

Functional Interaction of Common Allergens and a C-type Lectin Receptor, Dendritic Cell-specific ICAM3-grabbing Non-integrin (DC-SIGN), on Human Dendritic Cells^{*[5]}

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Fucosylated glycans on pathogens are known to shape the immune response through their interaction with pattern recognition receptors, such as C-type lectin receptors (CLRs), on dendritic cells (DCs). Similar fucosylated structures are also commonly found in a variety of allergens, but their functional significance remains unclear. To test a hypothesis that allergen-associated glycans serve as the molecular patterns in functional interaction with CLRs, an enzyme-linked immunosorbent assay-based binding assay was performed to determine the binding activity of purified allergens and allergen extracts. THP-1 cells and monocyte-derived DCs (MDDCs) were investigated as a model for testing the functional effects of allergen-CLR interaction using enzyme-linked immunosorbent assay, Western blotting, and flow cytometry. Significant and saturable bindings of allergens and allergen extracts with variable binding activities to DC-specific ICAM3-grabbing non-integrin (DC-SIGN) and its related receptor, L-SIGN, were found. These include bovine serum albumin coupled with a common glycoform (fucosylated glycan lacking the α 1,3-linked mannose) of allergens and a panel of purified allergens, including BG60 (Cyn dBG-60; Bermuda grass pollen) and Der p2 (house dust mite). The binding activity was calcium-dependent and inhibitable by fucose and Lewis-x trisaccharides (Le^x). In THP-1 cells and human MDDCs, BG60-DC-SIGN interaction led to the activation of Raf-1 and ERK kinases and the induction of tumor necrosis factor- α expression. This effect could be blocked, in part, by Raf-1 inhibitor or anti-DC-SIGN antibodies and was significantly reduced in cells with DC-SIGN knockdown. These results suggest that allergens are able to interact with DC-SIGN and induce tumor necrosis factor- α expression in MDDCs via, in part, Raf-1 signaling pathways.

It has been established that recognition of heteroglycans on various pathogens is critical in innate and adaptive immunity (1, 2). Dendritic cells (DCs)³ are known to utilize their innate receptor repertoire of molecular pattern-recognition TLRs and CLRs to generate innate immunity and influence subsequent adaptive response (3, 4).

TLRs are transmembrane proteins that can interact with certain microbial patterns to direct the subsequent immune response (4). CLRs, on the other hand, are crucial in recognition of complex glycan structures on various pathogens and have evolved to facilitate the endocytosis and presentation of pathogens (5, 6). For example, human DC-SIGN (CD209) and a related receptor, DC-SIGNR (L-SIGN; CD299), contain a C-type carbohydrate-recognition domain and are known to bind to high mannose oligosaccharides and several fucosylated Lewis glycoforms with different binding patterns (7, 8). Several human pathogens, including *Helicobacter pylori* and *Schistosoma mansoni*, are known to express fucosylated glycans, which are often associated with pathogen-induced Th2-biased adaptive immunity (7). We have previously shown that a neoglyco-antigen coupled with Lewis-x (Le^x) trisaccharides elicited potent Th2-biased immune response through their ability to modulate IL-12p70 expression (9). Studies of pathogen- and neoglycoprotein-DC interactions have, therefore, provided crucial information regarding the role of CLRs in generating distinct functional pathways to generate host immunity, facilitate the evasion mechanism of pathogens, or induce a state of tolerance.

Thus far, the direct interaction between allergens and CLRs has not been clearly demonstrated, and the potential role of CLRs in the regulation of allergic responses has not been investigated, but the mere fact that most allergens contain complex

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³ The abbreviations used are: DC, dendritic cell; MDDC, monocyte-derived DC; CLR, C-type lectin receptor; DC-SIGN, DC-specific ICAM3-grabbing non-integrin; L-SIGN, DC-specific ICAM3-grabbing non-integrin-related; miRNA, microRNA; ERK, extracellular signal-regulated kinase; TNF, tumor necrosis factor; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; IL, interleukin; Abs, antibodies; BSA, bovine serum albumin; PMA, phorbol 12-myristate 13-acetate; LV, lentivirus; MAPK, mitogen-activated protein kinase; TLR, Toll-like receptor.

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glycan structures raises the possibility that the allergen-CLR interaction may modulate DCs and subsequent immune response. Indeed, complex glycans bearing $\alpha(1-3)$ -fucosylated and xylosylated glycans are commonly present in plant, insect, and food allergens and have contributed to much of the cross-reactivity in IgE recognition (10–12), although these glycan-specific IgE Abs may not be clinically relevant and the immunological significance of these allergen-associated glycans remains still obscure. Three glycoforms, MMXF, MOXF and MMF, are commonly found in allergens based, in part, on the analysis of glycan-specific Abs and, in a few cases, of glycan structures in purified allergens. One non-mammalian type N-glycan structure, MMXF with $\alpha 1,3$ -linked fucose, is found in allergens such as those in Timothy grass, Phl p 1 (13), whereas in some cases, an MOXF glycan lacking the $\alpha 1,3$ -linked mannose is noted, for example, for bromelain (10). In contrast, allergens of Bermuda grass pollens, such as BG60, carry MMF-type structure without xylose linkage to the terminal mannose (14). Some allergens, such as peanut Ara h 1, lack the core fucosyl residue (15), whereas a hazelnut allergen, Cor a11, carries a mixed type of glycan structures, MMX and MMXF (16).

It is, thus, likely that those common glycans on allergens may, in fact, serve as a critical molecular pattern in CLR recognition in DCs. To begin testing this possibility, we have designed experimental strategies to address a specific hypothesis that allergens interact with CLRs and subsequently modulate DC functions. Evidence is provided herein supporting the existence of a functional allergen-CLR axis in human DCs.

EXPERIMENTAL PROCEDURES

Reagents—Bovine serum albumin (BSA) and α -L-fucose were purchased from Sigma. Lewis-x-BSA (Le^x-BSA), Le^x, and N-acetyllactosamine were obtained from V-LABS (Covington, LA). Natural and recombinant Der p2 and natural Asp f1, a mold allergen, were purchased from INDOOR Biotechnologies, Inc. (Charlottesville, VA). A neoglycoprotein, MOXF₃-BSA, was prepared by cross-linking BSA with a bromelain glycopeptide containing MOXF₃ glycan that lacks the $\alpha 1,3$ -linked mannose (10). Mannosylated BSA with 51 mannosides coupled to BSA (Man₅₁-BSA) was prepared as described (17) and served as a positive control. A panel of common allergen extracts used for skin testing was purchased from Greer Laboratories Inc. (Lenoir, NC).

