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The Mannose Receptor Mediates the Uptake of Diverse Native Allergens by Dendritic Cells and Determines Allergen-Induced T Cell Polarization through Modulation of IDO Activity

Pierre-Joseph Royer,* Mohamed Emara,* Chaoxing Yang,* Abeer Al-Ghouleh,* Paddy Tighe,* Nick Jones,[†] Herb F. Sewell,* Farouk Shakib,*[‡] Luisa Martinez-Pomares,*[‡] and Amir M. Ghaemmaghami*[‡]

The mannose receptor (MR) is a C-type lectin expressed by dendritic cells (DCs). We have investigated the ability of MR to recognize glycosylated allergens. Using a gene silencing strategy, we have specifically inhibited the expression of MR on human monocyte-derived DCs. We show that MR mediates internalization of diverse allergens from mite (Der p 1 and Der p 2), dog (Can f 1), cockroach (Bla g 2), and peanut (Ara h 1) through their carbohydrate moieties. All of these allergens bind to the C-type lectin-like carbohydrate recognition domains 4–7 of MR. We have also assessed the contribution of MR to T cell polarization after allergen exposure. We show that silencing MR expression on monocyte-derived DCs reverses the Th2 cell polarization bias, driven by Der p 1 allergen exposure, through upregulation of IDO activity. In conclusion, our work demonstrates a major role for MR in glycoallergen recognition and in the development of Th2 responses. *The Journal of Immunology*, 2010, 185: 000–000.

Type I IgE-mediated hypersensitivity reactions are initiated by the recognition of allergens by APCs, such as dendritic cells (DCs), and culminate in Th2 cell differentiation, IgE Ab production, and mast cell sensitization and triggering. DCs have been shown to play a key role in the induction and re-elicitation of Th2-mediated inflammation in allergic diseases (1, 2); however, the molecular processes underpinning these events have remained elusive. In particular, little information is available regarding the mechanism(s) used by DCs to recognize and internalize allergens and how these processes lead to Th2 cell polarization. DCs sample their environment using a plethora of receptors, such as C-type lectin receptors, scavenger receptors, and TLR, which increase their internalization efficiency and deliver information regarding the presence of danger signals. The mannose receptor (MR), a C-type lectin, is a multifunctional endocytic receptor with two

distinct lectin activities mediated by its extracellular region (3). The cysteine-rich (CR) domain recognizes sulfated sugars, whereas mannose (together with fucose and *N*-acetylglucosamine) recognition is mediated by the multiple C-type lectin-like carbohydrate recognition domains (CTLDs) (4, 5). MR is firmly placed at the interface of immunity and homeostasis. It mediates clearance when expressed by macrophages and selected endothelia and Ag presentation when expressed by professional APCs (6–10). Several studies have implicated MR in the modulation of DC function. In particular, Chieppa et al. (11) demonstrated that engagement of MR on DCs by selected ligands can lead to the generation of cells with a “regulatory” phenotype that are able to produce IL-10 and IL-1R antagonist and express the nonsignaling type II IL-1R. These DCs were unable to polarize Th1 effector cells and did not secrete the chemokines CXCL10 and CCL19 but produced large amounts of CCL22 and CCL17, thus favoring the amplification of Th2 circuits. These observations, together with others showing 1) that the MR gene is a candidate for allergen-induced airway hyperresponsiveness (12, 13), 2) higher MR expression in atopic patients compared with that in healthy donors (14), and 3) MR contribution to the inhibitory effect of mannose-capped lipoarabinomannans on IL-12 production by DCs in response to LPS (15), suggest a putative role for MR in Th2 polarization. Interestingly, a recent study (14), using blocking carbohydrates, suggested that MR is responsible for 40–50% of the uptake of the major house dust mite allergen Der p 1 by human monocyte-derived DCs (Mo-DCs).

MR could therefore potentially serve as a common receptor for allergens and play a role in Th2 polarization following allergen recognition by DCs. In this study, we show that MR serves as a receptor for a range of diverse allergens, including those from mite (Der p 1 and Der p 2), dog (Can f 1), cockroach (Bla g 2), and peanut (Ara h 1). Furthermore, we show that MR plays a crucial role in allergen-induced Th2 cell polarization as demonstrated by a biased Th1 response when MR-deficient DCs were exposed to Der p 1. Finally, we demonstrate that the reversal of Th1/Th2

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Abbreviations used in this paper: AGE, advance glycation end product; CR, cysteine-rich; CT, control; CTLD, C-type lectin-like carbohydrate recognition domain; DC, dendritic cell; DC-SIGN, dendritic cell-specific intercellular adhesion molecule 3 grabbing nonintegrin; DSA, *Datura stramonium* agglutinin; FNII, fibronectin type II; Gal, galactose; GNA, *Galanthus nivalis* agglutinin; 3HAA, 3-hydroxyanthranilic acid; KYN, L-kynurenine; MAA, *Maackia amurensis* agglutinin; MFI, median fluorescence intensity; MR, mannose receptor; Mo-DC, monocyte-derived dendritic cell; MTHT, methyl-thiohydantoin-tryptophan; PAA, polyacrylamide; PNA, peanut agglutinin; siRNA, small interfering RNA; SNA, *Sambucus nigra* agglutinin; SO₄-3-Gal-PAA, SO₄-3-galactose-PAA; Treg, regulatory T cell.

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Table I. Donor characteristics

Age, y	38 ± 4
Gender (male/female), n	6/3
Total IgE, kU/l	690 ± 396
Specific IgE, kU/l	29 ± 14
Positive skin prick test to house dust mite (wheal >3mm)	All donors

balance in the absence of MR is mediated, at least partly, through upregulation of IDO activity in DCs. These data provide important insights into the molecular basis of allergic sensitization and identify potential targets for the rational design of new therapeutic strategies for treating allergic diseases.

Materials and Methods

PBMC preparation and human Mo-DC generation

Heparinized blood from house dust mite-sensitized donors was obtained after prior consent and ethical committee approval. The atopic status of donors was determined by skin prick test and by measuring total and specific IgE to house dust mite allergen Der p 1 using the ImmunoCAP test (Phadia Milton Keynes, U.K.). Der p 1-specific IgE concentration ranged from 0.35 kU/l to >100 kU/l (grade 1 to 6), and total IgE concentration ranged from 121 to 2462 kU/l (Table I). PBMCs were separated on a Histopaque (Histopaque-1077; Sigma-Aldrich, Gillingham, U.K.) density gradient. Monocytes were purified by positive selection using a magnetic cell separation

system (Miltenyi Biotec, Bisley, U.K.) and were then plated (1×10^6 cells per milliliter) on a 24-well plate (Corning Life Sciences, Amsterdam, the Netherlands) in RPMI 1640 medium (Sigma-Aldrich) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine (all from Sigma-Aldrich), and 10% low-endotoxin FCS (Autogen Bioclear, Calne, U.K.) (R10 medium). Monocyte differentiation into DCs was carried out for 6 d in the presence of GM-CSF (50 ng/ml) and IL-4 (250 U/ml) (both from R&D Systems, Abingdon, U.K.).

