunteers (lane: NC). The molecular weight of the bands was estimated using standards run along with the extracts.

on DC function. It is also possible that the interaction of non-IgE-binding soy proteins with DC-SIGN may promote regulatory responses contributing to less severe allergies in soy.

It is interesting to note that several IgE-inducing food-allergenic proteins also bind to DC-SIGN. However, not all of DC-SIGN-binding proteins induce IgE responses (as seen in IgE blots, Figs 1, 4 and 5). The identity and function of these non-IgE- but DC-SIGN-binding proteins are not known. Studies in grass pollen-allergic subjects showed that there is no clear correlation between antigen-specific Th2-cell responses and IgE levels (29, 30). It is well established that allergic inflammation can occur in the absence of IgE (31) and that humoral IgE responses have been shown to be critically dependent on Tfh-, but not Th2-, derived IL-4 (32). Recently, Schulten et al. (33) identified several Th2-polarizing non-IgE-inducing proteins in grass pollen allergens. In this light, it is tempting to speculate that the non-IgE-binding glycoproteins that interact with DC-SIGN are novel molecules that might provide the so-called antigen bridge where T-cell responses specific to an epitope provide help to B cells for other epitopes within the same antigen or even other proximal antigens when processed together (as in a pollen or virus particles). This con-

cept has been recently proposed for novel Timothy grass proteins that elicited Th2, but not IgE, responses (34).

Glycated DC-SIGN-binding proteins may be involved in Th1, Tfh, or regulatory T-cell responses, or even presented to CD8^+ T cells. The outcome of DC-SIGN engagement and signaling is pleiotropic depending on the nature of the cross-linking ligand, and the responses are tailored to specific antigen/pathogen (35–39), which also differ between humans and mice (1). DC-SIGN binds to a wide variety of pathogens such as HIV-1, Ebola, and several bacteria including *Mycobacterium tuberculosis*, *Lactobacillus*, and *Bifidobacterium* (37–39). DC-SIGN ligation with specific antibodies resulted in LPS-induced IL-10 and synergized with TNF- α for IL-10 secretion (40). DC-SIGN-mediated generation of Foxp3 $^+$ Tregs has been reported for the probiotic *Lactobacillus* as well as bacterial manLam (35, 36, 41). However, *Mycobacterium tuberculosis* can also induce Th1 while inhibiting Th17 responses through DC-SIGN (39).

In the context of food allergy, lack of SIGNR1, the DC-SIGN homologue in mice, results in oral tolerance to food-induced anaphylaxis (11). In this report, we demonstrate that the minor peanut allergen PNA binds to DC-SIGN (Fig. 3), similar to that shown for Ara h 1 (8). However, silencing of

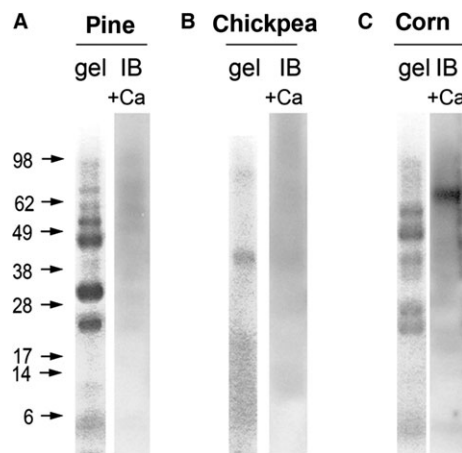


Figure 6 DC-SIGN binding to less-allergenic foods. Crude protein extracts from pine (A) or chickpea (B) and corn (C) were run on SDS-PAGE/Western blot. One lane of the gel was cut before transfer and stained with Coomassie Blue stain (lane: gel). The Western blots were probed with ^{125}I -DC-SIGN-Fc chimera in the presence of CaCl_2 followed by autoradiography (lanes: +Ca). The molecular weight of the bands was estimated using standards run along with the extracts.

DC-SIGN in DCs promotes Th2 phenotype in a Der p 1-preloaded DC-T-cell cocultures, but knocking down mannose receptors resulted in the opposite effect (i.e. Th1 skewing) (10). Thus, identification of additional DC-SIGN-binding proteins in peanut and in other allergenic foods leads us to ponder the nature of Th-cell induction with those individual proteins. Investigating T-cell responses to the novel proteins that bind DC-SIGN, but do not induce IgE antibody responses, may unravel specific immune mechanisms involved in allergen recognition.

It is likely that several other members of this C-type lectin receptor family such as dectins and mannose receptors, in addition to DC-SIGN, also bind to multiple proteins in allergenic foods during an immune response. It is possible that PNA, a plant lectin, may nonspecifically bind to DC surface protein (in addition to DC-SIGN) leading to membrane reorganization or steric hindrance of DC-SIGN binding leading to nonspecific responses. The relative balance of signaling through these receptors can ultimately determine the effector

Th response. In addition, we cannot rule out the possibility that the concentrations of allergen-reactive IgE to some of the DC-SIGN-binding proteins are very low in the serum of these allergic subjects and therefore not detected as IgE-binding proteins. Another limitation of this study is that due to the complex nature of glycoprotein interactions, the binding of DC-SIGN to ligands in food extracts may be prone to nonspecific signals even under carefully controlled experimental conditions. In addition, immunoblot analyses and MW determination alone are not sufficient to determine the exact identities of the DC-SIGN-binding proteins in food allergens, because the artifacts from cross-reactive contaminations cannot be fully excluded.

Yet, this study for the first time sheds light on the presence of a number of DC-SIGN-binding proteins in allergenic foods. A total of 43 proteins bands from six food protein extracts were detected in DC-SIGN immunoblots (Table 1). Of them, the identities of at least 15 proteins are not known based on the estimated MW. Exact determination of DC-SIGN-binding proteins will need to be performed independently for each allergenic food. Further functional characterization of these IgE-binding and non-IgE-binding, DC-SIGN-interacting molecules may provide new openings in our understanding of the pathology of food allergy and even novel therapeutic targets.

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Author contributions

MM and HAS designed the experiments and analyzed the data; MM, MK, LC, and GG performed the experiments; MM, MK, and GG wrote the manuscript.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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