The mannose receptor negatively modulates the TLR4-AhR-IDO axis in dendritic cells affecting T helper cell polarization

Fabián Salazar, MSc,[a] Laurence Hall, BSc,[a] Ola H. Negm, PhD[a,b], Dennis Awuah, MSc,[a] Patrick J Tighe, PhD,[a] Farouk Shakib, PhD, FRCPath,[a] and Amir M. Ghaemmaghami, MD, PhD[a]

[a] Division of Immunology, School of Life Sciences, Faculty of Medicine and Health Sciences, University of Nottingham, United Kingdom.

[b] Medical Microbiology and Immunology Department, Mansoura University, Egypt.

Address correspondence and reprint request to Dr. Amir M. Ghaemmaghami, Division of Immunology, School of Life Sciences, Faculty of Medicine and Health Sciences, West Block, A Floor, Queen's Medical Centre, University of Nottingham, Nottingham NG7 2UH, UK. Phone: +44 115 82 30730. Fax: +44 115 82 30759. Email: amg@nottingham.ac.uk.

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Abstract

Background: Dendritic cells (DCs) are key players in the induction and re-elicitation of Th2 immune responses to allergens. We have previously shown that different C-type lectin receptors on DCs play a major role in allergen recognition and uptake and downstream events leading to allergic sensitization. In particular, mannose receptor (MR), through modulation of TLR4 signalling, can regulate indoleamine 2,3 dioxygenase (IDO) activity favouring Th2 immune responses. Interestingly, the aryl-hydrocarbon receptor (AhR), a ligand-dependent transcription factor with an emerging role in immune modulation, has been implicated in IDO activation in response to TLR stimulation.

Objective: Here we investigated how allergens and lectins through MR can modulate the TLR4-AhR-IDO axis in human monocyte-derived DCs.

Methods: Using a combination of genomics, proteomics techniques and immunological studies, we investigated the role of MR and AhR in IDO regulation and its impact on T helper cell differentiation.

Results: We have demonstrated that LPS induces both IDO isoforms i.e. IDO1 and IDO2 in human DCs with partial involvement of AhR. Additionally, we found that like mannan airborne allergens from diverse sources can effectively down-regulate the TLR4-induced IDO1 and IDO2 expression, most likely through biding to the MR. Mannose-based ligands were also able to down-regulate IL-12p70 production by DCs affecting T helper cell polarization. Interestingly, AhR and some key components of the non-canonical NF-κB pathway were shown to be down-regulated after MR engagement, which could explain regulatory effects of the MR on IDO expression.

Conclusion: Our work demonstrates a key role for MR in the modulation of the TLR4-IDO-AhR axis, which clearly has a significant impact on DC behaviour and the development of immune responses against allergens.

Key messages

- TLR4 induction of IDO1 and IDO2 is down-regulated by airborne allergens through the mannose receptor in human dendritic cells changing their immune-regulatory properties.
- IDO regulation in human DCs is partially dependent on the aryl-hydrocarbon receptor, a ligand-dependent transcription factor involved in sensing intracellular or environmental changes, with participation of the non-canonical NF-κB pathway.

Capsule summary

This article demonstrates that several airborne allergens down-regulate both IDO isoforms in human DCs with a mechanism likely through binding to the mannose receptor. This process involves the aryl-hydrocarbon receptor and the NF-κB pathway. This work therefore highlights novel targets that can be used for modulating allergen-driven Th2 immune responses.

Key words

Dendritic cells, allergy, T helper 2, indoleamine 2,3-dioxygenase, C-type lectin receptor, mannose receptor, TLR4, aryl-hydrocarbon receptor, NF-κB.

Abbreviations

AhR, aryl-hydrocarbon receptor; BGP, Bermuda grass pollen; CLR, C-type lectin receptor; DC, Dendritic cell; DC-SIGN, dendritic cell-specific intracellular adhesion molecule 3-grabbing non-integrin; GC, German cockroach; HDM, house dust mite; IDO, indoleamine 2,3-dioxygenase; KYN, kynurenine; Man-LAM, mannose-capped lipoarabinomannans; monocyte-derived dendritic cell; NF-κB, nuclear factor-kappaB; MR, mannose receptor; Treg, regulatory T cells; TRP, tryptophan.

Introduction

Allergic diseases are typically characterized by an exacerbated immune response against allergens leading to Th2 polarization and production of allergen-specific IgE. Despite recent advances, the early events leading to allergic sensitization and the elicitation of Th2 immune responses are still unclear. Dendritic cells (DCs) are specialized antigen presenting cells that play an important role in the induction and re-elicitation of Th2-allergic immune responses (1-4). Recent work by us and others have shown that C-type lectin receptors (CLRs) such as mannose receptor (MR) and Dendritic cell-specific intracellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) play a major role in the recognition and uptake of allergens, the initial steps leading to allergic sensitization (1, 5-10). Interestingly, under certain conditions, DC-SIGN and MR ligation can have opposing effects on T cell polarization (5, 6). MR is a multifunctional endocytic receptor with two independent carbohydrate-binding domains that recognize sulfated and mannosylated structures, respectively (11). Several studies have demonstrated that CLR activation can modulate pattern recognition receptor-induced activation, particularly TLR4 signaling, and the downstream events leading to Th2 cell polarization (1, 5, 12-16). We have previously shown that Der p 1, the main allergen from house dust mite (HDM), in the presence of low levels of LPS down-regulates indoleamine 2,3-dioxygenase (IDO) through engagement of MR on human DCs leading to a Th2 immune response (5). This data shows the involvement of MR in inducing Th2-allergic immune responses through regulation of IDO activity in human DCs with the participation of TLR4 signaling however the exact mechanism remains unclear.

IDO is an enzyme that catalyzes the degradation of the essential amino acid tryptophan (TRP) into N-formyl-kynurenine, the first and rate-limiting step of TRP catabolism in the kynurenine pathway (17, 18). IDO has been demonstrated to be involved in diverse immune-regulatory processes in health and disease (17, 18). In particular, it has been shown that IDO

has a protective role in several models of experimental asthma (19-24). Furthermore, clinical studies have shown that asymptomatic non-atopic individuals have higher systemic IDO activity than symptomatic atopic individuals which might support the notion of IDO as a protector against allergy (25, 26). However, the mechanism of IDO regulation in these models particularly in a human context has remained elusive.

Aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor involved in sensing intracellular or environmental changes (27). Kynurenine (KYN), one of the main metabolites produced in the IDO-dependent TRP degradation pathway, can activate AhR leading to the generation of regulatory T cells (Treg) (28). In addition, previous observations in mouse DCs have suggested that AhR mediates IDO induction in response to TLR agonists (29, 30). Here we sought to investigate how allergens modulate the crosstalk between MR and TLR4 in the context of IDO and to establish whether AhR plays a role in IDO regulation in human DCs. We have demonstrated that different airborne allergens, most likely through biding to MR, can significantly down-regulate TLR4 induction of IDO and impair DC response to LPS as evidenced by suppression of IL-12 production and primingTh1 responses. Furthermore, we show for the first time the co-regulation of AhR and components of the non-canonical nuclear factor-kappaB (NF-κB) pathway with IDO expression, suggesting that a functional and/or physical association between them could be involved in IDO regulation in human DCs. These data further highlights the intrinsic immuno-modulatory properties of allergens through engaging CLRs and can help better understanding of how allergens can modulate DC behavior and bias T cell responses towards Th2 immune phenotype.