RNA interference

MR small interfering RNA (siRNA) was obtained from Invitrogen (Paisley, U.K.). The DNA-targeted sequence was 5'-TGGATGGATGATACCTGCGACAGTA-3' from 3299 to 3323bp. The control siRNA was the AllStars Negative Control siRNA purchased from Qiagen (Crawley, U.K.). Monocytes were transfected at day 1 with 37.5 nM siRNA using the HiPerFect Transfection Reagent (Qiagen). The inhibition of MR expression was assessed at day 6.

Glycosylation analysis

Native allergens (5 µg) (all from Indoor Biotechnologies, Wiltshire, U.K.) were separated via 12% SDS-PAGE and were transferred to nitrocellulose using standard procedures. Then, glycan chain analysis was performed using a Glycan Differentiation Kit (Roche, Welwyn Garden City, U.K.) according to the manufacturer's instructions.

Flow cytometry analysis

mAbs against IL-4 (clone 4D9), IFN-γ (clone 45.15), CD4 (13B8.2), CD80 (clone MAB104), CD86 (clone HA5.2B7), CD206 (clone 3.29B1.10), and

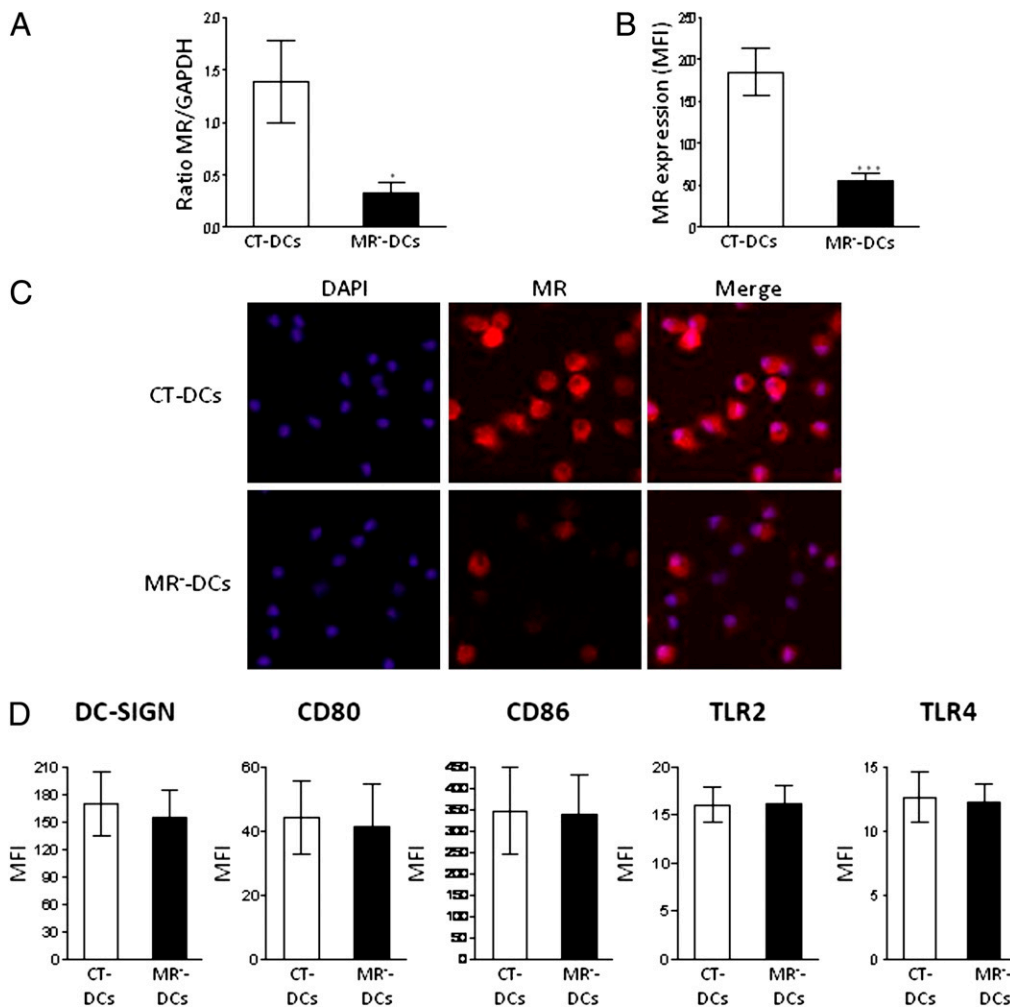


FIGURE 1. Specific downregulation of MR expression on human Mo-DCs by siRNA. *A*, Quantitative PCR analysis of MR expression by CT or MR-deficient DCs. Relative expression of MR is compared with that of GAPDH ($n = 6$). *B*, Analysis of MR expression by flow cytometry. MFI is shown. Student *t* test ($n = 12$). *C*, Fluorescence microscopy analysis of MR inhibition. Human Mo-DCs were stained for MR (red). Nuclear DNA is stained with DAPI (original magnification $\times 40$). *D*, Analysis of DC-SIGN, CD80, CD86, TLR4, and TLR2 expression by flow cytometry. MFI, median fluorescence intensity.

CD209 (clone AZND1) were purchased from Beckman Coulter (High Wycombe, U.K.). mAbs against TLR2 (clone 11G7) and TLR4 (clone HTA125) were from Serotec (Oxford, U.K.). Nonreactive isotype-matched Abs were used as a control. For IDO expression analysis, sheep anti-human IDO (HP5004; HyCult Biotechnology, Uden, The Netherlands) and rabbit anti-sheep-FITC (Beckman Coulter) Abs were used. Cells were stained for 30 min at 4°C in PBS containing 0.5% BSA and 0.1% sodium azide and washed before analysis on an ALTRA flow cytometer (Beckman Coulter). For intracellular staining, cells were fixed overnight at 4°C with 0.5% formaldehyde. They were then permeabilized for 15 min at room temperature in PBS containing 0.1% BSA and 0.1% saponin, stained for 45 min at 4°C in PBS containing 0.1% BSA and 0.1% saponin, and washed twice before analysis.