Binding Analysis—In the solid-phase binding assay, the microplates were first coated with varying concentrations of the test antigens in 0.05 M carbonate-bicarbonate buffer (pH 9.6) and blocked with assay buffer (10 mM Tris-Cl, 50 mM CaCl₂, 150 mM NaCl, and 2.5% goat serum). The plates were then incubated with 0.5 μ g/ml of recombinant human DC-SIGN-Fc or L-SIGN-Fc chimeras (R&D Systems, Minneapolis, MN) at room temperature for 2 h followed by incubation with peroxidase-conjugated goat anti-human IgG Fc Abs (Pierce) for 1 h at room temperature and by the addition of a substrate, tetramethylbenzidine (R&D Systems). The reactions were stopped by the addition of 2 N H₂SO₄ and read in a microplate reader. The relative binding activity was expressed as optical density for each test antigen after subtracting the values from the background. In some cases, blocking reagents, fucose (2.5

mm), Le^x (2.5 mm), N-acetyllactosamine (2.5 mm), and a calcium chelator, EDTA (10 mm), were added in conjunction with the addition of DC-SIGN-Fc fusion proteins for 2 h. Also, the relative binding affinities of allergens for DC-SIGN were assessed by an inhibition assay, wherein varying concentrations of allergens were used as competitors for binding 25 nM europium-labeled Man₅₁-BSA (Eu-Man₅₁-BSA; Ref. 18) to DC-SIGN-Fc fusion proteins. In this assay, 3 μ g/ml of goat anti-human IgG was coated onto microtiter plates and incubated with 1 μ g/ml DC-SIGN-Fc chimera for 1 h at room temperature. After washing, varying concentrations of allergens and 50 nM Eu-Man₅₁-BSA were added in buffer containing 20 mM CaCl₂ for 1 h at room temperature. Europium fluorescence was read using the time-resolved mode in a Wallac Multilabel counter (model 1420), and the IC₅₀, the concentration of inhibitor that causes 50% reduction in the amount of reporter ligand bound, was determined.

THP-1 Cells and Human Monocyte-derived Dendritic Cells (MDDCs)—The study of human subjects was approved by the Institutional Review Board of Kaohsiung Medical University, Taiwan; all study subjects gave informed consent before blood collection. To generate MDDCs, monocytes were first separated from the peripheral blood mononuclear cells of eight study subjects by magnetic beads conjugated with anti-CD14 (MACS Miltenyl Biotec). A human monocytic cell line, THP-1, was cultured in the absence or presence of PMA (20 ng/ml) and IL-4 (20 ng/ml) for 3–5 days to induce DC-SIGN expression. To generate monocyte-derived immature DCs (MDDCs), monocytes were cultured in medium containing IL-4 (10 ng/ml) and granulocyte monocyte colony-stimulating factor (50 ng/ml) for 7 days. In all cases MDDCs used in this study were CD14-negative but significantly expressed CD11c (>97%) and DC-SIGN (>70%) by flow cytometry. THP-1 cells, PMA/IL-4-stimulated THP-1 cells, and human MDDCs were treated with various stimuli including lipopolysaccharide (10–100 ng/ml; *Escherichia coli*, L0127:B8, Sigma), BG60 (5–20 μ g/ml), or Der p2 (5–20 μ g/ml). After 4 and 24 h, the supernatants were collected, and the levels of TNF- α and IL-12p70, respectively, were measured by enzyme-linked immunosorbent assay according to the manufacturer's instruction (eBiosciences, San Diego, CA). For blocking the allergen-mediated effect through DC-SIGN, cells were cultured in the presence of anti-DC-SIGN Abs (1 μ g/ml, R&D Systems) for 30 min before the stimulation of the cells with allergens. The concentrations of allergens used were optimal in the induction of cytokine expression as determined before the execution of this study. In some cases, the cells were treated in the presence or absence of a Raf-1 kinase inhibitor (Calbiochem) or the vehicle control, DMSO, for 1 h before stimulation.

Flow Cytometry Analysis—For cell surface expression of DC markers and CLRs, 2×10^5 cells were incubated with each of the fluorescence-labeled primary Abs, including anti-human CD11c and CD209 (DC-SIGN), at 4 °C for 20 min, and then the cells were washed twice with phosphate-buffered saline containing 1% BSA followed by the flow analysis with a FACSCalibur (BD Biosciences). Data were processed with CellQuest software (BD Biosciences).

Lentivirus-mediated Gene Silencing for DC-SIGN—To knock down the expression of DC-SIGN, a total of four miRNA

sequences directed against DC-SIGN mRNA were designed and purchased from Invitrogen and cloned in expression vectors driven by RNA polymerase II promoter (BLOCK-iT Pol II miRNA vectors, Invitrogen). All recombinant lentiviruses (LV), including a mock virus control, were produced by transient transfection of 293T cells according to the manufacturer's instruction and references therein (Invitrogen). In the initial studies only two of the four miRNA sequences showed gene knockdown and were used for the subsequent analysis (see below). THP-1 cells were transduced with recombinant lentivirus expressing each of the two miRNA sequences (LV-DC-SIGN3, 5'-TGCTGTGATGGAGTCGTGCCAGTTCCGTTTGGCCACTGACTGACGGAAGTGGCGACTCCATC; and LV-DC-SIGN4, 5'-TGCTGACTTCTGGAAGACTGCAGCTGGTTTTGGCCACTGACTGACCAGCTGCACTTCCAGAGT) or mock control sequences (LV-mock) at a multiplicity of infection of 20 for 24 h followed by treatment of the cells with PMA and IL-4 as above for 5 days, analyzed by Flow cytometry, and used in subsequent assays. Also, MDCCs were transduced with the same recombinant viral materials, harvested at day 6, and analyzed.

Western Blot Analysis—For analysis of BG60-induced signaling events, the cells were stimulated with BG60 or Der p2 (20 μ g/ml) for 15 min with or without the treatment with anti-DC-SIGN Abs for 30 min. After stimulation, the total cellular extracts were subjected to gel electrophoresis and probed with Abs against various kinases and phosphorylated kinases as previously described (19). The Abs used were rabbit anti-Raf-1 Ser-338, anti-Raf-1 Ser-259, and total Raf-1 Abs as well as Abs for members of the MAPK family (Cell Signaling Technology, Beverly, MA, and Millipore, Temecula, CA). The relative levels of phosphorylated proteins were quantified by densitometric analysis of the band intensities and normalized to those of β -actin.

Statistical Analysis—Data are expressed as the mean $\pm$ S.E. Statistical analysis was performed using Student's unpaired *t* test and the Mann-Whitney *U* test.