Material and Methods

Generation of human DCs from blood samples

Buffy coat samples were obtained from healthy volunteers after obtaining informed written consent and approval of local ethics committee (National Blood Service, Sheffield, UK). DCs were generated as described before (5, 31). Briefly, peripheral blood mononuclear cells were separated by density gradient centrifugation on Histopaque (Sigma-Aldrich). $CD14^+$ monocytes were isolated from PBMCs by positive selection using a magnetic cell separation system (Miltenyi Biotech, UK) (purity >95%) and were then seeded in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10%v/v heat inactivated Foetal Bovine Serum, 100 U/ml penicillin - 100 µg/ml streptomycin and 2 mM L-Glutamine (all from Sigma-Aldrich). Monocyte differentiation into DCs was carried out for 6 days in the presence of 50 ng/ml GM-CSF and 250 U/ml IL-4 (both from Miltenyi Biotech). Human peripheral blood myeloid DCs were isolated using the $CD1c^+$ dendritic cell isolation kit (Miltenyi Biotech) according to manufacturer's instructions.

Quantification of IDO activity

DCs ($2.5x10^5$ cells/ml) were seeded in a 24-well plate with complete media supplemented with 100 µM L-TRP (Sigma-Aldrich). After stimulation, IDO activity was measured by quantification of the levels of KYN in the culture supernatant using a colorimetric assay as we have described previously (5). Allergen extracts were purchased from Greer Labs, USA. Typical exotoxin content of HDM preparations was 26.1 EU/mg protein.

Flow cytometry analysis

Antibodies (Abs) against IFN-γ (clone B27), IL-4 (clone MP4-25D2) and MR (clone 19.2) were purchased from Biolegend, UK. Abs against CD80 (clone MAB104), CD83 (clone

HB15a), CD86 (clone HA5.2B7), MHC-II (clone Immu-357), CD4 (clone 13B8.2) and DC-SIGN (clone AZND1) were purchased from Beckman coulter, UK. Abs against PDL2 (clone MIH18), PDL1 (clone MIH1) and ICOSL (clone 2D3/B7-H2) were purchased from BD Biosciences. Ab against AhR (clone FF3399) was purchased from eBioscience, UK. Ab against IDO1 (clone 700838) was purchased from R&D Systems, UK. Ab against TLR4 (clone HTA125) was purchased from AbD serotec, UK. Nonreactive isotype-matched Abs were used as controls. Briefly, cells were harvested and washed twice with PBA (PBS containing 0.5% BSA and 0.1% sodium azide) (all from Sigma-Aldrich). At this point surface staining was done for 20 min at 4°C. For intracellular staining, cells were fixed for 10 min at room temperature with formaldehyde 4% (Sigma-Aldrich). They were then washed twice with permeabilization/wash buffer (PBA containing 0.5% saponin (Sigma-Aldrich)), stained for 30 min at 4°C and washed twice in the same buffer before analysis in an FC 500 Flow Cytometer (Beckman Coulter) (32). Intracellular staining for cytokines was analysed in a MoFlo XDP Flow Cytometer (Beckman Coulter). All data analysis was done using Weasel Software.

mRNA isolation, cDNA synthesis and PCR

Cells were washed twice in ice-cold PBS and total RNA extraction was carried out using the RNeasy Plus Minikit (Qiagen, UK) according to manufacturer's instructions. Samples were then DNase treated using the TURBO DNA-free kit (Thermo Fisher Scientific) and total RNA was concentrated through ethanol precipitation. cDNA was synthesized from total RNA using superscript III first-strand synthesis kit (Thermo Fisher Scientific) according to manufacturer's instructions.

Conventional PCR was carried out in a TC-312 PCR Thermocycler (Bibby Scientific Ltd, UK) using the Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific).

Cycling was initiated at 98°C for 10 secs, followed by 30 cycles of 98°C for 1 sec, 64°C for 5 secs and 72°C for 15 secs, the final extension was done at 72°C for 1 min. Then, the PCR products were analysed in an E-gel pre-cast 2% agarose electrophoresis system (Thermo Fisher Scientific).

Quantitative real time PCR was performed in a Strategene MxPro 3005P qPCR System with the Brilliant III Ultra-Fast SYBR Green qPCR Master Mix (Agilent Technologies, USA). Cycling was initiated at 95°C for 3 min, followed by 40 cycles of 95°C for 20 secs and 60°C for 20 secs, a melting curve was done at the end. Samples were run in triplicates, and relative expression was calculated using the comparative threshold cycle method, also known as the ΔΔCt method, normalized to GAPDH (33, 34).

Primers were obtained from Eurofin, UK: Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) Forward (5`-GAGTCAACGGATTTGGTCGT-3`), GAPDH Reverse (5`-GACAAGCTTCCCGTTCTCAG-3`), IDO1 Forward (5`-GGCACACGCTATGGAAAACT-3`), IDO1 Reverse (5`- GAAGCTGGCCAGACTCTATGA-3`), IDO2 Forward (5'-CTGATCACTGCTTAACGGCA-3'), IDO2 Reverse (5'-TGCCACCAACTCAACACATT-3'), AhR Forward (5'-ATCACCTACGCCAGTCGCAAG-3'), AhR Reverse (5'-AGGCTAGCCAAACGGTCCAAC-3'), CYP1A1 Forward (5'-CACAGACAGCCTGATTGAGCA-3'), CYP1A1 Reverse (5'-GTGTCAAACCCAGCTCCAAAGA-3'), RelB Forward (5'-TCGTCGATGATCTCCAATTCAT-3'), RelB Reverse (5'-CCCCGACCTCTCCTCACTCT-3'), MR Forward (5'-CGTTTACCAAATGGCTTCGT-3') and MR Reverse (5'-CCTTGGCTTCGTGATTTCAT-3').

Cytokine measurements

Cell-free supernatants were collected and stored at -20°C before analysis. Cytokine (IL-6, IL-10, IL-12p70, TGF-β and IFN-α) concentration was analysed using the ProcartaPlex Multiplex Immunoassay system (eBioscience) according to manufacturer's instructions.

RNA interference

Small interfering RNA (siRNA) was carried out as previously described with slight modification (5, 6). MR and AhR siRNA were the SMARTpool: ON-TARGETplus siRNA from GE Healthcare, UK. The control siRNA was the ON-TARGETplus Non-targeting control from GE Healthcare. Monocytes were transfected on day 0 with 50 nM siRNA using the DharmaFECT 2 Transfection Reagent (GE Healthcare). The inhibition was assessed at day 6.

Reverse phase protein microarray

After stimulation, DCs were washed twice with ice-cold PBS and lysed in 60 µl of RIPA buffer containing protease and phosphatase inhibitors (all from Thermo Fisher Scientific). For reverse phase protein microarray the procedure described in Negm *et al*. was followed (35). After denaturation, samples were spotted onto nitrocellulose-coated glass slides (Grace Bio-labs) with a microarray robot (MicroGrid 610, Digilab). Then, slides were incubated overnight in blocking solution (0.2% I-Block (Thermo Fisher Scientific), 0.1% Tween-20 in PBS (Sigma-Aldrich) at 4ºC. After washing, slides were incubated with primary Abs overnight at 4ºC. β-actin Ab was included as a loading control. All Abs were purchased from Cell Signaling Technologies. After washing, slides were incubated with infrared Licor secondary abs for 30 min at room temperature in the dark. Finally, slides were scanned with a Licor Odyssey scanner (LI-COR, Biosciences). The resultant TIFF images were processed with Genepix Pro-6 Microarray Image Analysis software (Molecular Devices Inc.). Protein signals were finally determined with background subtraction and normalization to the internal

housekeeping targets. Signal values represented on the colour scale for the heat map are log2 transformed from the arbitrary fluorescence units (AFU) and normalized by using the standard deviation. Heat maps were generated using TMEV software.