Quantitative RT-PCR

DCs were washed in ice-cold PBS, and mRNA extraction and cDNA synthesis were performed using the μ MACS One-Step cDNA Kit (Miltenyi Biotec) according to the manufacturer's instructions. Real-time PCR was performed in a Stratagene MxPro 3005P qPCR System with the Brilliant SYBR Green qPCR Master Mix (Stratagene, La Jolla, CA). Primer sequences were as follows: GAPDH (forward) 5'-GAGTCAACGGATTGGTTCGT-3', GAPDH (reverse) 5'-GACAAGCTTCCCGTTCTCAG-3'; MR (forward) 5'-CGTTTACCAATGGCTTCGT-3', MR (reverse) 5'-CCTTGGCTTCGTGATTTCAT-3'. Cycling was initiated at 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min. Samples were run in triplicate, and relative expression of MR was calculated using the comparative threshold cycle method normalized to GAPDH.

Fluorescence microscopy

DCs were allowed to adhere on poly-L-lysine-coated coverslip. After 30 min, cells were fixed with 0.5% formaldehyde for 15 min at room temperature and permeabilized with 0.1% Tween 20 in PBS. Cell staining was then performed for 45 min at room temperature in PBS containing 0.1% Tween 20 and 0.1% BSA. Cells were washed and incubated with 1 μ g/ml DAPI (Sigma-Aldrich) for 5 min. After three washes, coverslips were mounted with the SlowFade mounting medium (Invitrogen) before analysis on a LSM 510uv META Combi system (Zeiss, Oberkochen, Germany).

Analysis of Ag uptake

Allergens were labeled with Cy3 or Cy5 (Amersham Biosciences, Little Chalfont, U.K.) or with the Fluoro-Trap Fluorescein (Innova Biosciences, Cambridge, U.K.) labeling kits according to the manufacturers' instructions. The uptake buffer was R10 medium with 30% PBS with Ca^{2+} and Mg^{2+} . A total of 5×10^4 DCs were washed and resuspended in 50 μ l uptake buffer. In some experiments, DCs were treated with various concentrations of mannan (Sigma-Aldrich), SO_4 -3-galactose-polyacrylamide (PAA), or galactose-PAA (both from Lectinity, Moscow, Russia) for 20 min at 37°C prior to the addition of allergens. Labeled allergens (5 μ g/ml) were then added to the cells. After 25 min of incubation at 37°C, DCs were washed in ice-cold PBS and fixed with 0.5% formaldehyde before analysis by flow cytometry.

Allergen-MR binding ELISA

All of the washes and incubations were carried out in 10 mM Tris-HCl (pH 7.5), 10 mM Ca^{2+} , 154 mM NaCl, and 0.05% Tween 20. Different native

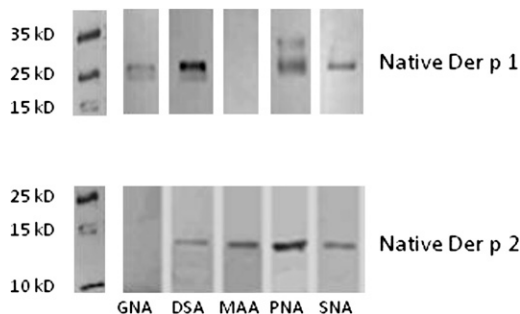


FIGURE 2. Analysis of glycan content of Der p 1 and Der p 2 allergens. Sugar binding specificities of lectin used for glycosylation analysis are as follows: GNA, $\text{Man}\alpha 1,2\text{Man}$, $\text{Man}\alpha 1,6\text{Man}$, $\text{Man}\alpha 1,3\text{Man}$; DSA, $\text{Gal}\beta 1,4\text{GlcNAc}$; MAA, $\text{NeuAc}\alpha 2,3\text{Gal}$; PNA, $\text{Gal}\beta 1,3\text{GlcNAc}$; SNA, $\text{NeuAc}\alpha 2,6\text{Gal}$. DSA, *Datura stramonium* agglutinin; GNA, *Galanthus nivalis* agglutinin; MAA, *Maackia amurensis* agglutinin; PNA, peanut agglutinin; SNA, *Sambucus nigra* agglutinin.

Table II. Glycosylation patterns of the allergens

Allergen	GNA	DSA	MAA	PNA	SNA
Ara h 1	+++	—	+	++	++
Bla g 2	+++	++	+	++	++
Can f 1	+	—	+	—	++
Der p 1	+	+++	—	++	++
Der p 2	—	+	++	+++	++

allergens as well as control carbohydrate ligands were coated onto the wells of MaxiSorb ELISA plates (Nunc, Roskilde, Denmark) by overnight incubation in PBS at 4°C. The plates were washed three times, and the different MR-Fc constructs were incubated at a concentration of 2 μ g/ml for 2 h at room temperature. After three washes, the binding was detected by incubation with anti-human IgG γ -chain-specific alkaline phosphatase conjugate (Sigma-Aldrich). Plates were washed three times and developed with *p*-nitrophenyl phosphate substrate (Sigma-Aldrich) in 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, and 5 mM MgCl_2 . Absorbance was measured at 405 nm. All of the assays were carried out in duplicate or triplicate.

Quantification of IDO activity

Cells (2.5×10^5 cells per milliliter) were resuspended in R10 medium with GM-CSF (50 ng/ml) and IL-4 (250 U/ml) and supplemented with 100 μ M L-tryptophan (Sigma-Aldrich). After 24 h, IDO activity was quantified by measuring the production of L-kynurenine (KYN) in the culture supernatant using a colorimetric assay (16). Briefly, 50 μ l 30% trichloroacetic acid was added to 100 μ l culture supernatant. The mixture was vortexed and centrifuged for 5 min at full speed in a benchtop centrifuge. Then 100 μ l soluble phase was added to an equal volume of Ehrlich's reagent: 100 mg *p*-dimethylbenzaldehyde in 5 ml glacial acetic acid (both from Sigma-