RESULTS

To test the hypothesis that glycan-containing allergens are natural ligands for CLRs, experiments were designed first to examine the relative binding activity of a battery of purified allergens and crude allergen extracts commonly used as skin-testing reagents to two members of the CLRs, DC-SIGN and L-SIGN. Fig. 1, *A* and *B*, shows the results of representative binding experiments wherein the binding of soluble human DC-SIGN (0.5 μ g/ml) and L-SIGN (0.5 μ g/ml), respectively, to a serial 3-fold dilution of 5 μ g/ml concentrations of purified allergens, BG60, Cyn d1, Asp f1, and a neoglycoprotein, MOXF₃-BSA, carrying a common glycoform of allergens, was analyzed. From these experiments, a significant and saturable binding of BG60, Cyn d1, and MOXF₃-BSA to both DC-SIGN and L-SIGN was observed, whereas Asp f1 (mold allergen) showed only trace binding activity and the binding of BSA was negligible. Moreover, in separate assays, binding analysis of two additional purified allergens, Cor a11 and Der p2, demonstrated differential binding activity of naturally derived allergens *versus* their recombinant, nonglycosylated counterparts

(from *E. coli*) to either DC-SIGN or L-SIGN (Fig. 1, *C* and *1D*, respectively).

To compare the relative binding affinity of BG60 and Der p2 allergens, binding competition assays of labeled Man₅₁-BSA (25 nM) with varying concentrations of BG60 and nDer p2 as competitors were performed, and relative IC₅₀ values calculated. The results (Fig. 2*A*) showed that BG60 and Der p2 had substantially higher IC₅₀ values (0.3 and 4 μ M, respectively) when compared with that of unlabeled Man₅₁-BSA (IC₅₀ value of 40 nM), and for non-glycosylated rDer p2, no significant competition was found (data not shown). To demonstrate carbohydrate specificity, inhibition assays of allergen binding to DC-SIGN were performed using various inhibitors. As seen in Fig. 2*B*, additions of fucose and Le^x inhibited the binding activities of MOXF₃-BSA and allergens, BG60, nCor a11, and nDer p2, to DC-SIGN when compared with that seen in the absence of inhibitors, whereas *N*-acetylglucosamine showed no inhibitory effect, suggesting the involvement of fucose in DC-SIGN recognition. Also, the binding of allergens to DC-SIGN is dependent on calcium, as the addition of EDTA inhibited their binding activities (Fig. 2*B*).

To examine the extent to which crude allergen extracts would be able to bind to CLRs, a panel of these extracts was examined using similar solid-phase binding analyses as above. MOXF₃-BSA and BSA were used as positive and negative controls, respectively. The results showed that similar to those found for purified allergens, whereas varying levels of relative binding activity were noted for the test allergen extracts, significant binding was seen for those from molds, cockroaches, and dust mites to both DC-SIGN and L-SIGN (supplemental Fig. 1, *A* and *B*, respectively). When allergen extracts from a variety of foods were analyzed, their relative binding activities tended to be lower when compared with those found for MOXF₃-BSA, except for those from mushroom and egg white. Three nut-related allergen extracts, coconut, hazelnut, and walnut, and those from celery and potato showed reproducible binding activity to DC-SIGN but not to L-SIGN (supplemental Fig. 1, *C* and *D*).

Modulation of TNF- α Expression in THP-1 Cells Stimulated with BG60—To examine the functional consequences of allergen-CLR interaction, a Bermuda grass allergen, BG60, was tested as a primary model allergen for two reasons; 1) BG60 is a common and important allergen and does not possess proteolytic activity, which may simplify the interpretation of the outcome measurement, and 2) its glycan structures have been characterized, and the major glycans are known to contain L-fucose attached to GlcNAc in the α (1,3)-linkage (14). The expression of an activation marker, TNF- α , in THP-1 cells was first analyzed as a model. These cells at the resting stage did not show any significant DC-SIGN expression, whereas induction of DC-SIGN expression was clearly evident after stimulation of the cells with PMA and IL-4 for 3–5 days both in the “floating” and “adherent” subpopulations of the cells (Fig. 3*A*). To examine the functional interaction of BG60 and DC-SIGN, resting or PMA/IL-4-stimulated THP-1 adherent cells were stimulated with BG60 in the presence or absence of DC-SIGN-blocking Abs, and TNF- α expression was determined. The results showed that although stimulation of the cells with lipopolysac-

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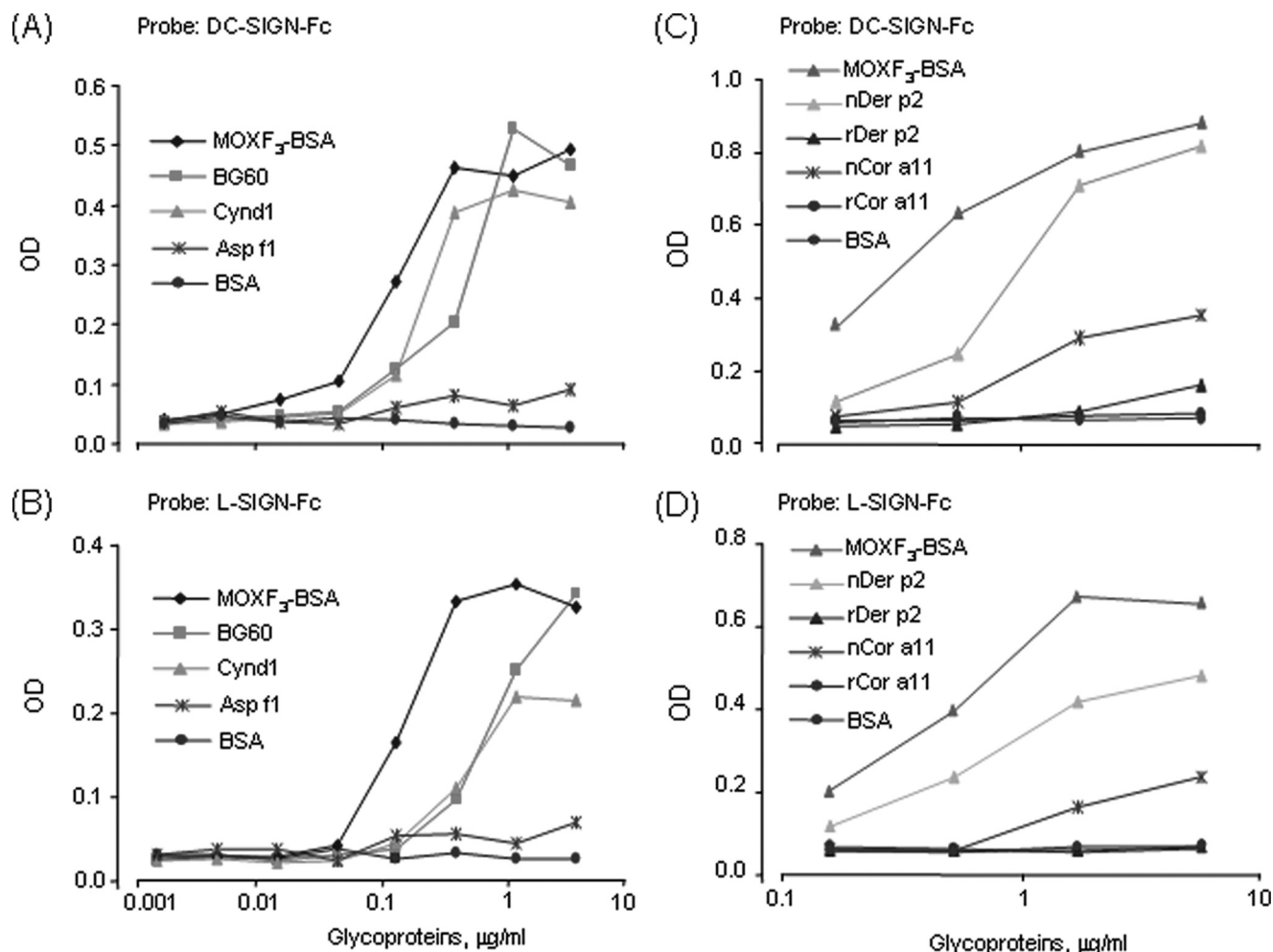