DC-T cell co-culture

Human DCs were treated or not with mannan (10 µg/ml) (Sigma-Aldrich) and co-cultured in the presence of LPS (0.01 µg/ml) (Sigma-Aldrich) in 96-well U-bottom plate (Corning Life Sciences) with $CD3^+CD45RA^+$ autologous naïve T cells (DC-Tc ratio 1:10) purified by immunomagnetic cell sorting (Miltenyi Biotech) in RPMI 1640 supplemented with 5% human AB serum, 100 U/ml penicillin - 100 µg/ml streptomycin and 2 mM L-Glutamine (all from Sigma-Aldrich). After 3-4 days, IL-2 (5 ng/ml) (R&D Systems) with fresh media was added to the co-culture. After another 3-4 days, T cells were restimulated with anti-CD3 (Sigma-Aldrich) and anti-CD28 (2 µg/ml) for 18 hrs (AbD serotec). For intracellular staining, brefeldin-A (10 µg/ml) (Sigma-Aldrich) was added after 2 hrs and the production of IL-4 and IFN-γ was detected on $CD4^+$ cells using specific abs. Quadrants were set in a way that 99.5% of the cells were in the bottom or left quadrant in the fluorescence minus one (FMO) controls accordingly.

Statistical analysis

Values of the mean ± SEM are shown unless otherwise stated. ANOVA or Student t Test was applied: *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$. For all statistical analysis GraphPad Prism 5 Software was used.

Results

TLR4 agonist induce IDO1 and IDO2 in human dendritic cells

Our data clearly show that LPS on its own can effectively induce the expression of both IDO isozymes (IDO1 and IDO2) and IDO activity in human monocyte-derived DCs as well as in peripheral blood myeloid DCs (Fig. 1A, B, C, D, E). This is in contrast with previous reports where co-stimulation with either IFN-γ or prostaglandin E2 was deemed to be essential (36-39). Additionally, this effect was observed with LPS from different bacterial sources, such as *E.coli* and *S.minnesota* (Fig. 1A and data not shown). Some LPS preparations are thought to be contaminated with small quantities of other bacterial components that could also activate TLR2 signaling. To confirm that the outcome on IDO was mediated by TLR4 engagement, we tested the effect of ultrapure LPS as well as the synthetic diacylated lipoprotein (FSL-1) as a specific TLR2 agonist. Our data showed similar dose-dependent effect induced by increasing concentrations of ultrapure LPS (Fig. 1B), while FSL-1 did not affect IDO activity in human DCs (data not shown). Interestingly, our data also show a 'bell-shaped' dose response for IDO activity after TLR4 activation with LPS from *E.coli*, with high levels (10 μg/ml) inducing less IDO activity than low levels (0.1 μg/ml) in human DCs. However, this was not the case for ultrapure LPS, where we observed a dose dependent induction of IDO activity in human DCs. The effect of LPS was not associated with cellular death as high levels of LPS did not affect cell viability quantified by the presence of annexin-V and propidium iodine (data not shown).

TLR4-induction of IDO1 and IDO2 is down-regulated by allergens from diverse sources

After establishing the role of LPS in IDO regulation, we investigated how different allergen extracts i.e. House Dust Mite (HDM) extract from *Dermatophagoides pteronyssinus*, German Cockroach (GC) extract from *Blattella germanica* and Bermuda Grass Pollen (BGP) extract

from *Cynodon dactylon* can modulate IDO activity in the presence and absence of LPS. A slight down-regulation in IDO activity levels were observed when human DCs were exposed to allergen extracts alone but this did not reach statistical significance. However, allergen extracts from HDM, cockroach and pollen were able to significantly reduce LPS-driven up-regulation of IDO in human DCs (Fig. 2A).

We have previously shown that many allergens from diverse sources (including HDM and cockroach) are heavily mannosylated (7) and such sugar moieties play a key role in CLR mediated allergen recognition and uptake by DCs (1, 7) . Accordingly, we studied the impact of highly mannosylated sugars in IDO regulation using mannan as a prototypic high mannose carbohydrate that can be recognized by MR (40, 41). Here we have shown that mannan is able to down-regulate both IDO1 and IDO2 gene expression and activity in human DCs (Fig. 2B, C). This data suggests a key role for carbohydrates in allergen preparations in modulating IDO in human DCs.

Mannose-based ligands down-regulate TLR4-induced IL-12p70 production in human dendritic cells and affect T helper cell polarization

Several cytokines have been linked with IDO regulation. Particularly, IL-10, TGF-β and type-I IFNs have been associated with IDO induction (18, 25, 42), while IL-6 has been shown to be involved in IDO degradation (18). Additionally, it has been suggested that specific MR agonists, such as the mannose-capped lipoarabinomannans (Man-LAM), can negatively regulates TLR4-dependent IL-12 production in mouse macrophages (43). Accordingly, we studied how these cytokines are regulated by mannan in human DCs. No significant differences were found when DCs were stimulated with mannan alone compared to un-stimulated DCs (Fig. 3A). However, the presence of mannan significantly reduced IL-12p70 production after LPS stimulation compared with LPS alone (Fig. 3A). Similar results were

found after stimulating DCs with LPS in the presence of HDM, cockroach and pollen extracts (Fig. 3B). Subsequently, we evaluated how different costimulatory receptors were regulated under these conditions. No significant differences were found for most of the receptors tested, except a significant down-regulation in CD86 expression (Fig. 3C).

We then performed co-culture experiments using DCs pre-stimulated with either mannan and/or LPS prior to co-culture with autologous naïve T cells. Our data shows a significant reduction in IFN-γ production by T cells that were co-cultured with 'mannan+LPS' primed DCs compared to LPS only controls (Fig. 3D, E, F). This reduction in IFN-γ was not due to reduced cell viability and was reflected in both percentage of IFN-γ producing cells and level of IFN-γ measured in the culture supernatant (Fig. 3D, E). Given the non-atopic status of donors, not surprisingly we did not detect high levels of IL-4 producing T cells except in one donor where there was a significant increase in the percentage of IL-4 producing cells in 'mannan+LPS' condition (data not shown). Taken together, these results show that allergen extracts and mannan can down regulate IDO in DCs and suppress Th1 polarization.

Mannose receptor mediates the IDO down-regulation by mannose-based ligands

Since MR is not the only receptor involved in the recognition of mannosylated structures on DCs, we sought to determine the role of MR in mannan-mediated modulation of IDO activity in human DCs. Using small interfering RNA (siRNA) we could knockdown MR expression on DCs by up to 80% similar to our previous work (5) (Suppl. Fig. 1). Control (CT) and MR^{low}-DCs were stimulated with mannan with or without LPS and IDO activity was measured after 24 hrs culture. This data clearly show an increase in IDO activity in MR^{low}-DC compared with CT-DC indicating that MR plays a key role in the IDO down-regulation induced by mannan in human DCs (Fig. 4A). Additionally, we found an inverse correlation between the expression of MR and IDO1 in DCs stimulated with mannan and LPS (Fig. 4B).

A reduction in IDO1 levels was associated with an increase in MR expression in DCs stimulated with mannan. These data suggest that mannosylated allergens down-regulate IDO through MR engagement in human DCs.

IDO regulation in human dendritic cells is partially dependent on AhR

In order to study the role of the receptor-transcription factor AhR in the induction of IDO in human DCs, we generated AhR^{low}-DCs through gene silencing (Suppl. Fig. 2) and analyzed IDO activity after TLR4 stimulation compared with CT-DCs. Our data shows a lack of IDO up-regulation in AhR^{low}-DCs compared with CT-DCs after TLR4 engagement (Fig. 5A), which suggests that LPS-mediated IDO induction in human DCs is partially dependent on AhR expression. Accordingly, we next sought to evaluate how AhR expression and activity were regulated after stimulating DCs with mannan in the presence and absence of LPS. When DCs were stimulated with mannan and LPS, AhR expression and activity, assessed by measuring the expression of one of its target genes i.e. cytochrome P450 1A1 (CYP1A1), were significantly reduced compared with DCs stimulated with LPS alone (Fig. 5B). This shows that MR engagement can interfere with TLR4 signaling by modulating the AhR-IDO axis in human DCs.

Furthermore, we found that IL-10 production, as well as IDO activity, were significantly lower in AhR^{low}-DCs than in CT-DCs, while IL-12p70 production, a key cytokine involved in Th1 polarization, was not affected (Fig. 5C). This data shows, for the first time, the important role of AhR in TLR4 signaling in human DCs, specifically in their regulatory functions, such as those mediated by IDO and IL-10 production.