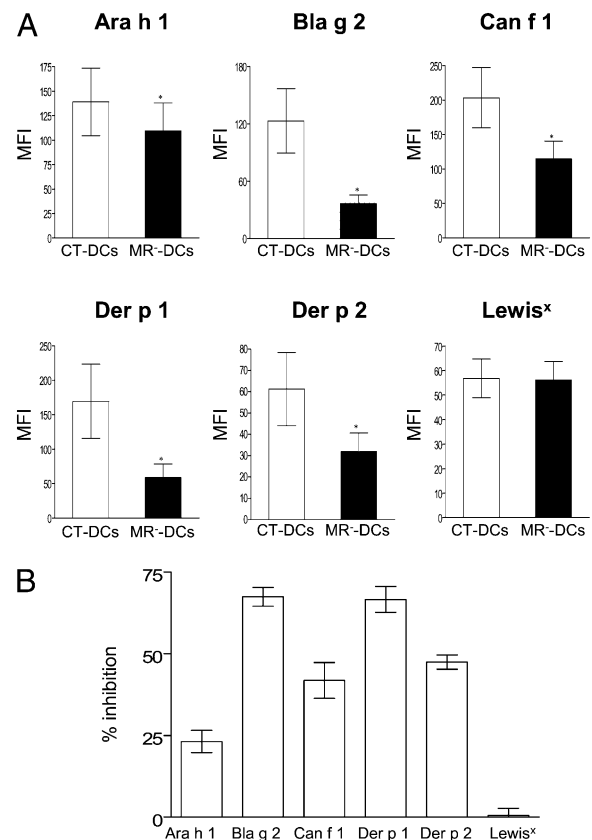


FIGURE 3. Allergen uptake by CT or MR-deficient DCs. *A*, Human CT or MR-deficient Mo-DCs were incubated for 20 min with 5 μ g/ml Der p 1 (labeled with Cy5), Ara h 1, Can f 1 (labeled with Cy3) and Bla g 2, Der p 2, or Lewis^x (labeled with fluorescein). The uptake of the allergens and Lewis^x Ag was determined by flow cytometry ($n \geq 4$). *B*, Percentage of uptake inhibition after MR knockdown. The percentage of inhibition was calculated as follows: $[(\text{MFI CT DCs} - \text{MFI MR}^- \text{DCs}) / \text{MFI CT DCs}] \times 100$.

Aldrich) in a 96-well microtiter plate. The OD was measured at 492 nm, and KYN concentration was calculated by referral to a KYN (Sigma-Aldrich) standard curve.

DC-T cell coculture

Mo-DCs were treated for 6 h with Der p 1 (10 µg/ml) in the presence of LPS (50 ng/ml) (Sigma-Aldrich). In some experiments, Mo-DCs were treated with the IDO inhibitor methyl-thiohydantoin-tryptophan (MHTT) (100 µM) or with the diluent alone (DMSO) (both from Sigma-Aldrich). Then Mo-DCs were washed and cultured in 96-well U-bottom plates with CD3⁺ autologous T cells (DC/T cell ratio of 1:5) purified by immunomagnetic cell sorting (Miltenyi Biotec). Medium used for the coculture was RPMI 1640 supplemented with penicillin/streptomycin and 5% human AB serum (Sigma-Aldrich). In some experiments L- KYN or 3-hydroxyanthranilic acid (3HAA) (both from Sigma-Aldrich) were added to a final concentration of 10 µM at the beginning of the coculture. At day 3, IL-2 (20 IU/ml; R&D Systems) was added, and from then feeding with fresh medium and IL-2 was performed every 3–4 d. After 15 d, T cells were restimulated for 8 h with PMA (15 ng/ml) and ionomycin (1 µg/ml) (both from Sigma-Aldrich). For intracellular staining, brefeldin A (10 µg/ml; Sigma-Aldrich) was added for the last 6 h, and the production of IL-4 and IFN-γ was detected on CD4⁺ cells (labeled with CD4-PC5) by intracellular staining using anti-IL-4-PE and anti-IFN-γ-FITC mAbs.

Cytokine measurement

Supernatants were collected and stored at –20°C before analysis. Cytokine secretion was analyzed with the FlowCytomix kit (Bender MedSystems, Vienna, Austria) according to the manufacturer's instructions.

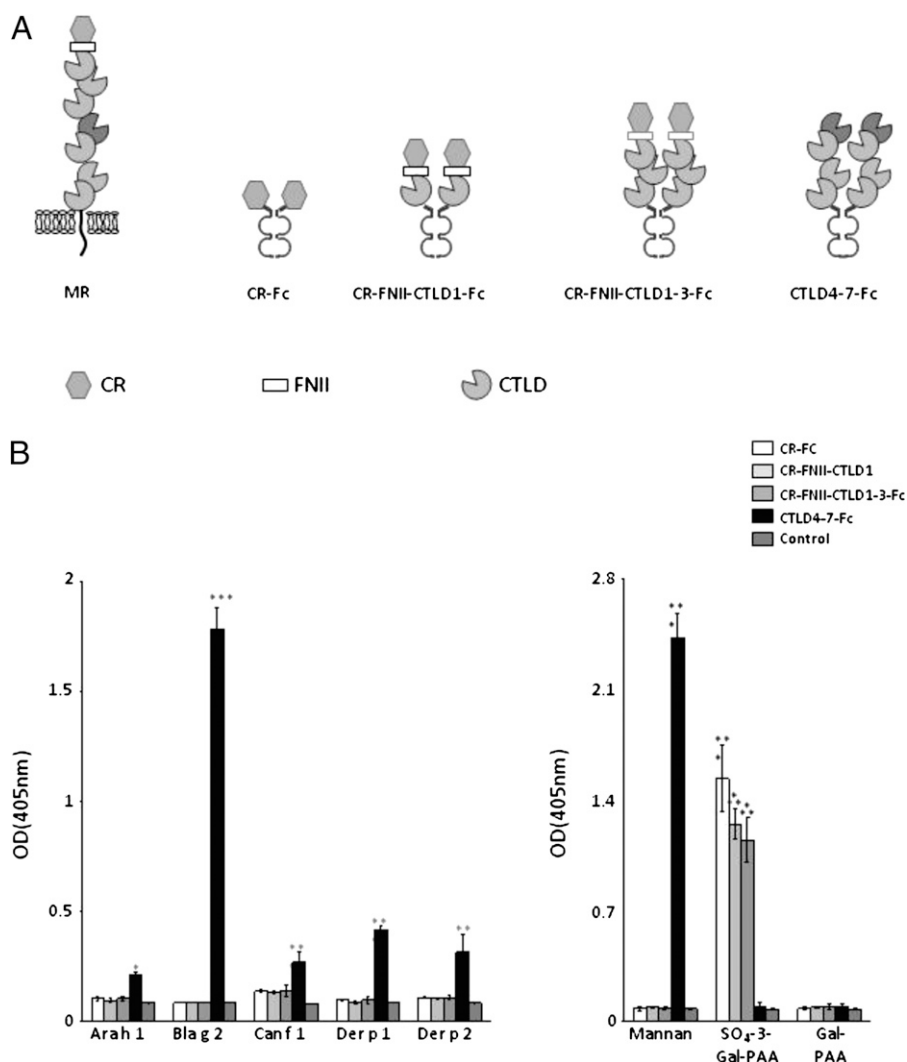
Statistical analysis

Values of the mean ± SEM are shown. ANOVA or Student *t* test was applied: **p* ≤ 0.05; ***p* ≤ 0.01; ****p* ≤ 0.001.