FIGURE 1. Representative binding analyses of purified allergens and soluble CLRs ($n = 4-8$ experiments). A serial 3-fold dilution of purified allergens, including natural (nCor a11 and nDer p2) or recombinant (rCor a11 and rDer p2) allergens and MOXF₃-BSA was probed with soluble DC-SIGN-Fc (A and C) and soluble L-SIGN-Fc (B and D), with binding activity expressed as optical density (OD).

charide as a positive control was able to induce significant levels of TNF- α expression in both THP-1 and PMA/IL-4-treated THP-1 cells, BG60 at 20 $\mu\text{g/ml}$ was able to induce TNF- α expression only in PMA/IL-4-treated THP-1 cells (Fig. 3B). Also, the addition of anti-DC-SIGN blocking Abs was able to inhibit BG60-induced TNF- α expression (Fig. 3B), and the addition of polymyxin B, an endotoxin inhibitor, did not inhibit BG60-induced TNF- α production, whereas lipopolysaccharide-induced TNF- α expression was inhibited. These results suggest, therefore, the importance of DC-SIGN as a receptor for BG60-mediated TNF- α expression in PMA/IL-4-stimulated THP-1 cells.

To further confirm the involvement of DC-SIGN in binding to allergens, a lentivirus-mediated gene-silencing approach was used. As seen in Fig. 3C, transduction of the cells with either LV-DC-SIGN3 or LV-DC-SIGN4 yielded significant knockdown of DC-SIGN expression, as compared with that of mock control (LV-mock)-transduced cells. To determine the effect of the DC-SIGN knockdown, lentivirus-transduced, PMA/IL-4-treated THP-1 cells were stimulated with BG60, and TNF- α levels were analyzed. The results showed that although mock control revealed no significant impact on BG60-induced TNF- α expression, the cells with DC-SIGN knockdown

showed no apparent induction of TNF- α expression above the base line (Fig. 3D).

Modulation of TNF- α Expression in MDDCs Stimulated with Allergens—To examine the functional effect of BG60 on the activation of human DCs, MDDCs from a total of eight subjects were analyzed as a model for testing the DC response to allergens, as MDDCs have been extensively examined as an *in vitro* model for immature DCs and are easily accessible with sufficient cell numbers for detailed functional analysis. The results showed that BG60 at 20 $\mu\text{g/ml}$ was able to induce significantly the expression of TNF- α (Fig. 4A) and IL-12p70 (Fig. 4B) in MDDCs from all study subjects. Furthermore, the addition of anti-DC-SIGN blocking Abs at 1 $\mu\text{g/ml}$ was able to inhibit BG60-induced TNF- α and IL-12p70 expression (Fig. 4, A and B). Also, stimulation of the cells with Der p2 (20 $\mu\text{g/ml}$) induced TNF- α expression, which was inhibitable by the addition of anti-DC-SIGN Abs (Fig. 4C). Using a similar gene knockdown approach, MDDCs transduced with LV-DC-SIGN3 or LV-DC-SIGN4 showed reduced levels of DC-SIGN expression (Fig. 4D) when compared with those seen in mock control-transduced cells. Significantly, no apparent induction of TNF- α expression was noted in DC-SIGN-silenced cells after the stimulation with BG60, as compared with that seen in mock controls (Fig. 4E).

These results suggest, therefore, that MDDCs from the study subjects are responsive to allergen stimulation through, at least in part, their interaction with DC-SIGN.

BG60-induced Signaling Events in MDDCs—Next, to delineate the underlying mechanisms of BG60-induced TNF- α expression in MDDCs subsequent to allergen-DC-SIGN interaction, a series of Western blotting experiments were pursued to uncover the signaling events in the BG60-DC-SIGN axis. It is known that treatment of cells with mitogen activates Raf-1 kinase and subsequent interaction with RAS/GTP (20, 21), where Raf-1 phosphorylation at serine 338 promotes Raf-1 kinase activity, whereas phosphorylation at serine 259 serves as a negative regulatory site. Activation of Raf-1 leads to the induction of MAPK/ERK signal transduction pathway. Of interest to note, a recent report by Gringhuis *et al.* (22) suggested that a mycobacterial cell wall component, ManLAM, interacts with DC-SIGN and triggers Raf-1 and NF κ B p65 activation, where activation of NF κ B p65 was suggested to be associated with the ManLAM-TLR axis. To investigate the potential involvement of the RAF/MEK/ERK signaling pathways in coupling with the allergen-DC-SIGN axis, the phosphorylation status of Raf-1 kinase in BG60-stimulated MDDCs was examined. The results showed that MDDCs stimulated with BG60 revealed significantly increased levels of Ser-338-, but not Ser-259-phosphorylated c-Raf (Fig. 5, A and B). In contrast, significantly reduced activation of c-Raf Ser-338 was noted in MDDCs in the presence of anti-DC-SIGN Abs (Fig. 5, A and B). The activation of Raf-1 was also observed in cells after Der p2 stimulation (Fig. 5C).