The NF-κB pathway is negatively regulated by MR in human dendritic cells

The NF-κB pathway plays a central role in driving immunity and inflammation. TLR4 signaling induces canonical NF-κB pathway resulting in p65 phosphorylation, nuclear translocation and induction of pro-inflammatory cytokine expression that activate the immune response (44). On the other hand, IDO induction has been shown to be under the control of the non-canonical NF-κB pathway (36, 42, 45) involving the generation of p52-RelB complexes (44, 46). Accordingly, we evaluated how MR modulates both the canonical and non-canonical NF-κB pathway in human DCs. In order to understand how different components of the NF-κB pathway were regulated upon TLR-4 activation (in the presence and absence of mannan) we used a reverse phase protein array approach (35) that enables simultaneous evaluation of protein expression as well as post-translational modifications. First, we evaluated a panel of 14 different targets at different time points (10, 30, 90, 360 and 1080 min), using β-actin as a housekeeping control. Green (low expression) to red (high expression) heat maps represent the relative abundance of proteins upstream of the canonical and non-canonical NF-κB signalling pathway (Fig. 6A). DCs stimulated with LPS exhibited a rapid induction in p65 phosphorylation with a peak at 90 min (canonical NF-κB activation), which was followed by NIK accumulation between 6 to 18 hrs (a classical indicator of non-canonical NF-κB activation) (Fig. 6A, B). However, mannan on its own did not induce NF-κB activation (data not shown). Main differences were found in the levels of phospho-p65 as well as the expression of RelB in DCs stimulated with mannan plus LPS compared to LPS only (Fig. 6 A, B, C). DCs stimulated with mannan and LPS showed a significant decrease in a component of the non-canonical NF-κB pathway namely RelB (Fig. 6B, C), which was further confirmed at gene level by qRT-PCR (Fig. 6D). In addition, a decrease was observed in phospho-p65 at late time points (Fig. 6B, C). Taken together, these data show that MR ligation in human DCs impairs NF-κB activation in the presence of LPS. This antagonistic effect of MR on TLR4 activation of non-canonical NF-κB pathway could explain the effect

on the AhR-IDO axis described above, which perhaps might involve a physical and functional association between AhR and RelB in modulating IDO levels in human DCs.

Discussion

Asthma is a common chronic inflammatory disease of the conducting airways that affects millions of people worldwide. Allergic asthma is characterized by Th2 cell differentiation and the presence of IgE antibodies against common inhaled allergens. DCs have been shown to be pivotal in the early events such as allergen recognition and uptake which lead to Th2 polarization and ultimately allergic sensitization, however, how allergens modulate DCs functions and downstream T cell responses is not clear (1-4). Previous observations have shown that TLR4 signaling is crucial for inducing Th2-mediated inflammation and asthma (47-49) which is dependent on the levels of LPS exposure (49). It is therefore reasonable to assume that allergens could potentially modulate DC response to LPS in favor of Th2 responses. LPS-induced programming of human DCs is characterized by a rapid increase in pro-inflammatory cytokines, followed by induction of anti-inflammatory mediators that help to resolve inflammation. One of the key molecules that mediate immuno-regulatory function in DCs is the TRP-metabolizing enzyme IDO (17, 18). In this study we first established how IDO is regulated in human DCs in response to TLR-4 agonist LPS showing IDO up-regulation in response to relatively low concentrations of LPS (up to 100 ng/ml) followed by reduction in IDO at higher concentrations (Fig. 1A, B, C, D, E). These data were confirmed after using ultrapure LPS establishing the involvement of TLR4 pathway only (Fig. 1B). Two IDO isozymes have been identified namely IDO1 and IDO2 (50), with IDO1 being the most extensively studied. Both genes have similar structures and are situated adjacent to each other on human chromosome 8. Although both proteins have similar enzymatic activity, there are different expression patterns in some pathological condition (51-53). Here we have shown for the first time that both IDO isozymes are induced after TLR4 engagement (Fig. 1D).

We and others have previously shown that CLRs such as MR and DC-SIGN are key receptors involved in allergen recognition by DCs and in downstream events leading to Th2

cell polarization (1, 5, 6, 8, 10, 54). In the case of MR this is likely to be mediated through down-regulation of IDO activity (5) however the molecular mechanisms of how MR ligation modulates IDO activity remained unclear. In the current study, using siRNA (Suppl. Fig. 1), we have shown that MR mediates the allergen (and mannan) induced down-regulation of IDO activity in LPS stimulated DCs (Fig. 4A, B). This data demonstrates the ability of a number of clinically relevant allergen extracts from diverse sources, such as HDM, cockroach and pollen in down-regulating LPS induced IDO activity and the importance of carbohydrates in this process (Fig. 2A, B, C). Admittedly not all allergens are necessarily glycosylated (e.g. lipocalins) however it is still worth investigating IDO regulation by such families of allergens given their proven ability in enhancing innate immune signaling by modulating TLR4 activation (55, 56).

Furthermore, we studied how MR engagement might modulate other aspects of DC function. Our data showed that mannosylated sugars can particularly down-regulate TLR4-mediated IL-12p70 production, a key cytokine in Th1 polarization (Fig. 3A). This shows an antagonistic effect between MR and TLR4 which was in line with previous observations (41, 43, 57, 58). A similar pattern was found with all the allergen extracts tested suggesting that the mannosylated sugars on them are responsible for the modulation of IL-12p70 production (Fig. 3B). In terms of costimulatory molecules, we found a significant down-regulation in the levels of CD86 in DCs stimulated with mannan (Fig. 3C). It is interesting to note that CD86 is one of the B7 family proteins, that has been previously associated with IDO induction (59, 60). Furthermore, we have shown that MR engagement by mannan impairs Th1 cell priming induced by LPS, as evidenced by a significant suppression in IFN-γ production, which could bias T cell responses towards a Th2 profile (Fig. 3D, E, F). These data clearly suggest that reduction in IDO, through MR, could promote Th2 immune responses.

IDO has been well defined for its role in Th1 cell-mediated immune responses; however, its role in Th2 immune responses particularly in human has remained controversial (61). Some studies have shown that IDO can have a protective effect in different models of experimental asthma (19-24). This is in line with clinical studies showing that asymptomatic non-atopic individuals have higher systemic IDO activity than symptomatic atopic individuals (25, 26). Paradoxically, IDO expression might also contribute in mediating inflammatory responses in established Th2-mediated airway diseases. For example, a study with IDO knockout mice demonstrated that lack of IDO provide a significant relief from establishment of allergic airway disease (62), which could be due to impaired DC function in IDO deficient DCs (63, 64).

The aryl-hydrocarbon receptor (AhR) is a ligand-dependent transcription factor and a member of the Per-Arnt-Sim (PAS) superfamily of proteins, which are involved in the detection of environmental or intracellular changes. The interaction of AhR with Ah receptor nuclear translocator protein (ARNT) allows it to bind specific enhancer sequences present in target promoters called dioxin responsive elements (DREs) (27). Previously, AhR has been shown to have a protective role in allergy (65-68). Accordingly, in an attempt to elucidate the mechanism of TLR-4 and MR-mediated IDO modulation we studied the potential link between AhR and IDO in human DCs. Here, we have shown that TLR4-mediated induction of IDO is partially dependent on AhR expression (Fig. 5A). In addition, we have shown that the anti-inflammatory cytokine IL-10 is under AhR control, which highlights a central role for AhR in modulating DC mediated immune-suppression/regulation (Fig. 5C). Furthermore, we demonstrated that MR can down-regulate AhR expression and activity in the presence of LPS in human DCs (Fig. 5B), which could explain its effect on IDO expression (Fig. 4) and suppression of Th1 responses (Fig. 3). Interestingly, it has been shown that exogenous AhR ligands like 2,3,7,8-Tetrachlorodibenzodioxin (TCDD) can impair DC phenotype and

448 function (68-72) suppressing allergic sensitization (66, 68). Furthermore, metabolites
449 produced in the IDO-dependent TRP degradation pathway, such as KYN and kynurenic acid,
450 can activate AhR leading to Treg or Th17 differentiation depending on the immunological
451 context (28, 73-75). Accordingly, we can speculate that IDO metabolites might have
452 disparate effects in allergic responses, which will require further investigation.