Results

MR mediates the uptake of allergens from diverse sources by DCs

Previous blocking experiments suggested a role for MR in the uptake of the house dust mite allergen Der p 1 (14). We therefore sought to investigate the contribution of MR to allergen uptake using a gene silencing strategy to specifically inhibit the expression of MR on human Mo-DCs. We assessed the expression of MR on control (CT) and MR siRNA-treated cells by real-time PCR (Fig. 1A), flow cytometry (Fig. 1B), and fluorescence microscopy (Fig. 1C). After MR siRNA treatment, mRNA transcription was inhibited by an average of 77 ± 7%, and MR expression on cell surface was inhibited by 68 ± 2%. The analysis of phenotypic markers, such as CD80 and CD86 or DC-specific intercellular adhesion molecule 3 grabbing nonintegrin (DC-SIGN), confirmed the specific inhibition of MR (Fig. 1D). Given that in subsequent experiments DCs were stimulated in the presence of LPS, it was important to rule out potential downregulation of relevant TLRs following siRNA treatment. Hence, we investigated TLR2 and TLR4 expression, which showed similar levels of expression in CT and MR siRNA-treated DCs (Fig. 1D).



We then investigated the uptake of a number of clinically relevant native airborne (Bla g 2, Can f 1, Der p 1, and Der p 2) and food (Ara h 1) allergens. The carbohydrate content of these allergens was first analyzed (Fig. 2, Table II). Whereas different patterns of glycosylation can be observed, all of the allergens tested are endowed with glycan chains potentially recognized by MR. In particular, as shown by the positive reaction with *Galanthus nivalis* agglutinin, high mannose-type carbohydrates are present on all of the allergens tested except Der p 2. By contrast, this allergen possesses a high level of *N*-acetylglucosamine (Gal β 1,3GlcNAc). We observed a significant reduction in the uptake of all of the allergens by the MR-deficient cells (Fig. 3), with the percentage decrease ranging from $23 \pm 3\%$ for Ara h 1 to $>65\%$ for Bla g 2 and Der p 1. As a control, we used a PAA polymer bearing Lewis^x Ag, a specific ligand for DC-SIGN, and as expected, no diminution of uptake was noticed with this molecule.

MR recognizes Ara h 1, Can F 1, Der p 1, Der p 2, and Bla g 2 via the CTLD4–7 domain

MR has two lectin activities mediated by the CR domain and the multiple CTLD domains. We used four different murine MR constructs, CR-Fc, CR-fibronectin type II (FNII)-CTLD1-Fc, CR-FNII-CTLD1–3-Fc, and CTLD4–7-Fc (17), in binding assays to determine whether MR could interact directly with the different allergens and which MR domains were involved (Fig. 4). Galactose-PAA was used as a negative control, and mannan and SO₄-3-galactose-PAA were used as ligands for the CTLD4–7 and the CR domains of the MR, respectively. Binding to the CTLD4–7 region was observed with Ara h 1, Bla g 2, Can f 1, Der p 1, and Der p 2 (Fig. 4B).

To further confirm the nature of the allergen–MR interaction, we investigated the ability of selected carbohydrates to inhibit the uptake of these allergens by Mo-DCs. As expected from the MR subfragment binding assays, a strong inhibition of Der p 1 uptake was observed with increasing doses of mannan (Fig. 5A). Additionally,

the uptake of Ara h 1, Bla g 2, Can f 1, and Der p 2 was significantly inhibited in the presence of 200 μ g/ml mannan (Fig. 5B).

MR is involved in Th2 polarization after exposure to Der p 1

We next sought to determine whether MR-mediated allergen recognition could modulate DC activity and support Th2 polarization. We thus compared the cytokine profile of T cells cultured with CT or MR-deficient autologous DCs. CD3⁺ T cells from house dust mite-sensitized donors were cocultured with Der p 1 plus LPS-exposed DCs for up to 15 d. LPS concentrations used in these experiments (i.e., 50 ng/ml) have been shown to be required for promoting Th2 responses to allergens (18). The percentage of IL-4 and IFN- γ -producing cells was determined by intracellular staining after restimulation with PMA and ionomycin for 8 h. The stimulation of T cells with untreated CT or MR-deficient DCs led to T cell populations with similar percentages of IL-4- and IFN- γ -producing cells, respectively (Fig. 6). As expected, LPS treatment slightly enhanced the production of IFN- γ in both sets of cocultures. After exposure of CT DCs to Der p 1, we observed an upregulation of IL-4 production. However, MR-deficient DCs treated with Der p 1 were not capable of inducing such a Th2 skewing. By contrast, they strongly supported IFN- γ production (Fig. 6). Furthermore, IFN- γ , IL-4, IL-5, and IL-13 production by T cells, after PMA and ionomycin restimulation, was determined in the culture supernatants, which showed a substantial decrease in IL-4, IL-5, and IL-13 concentrations in the MR-deficient condition as opposed to augmented IFN- γ production (data not shown). We also attempted measuring IL-10 and TGF- β concentrations in the supernatants; however, under current experimental conditions, the levels of both cytokines in CT and MR-deficient DC–T cell cocultures were just above the detection level. Thus, the presence or absence of MR on Mo-DCs, after allergen encounter, seems to determine the pattern of cytokine secretion by the T cells. Collectively, these results demonstrate the role of MR in Der p 1-driven Th2 polarization.