Moreover, activation of Raf-1 kinase in BG60-stimulated MDDCs led to the phosphorylation and activation of its downstream kinase, ERK1/2, a member of the MAPK family. As shown in Fig. 5A, activation of ERK1/2 was apparent in cells stimulated with BG60, which was

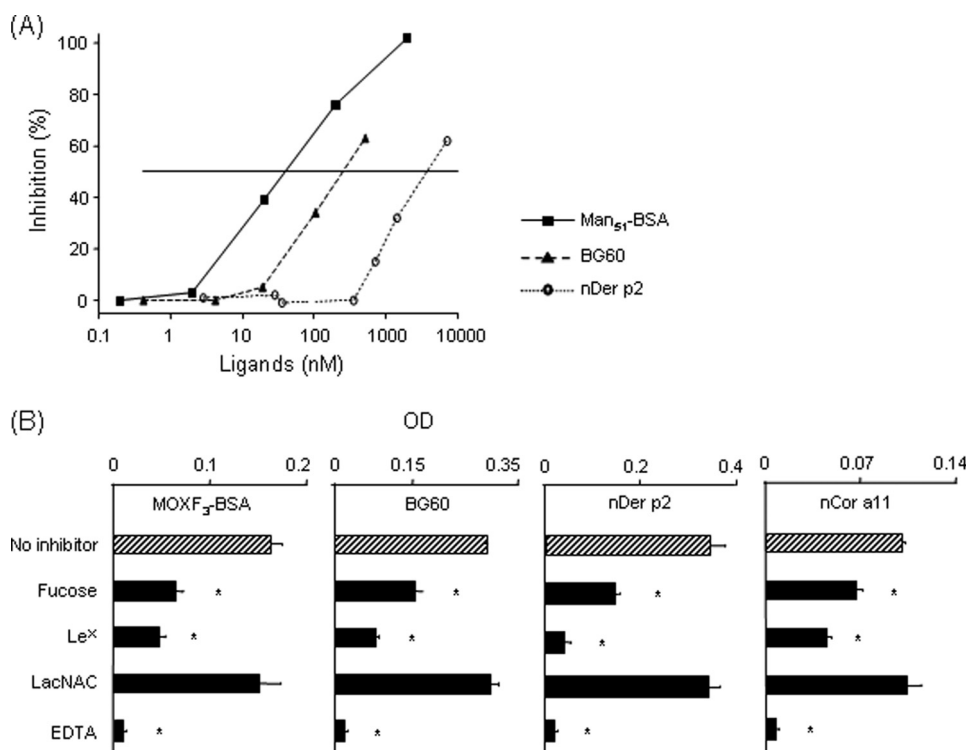


FIGURE 2. **Representative inhibition assays** ($n = 2-3$ experiments). *A*, shown are competitive inhibition assays with europium-labeled Man₅₁-BSA and purified allergens, BG60 and Der p2, as competitors. A line at 50% inhibition intercepting the inhibition curves is indicated. *B*, shown is inhibition of DC-SIGN-Fc binding to MOXF₃-BSA and purified allergens by various inhibitors as indicated. *, $p < 0.05$ versus bindings without the addition of inhibitors.

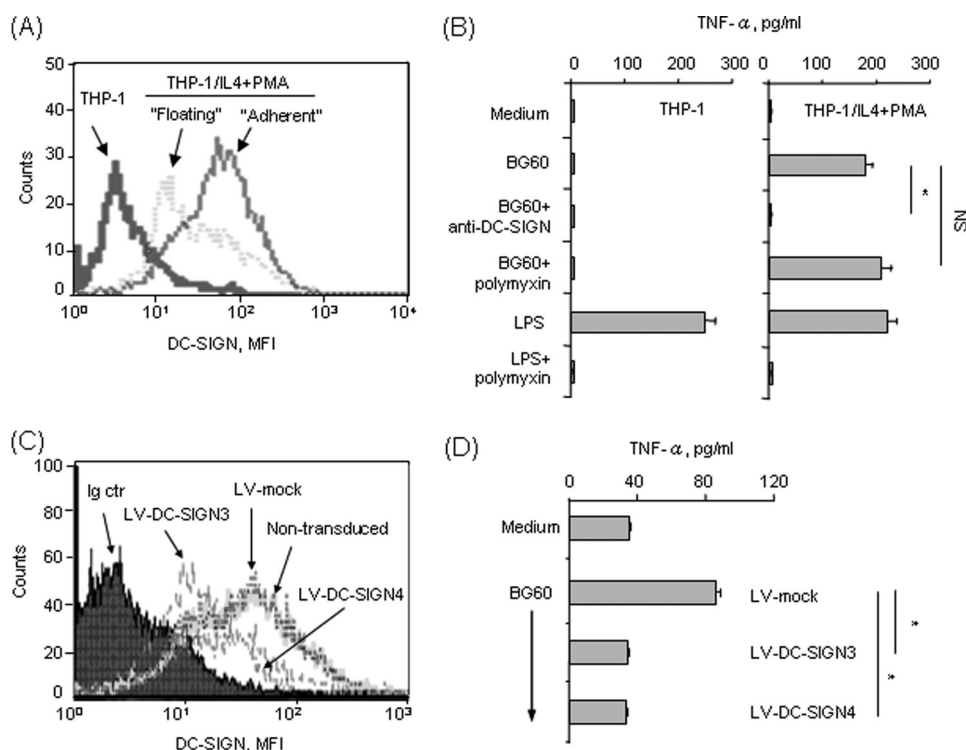


FIGURE 3. **Analysis of BG60-induced response in THP-1 cells and PMA/IL-4-stimulated THP-1 cells.** *A*, shown is analysis of DC-SIGN (CD209) expression. *B*, shown is BG60-induced TNF- α expression. *LPS*, lipopolysaccharide. *, $p < 0.05$; NS, not significant. *C*, flow analysis of DC-SIGN expression is measured as mean fluorescence intensity (MFI) in cells transduced with recombinant lentivirus expressing mock control (LV-mock) or two independent DC-SIGN miRNA sequences, LV-DC-SIGN3 and LV-DC-SIGN4. *Ig ctr*, isotype control Abs. *D*, analysis of TNF- α expression in cells with DC-SIGN knockdown is shown.