453 IDO induction has been shown to be under the control of the non-canonical NF-κB pathway
454 (36, 42, 45). Additionally, the NF-κB together with AhR has been shown to be pivotal in
455 TLR4 signaling (44, 76-78). Therefore, we evaluated the role of the NF-κB pathway in IDO
456 regulation by MR. Using a protein microarray approach (35), we evaluated the expression of
457 key components of the NF-κB pathway after stimulation with LPS in the presence and
458 absence of mannan. Our data showed that stimulation with mannan and LPS can significantly
459 down-regulate some key components of the non-canonical NF-κB pathway such as RelB, as
460 well as the canonical NF-κB pathway such as phospho-p65 (Fig. 6A, B, C, D). Although a
461 signalling motif has not been identified in MR cytoplasmic domain, members of the NF-κB
462 signaling pathway have been previously implicated in MR-mediated signaling (43, 79). For
463 instance, Man-LAM, most likely through binding to MR, mediates IRAK-M induction which
464 acts as a negative regulator of the NF-κB pathway (43). Interestingly our preliminary data
465 also shows that mannan stimulation reduces IRAK-M expression in the presence of LPS in
466 human DCs (data not shown), which is in line with previous observations showing that
467 IRAK-M might protect from complications of asthma, as Th2 cytokines decrease IRAK-M
468 expression in macrophages (80). Collectively these observations highlight the role of the NF-
469 κB pathway in MR mediated IDO regulation in DCs. It is important to note that KYN via the
470 AhR/SOCS2-dependet pathway can induce proteasome-mediated degradation of TRAF6,
471 which might potentially inhibits TLR signalling favouring non-canonical NF-κB pathway
472 (81). Additionally, an association between AhR and RelB has been suggested (82-84) that

might contribute to the stabilization of RelB complexes (85, 86). Accordingly, it is reasonable to suggest that AhR together with RelB might be involved in IDO regulation in human DCs, a pathway that is interfered after MR engagement. Future studies should aim at elucidating the potential physical association between AhR and RelB in regulating IDO levels.

In conclusion, we have demonstrated that diverse airborne allergens can down-regulate IDO after TLR4 engagement and this effect was mainly mediated by MR. In addition, we have showed that AhR and RelB are implicated in MR-mediated IDO down-regulation suggesting a new pathway involved in inserting immune regulatory properties of allergens (Fig. 7). These data provide new insight into the initial steps of allergic sensitization which could pave the way for developing more effective therapeutic strategies targeting early events in the allergic cascade.

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Figure Legends

Figure 1. TLR4 agonist induces IDO1 and IDO2 in human dendritic cells. **A.** IDO activity in human DCs stimulated 24 hrs with increasing concentrations of LPS from *E.coli* O111:B4 (n=5). **B.** IDO activity in human DCs stimulated 24 hrs with increasing concentrations of ultrapure LPS (n=2). **C.** IDO activity in human peripheral blood myeloid DCs stimulated 24 hrs with LPS (0.01 μg/ml) (n=2). **D.** Conventional PCR analysis of IDO1 and IDO2 gene expression in human DCs stimulated 24 hrs with LPS 0.01 μg/ml. GAPDH was used as housekeeping gene. IDO1 (n=5), IDO2 (n=3). **E.** Flow cytometry analysis of IDO1 protein expression in human DCs stimulated 24 hrs with LPS 0.1 μg/ml. Grey filled histogram represent autofluorescence, black line represent isotype control and red histogram represent stained sample (n=5).

Figure 2. TLR4-induction of IDO1 and IDO2 is down-regulated by allergens from diverse sources. **A.** IDO activity in human DCs stimulated 24 hrs with diverse allergen extracts (10 μg/ml) in the presence and absence of LPS (0.01 μg/ml). German Cockroach (GC) extract from *Blattella germanica*, House Dust Mite (HDM) extract from *Dermatophagoides pteronyssinus*, Bermuda Grass Pollen (BGP) extract from *Cynodon dactylon* (n≥4). **B.** IDO activity in human DCs stimulated 24 hrs with mannan from *Saccharomyces cerevisiae* (M) (10 μg/ml) in the presence and absence of LPS (0.01 μg/ml) (n=5). **C.** qRT-PCR analysis of IDO1 and IDO2 gene expression in human DCs stimulated 24 hrs with M (10 μg/ml) in the presence of LPS (0.01 μg/ml). Relative expression of IDO1 and IDO2 were compared with that of GAPDH (n=3). In co-stimulation experiments, cells were stimulated with LPS followed immediately by allergen extracts.

Figure 3. Mannose-based ligands down-regulate TLR4-induced IL-12p70 production by human dendritic cells and affect T helper cell polarization. **A.** Cytokine production by human

DCs stimulated or not with M followed by LPS for 24 hrs (n=3). **B.** IL-12 production by human DCs stimulated with several allergen extracts and LPS (n=3). **C.** Flow cytometry analysis of costimulatory molecules expression in human DCs stimulated 24 hrs with M and LPS (n=3). **D.** Percentage of IFN-γ positive cells (n=3). **E.** IFN-γ production by human T cells co-cultured with DCs stimulated or not with M followed by LPS (one experiment representative of three). **F.** DCs were stimulated or not with M and co-cultured in the presence of LPS with $CD3^+CD45RA^+$ naïve T cells. Polarization was assessed at day 6-8 by measuring IL-4 and IFN-γ production on $CD4^+$ gated cells after re-stimulation with anti-CD3/CD28 (one experiment representative of three).

Figure 4. Mannose receptor mediates the IDO down-regulation by mannose-based ligands. **A.** IDO activity in CT and MR^{low}-DCs stimulated with M and LPS for 24 hrs (n=3). **B.** Flow cytometry analysis of MR and IDO1 protein expression in human DCs stimulated or not with M and LPS for 24 hrs. Median fluorescence intensity (MFI) values were normalized to control unstimulated sample (n=3).

Figure 5. IDO regulation in human dendritic cells is partially dependent on AhR. **A.** IDO activity in CT and AhR^{low}-DCs stimulated or not with LPS for 24 hrs (n=3). **B.** qRT-PCR analysis of AhR and CYP1A1 gene expression in human DCs stimulated or not with M followed by LPS for 24 hrs. Relative expression of AhR and CYP1A1 were compared with that of GAPDH (n=3). **C.** IL-12p70 and IL-10 production by CT and AhR^{low}-DCs stimulated or not with LPS for 24 hrs (one experiment representative of three).

Figure 6. The NF-κB pathway is negatively regulated by MR in human dendritic cells. **A.** Heat maps representing the relative abundance of proteins upstream the NF-κB signalling pathway using human DCs stimulated with M and LPS for different time points. **B.** Protein expression of phospho-p65, NIK and RelB in human DCs stimulated with M followed by

LPS. Data are shown as geometric mean of three independent experiments. **C.** Protein expression of RelB (90 min), NIK (90 min), phosphor-p65 (18 hrs) and TRAF3 (18 hrs) in human DCs stimulated with M followed by LPS. All fluorescent signals are reported as AFU with β-actin normalisation. **D.** qRT-PCR analysis of RelB gene expression in human DCs stimulated with M followed by LPS for 24 hrs. Relative expression of RelB was compared with that of GAPDH (n=2).

Figure 7. MR-mediated IDO down-regulation in human DCs involved the transcription factors AhR and RelB, having an impact on T helper cell polarization.