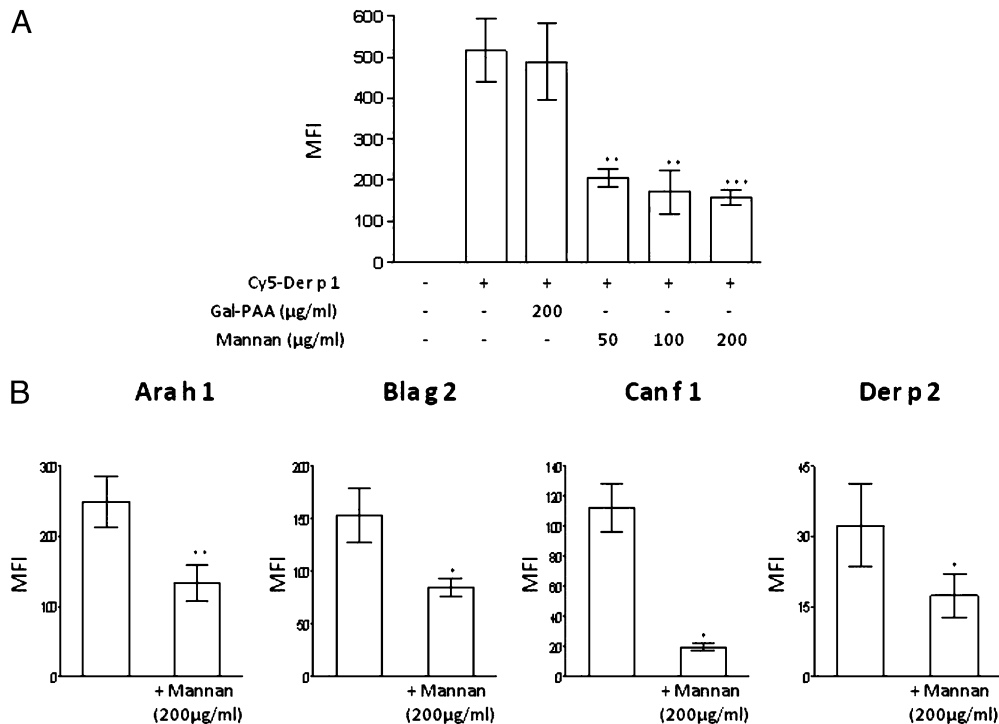
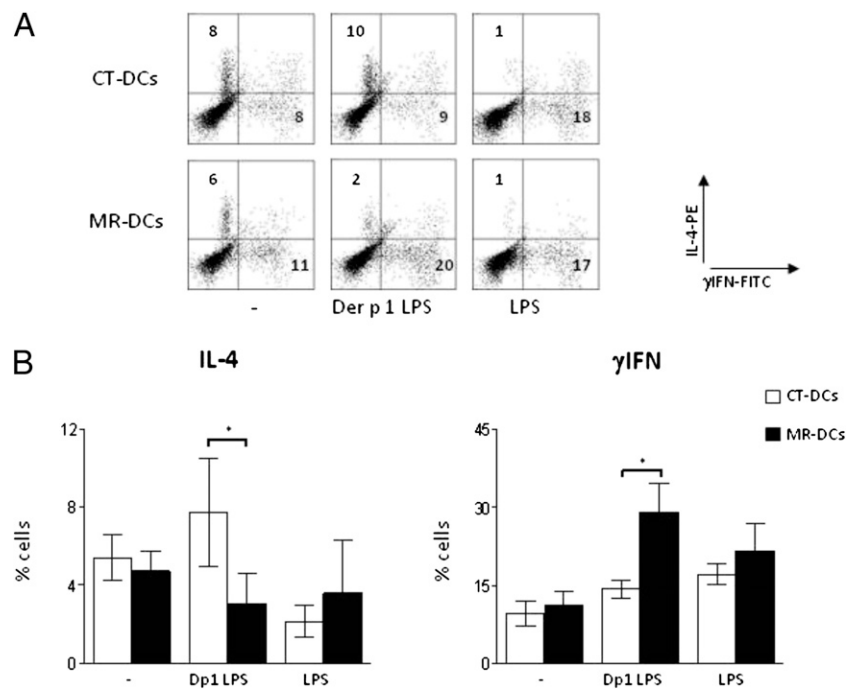


FIGURE 5. Inhibition of allergen uptake by DCs with MR ligands. *A*, Human Mo-DCs were incubated for 25 min with 5 μ g/ml Cy5-labeled Der p 1 in the presence of increasing doses of mannan. Galactose-PAA was used as a control ($n \geq 3$). *B*, Inhibition of Ara h 1, Bla g 2, Can f 1, and Der p 2 uptake by mannan ($n \geq 3$).

FIGURE 6. MR is involved in Th cell polarization after exposure to Der p 1. Analysis of Der p 1-induced T cell polarization in DC–T cell cocultures. CD3⁺ T cells were cultured with autologous CT or MR-deficient DCs exposed to LPS or LPS and Der p 1. T cell polarization was investigated at day 15 by intracellular IL-4 and IFN- γ staining on CD4⁺ gated cells after restimulation with PMA and ionomycin. *A*, One representative experiment is shown. *B*, Summary of five independent experiments.



CT or MR-deficient DCs induce T cell polarization differently through modulation of IDO activity

We investigated the expression of the costimulatory molecules CD80 and CD86 and production of the cytokines IL-6, IL-10, and IL-12p70 after Der p 1 and LPS treatment. Despite the well recognized involvement of these signals in T cell polarization, no differences were observed between the CT and the MR-deficient DCs (data not shown). Several studies have recently reported the potential involvement of the tryptophan-degrading enzyme IDO in the control of allergic reactions (19–21). In particular, Maneechote-suan et al. (19) have shown that upregulation of IDO activity in DCs could inhibit the development of Th2 cell clones. We therefore sought to measure IDO activity in CT and MR-deficient DCs. Compared to control DCs, we found an upregulation of IDO activity in MR-deficient cells after LPS treatment that reached significance in the presence of Der p 1 (Fig. 7A). Interestingly, despite significant differences in activity level, flow cytometry experiments showed similar IDO expression in CT and MR-deficient cells in response to Der p 1 and LPS (Fig. 7B). In support of these observations, we also observed an inhibition of IDO activity in response to LPS after MR engagement with specific anti-MR Ab or with mannan (data not shown). Thus, IDO activity seems to be downregulated upon MR binding.

We then hypothesized that the variation in IDO activity between the CT and the MR-deficient DCs could account for the observed differential T cell polarization. To verify this, we performed coculture experiments in which 1) we blocked IDO activity in the MR-deficient DCs, using the specific inhibitor MTHIT (22, 23), or 2) we simulated its activity, in the CT condition, by the addition of the tryptophan metabolites KYN and 3HAA. MTHIT treatment resulted in ~80% suppression of IDO activity in MR-deficient DCs treated with Der p 1 and LPS (data not shown). Although the suppression of IDO activity in the MR-deficient cells specifically led to a reversal of polarization toward Th2, as demonstrated by an increase in the percentage of IL-4-producing cells along with a decrease in IFN- γ production, it had no effect in CT cells (Fig. 8A).

Tryptophan metabolites, namely, KYN or 3-HAA, have been shown to mediate most of the immunological effects of IDO

(24). We thus investigated the impact of these molecules on T cell polarization. In line with the results obtained for the inhibition of IDO activity, we observed that treatment with these tryptophan metabolites inhibited Th2 polarization after Der p 1 treatment, with a significant decrease in IL-4 production and a slight increase in IFN- γ production (Fig. 8B).

Taken together, these results show that changes in IDO activity after Der p 1 and LPS treatment account for the contrasting T cell

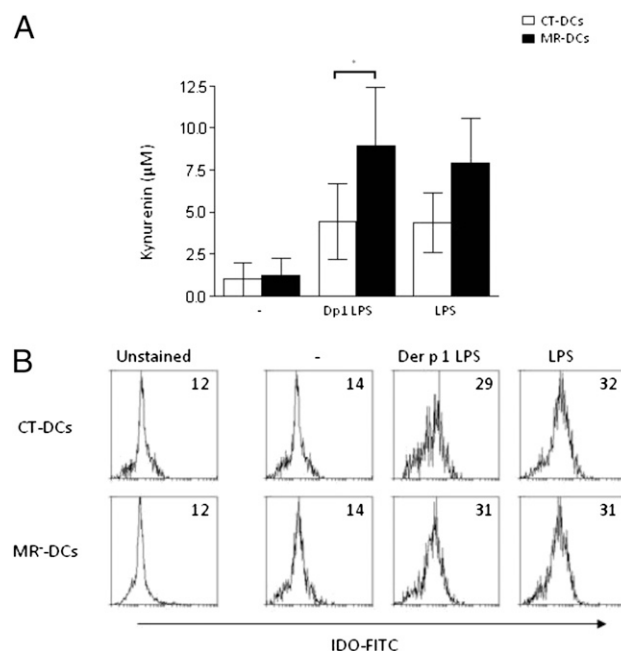


FIGURE 7. IDO activity and expression on CT and MR-deficient cells. *A*, CT or MR-deficient DCs were incubated for 24 h with LPS or Der p 1 and LPS in R10 medium with GM-CSF, IL-4, and 100 μ M L-tryptophan. IDO activity was determined by the dosage of KYN in DC supernatant ($n = 5$). *B*, Assessment of IDO expression by flow cytometry. CT or MR-deficient DCs were collected after 24 h of culture, and IDO expression was investigated by flow cytometry. MFI is indicated on each histogram. One representative experiment of four is shown.