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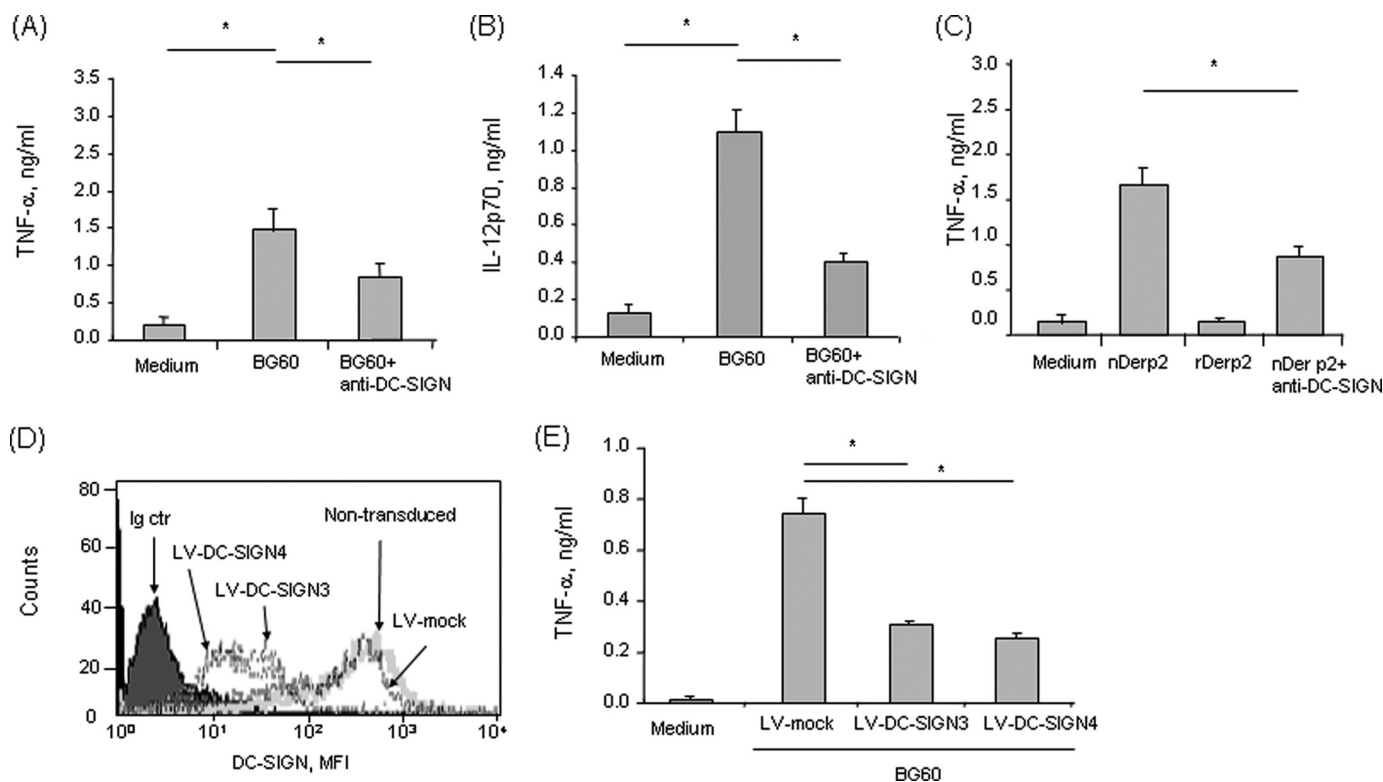


FIGURE 4. Allergen-induced response in human MDDCs. BG60-induced TNF-α (A) and IL-12p70 (B) levels in MDDCs ($n = 8$ subjects) are shown. C, Der p2-induced TNF-α expression is shown. D, flow analysis of DC-SIGN expression is measured as mean fluorescence intensity (MFI) in MDDCs with DC-SIGN knockdown as in Fig. 3C. Ig ctr, isotype control Abs. E, shown is BG60-induced TNF-α expression in DC-SIGN-silenced MDDCs.

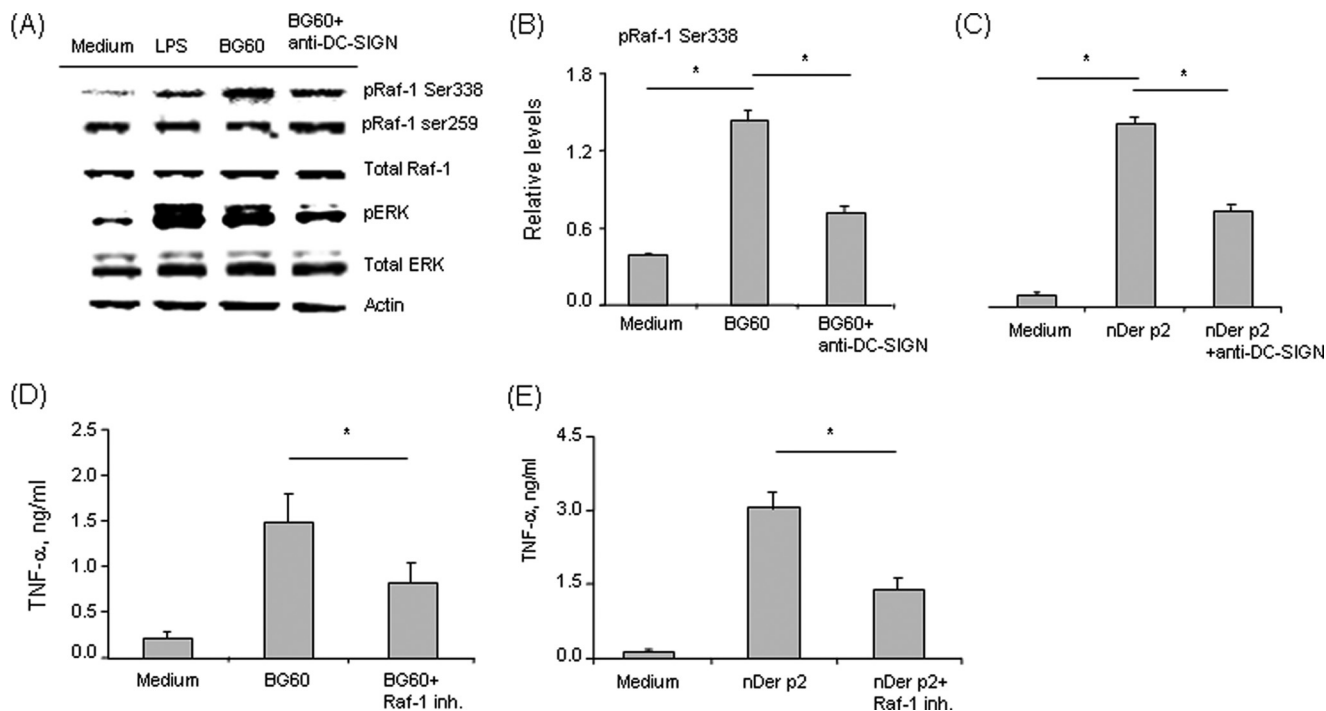


FIGURE 5. Analysis of signaling events in MDDCs. A, shown is a Western blot analysis of BG60-induced activation of Raf-1 (pRaf-1 Ser-338) and pERK as described under "Experimental Procedures." Shown are relative levels of phosphorylated Raf-1 Ser-338 ($n = 5$ subjects) in BG60- (B) and Der p2-stimulated MDDCs (C). *, $p < 0.05$. BG60 (D)- and Der p2 (E)-induced TNF-α expression in MDDCs treated with a Raf-1 inhibitor is shown. *, $p < 0.05$.

blocked by the addition of anti-DC-SIGN Abs. To confirm the involvement of Raf-1 kinase activation in BG60-induced TNF-α expression in MDDCs, the cells were pretreated with a Raf-1 kinase inhibitor for 30 min and then stimulated with or

without BG60 or Der p2, and the TNF-α levels were analyzed. As seen in Fig. 5, D and E, MDDCs from all study subjects showed significantly reduced levels of TNF-α when the cells were pretreated with Raf-1 kinase inhibitor as compared with

cells without pretreatment with inhibitor. These results suggest that Raf-1 kinase activation may play a role in determining the stimulatory activity of allergens in MDDCs via DC-SIGN ligation.