Supplementary Figures

Supplementary Figure 1. MR down-regulation by gene silencing. **A.** qRT-PCR analysis of MR gene expression in CT and MR^{low}-DCs. Relative expression of MR was compared with that of GAPDH (n=2). **B.** Flow cytometry analysis of MR protein expression. MFI is shown (n=5). **C.** Flow cytometry analysis of DC-SIGN, HLA-DR, TLR4, CD14, MD-2 and AhR protein expression. MFI is shown (n≥2).

Supplementary Figure 2. AhR down-regulation by gene silencing. **A.** qRT-PCR analysis of AhR gene expression in CT and AhR^{low}-DCs. Relative expression of AhR was compared with that of GAPDH (n=2). **B.** Flow cytometry analysis of AhR protein expression in human CT-DCs compared with AhR^{low}-DCs. MFI is shown (n≥3). **C.** Flow cytometry analysis of MR, DC-SIGN, TLR4, CD14, MD-2 and CD86 protein expression. MFI is shown (n≥2).

Figure 1

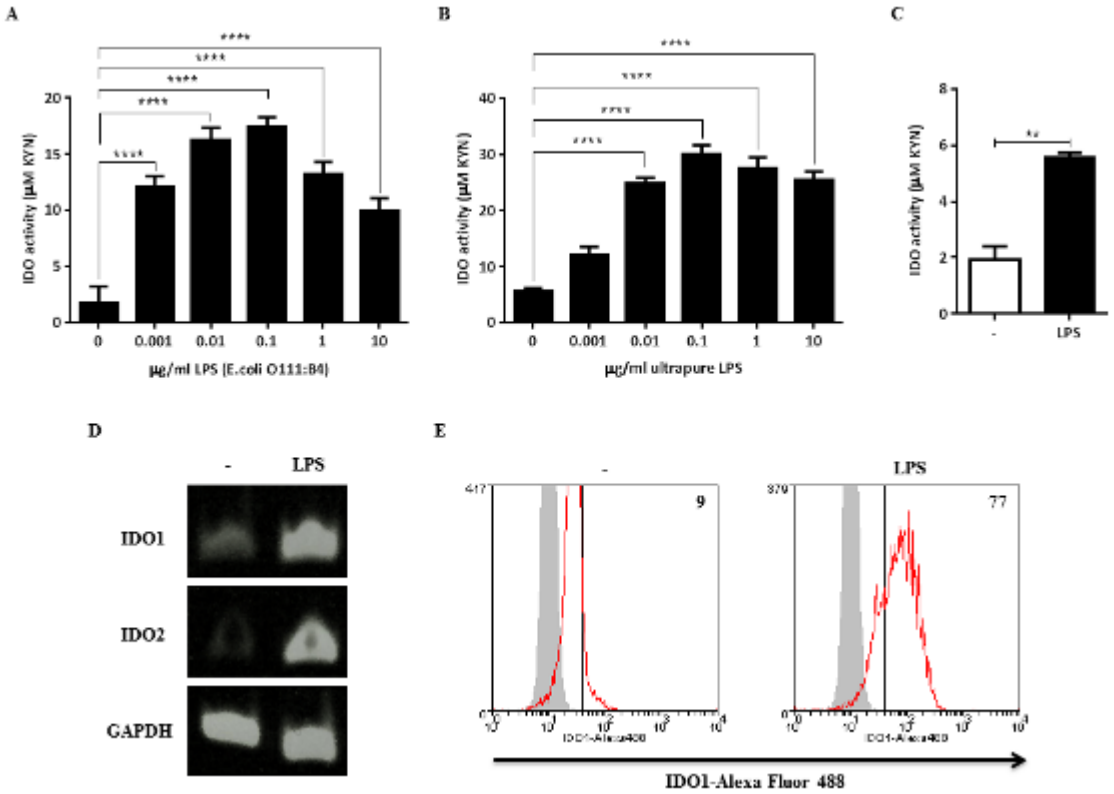
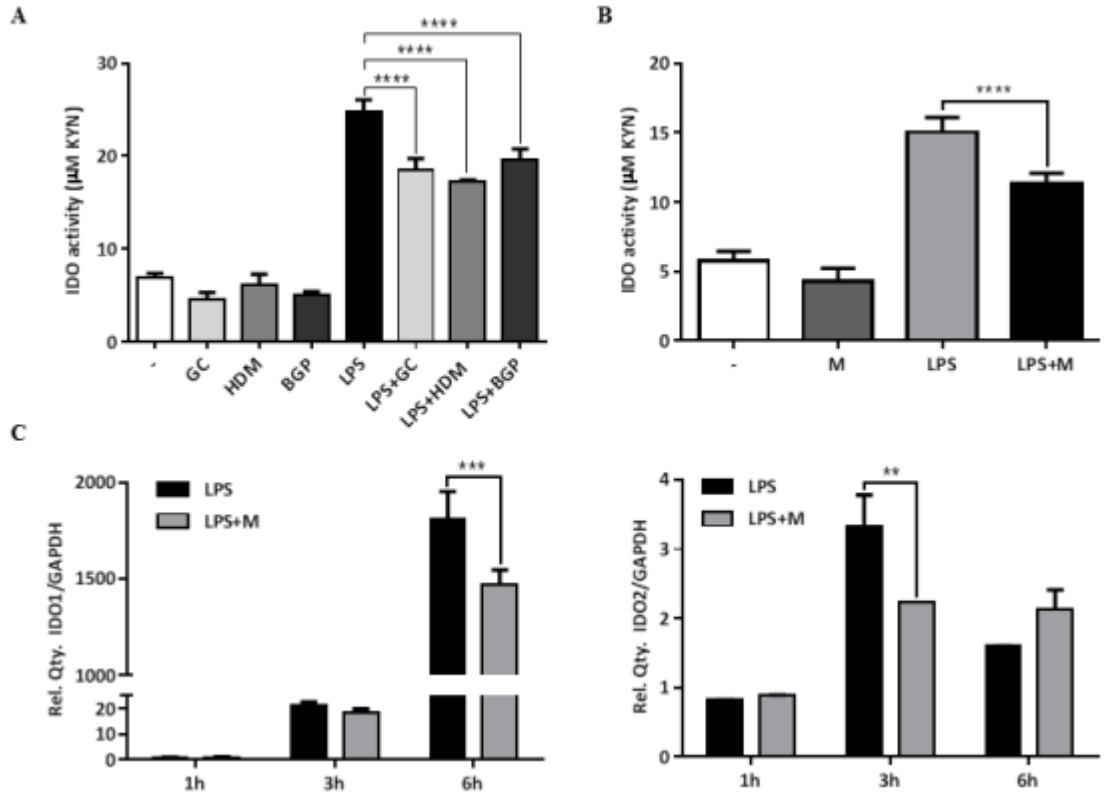