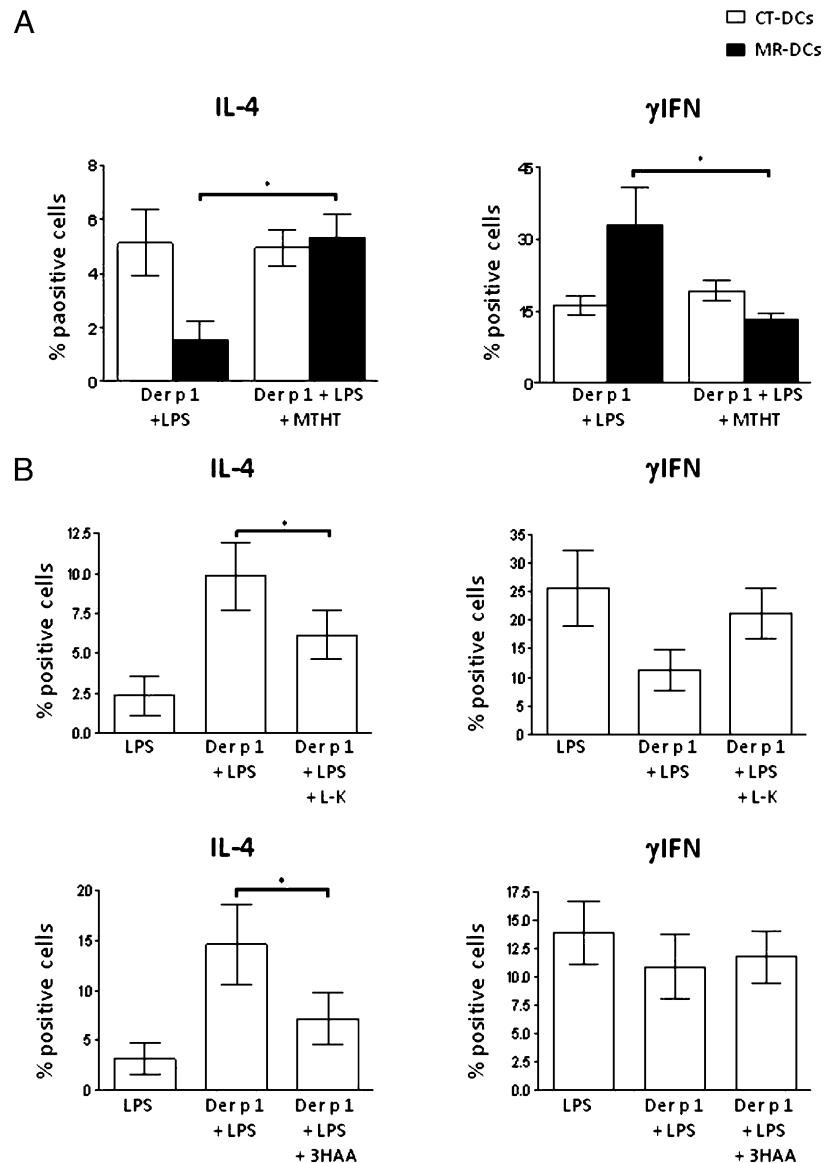


FIGURE 8. T cell polarization is regulated through the modulation of IDO activity. *A*, CT and MR-deficient DCs were exposed to Der p 1 and LPS in the presence or absence of the IDO inhibitor MTHT. DCs were then cocultured with T cells, and polarization was assessed at day 15 by measuring IL-4 and IFN- γ production on CD4⁺ gated cells after restimulation by PMA and ionomycin ($n = 4$). *B*, The IDO activity is mediated by the tryptophan metabolites KYN and 3HAA. CT DCs exposed to Der p 1 and LPS were cocultured with T cells in the presence or absence of 10 μ M KYN or 3HAA. After 15 d, T cell polarization, on CD4⁺ gated cells, was determined by analysis of IL-4 and IFN- γ production ($n \geq 3$).

polarization outcomes observed between the CT and the MR-deficient DCs.

Discussion

DCs are sentinels of the immune system with a superior ability for Ag recognition and uptake, as well as for T cell stimulation or priming (25). Microbial recognition by DCs and the development of subsequent immune responses, including the differentiation of Th1 and Th17 cells, are processes that are relatively well understood. However, despite the well recognized role of DCs in the initiation and maintenance of allergic sensitization (1, 2), their interaction with allergens and how subsequent Th2-type responses are established remained scarcely defined. The molecular definition of the mechanisms involved in allergen recognition and uptake by DCs is crucial for our understanding of the nature of the trigger for allergic responses.

The partial involvements of MR and DC-SIGN in the uptake of Der p 1 and Ara h 1, respectively, have been reported (14, 26); however, for most allergens, no receptors have been described yet. Glycosylation is a common feature of many allergens (27, 28), and it is therefore reasonable to assume that MR, through its carbohydrate recognition domains, could act as a common receptor for

diverse allergens. Because the carbohydrate specificity of MR is partially shared by other lectin receptors, such as DC-SIGN, we chose a gene silencing strategy to specifically target MR expression. MR-deficient DCs displayed a reduced capacity to internalize various airborne (Bla g 2, Can f 1, Der p 1, and Der p 2) and food (Ara h 1) allergens. Although other molecules are probably also involved in allergen recognition, the considerable decrease ($\geq 50\%$) of Bla g 2, Der p 1, and Der p 2 allergen uptake observed with the MR-deficient DCs suggests that MR may be the main endocytic receptor for these allergens. Accordingly, we have also demonstrated that MR directly binds to these allergens. The two sugar binding sites of MR, the CR domain and the CTLD4–7 region, were assessed for their allergen binding properties. The binding of all of the allergens tested to MR was mediated by the CTLD4–7 domains. This was in agreement with the glycosylation pattern of the allergens and in particular the presence of mannose or *N*-acetylglucosamine chains.