DISCUSSION

In this report evidence is provided supporting the existence of a functional allergen-DC-SIGN axis involving activation of Raf-1 and ERK kinases, leading to the induction of TNF- α expression in MDDCs. These results suggest that similar to that found in recognition of pathogens, the glycan structures on allergens, such as BG60, may serve also as a molecular (glycan) pattern for recognition by CLRs on DCs. Indeed, the results from binding experiments of purified allergens, including BG60, Der p2, Cyn d1, Cor a11, and a great majority of the test allergen extracts, lend further support to this hypothesis. Moreover, the results from the use of various DC-SIGN antagonists and of the cells with DC-SIGN knockdown provided experimental proof validating a direct allergen-CLR interaction.

Both BG60 and Cor a11 are known to contain fucosylated glycan (14, 16), a preferred ligand of DC-SIGN. In addition to DC-SIGN, a number of CLRs are known to be expressed on circulating DCs, except for L-SIGN, which is a CLR primarily expressed in lymphoid endothelium. It is, thus, likely that other CLRs expressed on MDDCs may also contribute to allergen binding. Although this possibility cannot be ruled out at this time and remains to be investigated, the fact that DC-SIGN blockade and gene knockdown inhibited the functional effect of BG60 suggests the primary role of DC-SIGN in allergen interaction in MDDCs. It is noteworthy that BG60 showed no apparent binding activity to mannose receptor and CLEC5a in our binding analysis (data not shown).

There have been a number of publications documenting DC-SIGN-mediated signaling events, but no consensus can be made at this time. In these studies the cell models and the modes of activation vary in different studies, and consequently the signaling events associated with DC-SIGN-ligand interaction are different among these published studies, and the effects on the expression of cytokines, including TNF- α , IL-10, and IL-12, are variable in different studies. It has recently been suggested that mannosylated glycoproteins, such as ManLAM, induce distinct "signalosome" from that of fucosylated antigens, such as synthetic Lex-PAA (23). Our results showed that activation of Raf-1 Ser-338 was evident in MDDCs stimulated with BG60, a fucosylated allergen, whereas no apparent activation of Raf-1 Tyr-340/341 and Src was noted in MDDCs (supplemental Fig. 2). Also, MAPK, JNK (c-Jun N-terminal kinase) and p38 were not activated, and no apparent increase in phosphorylated phospholipase C γ was found (supplemental Fig. 2). Therefore, it is likely that the difference in DC-SIGN signaling shown in different models may be attributed to the difference in glycan structures and their relative binding affinities to DC-SIGN.

It is noted also that BG60-induced cytokine expression was inhibited only partially by the addition of anti-DC-SIGN blocking Abs and of Raf-1 and/or MEK inhibitors. Although the involvement of DC-SIGN and Raf-1 kinase was clearly evident, the partial inhibition noted in various assays may reflect the

difference in the dosage of inhibitors used and/or the existence of other potentially important members of the CLRs and signaling molecules. Further systemic investigation of CLR involvement and signaling events is clearly needed.

Furthermore, it may be argued that allergen-induced TNF- α expression in MDDCs is mediated through the cross-linking of surface-bound, allergen-specific IgE with its high affinity receptor, known to be present on human circulating monocytoïd DCs and plasmacytoïd DCs (24). However, this possibility is unlikely, as in our study MDDCs did not show detectable levels of the α subunit of the IgE receptor (data not shown). Also, the response of MDDCs may still, however remote the possibility, involve endotoxin contamination in the allergen preparations, as endotoxin is known to be ubiquitous and difficult, if not impossible, to be removed. It is unlikely, however, because in the experiments using BG60, the addition of anti-DC-SIGN Abs produced inhibition of TNF- α release, and the addition of polymyxin B could not inhibit BG60-induced TNF- α expression.

The primary purpose of the current study is to provide biochemical and functional evidence supporting the interaction of allergens and CLRs. The primary study outcome is the level of a pro-inflammatory cytokine, TNF- α , in MDDCs, which serves as a marker for early activation. It is unknown at present regarding the extent to which the allergen-CLR axis influences the expression of other DC-derived cytokines and subsequent adaptive T-cell immunity, which is a limitation of our current analysis. Although this is an important functional aspect of DCs, the fact is that in addition to their antigen-presenting function, DCs are known to be involved in the regulation of innate immunity and inflammatory response. The ability of allergens to directly trigger the DC cytokine release, such as TNF- α , a critical pro-inflammatory cytokine, would be significant in and of itself. As a corollary, an earlier study by Okano *et al.* (25) showed that Cry j1 allergen (Japanese cedar) is able to modulate DC function, which can be blocked by the addition of mannan in allergen-treated culture, suggesting a possible interaction of Cry j1 and CLRs, although the nature of the mannan-inhibitable interaction was not clarified. Also, a recent study of peanut allergens has provided suggestive evidence that one of the major allergens, Ara h1, is able to polarize Th2 response via its likely interaction with DC-SIGN on MDDCs (26). Our data from analysis of DC-SIGN binding to crude peanut allergen extracts showed a detectable, albeit low level, but the significance of this trace binding activity at 10 μ g/ml is uncertain (see supplemental Fig. 1). It is also noted in the Shreffler *et al.* study (26) that the concentration of crude peanut extracts and purified Ara h1 tested for binding and functional assays was relatively high with the test concentrations ranging from 100 to 500 μ g/ml. Although it is uncertain whether at much lower doses peanut allergens still exhibit their functional activities, the relevance of such high doses at the cellular level of Ara h1 is uncertain. Although these issues require further validation, it is worth noting that Ara h1 is known to contain complex mannose- and xylose-type structures but not DC-SIGN-preferred fucosylated glycan structure (15).

Moreover, currently available purified allergens are limited, and the glycan structures have been characterized for only a