Figure 2



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895 **Figure 3**

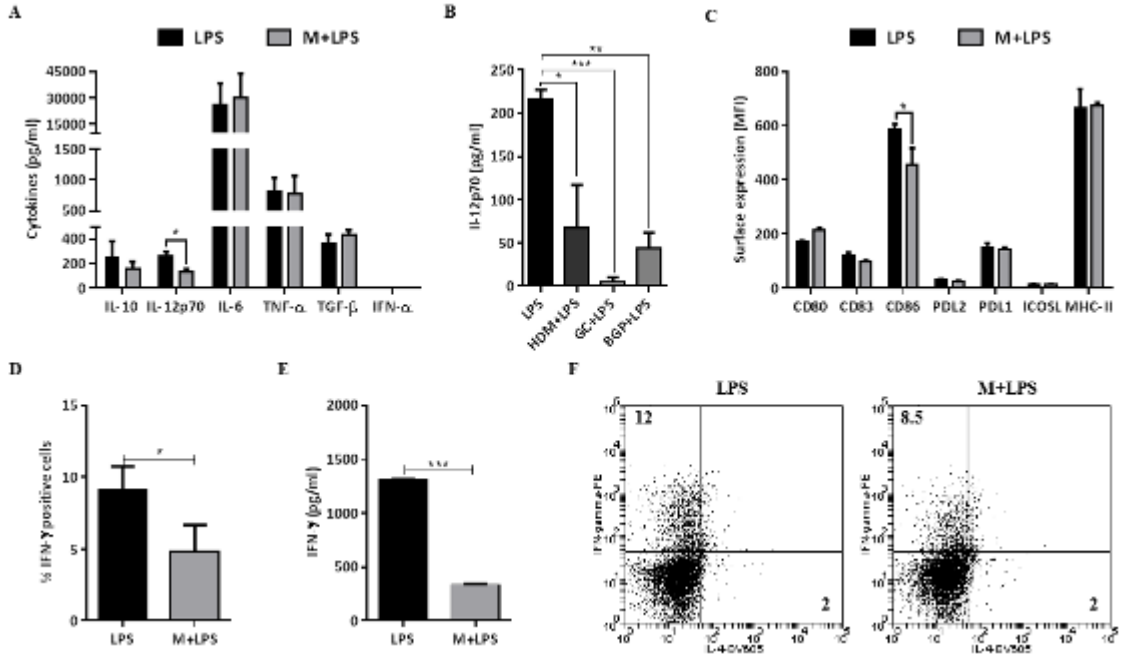

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Figure 4

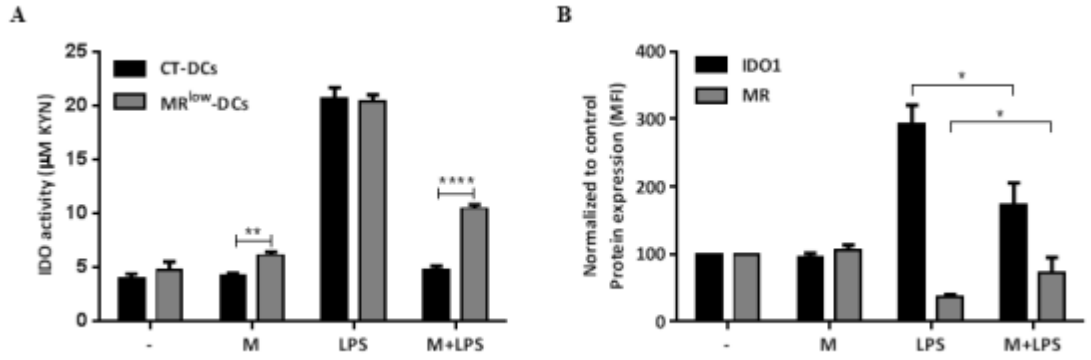


Figure 5

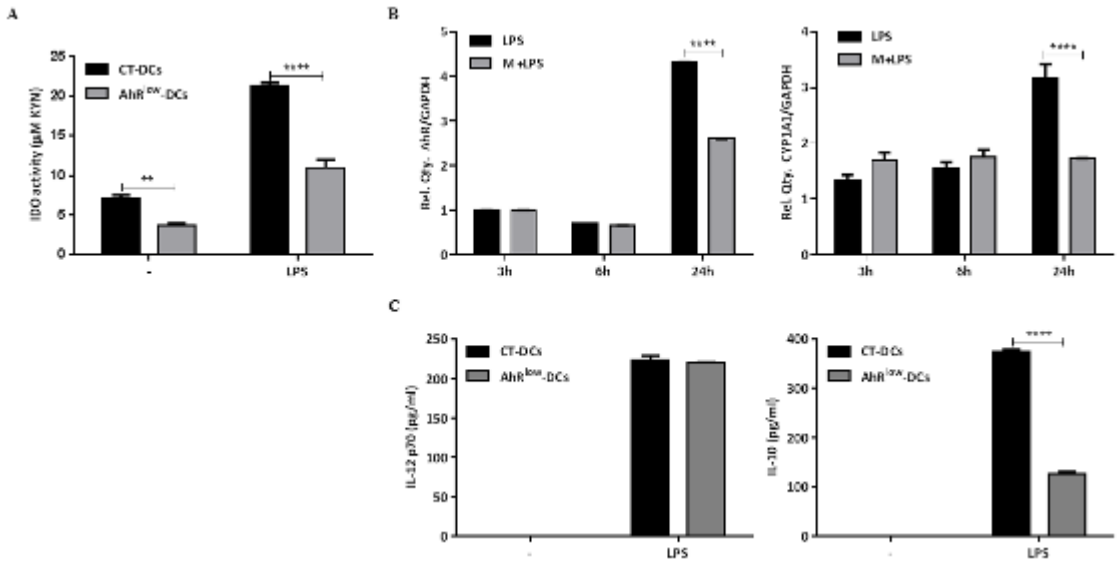
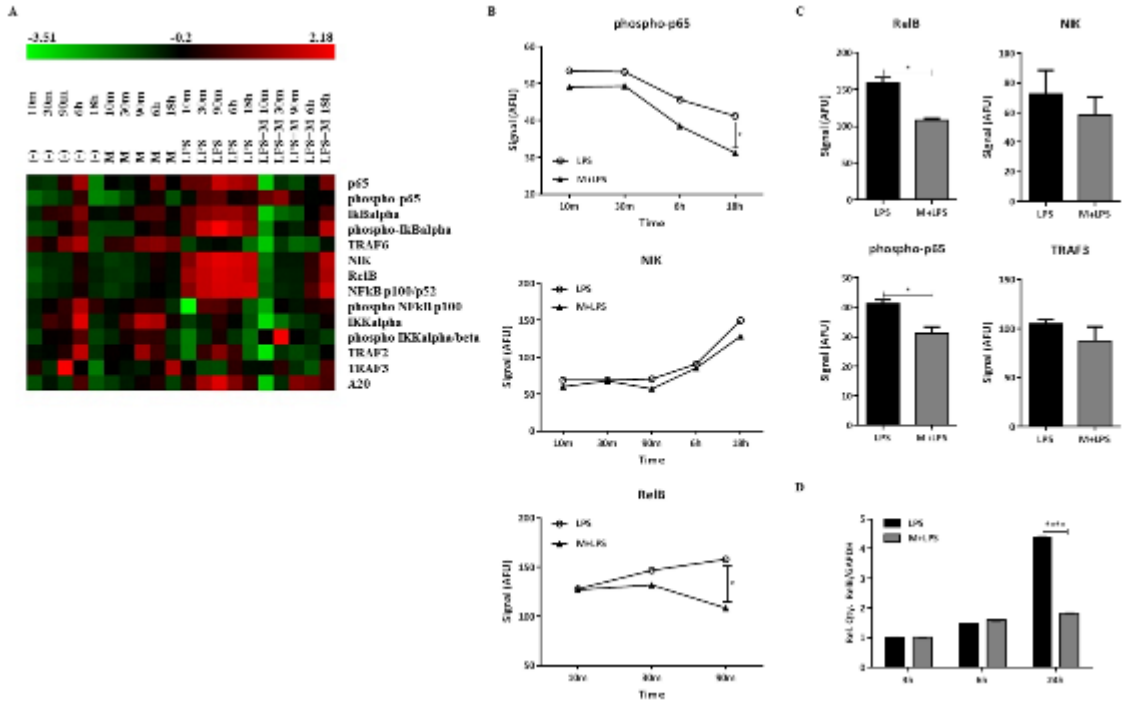