The present data clearly show the binding of Der p 2 to MR as well as MR-mediated uptake of Der p 2 by human DCs. This is particularly interesting, because recent work has shown the ability of Der p 2 to activate TLR4 signaling through its homology with the LPS binding molecule MD-2 (29). It is therefore

reasonable to suggest that MR could synergize with TLR4 in recognition of Der p 2 and subsequent signaling events that lead to Th2 polarization.

Having identified MR as a major receptor for diverse glycoallergens, we investigated its role in the development of the allergic response to the major house dust mite allergen Der p 1. Our results demonstrate for the first time the involvement of MR in Th2 polarization after allergen exposure, as demonstrated by the development of an IL-4 response that was dependent on the expression of MR on DCs. The secretion of IL-5 and IL-13, two other key type 2 cytokines (30, 31), was also dampened by MR deficiency. By contrast, the production of IFN- γ was boosted after T cell stimulation by MR-deficient DCs. These data provide new perspectives with regards to allergen-induced T cell polarization. MR expression has been shown to be higher on DCs from atopic donors (14), and as such, it is reasonable to assume that the allergen-induced Th2 cell polarization seen in atopic donors could, at least in part, be due to a more efficient uptake of allergen through MR. Within this context, it is interesting to note that mapping analyses in mouse (13) and human (12) have identified *Mrc1* (MR C-type 1) as a positional candidate gene for allergen-induced hyperresponsiveness, thus showing a clear association between MR and asthma. In a recent study, Hilmenyuk et al. (32) have shown the impact of glycation, and in particular the presence of advance glycation end products (AGEs), on the immunogenicity and allergenicity of the model allergen OVA. Interestingly, they show that the presence of AGEs on OVA favors its uptake by Mo-DCs, and experiments showing the inhibition of OVA uptake by mannan suggest that MR could be involved in AGE-OVA recognition. These observations clearly support our own results regarding the lower uptake of glycosylated allergens by MR-deficient DCs. The authors also observe a higher secretion of IL-6 by DCs after AGE-OVA treatment. This is in contrast with our data showing similar production of IL-6 by CT and the MR-deficient cells. It is very likely that this is due to the different conditions used to induce DC maturation in these two studies [i.e., TNF- α , IL-1 β , and PGE₂ in Hilmenyuk et al. (32) versus LPS in our work]. In particular, PGE₂ has been shown to enhance IL-6 levels (33), which might explain the relevance of this cytokine in the Hilmenyuk et al. (32) model.

Despite having comparable levels of IDO expression, we observed a significant difference in IDO activity after Der p 1 and LPS treatment between CT and MR-deficient DCs. Moreover, the stimulation of MR with specific Ab or with mannan led to a reduction in IDO activity after LPS treatment (data not shown). These results, together with our other data showing similar levels of TLR4 expression by CT and MR-deficient cells, suggest that MR engagement may contribute to the maintenance of a lower IDO activity. Translational or posttranslational regulations of IDO activity are still poorly understood. Braun et al. (34) have reported the need for TLR or TNF signaling for the production of the active form of IDO. In our experiments, DCs were treated with LPS or with a combination of Der p 1 and LPS. Therefore, further work is required to determine how Der p 1 or LPS interferes, through MR engagement, with TLR4 signaling and whether they are able to modulate IDO activity through binding to another receptor.

Although the immunoregulatory role of IDO in animal models is now well described, many aspects of its expression and activity in the human system remain unclear (35, 36). IDO has been shown to exert its immunoregulatory activity through different mechanisms, including the induction of regulatory T cells (Tregs) or the depletion of effector T cells. The ability of IDO to inhibit asthma development in mouse models has been recently reported by different groups (20, 21, 37, 38). However, the role of IDO in asthma seems to be dependent on the model used or on

the cell type investigated, because another study associated IDO activity with Th2 responses and the development of allergic reactions (39). In humans, a low IDO activity in serum correlates with the atopic status of the donors (40). Moreover, in vitro experiments have demonstrated the suppressive effect of IDO on the proliferation of Der p 1-specific Th2 cell clones (19). The factors involved in Th2 cell differentiation are still elusive; in particular, the absence of Th2 cytokine (such as IL-4) production by DCs has led to the view that Th2 responses develop only in the absence of Th1 signals (2). In this study, we show that IDO activity plays an important role in regulating the balance between Th1 and Th2 cells. This effect was notably mimicked by the tryptophan metabolites KYN and 3HAA. Because 3HAA has been shown to induce the death of Th2 cells (41), it is reasonable to consider that IDO activity, through the production of tryptophan metabolites, induces the death of Th2 cells and consequently favors the development of Th1 cells. Nevertheless, further experiments will clearly need to be performed to define how these tryptophan metabolites preferentially affect Th2 cells and to determine also if tryptophan depletion takes part in this process. It will be of particular interest to determine if IDO activity results in the inhibition of early IL-4 production by T cells or other immune cells involved in Th2 differentiation (42). Moreover, IDO has been shown to support Treg expansion (43, 44); thus, it will be interesting to define whether IDO activity directly inhibits Th2 cell differentiation or acts through the development of Tregs. To this end, our initial observations indicate that higher IDO production by MR-deficient DCs does lead to an increase in the number of FOXP3⁺ cells; however, the cytokine profile and the regulatory function of these cells have yet to be fully investigated.

The role of DCs in Th2 cell polarization was recently challenged by mouse in vivo experiments showing that Th2 responses could be induced in the absence of DCs. These studies have shown that basophils are essential and indeed sufficient for the initiation of Th2 responses to helminthic parasites, allergens, or Ag-IgE complexes (45–47). These are clearly very interesting observations; however, there are equally convincing data, including those presented in this study, demonstrating the key role played by DCs in the development and maintenance of allergen-driven Th2 responses (26, 31, 48–50). It is obvious that unlike Th1 and Th17 responses early events leading to Th2 cell differentiation are more complex, and there seems to be diversity dependent on the nature of the allergens initiating the immune response. Thus, although DCs may not be able to produce IL-4, other DC signals, including IDO activity, can drive Th2 cell polarization. Given their exceptional capacity for Ag recognition and uptake from exposed epithelial surfaces as well as for priming naive T cells, DCs still remain as key cells involved in the induction and re-elicitation of Th2-mediated inflammation in allergic diseases (1, 2), particularly in human.

In conclusion, we have demonstrated that MR is the main receptor on DCs for a number of glycoallergens from diverse sources and that MR plays a key role in the Th2 cell polarization observed after Der p 1 exposure through the regulation of IDO activity. The mechanisms of IDO regulation through MR- and IDO-dependent Th2 cell polarization are therefore of major interest for the understanding of allergic processes and for the development of new strategies for the treatment of allergic diseases.

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Disclosures

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