Allergens Interact with CLRs

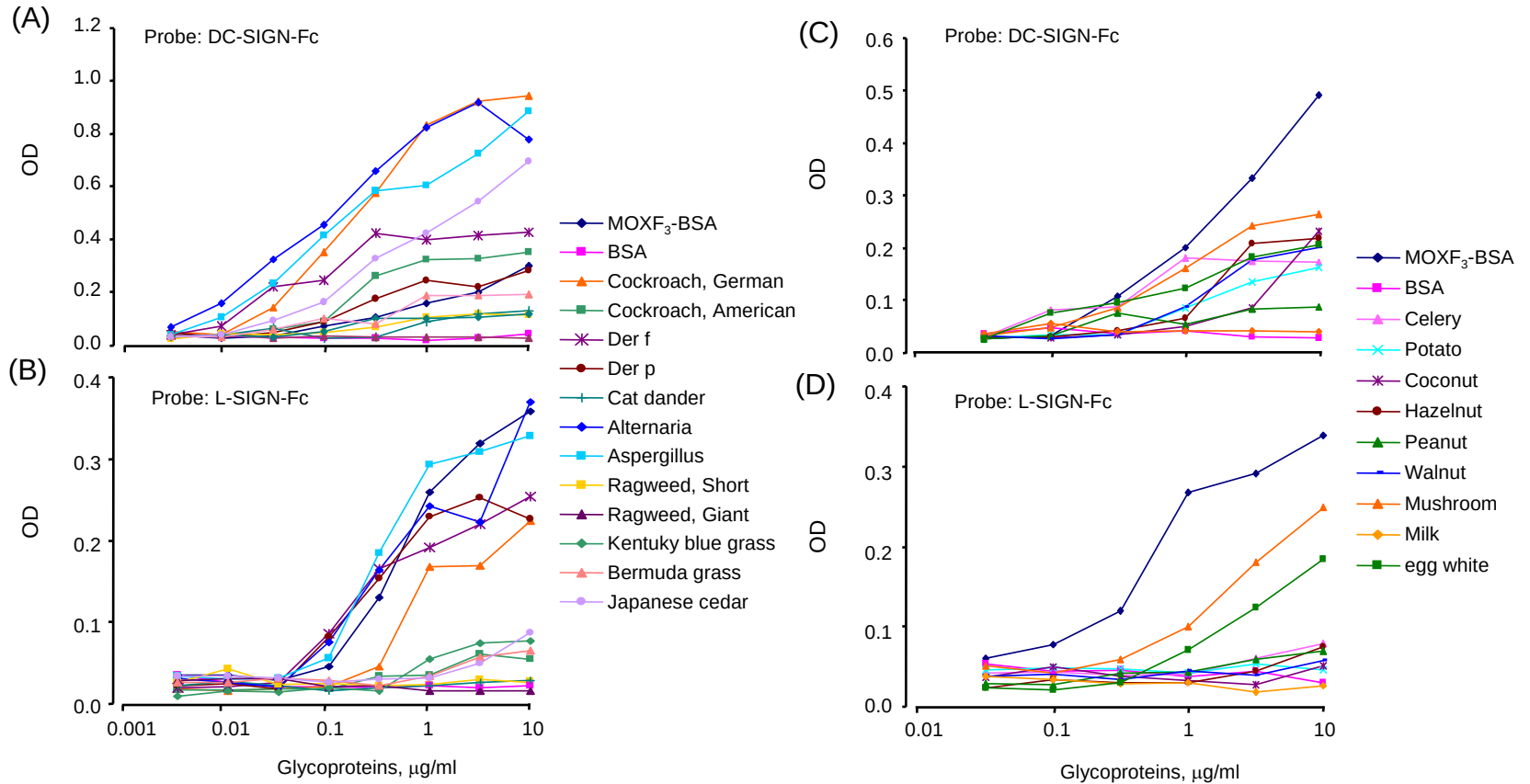
handful of allergens, which severely limits the extent to which the functional importance of the allergen-CLR axis can be evaluated. It is hoped that the current study of an *in vitro* model system would offer a basis for further pursuing more in-depth studies for ultimate discovery of the role of CLRs in allergic diseases. Recently, a few allergens, such as Der p1, have been shown to modulate cellular functions via their proteolytic enzyme activity and in some cases through their interaction with protease-activated receptor 2 (PAR-2; Ref. 27). Our results suggest the existence of an additional regulatory mechanism involving the allergen-CLR axis, adding a new dimension to the existing paradigm in host responses to allergens. Our results showing significant binding of house dust allergens (Der p) to both DC-SIGN and L-SIGN together with other findings that a major house dust allergen, Der p1, is a possible ligand for mannose receptor (28) and that DC-SIGN is an enzymatic substrate for Der p1 (29) suggest, therefore, the likelihood for an interactive effect of PAR-2 and CLR signaling. Furthermore, a significant degree of cross-regulation has been noted between CLRs and TLRs, as in the case of the combined functional effect of yeast zymosan and its interaction with both dectin-1 and TLR2 (30), mimicking more closely the natural exposure to pathogens. Therefore, to understand fully the regulatory role of allergen-CLR axis in allergic response, it would be prudent in the future to take into account the possible cross-regulation between CLRs, TLRs, and protease-activated receptors.

Taken together, current data provide evidence for the existence of a functional allergen-CLR axis in DC function. How DCs integrate the allergen-CLR signaling in controlling innate immunity and subsequent adaptive responses may, thus, represent an important mechanism in the genesis of and/or the progression in allergic diseases. Also, understanding the regulatory role of CLRs in allergic diseases may offer an opportunity for the design of novel, safer, and more efficacious therapies.

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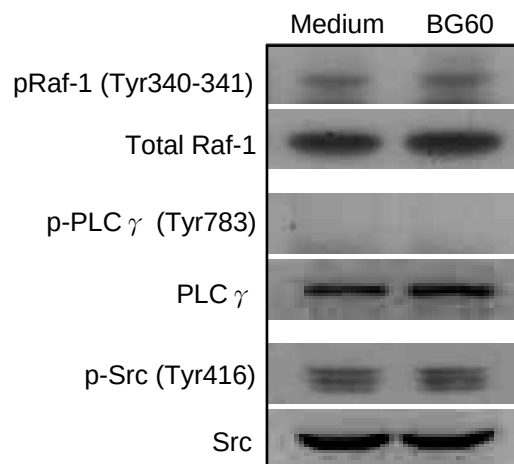
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Supplemental Figure 1.



Supplemental Figure 1. Relative binding activity of allergen extracts to DC-SIGN and L-SIGN. A serial 3-fold dilution of various allergen extracts was probed with (A and C) soluble DC-SIGN-Fc and (B and D) soluble L-SIGN-Fc, with binding activity expressed as optical density (OD) in separate experiments (N=3-5 experiments).

Supplemental Figure 2.



Supplemental Figure 2. Western blotting analysis of BG60-stimulated MDDCs. For analysis of BG60-induced signaling events, the cells were stimulated with BG60 (20 μ g/ml) for 15 min. Following stimulation, total cellular extracts from stimulated (BG60) and non-stimulated (medium) cultures were subjected to gel electrophoresis and probed with Abs against various kinases and phosphorylated kinases as previously described (ref. 19). The Abs used were rabbit anti-c-Raf (pTyr340, Tyr341) (Calbiochem), total Raf-1 Abs (Cell Signaling Technology), anti-phospho-PLC γ (Tyr783) (Millipore), anti-PLC γ (Millipore), anti-phospho-Src family (Tyr416) (Cell Signaling), and anti-Src (Upstate). Immunoreactive bands were visualized using horseradish peroxidase-conjugated secondary Abs and the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech). Results shown are representative of three independent experiments from analysis of MDDCs from three subjects.