Figure 6


A
-3.51
-0.2
2.18
p65
phospho p65
IkBalpha
phospho-IkBalpha
TRAF6
NIK
RelB
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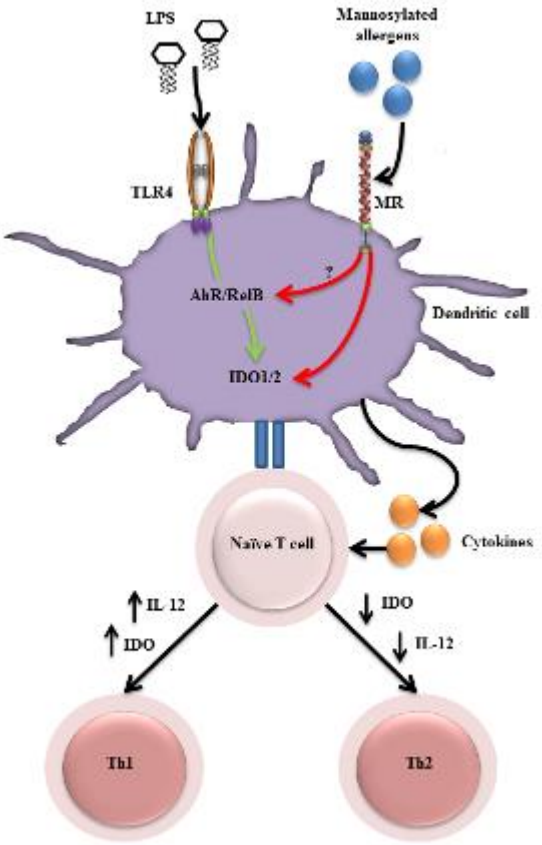
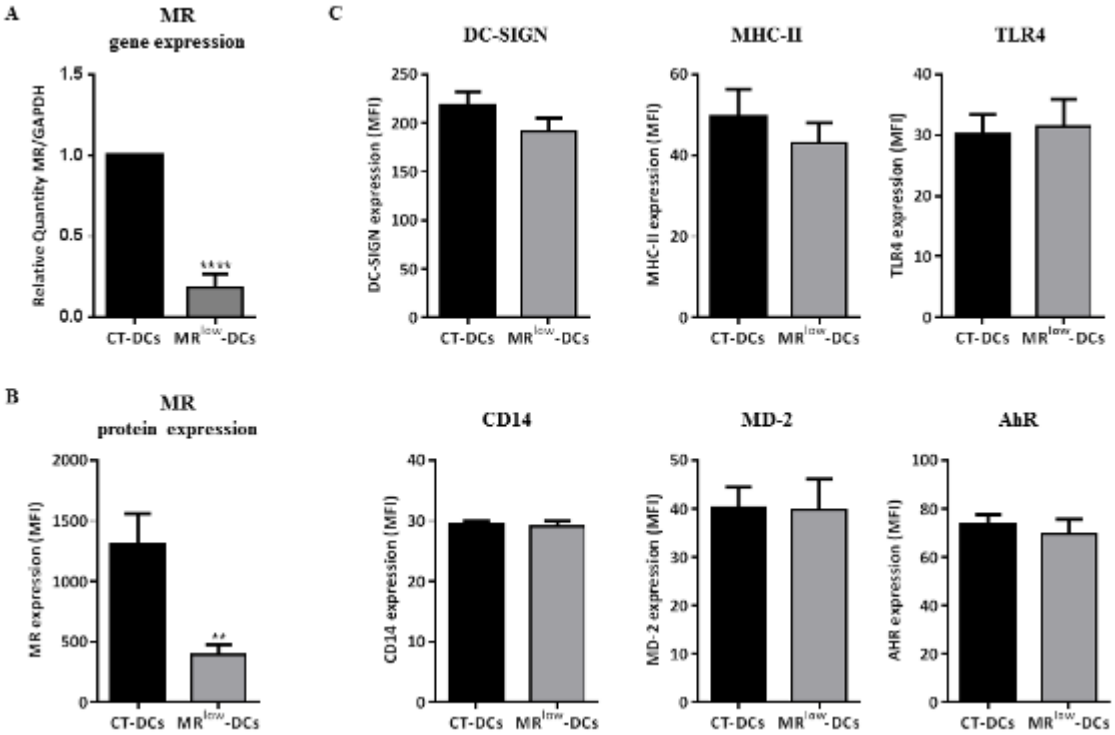
phospho NFkB p100
IKKalpha
phospho IKKalpha/beta
TRAF2
TRAF5
A20
B
phospho-p65
NIK
RelB
Signal (AFU)
Time
LPS
M+LPS
C
RelB
NIK
phospho-p65
TRAF5
D

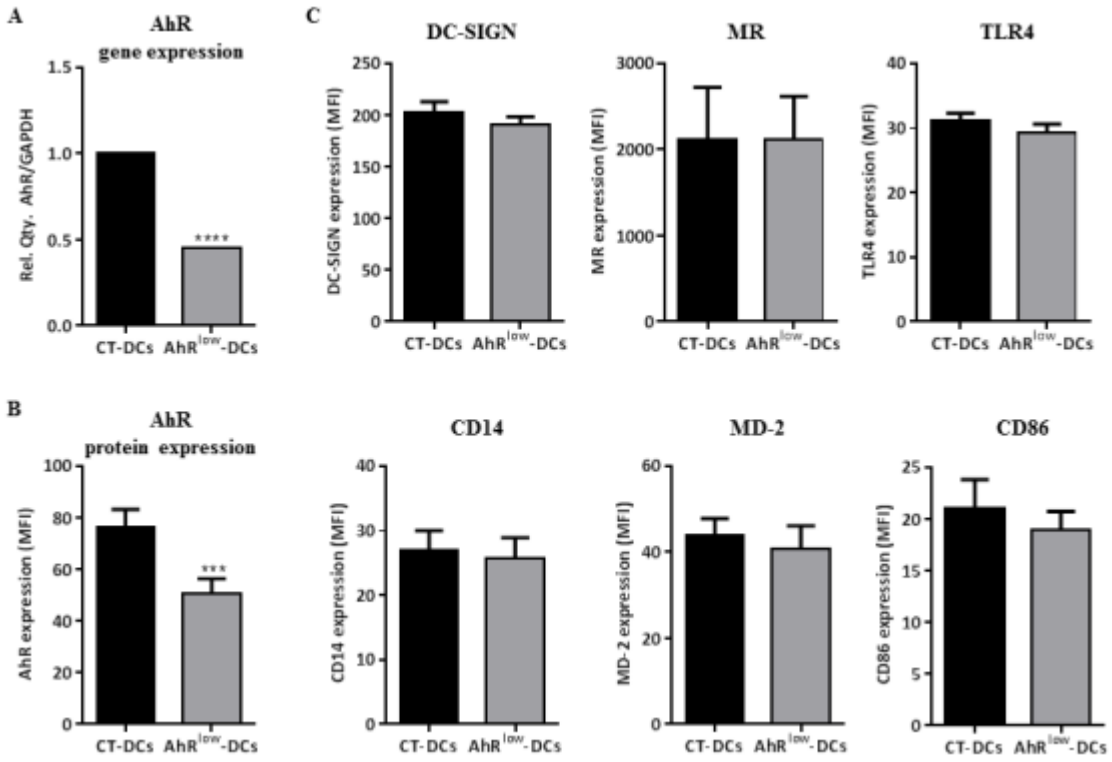
Figure 7


LPS
Mannosylated allergens
TLR4
MR
?
AhR/RelB
IDO1/2
Dendritic cell
Naïve T cell
Cytokines
IL 12
IDO
IDO
IL-12
Th1
Th2

Suppl Fig. 1



Suppl Fig 2.


A
AhR
gene expression
Rel. Qty. AhR/GAPDH
1.5
1.0
0.5
0.0
****
CT-DCs
AhR low -DCs
C
DC-SIGN
DC-SIGN expression (MFI)
250
200
150
100
50
0
MR
MR expression (MFI)
3000
2000
1000
0
TLR4
TLR4 expression (MFI)
40
30
20
10
0
B
AhR
protein expression
AhR expression (MFI)
100
80
60
40
20
0
***
CD14
CD14 expression (MFI)
40
30
20
10
0
MD-2
MD-2 expression (MFI)
60
40
20
0
CD86
CD86 expression (MFI)
25
20
15
10
5
0

941